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Micellar electrokinetic capillary chromatography for the determination of fluoxetine and its metabolite norfluoxetine in biological fluids ☆

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Abstract

A micellar electrokinetic capillary chromatography (MEKC) for determining fluoxetine and its metabolite (norfluoxetine) is proposed. Optimal conditions for the quantitative separation were investigated. A background electrolyte solution consisting of 5 m*M* phosphate buffer adjusted to pH 12.3 and 40 m*M* of 1-decanesulfonic acid sodium salt (DSS), hydrodynamic injection and 25 kV of separation voltage were used. Good linearity and precision were obtained for both compounds. Detection limits of 0.2 mg/l for fluoxetine and norfluoxetine were obtained. The developed method is rapid and it has been applied to determine fluoxetine and its metabolite in human serum and urine. The samples were purified and enriched by means of extraction–preconcentration step with a preconditioned C_{18} cartridge and eluting the compounds with methanol.

Keywords: Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine has been shown to be a specific serotonin (5-HT) reuptake inhibitor in man [1,2] and animals [3,4]. The serotonergic system has been implicated in the physiopathology of a variety of diseases, including depression [5], obesity and alcoholism [6]. Moreover, serotonin is important in modulating other neurophysiologic systems, including gastrointestinal function, analgesia, control of blood vessel tone and hemostasis. Thus, fluoxetine is now one of the most frequently prescribed antidepressants drugs [7]. Although the pharmacology of fluoxetine has been extensively studied and it is known to be metabolised to the selective 5-HT uptake inhibitor *N*-desmethylfluoxetine (norfluoxetine), much is still unknown about the metabolites and elimination of fluoxetine and its metabolites [8].

The degree of serotonin reuptake inhibition is correlated with the fluoxetine plasma concentration. Fluoxetine is well absorbed after oral administration and disappears from plasma with a half-time of 1–3 days; its metabolite norfluoxetine has a plasma half-time of 7–15 days. After administration of fluox-

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etine, approximately 65% of the administered dose of this drug is recovered in urine and about 15% in feces [8]. The therapeutic dosage for fluoxetine is 20 mg/day which is metabolised in the liver to norfluoxetine and other unidentified metabolites. Overdoses of fluoxetine have been reported to cause death. The plasma concentrations of the drug in these fatalities are 1.93–4.57 μg/ml [9].

Several methods for the determination of fluoxetine and norfluoxetine in biological samples have been published. Mostly based on liquid chromatography (LC) with ultraviolet [10–12] or fluorescence detection [13,14], gas chromatography–electron capture detection (GC–ECD) [15,16] or GC–mass spectrometry (MS) detection [17]. Others woks include the quantification of enantiomeric forms of both fluoxetine and norfluoxetine by LC [18] and GC–ECD [15] techniques. Fluoxetine together with other serotonin reuptake inhibitor (fluvoxamine) have been determined in pharmaceutical formulation by capillary zone electrophoresis [19]. But, only a capillary electrophoresis method has been published for the stereoselective determination of fluoxetine and norfluoxetine in plasma and serum using cyclodextrin-modified sodium phosphate buffer at pH 2.5 [20].

In this work, we propose an easy and fast method for micellar electrokinetic capillary chromatography (MEKC) to determine fluoxetine and norfluoxetine in serum and urine. We used, prior to the electrophoretic separation a previous extraction and preconcentration process on a C_{18} cartridge. The proposed method is simple, fast and with a wide scope because of the possibility to establish a general method for the fluoxetine and norfluoxetine analysis in different kind of biological samples.

2. Experimental

2.1. Reagents

The organic solvents were LC grade. Milli-Q water was used throughout the study.

Fluoxetine clorhidrate was purchased from Tocris Coolson Ltd. and distributed by Biogen Científica S.L. Norfluoxetine hydrochloride was purchased from Sigma-RBI.

Standard solution (200 mg/l) were prepared in water and stored in the refrigerator at 4 °C. Working standard solutions were daily prepared by diluting the stock standard solution containing also 25 m*M* phosphate buffer (pH=12.3) and 50% of methanol.

A 5 m*M* phosphate buffer (pH 12.3) and 40 m*M* 1-decanesulfonic acid sodium salt (DSS) solution were used as separation electrolyte and were daily prepared.

2.2. Apparatus

A Beckman System 5510 capillary electrophoresis equipped with a diode-array UV/Vis detector (DAD) and controlled by a Dell DIMENSION P133 V with P/ACE station software was used. The separation capillary was made from fused-silica a 57 cm×75 μm I.D. (50 cm to detector) maintained in a cartridge with a detection window of 100×800 μm.

The extraction and preconcentration process was achieved with a home-made device composed by a Waters manifold Millipore Vacuum sep-pack system coupled with a Gilson Minipuls 3 automatic pump.

Centrifugation of blood and urine was carried out by means of Roto-Silenta Hettich apparatus.

2.3. Biological samples treatment

Both fresh human blood and urine samples were obtained from three different volunteers.

Blood samples were collected in evacuated tubes (9.5 ml UT-109SAS, Venoject, Leuven, Belgium) containing a gel+silicone coated Z. The tubes were centrifuged (5000 rev./min, 15 min, 20 °C) and serum was transferred to 1.5-ml polypropylene tubes (Eppendorf, Hamburg, Germany) where it was kept frozen at −18 °C and defrosted just before the extraction and preconcentration process.

Fresh urine samples were directly submitted to the solid-phase extraction process after a preliminary centrifugation step (5000 rev./min, 15 min, 20 °C).

2.4. Extraction and preconcentration procedure in biological samples

The extraction of fluoxetine and norfluoxetine from the biological samples was performed in a reversed-phase cartridge C_{18} (Waters Sep-Pak Plus,

Milford, MA, USA). The cartridge was conditioned before use by means of 5 ml of methanol followed by 5 ml of 10 m*M* phosphate buffer solution (pH= 7.0).

Then, variable volumes of the biological samples were slowly loaded to the conditioned cartridge. After, the cartridge was washed with 8 ml of 10 m*M* phosphate buffer (pH=7.0) and 2 ml of a 30% methanol–water solution. Finally fluoxetine and norfluoxetine were eluted with 1.0 ml of methanol, this extract was diluted with 0.5 ml of 100 m*M* phosphate buffer (pH=12.3) and 0.5 ml of water. This sample was immediately injected into the capillary electrophoretic equipment.

2.5. Operating conditions

The capillary was conditioned before its first use by flushing with 0.1 *M* NaOH for 15 min, then with water for 5 min and finally with the electrolyte solution for 5 min. By means of preliminary experiments, we decide to rinse the capillary with the separation buffer for 2 min between sample injections.

The samples were injected by hydrodynamic injection for 16 s. The electrolyte and operating potential were varied according to the designed experiments. Separation was carried out at 25 °C. Electropherograms were recorded at 230 nm. Different sets of electrolytes were used for rinsing and separating operations in order to keep constant the electrolyte level on the anodic side.The set of separation vials was changed every six runs. Duplicate injections of the solutions were performed and average peak areas were used for the quantification.

3. Results and discussion

3.1. Optimization of the separation conditions

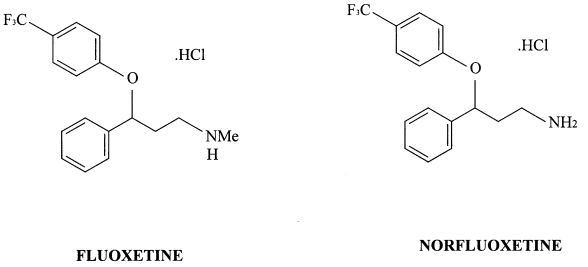
3.1.1. CZE: preliminary experiments

3.1.1.1. Effect of electrolyte pH. The pH of the running electrolyte had a significant impact on the ionisation of the acidic silanols of the capillary wall and on the electrophoretic mobilities of the compounds studied. Taking into account the structure of the basic analytes or acidic buffers could be used to promote their ionisation. In this way, some experiments were carried out in order to evaluate the influence of pH over the separation of fluoxetine and norfluoxetine. A pH range of 2.0–12.5 was tested with this object. Between 2 and 9 pH units, fluoxetine and norfluoxetine exhibited a cationic form due to the protonation of the amino groups of both molecules (Fig. 1), for this reason they showed lower migration times than the electrosmotic flow (EOF) and also very similar electrophoretic mobilities being not separated. In the pH range between 9 and 11.5, fluoxetine and norfluoxetine migrated with the same speed as the EOF as results of a non-ionic form. For pH higher than 11.5, the migration times of the two compounds are higher then the EOF as corresponded to the anionic form of the two drugs but, a very poor resolution between peak was observed. For these reason we tried this determination by MEKC.

3.1.2. MEKC: preliminary experiments

Several micellar additives including sodium dodecylsulfate (SDS) were tested. The best results were obtained when 1-decanesulfonic acid sodium salt (DSS) was used as micellar additive to the electrolyte and this compounds was selected as optimal micellar additive. The study of the influence of the additives was achieved in methanol–water (50%) solution of fluoxetine and its metabolite and also in the extract from the human serum and urine (spiked with fluoxetine and norfluoxetine) with the object to get a good method that discriminate both drugs in the fluids biological samples.

A phosphate buffer (Na_2HPO_4/Na_3PO_4) at pH 12.3 was chosen in our study for the following reasons: (a) at this pH it has a high buffer capacity



FLUOXETINE **NORFLUOXETINE**

Fig. 1. Structures of fluoxetine and norfluoxetine.

and (b) the two compounds showed only negative charges with a good resolution between the two peaks (both fluoxetine and norfluoxetine have basic functional groups). For pH values less than 9.0 the ion-pair interaction between the two compounds (positive) and the DSS anionic micelles produces a big interaction inside the capillary with longer migration times and poor resolution.

For these reasons, a pH 12.3 was selected as optimum in order to minimize analysis times with good resolution between peaks.

3.1.2.1. Effect of ionic strength of electrolyte. The study of this effect was performed on human serum and urine extracts spiked with 2 mg/l of fluoxetine and norfluoxetine. The optimal ionic strength of the electrolyte must be a balance between an acceptable low current to minimize the noise and a good peak resolution. The effect of the concentration of buffer solution from 4 to 15 m*M* with a constant concentration of DSS 50 m*M* was studied. This experiment showed that when the concentration of buffer increases the migration times of fluoxetine and norfluoxetine also increase. A buffer concentration of 5 m*M* was selected to maintain good peak shape and low current in order to minimize the noise and baseline aberrations.

A 50 m*M* phosphate buffer was selected for the preparation of standard and biological samples in order to reach a preconcentration analyte effect into the capillary (stacking).

3.1.2.2. Effect of micellar surfactant concentration. The influence of adding DSS to the electrolyte solution on migration time is given in Fig. 2. In this experience, the separation potencial was 25 kV and the operating temperature 25 °C. The obtained results demonstrate that although the DSS concentration has virtually no effect on the velocity of the EOF, the influence on the mobility of the two compounds is evident. As the number of micelles is increased, the concentration of solute in the micelles increases producing lower mobilities and longer migration times. Fluoxetine and norfluoxetine are negatively charged at the separation pH, so, the separation is achieved by effects like their hydrophobicity and to an electrostatic repulsive effect with the like-charged micelles. This effect results in a change in the migration times as a function of DSS concentration for the two compounds. Then, 40 m*M* of DSS was selected as optimum in order to minimize analysis times and to maintain a good resolution between peaks.

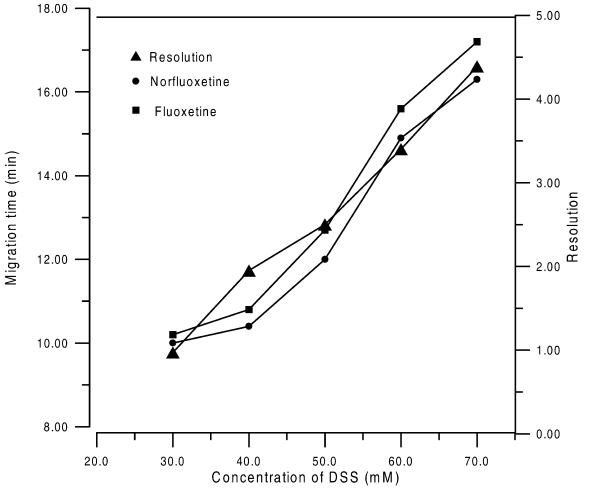


Fig. 2. Influence of DSS concentration on migration times and resolution between peaks. Operating conditions: 5 m*M* phosphate buffer pH 12.3, 25 kV as separation voltage, 25 °C of capillary temperature and 16 s of injection time.

The effect of different added DSS amounts on injected samples (both biological and standard ones) was tested, resulting in deformed and wider peaks in all cases. Therefore it was chosen not to add DSS in the injected samples.

3.1.2.3. Effect of voltage applied. The effect of the voltage applied from 5 to 30 kV was investigated using the same experimental conditions as above. A voltage of 25 kV yielded the best compromise in terms of run time, regenerated current and efficiency of separation. This voltage was used in subsequent stages of the method development.

3.1.2.4. Optimisation of injection time. In order to decrease the detection limits in the studied biological fluids, the injection time was varied between 3 and 30 s. As expected, when the injection time increased the peak area of both compounds also increased but for injection times higher than 16 s a loss of resolution between peaks was observed. For this

reason, 16 s of injection time was chosen as optimal value. The pressure of injection was always 0.5 p.s.i.

In consequence, the following electrophoretic conditions were selected:

- Electrolyte: 5 m*M* phosphate buffer pH 12.3, 40 m*M* of DSS
- Voltage: 25 kV
- Capillary: Fused-silica (57 cm×75 μm I.D.)
- Injection: hydrodynamic, 16 s
- Temperature: 25 °C
- Detection wavelength: 230 nm

3.1.3. Solid-phase extraction of the human urine and serum samples

First, the method was applied to the analysis of human urine and serum samples which had not been submitted to any special treatment, but due to the presence of a large quantity of various interferent compounds and the low concentration of fluoxetine and norfluoxetine, it was necessary to extract the compounds of interest in order to obtain a cleaner electropherogram. C_{18} cartridges were used to extract fluoxetine and norfluoxetine. Variables such as organic solvent, proportion and volume of organic solvent–water ratio in order to elute our analytes free from interferences were studied.

A cleaner electropherogram was obtained when, the cartridge charged with the urine or serum samples was previously washed with 8 ml of 10 m*M* phosphate buffer (pH 7.0) solution and 2 ml of 30% methanol–water solution in order to minimize the interferences. After the load of the sample fluoxetine and norfluoxetine were eluted with 1 ml of methanol. Finally this extract was diluted with 0.5 ml of 100 m*M* phosphate buffer (pH=12.3) and 0.5 ml of water. This procedure was found to be enough to elute quantitatively the analytes at the concentration levels present in the studied samples.

As examples, the electropherograms corresponding to the extracts from the spiked serum and urine are shown in Figs. 3 and 4, respectively.

The different migration times between urine and serum samples analytes may be caused by the different matrix effects of both samples, which were still lightly present in the injected samples despite the SPE step.

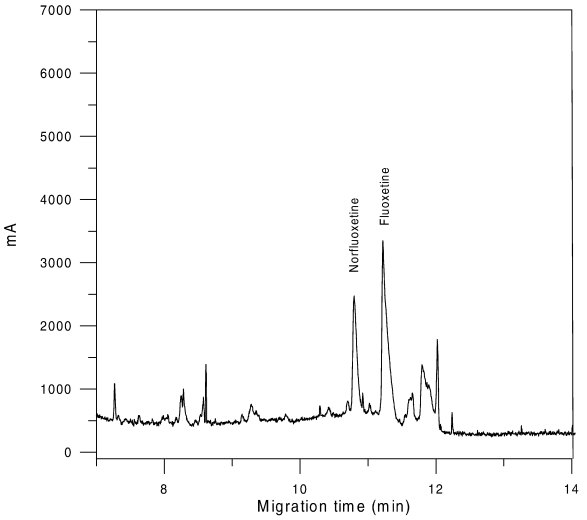


Fig. 3. Electropherograms in MEKC of a serum sample spiked to 2.0 mg/l of fluoxetine and norfluoxetine. Operating conditions: 5 m*M* phosphate buffer pH 12.3, 40 m*M* DSS, 25 kV as separation voltage, 25 °C of capillary temperature and 16 s of injection time.

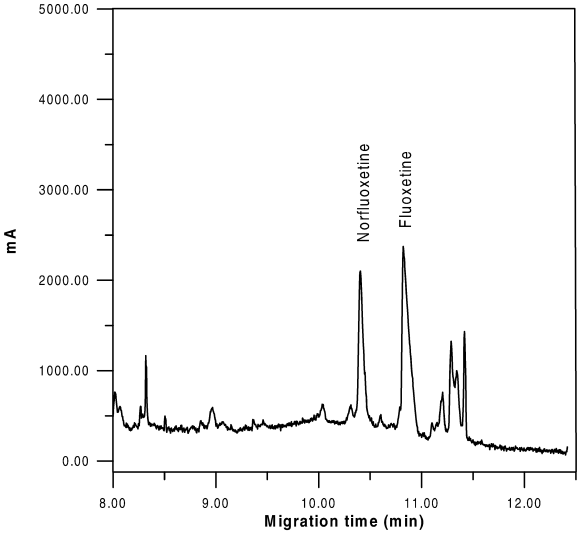


Fig. 4. Electropherograms in MEKC of a urine sample spiked to 2.0 mg/l of fluoxetine and norfluoxetine. Operating conditions: 5 m*M* phosphate buffer pH 12.3, 40 m*M* DSS, 25 kV as separation voltage, 25 °C of capillary temperature and 16 s of injection time.

3.2. Performance of the method

3.2.1. Stability of solutions

The stability of the standard solutions was determined by comparing the response factors (concentration/peak area) of duplicate solutions stored at room temperature and under diffuse light with those freshly prepared duplicate solutions.

The stability of spiked urine and serum extracts was evaluated by comparing the fluoxetine and norfluoxetine contents of these extracts kept at room temperature and under diffuse light at different time intervals with those of a freshly prepared standard solutions.

Less than a 0.2% concentration difference was found between the standard solution freshly prepared and the extracts prepared aged for 1 day. In the same way, stock standard solution of fluoxetine and norfluoxetine were checked and found to be stable for 3 months at least.

3.2.2. Linearity

The linearity of the response was examined by injection of seven fluoxetine and norfluoxetine spiked biological samples (serum and urine) after their submission of a SPE treatment. The linearity range was tested between 0.3 and 3.0 mg/l and the regression lines, calculated using the least-squares method, were:

URINE

Fluoxetine: $Y = (28.98 \pm 12.90) + (709.23 \pm 23.86)X \quad r^2 = 0.9977$

Norfluoxetine: $Y = (69.02 \pm 18.50) + (584.83 \pm 34.21)X \quad r^2 = 0.9932$

SERUM

Fluoxetine: $Y = (-17.50 \pm 38.02) + (787.27 \pm 62.73)X \quad r^2 = 0.9937$

Norfluoxetine: $Y = (-52.13 \pm 26.40) + (865.34 \pm 43.56)X \quad r^2 = 0.9950$

where Y represents the peak area, X the concentrations of standard solutions (mg/l) and r^2 denotes the correlation coefficient.

The confidence intervals were calculated with $P = 0.05$. Each point of the calibration graph corresponded to the mean value obtained from two independent area measurements. The satisfactory correlation coefficients showed that fluoxetine and norfluoxetine responses were linear over the studied concentrations range.

3.2.3. Recovery

In order to test the accuracy of the proposed method, several aliquots of fluoxetine and norfluoxetine standard solutions were added into human urine and serum samples. These samples were analysed using the extraction and electrophoretic procedures described in this work. Good results were obtained, as can be seen in Table 1. Every sample was injected three times.

The use of a photodiode detector allowed us to confirm the identity of the peak not only by its migration times, but also by the overlay of the UV–Vis spectra of the samples with a standard.

3.2.4. Specificity

Specificity can also be determined by measurement of peak homogeneity. The studied techniques for validating the peak purity corresponding to

Table 1
Recovery of human serum and urine sample

	Serum				Urine			
	Fluoxetine		Norfluoxetine		Fluoxetine		Norfluoxetine	
	Added	Recov. (%)	Added	Recov. (%)	Added	Recov. (%)	Added	Recov. (%)
S1	0.793	95.5	0.793	99.3	0.793	93.2	0.793	90.2
S2	0.636	92.8	0.636	93.7	0.530	91.8	0.530	94.7
S3	0.528	90.6	0.528	91.0	0.398	94.8	0.398	92.3
S4	0.397	91.3	0.397	91.0	0.319	94.0	0.319	91.5

fluoxetine and norfluoxetine in human urine and serum samples were [21]:

- normalising and comparing spectra from several peak sections;
- absorbance at two wavelengths.

Both techniques demonstrate in all cases that the peaks corresponding to fluoxetine and norfluoxetine in the analysed samples (Figs. 2 and 3) present a high level of purity.

3.2.5. Precision

The precision of the proposed method for determining fluoxetine and norfluoxetine is expressed in terms of relative standard deviation (RSD).

In order to test the precision of the electrophoretic procedure, eight injections of a standard of 4 mg/l of fluoxetine and norfluoxetine were carried out sequentially. This operation was repeated over 3 days. The precision of the migration times and peak area were good with RSD ($n=24$) of 0.9 and 1.0 for migration times and 2.0 and 1.7 for peak area of fluoxetine and norfluoxetine, respectively.

To evaluate the precision of extraction method, urine and serum samples spiked with 2 mg/l of fluoxetine and norfluoxetine were analysed independently four times. The average of the recoveries from the spiked urine sample was 92.5 ± 3.0 and 93.1 ± 2.5 for fluoxetine and norfluoxetine, respectively. The results for the spiked serum samples were 91.7 ± 2.6 and 90.4 ± 3.5 for fluoxetine and norfluoxetine, respectively.

3.2.6. Limits of detection and quantitation

The limits of detection (LODs) and quantitation (LOQs) were calculated by measuring six blanks, using the maximal sensitivity allowed by the system and calculating the standard deviation (SD) of this response. LODs was estimated by multiplying the SD by a factor of three. The LOQs was defined as ten times the SD.

The LODs and LOQs obtained considering a dilution factor of 4.0 for both studied compounds (from the extraction–preconcentration process) were 0.2 and 0.6 mg/l, respectively in biological samples.

4. Conclusions

In this work, a method is described for the extraction and determination of fluoxetine and its metabolite norfluoxetine in biological fluids by MEKC. Although fluoxetine and norfluoxetine have been previously determined in these kind of samples by several analytical techniques, this is the first report where the determination of these drugs is performed by MEKC. The obtained results concerning linearity, recovery and precision were highly satisfactory and comparable to those obtained by the proposed methods in the literature. It could be concluded that MEKC can be an alternative to traditional existing methods for the determination of fluoxetine and norfluoxetine in different biological fluids.

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