

# Development and validation method for determination of fluoxetine and its main metabolite norfluoxetine by nonaqueous capillary electrophoresis in human urine

J. Rodríguez Flores<sup>a,\*</sup>, J.J. Berzas Nevado<sup>a</sup>, G. Castañeda Peñalvo<sup>a</sup>,  
N. Mora Díez<sup>b</sup>

<sup>a</sup> Department of Analytical Chemistry and Foods Technology, UCLM 13071, Ciudad Real, Spain

<sup>b</sup> Department of Analytical Chemistry, University of Extremadura, Badajoz, Spain

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## Abstract

A simple, rapid and sensitive procedure using nonaqueous capillary electrophoresis (NACE) to measure fluoxetine and its main metabolite norfluoxetine has been developed and validated. Optimum separation of fluoxetine and norfluoxetine, by measuring at 230 nm, was obtained on a 60 cm × 75 µm capillary using a nonaqueous solution system of 7:3 methanol-acetonitrile containing 15 mM ammonium acetate, capillary temperature and voltage 25 °C and 25 kV, respectively and hydrodynamic injection. Paroxetine was used as internal standard. Good results were obtained for different aspects including stability of the solutions, linearity, and precision. Detection limits of 10 µg L<sup>-1</sup> were obtained for fluoxetine and its metabolite. This method has been used to determine fluoxetine and its main metabolite at clinically relevant levels in human urine. Before NACE determination, the samples were purified and enriched by means of extraction-preconcentration step with a preconditioned C<sub>18</sub> cartridge and eluting the compounds with methanol.

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**Keywords:** Nonaqueous capillary electrophoresis; Fluoxetine; Norfluoxetine; Metabolite; Antidepressant and human urine

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## 1. Introduction

Before 1980, antidepressant treatment principally consisted of the tricyclics, monoamine oxidase inhibitors and lithium. Since the early 90s, a new generation of compounds is available, having a different pharmacological profile and generally better tolerated adverse effects [1]. The first class introduced fluvoxamine, fluoxetine, sertraline, paroxetine and citalopram. A second class consists of venlafaxine and milnacipran.

Fluoxetine hydrochloride is an antidepressant [2] for oral administration; it is chemically unrelated to tricyclic, tetracyclic, or other available antidepressant agents. It is des-

ignated (±)-*N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)-oxy]propylamine hydrochloride, it is a cyclic secondary amine and has the empirical formula of C<sub>17</sub>H<sub>18</sub>F<sub>3</sub>NO·HCl.

Fluoxetine (Fig. 1) is in a new class of antidepressant medications that affects chemical messengers within the brain. These chemical messengers are called neurotransmitters. Many experts believe that an imbalance in these neurotransmitters is the cause of depression. Fluoxetine is suggested to work by inhibiting the release or affects the action of serotonin [3]. It is used to treat mental depression [4]. It is also used to treat obsessive-compulsive disorder, nervous bulimia, and premenstrual dysphoric disorder. Fluoxetine belongs to a group of medicines known as selective serotonin reuptake inhibitors (SSRIs). These medicines are thought to work by increasing the activity of a chemical called serotonin in the brain.

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\* Corresponding author. Tel.: +34-926295300.

E-mail address: [juana.rflores@uclm.es](mailto:juana.rflores@uclm.es) (J.R. Flores).

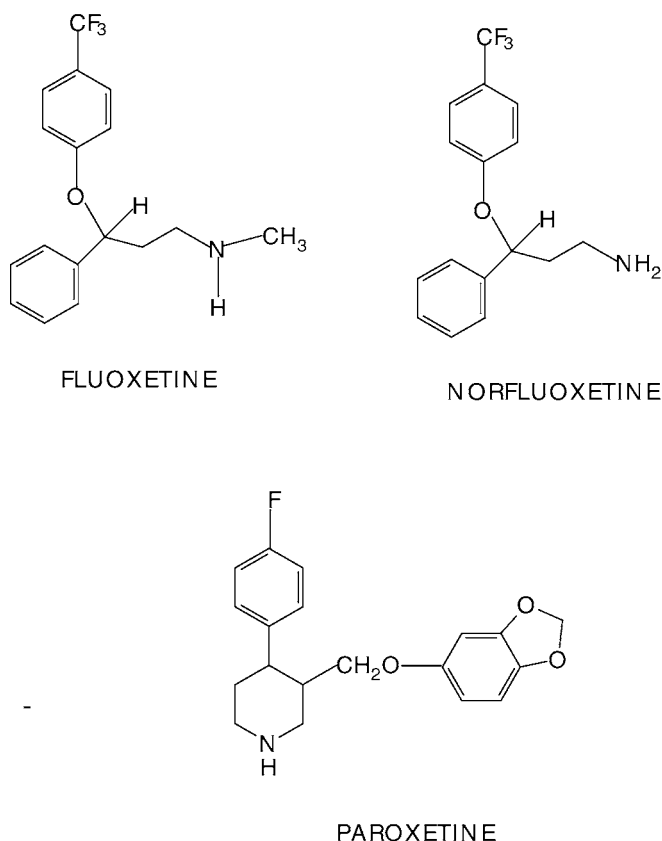


Fig. 1. Structures of the molecules.

Fluoxetine is extensively metabolized in the liver to norfluoxetine, and other, unidentified metabolites; but much is still unknown about the metabolites and elimination of fluoxetine and its metabolites [5]. Norfluoxetine (Fig. 1), a desmethyl metabolite, is also a serotonin reuptake inhibitor; its pharmacological activity being similar to that of the parent drug. Norfluoxetine contributes to the long duration of action of fluoxetine. Elimination of metabolites occurs primarily in the urine with a smaller amount also being present in the feces.

Several methods for the determination of fluoxetine and norfluoxetine in biological samples have been published. Mostly based on liquid chromatography (LC) with ultraviolet [6–8], fluorescence [9–11] or mass spectrometry detection [12], gas chromatography–electron capture detection (GC–ECD) [13,14] or GC–mass spectrometry (MS) detection [15]. Others works include the quantification of enantiomeric forms of both fluoxetine and norfluoxetine by LC [13] and GC–ECD [16] techniques. But only few works have been published by capillary electrophoresis. There are three papers about enantiomeric separation of fluoxetine and norfluoxetine [17–19]. Desiderio et al. [18] use a cyclodextrin-modified sodium phosphate buffer at pH 2.5, and Gatti et al. [19] made a comparison of the enantiorecognition ability of linear, neutral polysaccharides in acidic running buffer (pH 3.0). A micellar electrokinetic capillary chromatography [20]

has been proposed and applied in different kind of biological samples. Only one work [21] is reported by using non-aqueous electrophoresis coupled on-line with electrospray ionisation–mass spectrometry, although there is a work by nonaqueous capillary electrophoresis (NACE) where fluoxetine is employed as internal standard in the determination of paroxetine and its metabolites [22]. Another antidepressants have been determined by NACE [23,24].

The use of nonaqueous buffers to extend the application range of CE, has encountered growing interest [21]. Compared to aqueous buffer solutions, the different physico-chemical properties of organic solvents (viscosity, dielectric constant, polarity, autoprotolysis constant, electrical conductivity, etc.), induce selectivity modification a challenging task in the science of separation. In fact, organic solvents proved to be useful for the analysis of hydrophobic compounds as well as of drugs and metabolites, which are difficult to separate with aqueous buffers. Short analysis time, less Joule heating, and the possibility to increase analyte solubility are the main reasons for this success. Excellent reviews on non-aqueous capillary electrophoresis [25–29] have been published and can be consulted for a more systematic coverage of the field.

The purpose of this work was to perform a simple and fast separation of fluoxetine and its major metabolite norfluoxetine by non aqueous capillary electrophoresis in human urine samples by means of diode-array detection. Prior to the electrophoretic separation a previous extraction and preconcentration process on a  $C_{18}$  cartridge was used.

## 2. Experimental

### 2.1. Chemicals

Methanol (99.8%) (0.1% water) and acetonitrile (99.9%) (0.02% water) was purchased from PANREAC (Madrid, Spain).

Fluoxetine hydrochloride and its metabolite norfluoxetine were purchased from Tocris and Sigma, respectively.

Standard solutions ( $100 \text{ mg L}^{-1}$ ) were prepared in methanol and stored under refrigeration at  $4^\circ\text{C}$ . Working standard solution were prepared daily by dilution of the stock standard solutions with methanol.

### 2.2. Instrumentation

Analysis was performed with Beckman P/ACE System MDQ capillary electrophoresis equipment with diode-array detection (DAD) and controlled by means of a P/ACE System MDQ capillary electrophoresis software. The 60 cm (50 to the detector)  $\times$   $75 \mu\text{m}$  i.d. fused-silica separation capillary was maintained in a cartridge with a  $100 \mu\text{m} \times 800 \mu\text{m}$  detection window.

The use of photodiode detector allowed us to confirm the identity of the peaks, not only by its migration time, but also

by the overlay of the UV–Vis spectra of the samples with a standard.

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

### 2.3. Treatment of the urine samples, extraction and preconcentration procedure

Fresh human urine samples were obtained from different volunteers who had or had not taken fluoxetine.

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

The extraction of fluoxetine and norfluoxetine from the biological samples was performed in a reverse-phase C<sub>18</sub> cartridge (Waters Sep-Pak Plus, Milford, MA, USA). The cartridge was conditioned prior to use with 5 mL of methanol followed by 5 mL of 10 mM phosphate buffer solution (pH 7.0). In all the cases 0.5 mg L<sup>-1</sup> of paroxetine was added as internal standard.

Different volumes (between 2 and 10 mL) of urine were slowly loaded into the conditioned cartridge. The cartridge was then washed with 8 mL of 10 mM phosphate buffer (pH 7.0) and 2 mL of a 30% methanol–water solution. Finally, fluoxetine and norfluoxetine were eluted with 2 mL of methanol. This extract was immediately injected into the capillary electrophoresis equipment.

### 2.4. Operating conditions

Before the first use the capillary was conditioned by flushing with 0.1 M NaOH for 60 min, then with water for 30 min, and finally with the background electrolyte solution for 20 min.

Electrophoretic separation was performed using a non-aqueous solution of 7:3 (v/v) methanol-acetonitrile containing 15 mM ammonium acetate. Before use, the electrolyte solutions were filtered through a 0.45 µm microfilter and degassed by passing N<sub>2</sub> stream for 10 min at very slow flow. The temperature of the cartridge, the applied voltage and the injection time in the hydrodynamic way were 25 °C, 25 kV and 5 s, respectively.

Urine samples were kept at 20 °C inside the capillary electrophoresis equipment. Between measurements the capillary was conditioned for 2 min with nonaqueous solution because contamination from an unknown component of the urine samples was observed between consecutive injections. At the start of each sequence of analyses the capillary was washed for 5 min with 0.1 M NaOH, 5 min with water and 10 min with nonaqueous solution.

Duplicate injections of the solution were performed and relative peak areas (analyte area/Paroxetine area) were used for the quantification.

## 3. Results and discussion

### 3.1. Optimisation of the test electrophoretic procedure

#### 3.1.1. Effect of H<sub>2</sub>O/MeOH mixtures percentages

First it was study the effect of the presence of water in nonaqueous solution in the range 0–20% (v/v) assay of water in 5% steps, and it was found that by increasing the percentage of water, the peaks height decrease and the resolution was worse (Fig. 2). Then it was selected the no addition of water to nonaqueous solution.

#### 3.1.2. Effect of ACN/MeOH mixtures percentages

ACN/MeOH mixtures are widely used in NACE. The selectivity of the separation systems changed significantly with the ratio of ACN/MeOH, and the electrophoretic mobility varied according to the ACN/MeOH composition, with a maximal value at 75% [30]. The zeta potential in ACN is higher than in MeOH, if both solvents do not contain supporting electrolytes [31,32]. Therefore, changes in viscosity and dielectric constant predict a steadily increase of EOF with the ACN concentration. This was found for ACN/MeOH mixtures containing NR<sub>4</sub><sup>+</sup> and equal or slightly higher amounts of OAc<sup>-</sup> [33,34].

Several ACN/MeOH mixtures (0, 10, 30, 50 and 70% assay of acetonitrile (v/v)) containing 15 mM NH<sub>4</sub>OAc were tested for the separation of the studied compounds. When the % of ACN increases, the migration times of fluoxetine and its metabolite decrease, but the resolution became bad over 30% of acetonitrile (Fig. 3) and the current increases. Then, 30% ACN was chosen as optimum.

#### 3.1.3. Effect of ionic strength of electrolyte

As in aqueous CE, the nature of the electrolyte plays an important role during method optimization. Most organic solvents are capable of dissolving electrolytes, at least to some extent. One of the most commonly used as electrolytes in NACE is the mixture of acids and their ammonium salts. In our work, a mixture of HOAc and NH<sub>4</sub>OAc was investigated, because it is a suitable electrolyte for direct UV-detection of anions and cations in the most organic solvents, and the addition of these compounds to the organic solvent increases the conductivity and promotes a stable current. Therefore, in our case, the ionic strength of the electrolyte is depending on the concentration of NH<sub>4</sub>OAc and HOAc added to the background. In NACE it is suggested to work with level current lower than 20–25 µA because this avoids a bubble formation into the capillary, therefore it avoids the current courts.

The influence of increasing amounts of HOAc (0, 0.5 and 1% (v/v)) over resolution was studied maintaining 15 mM of NH<sub>4</sub>OAc constant. High values of percent HOAc in the buffer increase the current level and provoke current courts. No addition of HOAc was chosen as optimum (Fig. 4) in order to obtain the best resolution, the best sensitivity and a current lower than major percentages of HOAc, avoiding current courts.

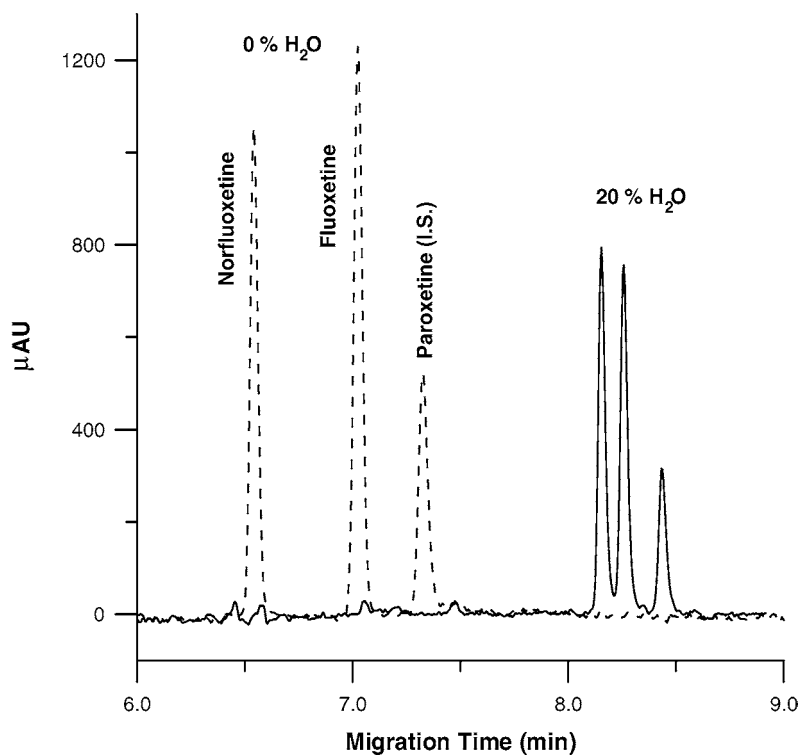


Fig. 2. Effects of percentage of water upon migration time. Migration order: norfluoxetine, fluoxetine and paroxetine. Experimental conditions: 15 kV, 3 s injection, 230 nm, 15 mM  $\text{NH}_4\text{OAc}$ ,  $2 \mu\text{g mL}^{-1}$  of each analyte and  $0.5 \mu\text{g mL}^{-1}$  of paroxetine.

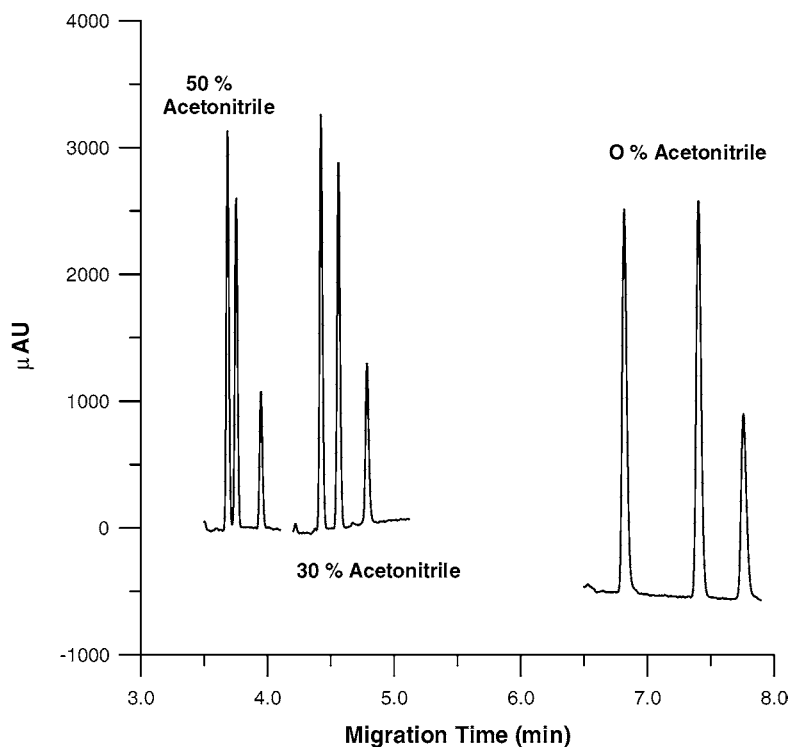


Fig. 3. Effects of percentage of acetonitrile upon migration time. Migration order: norfluoxetine, fluoxetine and paroxetine. Experimental conditions: 15 kV, 3 s injection, 230 nm, 15 mM  $\text{NH}_4\text{OAc}$ ,  $2 \mu\text{g mL}^{-1}$  of each analyte and  $0.5 \mu\text{g mL}^{-1}$  of paroxetine.

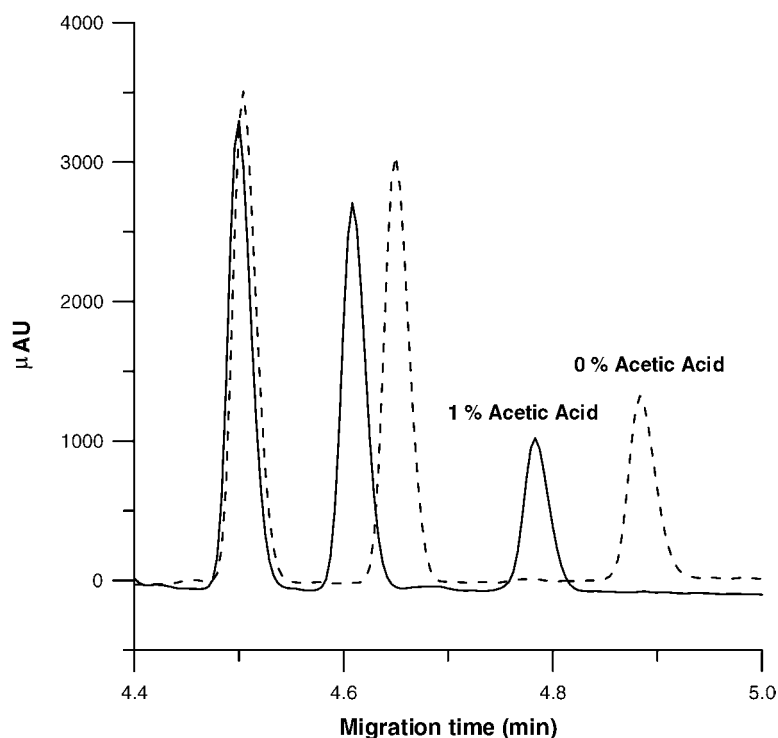


Fig. 4. Effects of percent acetic acid upon migration time. Migration order: norfluoxetine, fluoxetine and paroxetine. Experimental conditions: 15 kV, 3 s injection, 230 nm, 15 mM  $\text{NH}_4\text{OAc}$ ,  $2 \mu\text{g mL}^{-1}$  of each analyte and  $0.5 \mu\text{g mL}^{-1}$  of paroxetine, non-aqueous solution: 30% acetonitrile, 70% methanol.

In the same way, the effect of the concentration of  $\text{NH}_4\text{OAc}$  (10, 15, 20 and 30 mM) on the migration time of the investigated compounds was studied. As expected, when the concentration of  $\text{NH}_4\text{OAc}$  increases the migration times of fluoxetine and its metabolite also increase.

A concentration of 15 mM  $\text{NH}_4\text{OAc}$  buffer was selected as optimal since this value maintains good peak shape, low current ( $<20 \mu\text{A}$ ) and the better resolutions between peaks for all the studied drugs.

#### 3.1.4. Effect of the applied voltage

The effect of the voltage applied from 5 to 30 kV (steps of 5 kV) was investigated. A voltage of 25 kV yielded the best compromise in terms of lower migration times and higher corrected areas and resolutions. Voltages higher than 25 kV generate currents higher than  $20 \mu\text{A}$ , which permit a bubble formation into the capillary and current courts.

#### 3.1.5. Optimisation of injection time

In order to decrease the detection limits, the injection time was varied between 3 and 11 s (steps of 2 s). As expected, when the injection time increased the peak area of all compounds also increased, but for injection times higher than 5 s a loss of resolution between peaks was observed. For this reason, 5 s of injection time was chosen as optimal value. The pressure of injections was always 0.5 psi.

#### 3.1.6. Effect of temperature

Changes in capillary temperature can cause variations in efficiency, viscosity, migration times, injection volumes and detector response. The effect of temperature on the separation was investigated in the range  $18\text{--}30^\circ\text{C}$  (18, 20, 25 and  $30^\circ\text{C}$ ). As temperature increases, the viscosity of buffer decreases, so the resistance of the buffer decreases, and as the electric field is constant, the current increases. The decrease in the migration times of the drugs at higher temperatures results in poor resolutions between fluoxetine and norfluoxetine. The temperature selected was  $25^\circ\text{C}$  because it results the best resolution with a current level  $<20 \mu\text{A}$ .

#### 3.2. Solid-phase extraction (SPE) of the human urine samples

Due to the presence of a large quantity of various interfering compounds and the low concentration of the drugs under study, it was necessary to extract the compounds of interest in order to obtain a cleaner electropherogram.  $\text{C}_{18}$  cartridges were used to extract the studied drugs from the human urine. Variables such as organic solvent, washing stages using different solvents, organic-water ratio for elution of the analytes free from interferences, and final volume of the extract, were studied.

A cleaner electropherogram was obtained when the cartridge charged with the urine sample had been previously washed with 8 mL of 10 mM phosphate buffer (pH 7.0) and

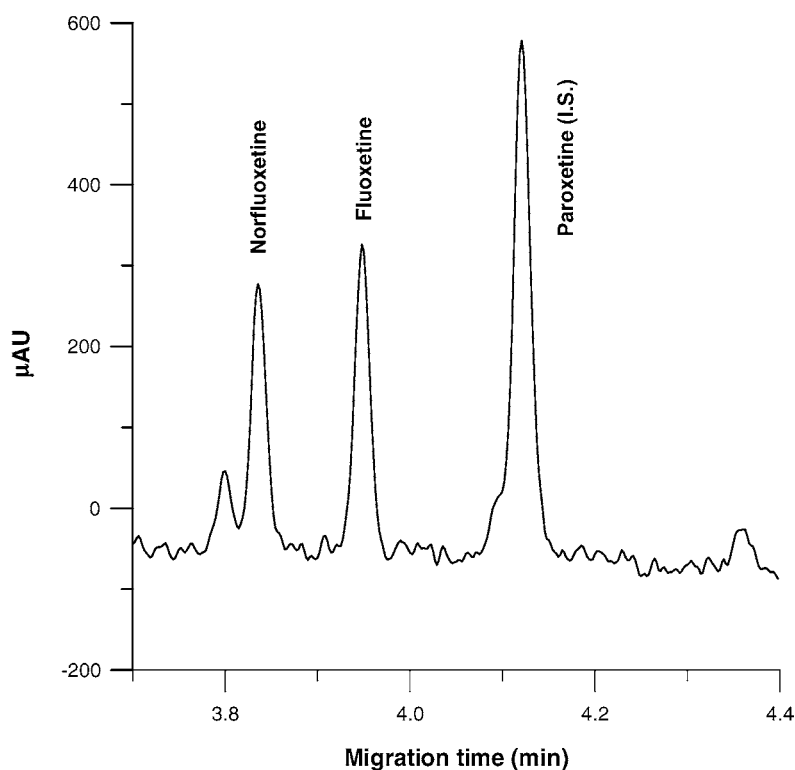


Fig. 5. Electropherogram of the extract from urine (6 mL) obtained with the final selected conditions, and spiked with  $0.1 \mu\text{g mL}^{-1}$  of fluoxetine and norfluoxetine, and  $0.5 \mu\text{g mL}^{-1}$  of paroxetine (I.S.). Experimental conditions: 25 kV, 5 s injection, 230 nm, 15 mM  $\text{NH}_4\text{OAc}$ , 7:3 (v/v) methanol-acetonitrile.

2 mL of a 30% methanol–water solution in order to minimise interferences. After that, fluoxetine and norfluoxetine were eluted with 2 mL of methanol. The maximal capacity of the cartridge was investigated and established 10 mL, therefore, it was possible to preconcentrate five times. Fig. 5 shows the electropherogram corresponding to the methanolic extracts from 6 mL of urine sample spiked with  $0.1 \mu\text{g mL}^{-1}$  of fluoxetine and norfluoxetine, and  $0.5 \mu\text{g mL}^{-1}$  of paroxetine (I.S.).

### 3.3. Validation of the electrophoretic procedure

#### 3.3.1. Stability of the solutions

The stability of the standard and test solutions of fluoxetine, its metabolite norfluoxetine and paroxetine (the internal standard), was determined by comparing the response factors (concentration/average peak areas) of triplicate solutions stored at  $4^\circ\text{C}$ , in darkness, with those of freshly prepared triplicate solutions. A concentration difference of less than 2% was found between the standard solution freshly prepared and the solutions prepared 7 days before. In this way, stock standard solutions of fluoxetine, norfluoxetine and paroxetine were checked and found to be stable for at least 2 months.

The stability of spiked urine extract containing the three compounds was evaluated by comparing the relative peak areas obtained at different time intervals with those of a freshly prepared extract. It was found that the extract was stable for at least 2 h. For this reason, it is recommended that NACE

analysis be carried out immediately after the extraction step.

#### 3.3.2. Linearity

The linearity of the response was examined by the injection of seven spiked urine samples after SPE treatment. The linearity was tested over the range from  $0.1$  to  $2.0 \text{ mg L}^{-1}$  for each analyte in the urine (6 mL of urine samples). In all the cases  $0.5 \text{ mg L}^{-1}$  of paroxetine was added as internal standard. The results were given in terms of relative peak areas. The linear regression equations obtained using the least-squares method and coefficients of correlation are presented in Table 1. The satisfactory coefficients of correlation confirm that fluoxetine and norfluoxetine responses were linear over the concentration range studied. The slope of this calibration graph was compared with another calibration graph in methanol by applying an ANOVA test, and it was found not significant differences between them for a significant level of 0.05, then it is possible to determine fluoxetine and norfluoxetine directly by using a calibration graph in methanol (there is not matrix effect).

#### 3.3.3. Precision

The precision of the proposed method is expressed in terms of relative standard deviation (R.S.D.).

In order to test the precision of the overall process (extraction, preconcentration and NACE step) five different urine samples spiked with  $1 \text{ mg L}^{-1}$  of fluoxetine and norfluoxetine

Table 1  
Linearity ( $n = 7$ ) in urine samples

Fluoxetine		Norfluoxetine
Equation <sup>a</sup>	$y = (-0.24 \pm 0.19) + (6.55 \pm 0.17) x$	$y = (-0.24 \pm 0.17) + (5.03 \pm 0.15) x$
Coeff. of determ. ( $r^2$ )	0.9967	0.9955
LOD ( $\mu\text{g L}^{-1}$ ) 6 mL urine	10	10
LOQ ( $\mu\text{g L}^{-1}$ ) 6 mL urine	32	35
LOD ( $\mu\text{g L}^{-1}$ ) 10 mL urine	4	5
LOQ ( $\mu\text{g L}^{-1}$ ) 10 mL urine	13	16

LODs and LOQs for the two studied drugs.  $y = (a \pm Sa) + (b \pm Sb) x$ . a, intercept; Sa, standard deviation of intercept; b, slope; Sb, standard deviation of slope.

<sup>a</sup> Concentration ( $x$ ,  $\text{mg L}^{-1}$ ) vs. relative peak area ( $y$ ).

and  $0.5 \text{ mg L}^{-1}$  of paroxetine (I.S.) were carried out sequentially. The precision of the migration times and relative peak area were good with a RSD between 0.27 and 0.28% for migration times and between 2.59 and 3.10% for relative peak areas.

### 3.3.4. Specificity

Specificity can also be determined by measurement of peak homogeneity. Because of the different techniques available in a DAD are not equally effective for the detection of possible impurities or interferences in an electrophoretic peak, the use of several techniques is recommended [35].

In this work the techniques used to validate the peak purity of the studied compounds present in urine samples were [36]:

- Normalization and comparison of spectra from different peak sections.

- Absorbance at two wavelengths.

Both techniques proved a high level of purity of the peaks corresponding to the compounds studied in urine. Therefore, no interferences by matrix effect were observed.

### 3.3.5. Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection (LODs) and quantification (LOQs) were calculated by measuring the noise in different blanks, and taking into account a factor of 3 and 10 for LODs and LOQs, respectively, and by using standards obtained in order to convert to concentration units.

The LODs and LOQs have been calculated taking into account the overall process (extraction, preconcentration and NACE step), and by passing 6 and 10 mL of urine samples (Table 1).

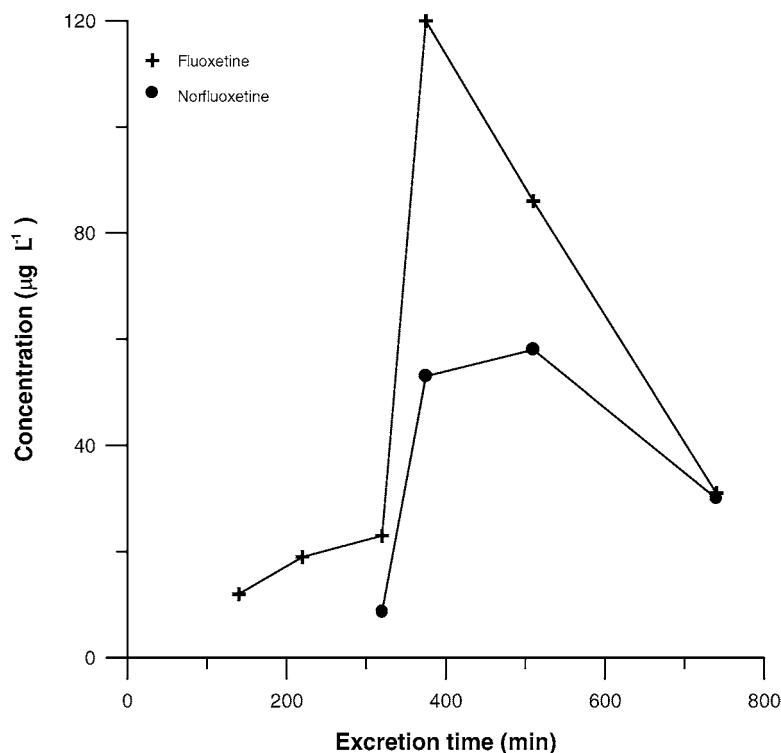


Fig. 6. Time course of urine fluoxetine and metabolite levels in a volunteer receiving 20 mg of the drug orally. Experimental conditions: 25 kV, 5 s injection, 230 nm, 15 mM  $\text{NH}_4\text{OAc}$ , 7:3 (v/v) methanol-acetonitrile.



Table 2  
Recovery of human samples

Sample	Added ( $\mu\text{g mL}^{-1}$ )		% Recovery	
	Fluoxetine	Norfluoxetine	Fluoxetine	Norfluoxetine
1	1.0	1.0	89	90
2	1.0	0.4	85	99
3	0.4	1.0	93	91
4	0.6	1.2	94	92
5	1.2	0.6	87	92
6	0.4	0.4	99	97

### 3.3.6. Recovery

In order to test the accuracy of the proposed method, several aliquots of fluoxetine and norfluoxetine standard solutions were added into human urine samples of three women, two men and one pregnant woman. These samples were analysed using the extraction, preconcentration and electrophoretic procedures described above. As may be observed in Table 2, good results were obtained. Recoveries were calculated by using the calibration graph.

## 3.4. Applications

### 3.4.1. Pharmacokinetic study

An unique pharmacokinetic study was performed during a day by analysing urine samples of a volunteer receiving 20 mg of the drug orally (Prozac). The concentration of fluoxetine and norfluoxetine found using this method at different interval times are shown in Fig. 6.

For this study 10 mL of urine was loaded to the SPE cartridge and was eluted with 2 mL of methanol, in this way, the concentration was five times bigger than the original concentration.

## 4. Conclusion

A simple, rapid and sensitive electrophoretic method has been developed, for the analysis of fluoxetine and its metabolite norfluoxetine in human urine. Although both analytes have previously been determined by capillary electrophoresis, this is the first report that enables the determination of paroxetine and norfluoxetine in human urine by nonaqueous capillary electrophoresis with diode-array detection. NACE proved to be an effective technique for the simultaneous analysis of these antidepressant. The organic solvent composition and electrolyte concentration had a significant effect on resolution, sensibility and separation time. Compared to aqueous CE [20], NACE permit lower detection limits for these analytes (from 200 to  $10 \mu\text{g L}^{-1}$ , respectively). This fact suppose an increase of 20 times in the sensibility of the described method in this paper.

Prior to NACE analysis, samples are purified and concentrated by solid-phase extraction which permits quantification of fluoxetine and norfluoxetine at clinically relevant concentrations. The electrophoretic (NACE) method has been val-

idated for the analysis of them in human urine without any matrix interference. It has been demonstrate the reliability of the electrophoretic procedure for its desired application by means of the experimental results like linearity, accuracy, specificity, sensitivity and precision.

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