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Development and validation of new methods for the determination of melatonin and its oxidative metabolites by high performance liquid chromatography and capillary electrophoresis, using multivariate optimization

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ABSTRACT

Melatonin (N-acetyl-5-metoxytriptamine, MEL) has focused a lot of attention as consequence of its multiple functions. MEL is a potent endogenous antioxidant and a free radical scavenger that reacts with several sort of radicals generating various metabolites. Two of them are N1-acetyl-N2-formyl-5-methoxykynurenine (AFMK) and N1-acetyl-5-methoxykynurenine (AMK). These compounds are important because they have also antioxidant actions as well as other important biological properties. In the present work, we develop two methods to detect and quantify these compounds (MEL, AFMK and AMK) in the same sample. For this purpose we used an experimental design, and utilized high performance liquid chromatography (HPLC-DAD) and micellar electrokinetic chromatography (MEKC) techniques with diode array detector in both of them. The limit of detection/quantification for MEL, AFMK and AMK were respectively 44/94, 18/38 and 23/51 ng mL^{-1} by using HPLC and 13/44, 37/124 and 47/156 ng mL^{-1} by using MEKC. This is the first time that these compounds have been separated in the same chromatogram or electroferogram. The time of analysis was faster using MEKC. Furthermore, this technique showed better resolution but HPLC offered better limit of detection and quantification for metabolites. Both methods were validated and correlation coefficients were higher than 0.999 and the range of recovery of those methods were 99.6–103.7%. Precision was evaluated as repeatability and intermediate precision with relative standard derivation <5%. When a 5 μg mL^{-1} solution of these compounds were analyzed with both methods we do not observed any statistically significance differences. Moreover, we analyzed 3COHM (cyclic-3-hydroximelatonin), another known metabolite of melatonin, by using the same methods. The employment of these methods will offer a useful tool to contribute to answer the role of MEL, AFMK and AMK in biological system and both methods can be used in routine analysis for these compounds.

1. Introduction

N-acetyl-5methoxytriptamine or melatonin (MEL) is an indolamine discovered and isolated in 1958 from bovine pineal glands by Lerner et al. [1]. The indole is derived from tryptophan and in all vertebrates is mainly synthesized by the pineal gland. Its synthesis has been reported in other taxonomically distant groups of organisms; bacteria, fungi, and plants [2]. Additionally extrapineal melatonin synthesis has been reported. Thus, other organs including retina, Harderian gland, gut mucosa, cerebellum, airway epithelium, liver, kidney, adrenals, thymus, thyroid, pancreas, ovary, carotid body, placenta and endometrium or skin synthesize it as well [3]. In mammals, melatonin is a potent sleep-inducing mediator [4] and modulates seasonally reproductive cycles in some mamals [5]. Melatonin has several physiological functions including a role as inmunomodulator [6] and as neuroprotective agent in Alzheimer and Parkinson's disease models [7]. It also has a possible role in metal regulation in central nervous system and could form complexes with aluminium, cadmium, copper, iron and other metals [8]. Melatonin is a potent free radical scavenger and stimulates a number of cellular antioxidant enzymes including glutathione reductase and superoxide dismutases [9,10]. Melatonin has been shown to synergize with vitamins C and E among others to reduce free radical damage [11,12]. Melatonin overcome cardiac hypertrophy and oxidative alterations associated with the treatment of hyperthyroidism [13]. Since Tan et al. [14] demonstrated

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the ability of melatonin to scavenge hydroxyl radicals (•OH), the main metabolites resulted from this reaction are under investigation. N1-acetyl-N2-formyl-5-methoxykynurenine (AFMK) is considered one of the major metabolites of melatonin even more important than its urinary secretory product, 6-hidroxymelatonin sulfate. AFMK can also be synthesized either via enzymatic or non-enzymatic pathways both in vivo and in vitro (Fig. 1). AFMK exerts anti-inflammatory properties in macrophages [15] and has an important role in cell cycle modulation of malaria parasites [16]. As an antioxidant, AFMK is very effective in neutralizing free radicals because it transfers two electrons while other classic antioxidants such as vitamins C and E or glutathione can only transfer one [17]. There are not so many studies focused on the antioxidant capacity of other metabolite by melatonin, N-acetil-5-methoxykynurenine (AMK) but it has been reported that AMK inhibits lipid peroxidation [18], has anti-inflammatory properties [15] and interacts with several reactive molecules including $^1O_2$ [18]. AMK reduces the activity of nitric oxide synthase (NOS), the enzyme that catalyst the formation of radical NO• [19].

In order to study the biological properties of MEL, AFMK and AMK and their presence inside cells it is necessary to develop alternative analytic methods to detect and quantify these compounds in different type of biological samples. During the 1970s, melatonin analysis was carried out basically by bioassay [20] radio-inmune assay (RIA) [21] and by fluorometric methods [22]. Consequently, the analysis of MEL has focused research attention and in the last 40 years, several analytical techniques have been developed. In some of these, such as GC–MS a derivatization of MEL using silanizing agents or pentafluoropropionic anhydride to form trimethylsilyl or pentafluoropropionyl melatonin respectively prior to analysis is necessary [23–25]. Another technique employed to analyze MEL has been HPLC–MS–MS [26]. Although, this is the state-of-the-art method, it is difficult to use routinely in many laboratories. Some researchers use HPLC under different conditions, but an isocratic mobile phase with reversed phased is the most commonly used. In this case, there are several types of detectors such as fluorescence detector [27–30], electrochemical detector [31,32] and UV [33–35]. These detectors are useful in different matrixes and show different detection (LOD) and quantification limits (LOQ) [36]. The analysis of melatonin by capillary electrophoresis (CE), using either electrochemical [37,38] or UV detectors [39–41], is much less frequent. Kim et al. [40] obtained a LOD of 1 pg mL^{-1} for MEL using mouse blood samples by CE with UV detector but the best LOD were obtained by using electrochemical detection [37,38]. In the case of AFMK and AMK analytical methods are not enough described. Thus, AFMK is analyzed by HPLC–MS after melatonin reaction with ABTS [42] and by using a fluorescence detector in cerebrospinal fluid [43]. Siva et al. [44] used HPLC with UV detection to analyze AMK as well as fluorescence detector for AFMK [45]. For the separation of these compounds it has been necessary to use micellar electrokinetic chromatography (MEKC), which is one of the modalities of CE. MEKC allows to separate and determine simultaneously neutral and charged substances. Recently, the same group analyzed melatonin in pharmaceutical preparation [46] in human serum [39] employing MEKC-UV. Using UV and fluorometric detection, Pbozy et al. analyzed MEL in pharmaceutical tablets and biological samples [47].

In this paper we used the Factorial Analysis approach because using this type of experiment design allow us to understand the complex relation between variables that is not possible using the traditional univariant methods. Using the factorial analysis all variables are considered simultaneously. This technique is ideal to extract all information from data available and in this way we evaluate the importance of each variable when compared them to the estimated responses [48,49].

To date, there are no reported methods to analyze MEL, AFMK and AMK in a single analysis and because of the high important of these compounds in different biological systems and considering their antioxidant, antitumoral and anti-inflamatory properties among others, we believe is necessary to develop alternative, simple methods to analyze these compounds routinely in laboratories of different disciplines.

2. Experimental

2.1. Measurement by HPLC and MEKC

HPLC analysis was performed on a Shimadzu HPLC system (Shimadzu, Duisburg, Germany) equipped with two LC-10AD pumps, a UV–Vis SPD-M10AD photo-diode array detector (10 μl flow cell and 80 ms of response time), a Sil-10AD automatic injector, and a Gastor 150 LCD degasser on-line. A Tracer Extrasil ODS1 column (250 mm × 0.46 mm, 5 μm) (Teknokroma, Barcelona, Spain), operating at 35 °C, was used. An ODS guard column was placed previously to protect the analytical column. Mobile phase solution was always filtered through a 0.45 μm membrane filter. Identification of compounds was determined by their retention time (RT) and UV spectrum. Quantification was performed at 231 nm. All measurements were performed using Shidmadzu CLASS-VP software (Version 5.032). All the HPLC solutions were made by using double-filtered deionised Mili-Q water (Waters, Milfords, MA, USA).

MEKC analysis was performed on a Hewlett-Packard 3D CE system (Waldbronn, Germany) equipped with a UV–Vis photo-diode array detector using HP ChemStation software for quantification. Fused-silica capillary tubes (48.5 cm total length, 40 cm effective length, 75 μm inner diameter) were supplied by Merck (Darmstadt, Germany). Capillary was preconditioned prior to its first use by flushing with 1 M NaOH for 30 min and then, with pure water for 15 min. Before each analysis, the capillary was preconditioned with 0.1 M NaOH for 10 min, and rinsed with pure water for 5 min and then, with BGE (background electrolyte) solution for 15 min. Finally capillary was pre-equilibrated by applying voltage (−25 kV) for 10 min. Capillary was rinsed with BGE for 5 min prior to injection. In MEKC with reverse migration of micelles, sodium dodecyl sulfate (SDS) was employed as pseudostationary phase and reverse polarity was applied. The sample was introduced from the anodic end of the fused-silica capillary by 5 s hydrodynamic injection with a pressure of 50 mbar. The capillary was thermostatically kept at 30 °C. The determination of compounds was carried out at 215 nm and identification was performed using their migration time and UV spectra.

2.2. Chemicals

Ultra pure grade melatonin was a gift from Helsinn Chemical (Biasca, Switzerland). Acetic acid was obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol was purchased from Romil (Cambridge, UK) and glacial acetic acid was obtained from Panreac (Barcelona, Spain). Hydrochloric acid and sodium hydroxide were obtained from Probus (Badalona, Spain). The rest of chemicals used in the present study were purchased from Sigma–Aldrich, unless otherwise indicated.

2.3. Synthesis of AFMK and AMK

AFMK and AMK were synthesized by using the method recently reported by Tan et al. [50]. Briefly, H_2O_2 was diluted to 50 mM concentration in PBS (50 mM, pH 7.0) and mixed with desferoxamine at a final concentration of 1 mM to chelate any possible trace of free irons. Melatonin was then added to this solution to make a final concentration of 1 mM. The mixture was incubated at room

Table 1
Factors and levels used in factorial design for HPLC and MEKC.

HPLC			MEKC		
Variable	Low	High	Variable	Low	High
Methanol (%)	30.0	60.0	Phosphates (mM)	10.0	30.0
Sodium acetate (mM)	20.0	70.0	SDS (mM)	20.0	70.0
Flow rate (mL min^{-1})	0.5	0.9	Voltage (kV)	−25.0	−5.0
pH	4.0	5.5	pH	2.0	3.0

temperature for 2 h. This solution was then mixed with an equal volume of dichloromethane and shaken horizontally for 10 min. The water phase was discarded and the organic phase was dried under vacuum. The residue was redissolved in a small volume of methanol and fractionated by analytical thin layer chromatography with silica gel on polyester, fluorescent indicator, layer of 250 mm and 20 cm × 3 cm × 20 cm (TLC) using ethyl acetate as the solvent. The major spot (about 90% in all metabolites), which migrated with an RF of 0.2 (detected with UV lamp at 254 nm) was scraped from the TLC plate and extracted with methanol. The TLC purification was repeated two additional times. The purified product was identified to be AFMK by simple RMN-H.

For AMK synthesis: The above purified AFMK was further dissolved in PBS buffer (50 mM, pH 7.0) at a final concentration of 7 mM and incubated with catalase (2500 U/ml) at room temperature for 24 h. The solution was mixed with two parts of dichloromethane (per volume) and shaken horizontally for 10 min. The water phase was discarded and the organic phase was dried under vacuum. The residue was dissolved in a small volume of methanol and the enzyme metabolite was fractionated by analytical TLC using ethyl acetate as the solvent. The single metabolite produced by catalase was isolated from TLC plate as described above and identified to be the AMK by RMN-H.

2.4. Preparation of samples

Quantification was carried out by the "internal standard method" in both techniques. Different volumes of the stock solution (1 M in DMSO) of MEL, AFMK and AMK and 1 mL of 20 μg mL^{-1} solution of 5-methoxytriptophol (5-MT) were transferred into a 10 mL flask and raised up to volume with Milli-Q water. 5-MT was chosen as internal standard, following previous results [34]. The final concentrations of the MEL, AFMK and AMK in the solutions were ranging between 0.1 and 10 μg mL^{-1}, and the concentration of 5-MT was 2 μg mL^{-1}. This solution was filtered through a 0.22 μm membrane and degassed in an ultrasonic bath prior used. A volume of 0.5 mL of each sample was transferred to the vial and was then injected into CE or HPLC system. Each concentration was assayed at least three times.

2.5. Experimental design and data analysis

Column type, injection volume, temperature and other parameters established were based on previous experiments. The optimization for HPLC was performed using a Factorial Design to evaluate experimental parameters (i.e. time, resolution and efficacy), while the multivariate method was used to evaluate pH, flow, methanol percentages and acetates concentration in the mobile phase as variables. In MEKC, four quantitative factors (SDS concentration, background buffer pH, concentration and potential) were evaluated using the "Statgraphics" statistical package. The selected response variables were: (i) resolution between AFMK and 5-MT, (ii) migration time of the AFMK, and (iii) peak width AFMK. Resolution was calculated by measuring the difference in migration time and peak width of the two analytes [$Rs = 2((t_m)_B - (t_m)_A)/(W_A + W_B)$]. Variables selection was based on chemical and physical properties of the substances. In this way, we were able to fix levels of each factor studied to find out the optimum values and responses (Table 1).

3. Results and discussion

3.1. Experimental design for HPLC analysis

First, we studied different conditions in order to analyze MEL, AFMK, AMK and 5-MT by HPLC in the same analysis. We included 5-MT because we used this compound as internal standard [34]. To do so, we established the mobile phase formed with methanol and sodium acetate. C18 (reversed phase) was the column type employed and the temperature was set at 35 °C. We used experimental design to study intervals of several response variables such as methanol and acetate concentration in the mobile phase, flow rate and pH in order to find out the most accurate valor. Factorial program in one block with six degrees of freedom was applied and 21 experiences with randomly variation of the variables were necessary. After analyzed AFMK, AMK, MEL and 5-MT in all chromatographic conditions necessary for multivariate and predictive analysis, a chromatogram was obtained and employed for quantifying the influence of each variable. Results indicate that all parameters studied were important and their interactions contributed to modify the response of variables. According to results shown in Fig. 2A the most important parameters are Rs2 and "*t*". In the case of Rs2, that is separation of AMK and MEL, methanol and acetate concentration were very important variables, as it is shown in equations (Fig. 2C). In the estimated surface of time as it could be predicted, it is showed that when flow is elevated, less time is required and also when pH is low, the analysis is faster (Fig. 2A).

In order to optimize the analytical conditions and given the impact and weights of the parameters assayed, it became clear that the most important parameter is Rs2, and the value of impact and thus weight of this parameter is maximum, i.e. 5. Another important parameter is "*t*" which has an impact of 4, while both Rs1 and Rs3 have an impact of 3. On the other hand, these three parameters,

Table 2
Optimization of HPLC and MECK variables using factorial design.

HPLC			MEKC		
Variable	Optimal	Used	Variable	Optimal	Used
Methanol (%)	35.75	35.8	Phosphates (mM)	30.0	30.0
Sodium acetate (mM)	20.0	20.0	SDS (mM)	70.0	70.0
Flow rate (mL min^{-1})	0.896	0.9	Voltage (kV)	−25.0	−25.0
pH	5.076	5.1	pH	3.0	3.0

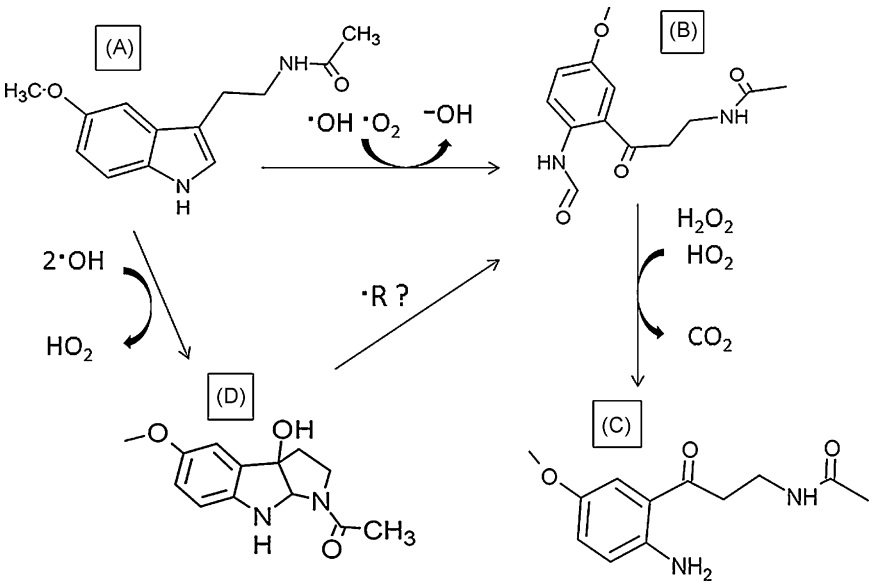
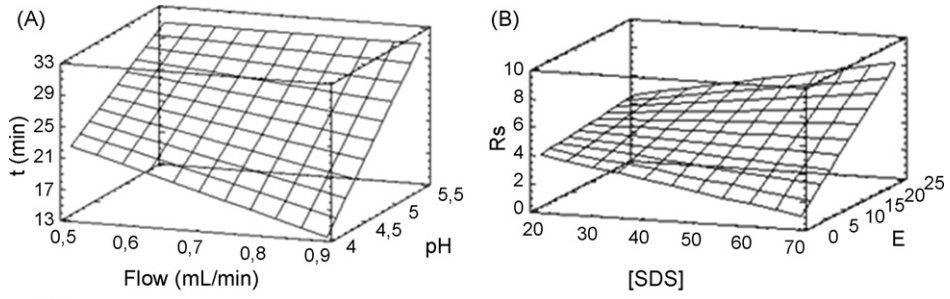


Fig. 1. Reaction of MEL (A) with reactive oxygen species to form AFMK (B), AMK (C) and 3COHM (D).

"*t*", Rs1, and Rs3, have 3 while "*w*" has 1 a weight of impact. Considering these conditions, the optimal parameters for the detection and correct separation of MEL are show in Table 2.

3.2. Final conditions of analysis by HPLC

We used all variables optimized (Table 2) and several wavelengths (190 up to 800 nm). We obtained the absorbance for all compounds in the same chromatogram and showed that the elution order was 5-MT, AMK, MEL and AFMK (Fig. 3). As it can be observed, MEL and 5-MT showed the same profile of absorbance and both had two maximum peaks of absorbance at 230 nm (absolute maximum) and 279 nm (relative maximum). AMK had a maximum peak of absorbance at 233 nm and displayed other relative maximum at 380 nm. AFMK showed absolute and relative maximum of absorbance at 233 and 337 nm respectively.



(C)

$t = -60{,}85 - 81{,}56 \cdot A + 19{,}39 \cdot B + 0{,}42 \cdot C + 2{,}78 \cdot D + 14{,}72 \cdot A \cdot B - 0{,}083 \cdot A \cdot C + 0{,}08 \cdot A \cdot D + 0{,}20 \cdot B \cdot C - 0{,}66 \cdot B \cdot D - 0{,}02 \cdot C \cdot D$

$w = 5{,}5410 - 4{,}1591 \cdot A - 0{,}3505 \cdot B + 0{,}0141 \cdot C - 0{,}0331 \cdot D + 0{,}4880 \cdot A \cdot B + 0{,}0043 \cdot A \cdot C + 0{,}0195 \cdot A \cdot D + 0{,}0050 \cdot B \cdot C - 0{,}0033 \cdot B \cdot D - 0{,}0007 \cdot C \cdot D$

$Rs1 = -17{,}95 + 7{,}21 \cdot A + 2{,}54 \cdot B + 0{,}01 \cdot C + 0{,}48 \cdot D + 1{,}26 \cdot A \cdot B - 0{,}04 \cdot A \cdot C - 0{,}20 \cdot A \cdot D + 0{,}03 \cdot B \cdot C - 0{,}08 \cdot B \cdot D - 0{,}01 \cdot C \cdot D$

$Rs2 = 6{,}075 - 1{,}939 \cdot A - 0{,}305 \cdot B + 0{,}017 \cdot C - 0{,}115 \cdot D + 0{,}400 \cdot A \cdot B - 0{,}020 \cdot A \cdot C + 0{,}025 \cdot A \cdot D - 0{,}004 \cdot B \cdot C + 0{,}005 \cdot B \cdot D + 0{,}001 \cdot C \cdot D$

$Rs3 = -9{,}26 + 4{,}99 \cdot A + 1{,}53 \cdot B + 0{,}03 \cdot C + 0{,}22 \cdot D - 0{,}10 \cdot A \cdot B - 0{,}03 \cdot A \cdot C - 0{,}04 \cdot A \cdot D + 0{,}02 \cdot B \cdot C - 0{,}04 \cdot B \cdot D - 0{,}01 \cdot C \cdot D$

(D)

$Rs = 8{,}82 - 0{,}19 \cdot A - 0{,}55 \cdot B - 0{,}58 \cdot C - 2{,}03 \cdot D + 3 \cdot 10^{-5} \cdot A \cdot B + 5{,}2 \cdot 10^{-4} \cdot A \cdot C - 0{,}02 \cdot A \cdot D + 2{,}2 \cdot 10^{-3} \cdot B \cdot C - 0{,}25 \cdot B \cdot D + 0{,}19 \cdot C \cdot D$

$tm = 29{,}67 - 0{,}42 \cdot A + 1{,}18 \cdot B - 0{,}39 \cdot C - 0{,}36 \cdot D - 0{,}01 \cdot A \cdot B + 2{,}9 \cdot 10^{-3} \cdot A \cdot C + 0{,}14 \cdot A \cdot D + 0{,}01 \cdot B \cdot C - 0{,}21 \cdot B \cdot D - 0{,}35 \cdot C \cdot D$

$w = 0{,}09 + 7{,}6 \cdot 10^{-3} \cdot A + 0{,}02 \cdot B + 0{,}01 \cdot C + 0{,}09 \cdot D + 2{,}3 \cdot 10^{-4} \cdot A \cdot B - 1 \cdot 10{-}5 \cdot A \cdot C - 3{,}0 \cdot 10^{-3} \cdot A \cdot D + 4{,}9 \cdot 10^{-4} \cdot B \cdot C - 6{,}1 \cdot 10^{-3} \cdot B \cdot D - 0{,}01 \cdot C \cdot D$

Fig. 2. Estimated surface of answer to time *vs.* pH and flow with 45% of methanol and 45 mM of acetate concentrations in HPLC (A) and Rs *vs.* SDS concentration and voltage when pH and phosphate concentration are constant, 2.5 and 20 mM respectively in MECK (B). Equations for different parameters show the influence of the variables analyzed, where *A*, *B*, *C* and *D* are methanol percentage, acetate concentration, pH and flow, respectively in HPLC (C) and phosphate concentration, SDS concentration, voltage and pH respectively in MECK (D).

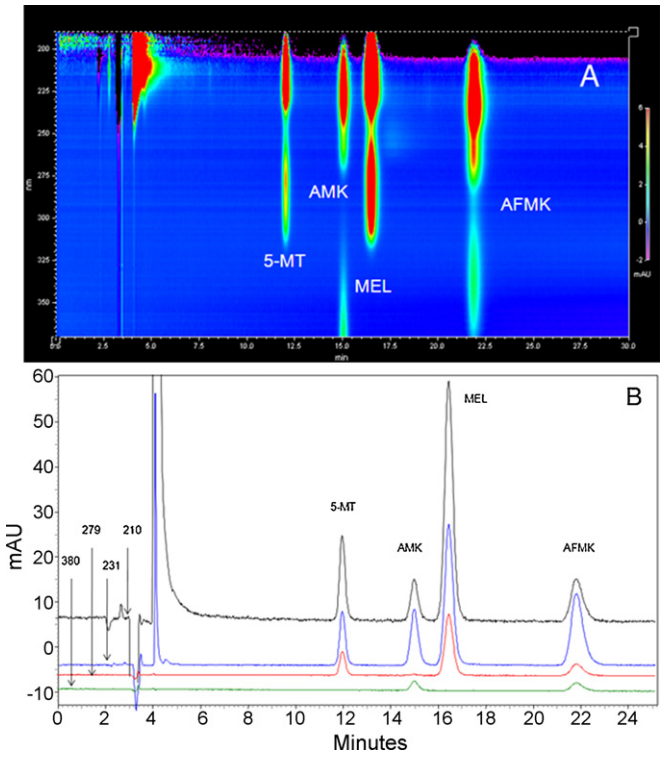


Fig. 3. Absorbance spectra at different wavelengths for 5-MT, AMK, MEL and AFMK when the analysis was performed with the optimized conditions using factorial design (A). Chromatogram at 210, 231, 279 and 380 nm of the same compounds (B).

3.3. Experiment design for MEKC analysis

Capillary dimensions, the type of running buffer surfactant and temperature were established by our previous experiences. Intervals of factors and the response variables were selected and applied from the factorial program in one block, with eight degrees of freedom. The experiment design program proposed 19 experiences with random variation of factors. Variables chosen were "resolution" for AFMK and 5-MT (Rs), "migration time" for AFMK (t) and "wide of peak" for AFMK (w). These parameters were obtained from electropherograms. Data collected from AFMK, AMK, MEL and 5-MT analysis, proposed by multivariate and predictive analysis, were used to quantify the influence of several variables and they are represented in Fig. 2.

In order to optimize the analytical conditions, we gave different impact and weights to the different parameters. Therefore, the most important parameter is time and the valor of impact and consequently weight for this parameter is maximum (5). Other important parameter is Rs, which has weight value of 3 since the resolution in all analysis is optimal. Finally, "w" impact and weight was 1. Considering all these conditions, the optimal parameters are shown in Table 2.

3.4. Final conditions of analysis by MEKC

We used the optimized variables obtained by multifactorial analysis (Table 2) using wavelengths between 199 and 300 nm. Therefore we decided to employ 215 nm of wavelength for all the compounds assayed. The electropherogram is shown in Fig. 4. As it can be observed, migration order is AMK, MEL, 5-MT and AFMK. In this electropherogram all peaks are perfectly resolved and the time of analysis is very fast when compared to HPLC analysis.

3.5. Method validation

Calibration lines were constructed by using five-point analysis, and the resulting plots followed a perfect linear regression in the concentration range from 1 mg L^{-1} at least up to 10 mg L^{-1} for MEL, AFMK and AMK. These analyses were performed in triplicates, adding 5-MT as internal standard. The calibration graphs for all the compounds displayed a good fit to a linear model regression between peak areas and analyzed concentrations. The regression coefficients were greater than 0.9992 in all cases. The linearity of the calibration graphs was also tested by mean of two different statistical tests: linearity and proportionality tests. For the former, the linearity of the method was confirmed showing that the slope of RSD values were lower than 2%, and the values obtained from the Fisher's test (Analysis of Variance, ANOVA) were always lower than tabulated values ($\alpha = 0.05$) (Table 3).

Proportionality test, demonstrated that interception was not statistically different from 0 and the Student's t-test values calculated were always lower than the tabulated values for the same level of significance. This indicates the absence of systematic error, being linearity therefore demonstrated. The precision of the method was assessed by expressing the relative standard deviation of several repeated measurements. Instrumental repeatability was estimated from six replicates at three concentrations (low, medium and high level) within the lineal interval. Values obtained ranged between 0.17% and 2.9% and always below the acceptance criteria ($\leq$3%). The estimation of repeatability was performed over a period of 10 h. The compounds were stable and showed no significant differences in the peak area after this time. Intermediate precision was determined by comparing the results obtained from the analysis of freshly prepared samples on two separate days. The results, ranging between 0.2% and 4.3%, were also lower than the acceptance criteria ($\leq$5%). Therefore, acceptable precision was obtained for all preparations.

Recovery experiments for all substances were performed in order to study the accuracy of the method. A mixture of known

Table 3
Values obtained of several parameters in the methods to analyze MEL, AFMK and AMK by HPLC or MEKC.

	HPLC/MEKC			
Range (mg L^{-1})	Specification	MEL	AFMK	AMK
Correlation coefficient	$\geq$0.997	0.9998/0.9996	0.9997/0.9996	0.9997/0.9992
Linearity test				
Slope RSD	$\leq$2%	1.00/1.30	1.37/1.29	1.36/1.30
ANOVA	$F_{exp} < F_{tab}$[a]	0.019/2.789	0.026/1.456	0.030/2.052
Instrumental repeatability (RSD%)	$\leq$2%	0.8–1.4/0.4–1.6	0.3–0.6/0.5–2.7	0.2–0.9/1.0–1.9
Intermediate precision (RSD%)	$\leq$5%	3.1–3.8/0.7–3.0	0.2–4.3/0.2–4.9	1.1–1.8/0.3–3.0
LOD (ng mL^{-1})[b]	–	44/13	18/37	23/47
LOQ (ng mL^{-1})[b]	–	94/44	38/124	51/156

[a] F_{tab} = 3.025 to MECK method and F_{tab} = 3.708 to HPLC method.
[b] LOD = limit of detection; LOQ = limit of quantification.

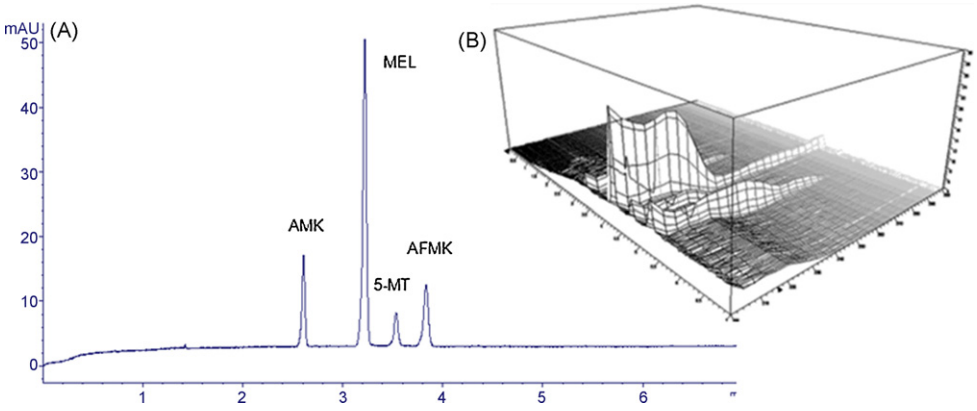


Fig. 4. Electroferogram at 215 nm for the same compounds (A). Absorbance at different wavelengths (200–300 nm) for AMK, MEL, 5-MT and AFMK when analysis was performed with optimized conditions after using factorial design (B).

concentrations of each solute was prepared and analyzed by this method at low, medium and high calibration ranges on the same day. All analyses were carried out in triplicates. The average recoveries obtained, which ranged between 99.6% and 103.7%, also testify the accuracy of method.

The detection and quantification limits for all substances assayed are shown in Table 4. These limits were determined by 10 repeated of measurements blank, followed by the preparation of calibration plots (peak height *vs.* concentration) from 1 to 10 mg L^{-1} of each component. The limits of detection/quantification for MEL, AFMK and AMK were respectively 44/94, 18/38 and 23/51 ng mL^{-1} using HPLC and 13/44, 37/124 and 47/156 ng mL^{-1} using MEKC.

3.6. Applications

3.6.1. Analysis of other metabolite than melatonin: cyclic 3-hydroxymelatonin

In addition to AFMK or AMK, other important melatonin metabolite is cyclic 3-hydroxymelatonin (3COHM). It was first isolated and structurally identified as an oxidative melatonin metabolite after its reaction with •OH [51] and the chemical structure is depicted in Fig. 1.

We used our method to analyze this compound. By using HPLC, 3COHM has a retention time of 8.1 min and at this time there are no other peaks in the chromatogram. It is noteworthy to mention that is possible to analyze the content of 3COHM in biological samples using these methods without changing any condition. In Fig. 5, we show the chromatogram of these compounds and a 4 min peak corresponds to solvent of stock solutions of standards, dimethyl sulfoxide. In this chromatogram it can be observed which good resolutions between all compounds.

3.6.2. Comparison between HPLC and MEKC

To evaluate both methods, HPLC and MEKC, we analyzed 5 samples with a concentration of 5 μg mL^{-1} of MEL, AFMK, AMK and 3COHM and 2 μg mL^{-1} of 5-MT. As it can be observed in Table 4 we show that HPLC method would be the method of choice for more accurate measurement. We used an independent *t*-test to compare the determined average samples using the two different assays. The test showed that concentration of MEL, AFMK and AMK assayed by both techniques were statistically similar. Additionally, results from replicates indicate that our methods are simple and adequate to analyze these compounds. As showed for 3COHM, the other melatonin metabolite analyzed, it has a statistically difference ($p < 0.001$). Probably this is because the 3COHM analyzed by MEKC is difficult to carry out and the peak in both methods is wide. Also, these methods are yet no validated for 3COHM assay.

Table 4
Analysis of 5 μg mL^{-1} of MEL, AFMK, AMK and 3COHM using HPLC and MECK methods. Five samples were analyzed and value of *t*-test to compare both techniques was calculated.

	MEL	AMK	AFMK	3COHM
HPLC (μg mL^{-1})	5.01 ± 0.04	4.99 ± 0.04	4.96 ± 0.03	4.93 ± 0.09
MECK (μg mL^{-1})	5.08 ± 0.08	4.98 ± 0.38	4.96 ± 0.13	4.70 ± 0.03
p-Value	0.118	0.955	1.000	<0.001

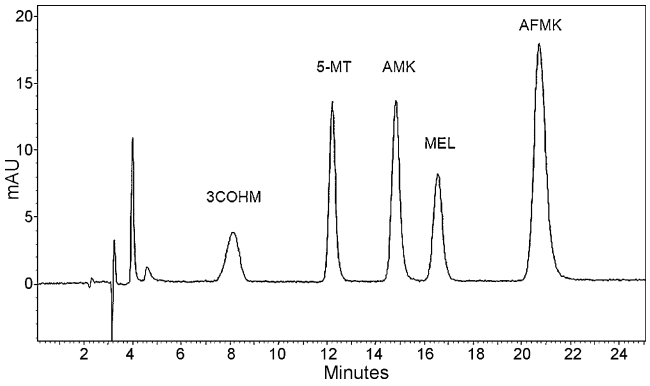


Fig. 5. Analysis of 5 μg mL^{-1} of MEL, AFMK, AMK, 5-MT and 3COHM by HPLC.

4. Conclusions

In this paper we demonstrate that experimental design program is an excellent tool to create analytical methods. We create two methods in order to analyze the neuroindole MEL and kynurenines AFMK and AMK by MEKC and HPLC, which can be applied for routine analysis in biological samples. Reproducibility and low cost of single analysis are central features for routine laboratory, and these methods are cheap and easily affordable by laboratories equipped with standard HPLC and CE systems. With both methods, researches may find a very useful tool to answer important questions regarding MEL, AFMK or AMK functions in biological samples. Furthermore with these methods we can analyze other metabolites of melatonin such as 3COHM.

Comparison between HPLC and MEKC analysis for melatonin and its metabolites, demonstrated that CE techniques could be considered a useful tool, principally by the speed of analysis and few solvent consumption. Whereas HPLC is the most sensitive technique to analyze these compounds, mainly by UV detector, in the case of CE it has a small optical pass and this might be a problem when samples are present at small concentrations due to low lim-

its of detection and/or quantification. For this reason according to the results shown here we propose the employment of MEKC when the concentration of compounds is not a problem. However when concentration is a limiting factor, HPLC should be used instead.

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