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Stability-indicating MEKC method for determination of milnacipran in capsules

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ABSTRACT

A stability-indicating method by micellar electrokinetic chromatography (MEKC) was developed and validated for determination of milnacipran (MNC) hydrochloride in capsules. The MEKC separation was performed in a fused-silica capillary (40 cm x 50 µm i.d.), 30 kV voltage applied at 30 °C, using as background electrolyte solution 20 mM borate buffer (pH 8.8), 20 mM sodium dodecyl sulfate and detection at 210 nm with a PDA detector. The parameters specificity, linearity (range 20-60 µg.mL^{-1}), precision, accuracy, and robustness were evaluated. The validation data showed that MEKC method is reproducible, providing an accurate (100.3 % to 101.2 %) and precise (RSD = 1.2 %) to quantify MNC hydrochloride in capsule. The results of accuracy and precision tests were compared to previously validated methods, second order derivative UV spectroscopic (UV-D^2) and liquid chromatography (LC), by ANOVA and Tukey. The results of accuracy showed non-significant difference between the three methods. For precision, the statistical analysis between MEKC method and LC method showed no significant difference. However, the comparison between MEKC method and UV-D^2 method showed significant difference.

Key words: Milnacipran, MEKC, LC, UV-D^2.

INTRODUCTION

Depression is characterized by the presence of two core symptoms, depressed mood and anhedonia (decreased pleasure or interest), and this disorder affects about 121 million people worldwide [1,2]. Decreased concentration, mental and physical slowing, loss of energy, lassitude, tiredness, and reduced self-care are all symptoms related to reduced noradrenergic activity. Depressed mood; loss of interest or pleasure; sleep disturbances; and feelings of worthlessness, pessimism, and anxiety are related to reduce activity of both serotonergic and noradrenergic neurotransmission [3].

MNC is a selective serotonin reuptake inhibitor (SSRI) and noradrenaline reuptake inhibitor (SNRI) with a (1R,2S)-2-(aminomethyl)-N,N-diethyl-1-phenylcyclopropane-1-carboxamide chemical structure [4] (Figure 1). MNC is an antidepressant drug and it is available in some European Countries and in Japan for the treatment of depression. It was approved by the Food and Drug Administration (FDA) for the management of fibromyalgia syndrome, characterized by widespread pain condition associated with fatigue, cognitive dysfunction, sleep disturbance, depression, anxiety, stiffness and decreased physical function [5,6].

LC methods were developed for the determination of MNC in bulk drug [7], pharmaceutical formulations [5,7-9] and MNC combined with other antidepressants in human plasma [10-14]. An UV-D^2 method was developed to assay this drug in pharmaceutical formulation [5]. A gas chromatography-mass spectrometry (GC-MS) method was used for quantitative analysis of MNC in human plasma [4] and it is combination with others antidepressant drugs [10]. A

MEKC method was developed for separation MNC and other antidepressants with their metabolites in blood and urine [15]. Simultaneous determination of MNC and some antidepressants, in plasma, was carried out by non-aqueous capillary electrophoresis-time of flight mass spectrometry [16].

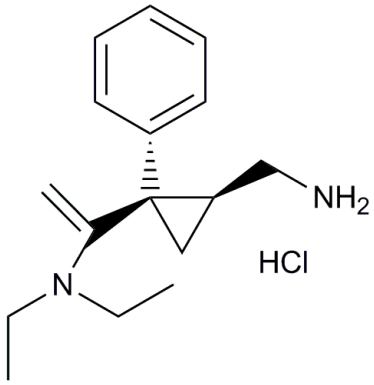

Figure 1: Chemical structure of MNC hydrochloride

MEKC has proved to be a method that can compete with LC with regard to efficiency and selectivity adjustment. It is a more scattered mode of electrokinetic chromatography in which the separation electrolyte contains the background electrolyte (BGE), mostly a buffer system and a surfactant dissolved in water or an aqueous and organic solvent mixture in a concentration higher than critical micellar concentration (CMC) [17,18].

Presently, there is no published MEKC method for the quantitative analysis of the MNC hydrochloride in pharmaceutical formulation. In this context, the objective of this work was to develop and validate a stability-indicating method using MEKC for the determination of this drug in capsules, according to the current International Conference on Harmonization (ICH) guidelines [19-20] performing a comparison with the previously validated LC and UV-D^2 methods developed, thereby contributing to the development of new alternatives techniques for the quality control of MNC.

EXPERIMENTAL SECTION

Chemical and Reagents

The MNC hydrochloride (assigned purity, 99.7%) used as reference substance (RS) was purchased from Synfine Research (Canada), salicylic acid (SA) RS (assigned purity, 99.9%), used as internal standard (IS), was purchased from United States Pharmacopeia (USP, Rockville, USA) and Dalcipran® (Pierre Fabre Médicament, Boulogne, França), containing 50 mg of MNC per dose was obtained from commercial sources within their shelf life period. The excipients contained in the dosage form are dibasic calcium phosphate, povidone, carboxymethylcellulose calcium, colloidal silicon dioxide, magnesium stearate and talc.

Both sodium dodecyl sulfate (SDS) and boric acid were acquired from Synth (São Paulo, Brazil). For all of the analyses and sample preparation, ultrapure water was used (Milli-Q Plus®, Millipore, Bedford, USA). All other reagents and chemicals used were of pharmaceutical or special analytical grade.

Apparatus

The CE instrument used was HP3D CE instrument Agilent Technologies (Santa Clara, USA) equipped with an autosampler, a photodiode array (PDA) detector, a temperature controlling system (4-60 °C), and power supply able to deliver up to 30 kV. The DAD was set at 210 nm. The capillary temperature was maintained constant at 30 °C. All experiments were carried out applying positive mode. CE ChemStation software (version A 09.01) was used for instrumentation control, data acquisition, and analysis. The separation was carried out using a conventional fused-silica capillary (40 cm x 50 μm i.d.) Agilent Technologies (Santa Clara, USA). The solvents were filtered in a 0.45 μm membrane filter Millipore (Belford, USA) and degassed daily.

The LC system consisted of a Shimadzu (Kyoto, Japan) LC-10ADVP liquid chromatography, SPD-M10AVP diode array detector, Rheodyne® manual injection, DGU-14A degasser, SCL-10AVP system controller and Class-VP chromatography data system; a reversed phase column Nucleosil C8 endcapped (150 × 4.6 mm, 100 Å, 5μm) from Macherey-Nagel (MN) and a guard column Phenomenex were used in the study.

The mobile phase contained a mixture of acetonitrile, water and triethylamine (70:30:0.085, v/v/v), at a constant flow-rate of 1.2 mL.min^{-1} and at room temperature (20 ± 2 °C). The pH value of the aqueous phase was adjusted to 7.5 with phosphoric acid. Aliquots of 20 µL were injected. The detector was operated at a wavelength of 210 nm.

A double-beam UV-visible spectrophotometer (SHIMADZU, Japan) Model UV-1601 PC was used. The software employed was UVPC personal spectroscopy software, version 3.9. For all the tested solutions the second derivative spectra (D^2) was recorded over the range 280-250 nm against solvent in a 1-cm quartz cells, fixing $\Delta\lambda$ at 4 nm and scaling factor at 200. The amplitude values of D^2 were measured at 268.5 nm for MNC hydrochloride, zero crossing of inactive ingredients.

Photodegradation studied was carried out in a photostability UV chamber (1.0 x 0.17 x 0.17 m) with mirrors and equipped with an UV-C lamp (Orion®, 254 nm, 30 W, 130 V) and UV cuvettes (Ultra Vette®, São Paulo, Brazil) were used as a container for samples. The Ultrabasic potentiometer (Denver Instrument, São Paulo, Brazil), was used to determine the pH of all solutions.

Electrophoretic Procedure

At the beginning of each working day, the capillary was conditioned by rinsing with 0.1 M NaOH for 30 minutes, water for 15 minutes, and finally with the buffer solution for 15 minutes. Between injections, the capillary was conditioned with 0.1 M NaOH (3 minutes), water (1 minutes), and a running BGE solution (3 minutes). Samples and standards were injected using the hydrodynamic injection for 5 s at 50 mbar and a constant voltage of 30 kV was applied during the analysis. Since electrolysis can change the electroosmotic flow (EOF) and affect the migration time, efficiency and selectivity, after each three injections the running electrolyte solution was replaced by a fresh solution.

Preparation of Standard Solutions and Pharmaceutical Samples

The stock solution of MNC hydrochloride RS was prepared to have a final concentration of 1000 µg.mL^{-1}. All solutions were prepared each day. The average mass of twenty units of pharmaceutical dosage form was calculated. An amount equivalent to 10 mg of MNC hydrochloride was transferred into a 10 mL volumetric flask. After adding 5 mL of water, the solution was sonicated for about 5 minutes, the samples were made up to volume with the same solvent, and filtered. An aliquot of the filtrate was diluted to yield a final concentration of 50 µg.mL^{-1}. The solutions were filtered through a 0.22 µm membrane filter (Millipore®). For all quantitative determination, a constant amount of SA IS (20 µg.mL^{-1}), previously solubilized in methanol was added to the drug solution.

Preparation of BGE Solution

The optimized BGE solution used in this analysis was prepared by separately weighing boric acid and SDS quantities, respectively equivalent to 20 mM. The pH was adjusted to 8.8 adding 0.1 M NaOH, and the volume completed to 50 mL with water. The solution was daily prepared and filtered. The sample dilutions were made with boric acid pH 8.0.

Method Validation

Validation to demonstrate the suitability of this method for pharmaceutical quality control was conducted according to the ICH guidelines [19-20] following the parameters: specificity, linearity, precision, accuracy, and robustness.

Specificity and Forced Degradation Studies

Electrophoretic runs of a placebo solution and forced degradation studies were performed to evaluate the specificity of the method. The placebo solution was prepared as described in the *Preparation of Standard Solutions and Pharmaceutical Samples* section, using an amount of excipients without MNC RS. Their concentration in these formulations was based on the literature [21] and calculated in relation of the median mass of the pharmaceutical form.

Forced degradations were performed to provide stability-indicating properties of the analytical method. A MNC RS solution (500 µg.mL^{-1}) was subjected to accelerated degradation by oxidative and photolytic conditions to evaluate the interference in the quantitation of the drug. Oxidative degradation was induced by treating the reference substance solution with 3.0 % H_2O_2 at room temperature for 24 h, protected from light. Photodegradation was induced by exposing the reference substance sample solution to UV-C radiation (254 nm) for 2 h, at room temperature. The samples were analyzed against a freshly prepared control sample (without degradation treatment).

Following the degradation period, all samples were prepared for analysis as previously described and the specificity of the method was established by determining the peak purity of MNC in the degraded samples using a PDA detector.

Linearity and limits of detection (LOD) and quantitation (LOQ)
Linearity was evaluated by constructing three calibration curves each one with 5 concentration levels of MNC RS (20-60 μg.mL^{-1}) on three different days. Five replicate injections of each reference substance solution spiked with SA IS, were made to verify the repeatability of the detector response. The peak area ratio of MNC RS to the IS, against the respective reference concentrations was used for plotting the graph, and the linearity evaluated by the least-square regression parameters (correlation coefficient, slope, intercept). The LOD and LOQ values were mathematically determined through calibration curves. The aforementioned factors (3.3 and 10) were multiplied by the ratio of the residual standard deviation and the slope, according to guidelines [20].

Precision
The precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) studies and was expressed based on the percent relative standard deviation (RSD %) of peak area measurements. To perform the repeatability studies, six samples containing 50 μg.mL^{-1} of MNC and 20 μg.mL^{-1} of SA, injected in duplicate, in the same day. Intermediate precision was tested of repeating the same procedure in two different days (n = 12) and comparing the results between them.

Accuracy
The accuracy of the method was evaluated by adding a known amount of MNC RS at three concentrations (10, 20, 30 μg.mL^{-1}) corresponding to 75, 100 and 125 % of the nominal analytical concentration (50 μg.mL^{-1}), and a fixed aliquot of a SA IS. Each solution was prepared in triplicate and injected 3 times. The concentrations and recoveries were calculated against the added concentration.

Robustness
Robustness of the proposed method was examined by evaluating the influence of small variations of the most important procedure variables such as buffer concentration (19.5 and 20.5 mM), SDS concentration (19.5 and 20.5 mM), BGE pH solution (8.6), voltage (28 KV), and temperature system (27 °C). Analyses were carried out with MNC RS and SA IS solutions at 50 μg.mL^{-1} and 20 μg.mL^{-1}, respectively, in triplicate. Only one parameter in the experiments was changed at a time, and the effects were studied based on RSD (%) values obtained among the parameters analyzed.

RESULTS AND DISCUSSION

The conditions established for the MEKC method for determination of MNC in capsules were chosen based on previously work published by Labat *et al* [15]. In this article the author developed a MEKC method for analysis of MNC and other antidepressants with their metabolites in blood and urine using a BGE solution of 20 mM sodium borate, pH 8.5, with 20 mM SDS, 15 % isopropanol, at an operating voltage of 25 kV, and capillary temperature 40 ºC with diode array detection. Thus, we decided to test these conditions with some modifications and without use of organic solvent in the BGE to determinate MNC in capsules.

During the developed method, the pH of the BGE exhibited a significant impact on the ionization of the silanol group of the capillary wall and on the electrophoretic mobility of the compounds. The effect of the pH was studied in the range from 7 to 10. The change of pH showed that it interferes on the peak resolution and on the migration time of MNC. With the increase of the pH the migration time reduced, however after pH 9.0 the resolution of the peaks decreased. Under these conditions, one that presented the best migration time and peak resolution was the one with pH 8.8.

The capillary temperature is important in order to avoid unwanted changes in EOF, efficiency, electrophoretic mobility and migration time. A temperature of 30 °C was chosen due to the short run time, peak symmetry and acceptable current. The effect of the voltage applied on the separation was studied through changes from 25 to 30 kV. Raising the voltage led to shorter analysis times (MNC RS: 3.5 minutes, SA IS: 2.4 minutes) and with an acceptable current. The use of 20 mM borate buffer and 20 mM SDS with pH value around 8.8 (at a temperature of 30 ºC, 30 kV) resulted in high sensitivity and good peak symmetry of MNC.

A stability-indicating method is defined as an analytical technique that accurately quantifies the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities [22].

Specificity of the method evaluated and shows that the degradation products of MNC do not interfere on the measurement of the samples. MNC was submitted under oxidative and photolytic stress conditions. Other conditions were not tested because as it was already being described MNC is stable to temperature, acid hydrolysis and radiation UV 352 [5].

Runs of placebo solution were also performed and no interference with the elution of MNC was found (Figure 2). Figure 3A shows that oxidative condition resulted in a decrease area with additional peak at 1.4 minute. The photolytic condition stress exhibited decrease of the area, and two additional peaks were detected at 1.3 and 2.1 minutes (Figure 3B). The MNC peak purity was analyzed with PDA detector, demonstrating that the proposed method is specific for the analysis.

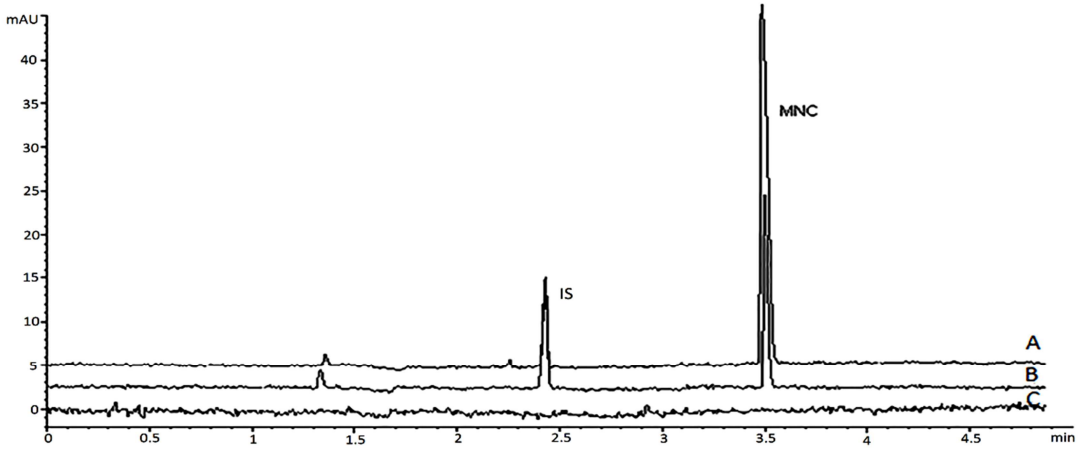

Figure 2: MEKC eletropherogram: (A) MNC hydrochloride RS; (B) MNC capsules; (C) Placebo solution

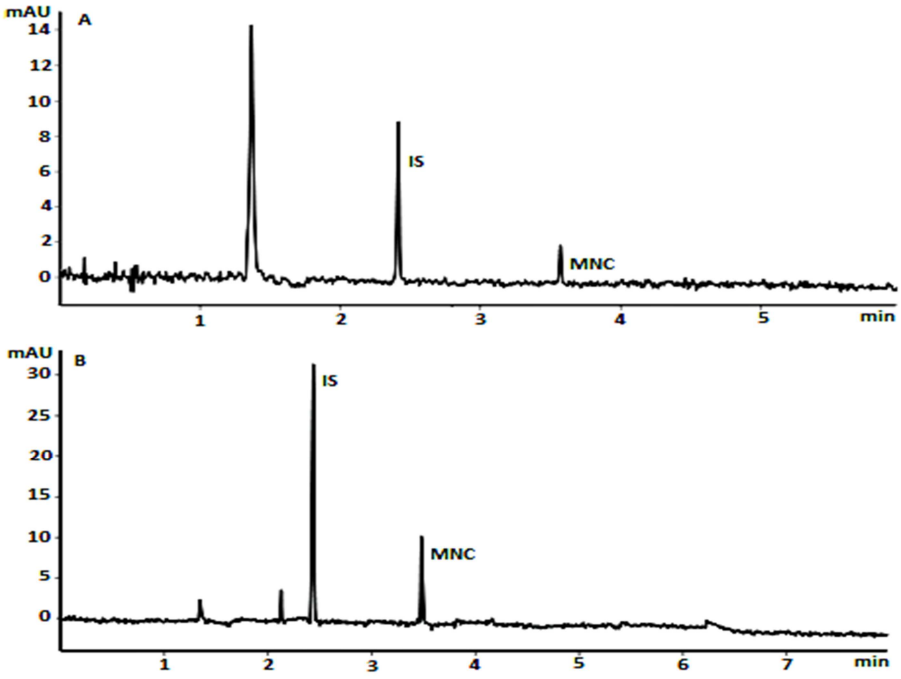

Figure 3: MEKC eletropherogram: (A) Oxidation; (B) Photolysis. MEKC conditions: electrolyte solution 20 mM borate buffer (pH 8.8), 20 mM SDS, and detection at 210 nm

Linearity was evaluated in concentration range 20-60 μg.mL^{-1} for MNC RS and 20 μg.mL^{-1} of SA IS was added as IS in all cases. The calibration curves constructed for MNC were found to be linear and the equation obtained was y = 0.0200 x + 0.1370, where y is the peak area ratio of MNC to IS, and x is the concentration of MNC in μg.mL^{-1}. The correlation coefficient was 0.9991. The validity of the assay was verified by means of analysis of variance (ANOVA), which demonstrated that the regression equation was linear ($F_{calculated} = 5.5.10^{2} > F_{critical} = 4.6$; $\alpha = 0.05$) with no deviation from linearity ($F_{calculated} = 3.3.10^{-1} < F_{critical} = 3.7$; $\alpha = 0.05$). The LOD and LOQ were estimated to be 4.2 and 12.9 μg.mL^{-1}, respectively, indicating suitable sensitivity of the method.

The repeatability of the method was determinate by calculating the RSD for six determinations of MNC, performed on the same day and under the same experimental conditions. The inter-day precision was assessed by analyzing six samples on two different days. These results are given in Table 1. The low RSD (%) obtained for the intra-day (<

2.0 %) and inter-day precision (1.2 %) for capsules, confirmed good precision of the MEKC method.

Table 1: Method repeatability/intermediate precision for MNC hydrochloride

% Label claim (50 µg mL^{-1})							Mean (%)	RSD[a](%)
Day I	102.4	101.3	103.1	101.5	105.4	103.7	102.9	1.5
Day II	103.5	101.2	101.3	101.4	102.2	101.5	101.9	0.8
Mean inter-assay (%) (*n* = 12)							102.4	
RSD (%)							1.2	

[a]*RSD: Standard Relative Deviation.*

The accuracy was calculated as the percentage of recovery by the assay of known added amounts of MNC RS in sample solutions using three concentration levels. The accuracy of the method ranged from 100.3 to 101.2 %, with RSD lower than 2.0 %, indicating that the assay is accurate (Table 2).

Table 2: Results for the recovery test for MNC hydrochloride RS

Concentration (%)	Amount added concentration (µg.mL^{-1})	Amount found concentration (µg.mL^{1})	Recovery (%)
75	10.0	10.0	100.3
100	20.0	20.2	101.2
125	30.0	30.3	101.1
Mean recovery (%)	100.9		
RSD[a] (%)	1.1		

[a]*RSD: Standard Relative Deviation.*

The RSD (%) of peak area ratio between MNC and SA was calculated for each parameter proposed (buffer, SDS and BGE concentrations, pH and temperature). The results of variables over the experimental range evaluated were within the acceptable deviation (RSD < 2.0 %). There were non-significant changes in migration time and peak area ratios, as well as in the electropherogram pattern compared to the optimized conditions, indicating that the proposed method is robust under the conditions tested. The system suitability was also tested during the analysis of these conditions and the parameters of tailing factor (≤ 2.0), theoretical plates (≥ 2000), resolution (≥ 2.0), and capacity factor (≥ 2.0) were within the specified limits.

The validated MEKC method was applied for the determination of MNC in capsules and the results obtained with the accuracy and precision tests were compared to those obtained using a previously validated UV-D^2 and LC methods [5].

The results of accuracy test obtained from MEKC method were compared statistically with the UV-D^2 method and LC method by ANOVA, using *F*-test, and showed non-significant difference between the methods. However when the results of precision test obtained from MEKC method were compared statistically with the UV-D^2 method and LC method by ANOVA, the experimental values obtained reveals significant difference between the methods (Table 3). Hence, became necessary to define which methods are statistically different.

Table 3: ANOVA for precision on determination of MNC hydrochloride in capsules by MEKC, UV-D^2 and LC method

Variation source	DF[a]	Sum of squares	Variance	$F_{calculated}$	$F_{critical}$ (5%)	$F_{critical}$ (1%)
Between	5	10.14	5.07	5.30*	3.29	5.31
Inside	33	31.58	0.96	0.80		
Total	35	41.72	1.19			

[a]*DF: Degree Freedom. * F for the 0.05 significance level.*

The Tukey test was applied and did not reveal discrepancy between the experimental values obtained by UV-D^2 method and LC method, and between MEKC method and LC method. However, when UV-D^2 method and MEKC method were statistically compared, the results obtained showed significant difference between the results (Table 4). But both methods, UV-D^2 and MEKC, showed to be equivalent to LC being the best method to compare the results.

Table 4: Comparison between the MEKC, UV-D^2 and LC method by Tuckey test

Method	Mean (RSD[a] %) Accuracy (*n*=9)	Mean	Mean (RSD[a] %) Precision (*n*=12)	Mean
UV-D^2	100.1 (1.1)	A	101.1 (0.7)	A
LC	99.7 (1.0)	A	99.7 (1.0)	A B
MEKC	99.7 (1.0)	A	102.4 (1.2)	B

[a]*RSD: Standard Relative Deviation.*

CONCLUSION

The method developed by MEKC has been validated under established conditions, proving to be linear, specific, precise and accurate for the quantitative analysis of MNC in capsules. The comparative analysis between the proposed methods LC/UV-D^2 and LC/MEKC showed no statistically significant difference between them, indicating their equivalence for the quantitative determination of MNC the capsules. The MNC in pharmaceutical formulation showed to be more sensitive to oxidation condition, with appearance of a peak corresponding to a major degradation product.

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