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Talanta 65 (2005) 155–162
Talanta
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Nonaqueous capillary electrophoresis method for the analysis of tamoxifen, imipramine and their main metabolites in urine

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Received 1 March 2004; received in revised form 13 May 2004; accepted 28 May 2004
Available online 29 July 2004

Abstract

The viability of nonaqueous capillary electrophoresis (NACE) was investigated for the simultaneous determination of tamoxifen, imipramine and their main metabolites (4-hydroxytamoxifen and desipramine, respectively). Baseline separation of the studied solutes was obtained on a 57 cm × 75 μm capillary using a nonaqueous solution composed of 17 mM ammonium acetate and 1.25% acetic acid in 80:20 (v:v) methanol–acetonitrile, temperature and voltage 22 °C and 15 kV, respectively, and hydrodynamic injection. Paroxetine was used as internal standard. Different aspects including linearity, accuracy, ruggedness and precision was studied. Detection limits between 9.0 and 15.0 $\mu g\,L^{-1}$ were obtained for all the studied compounds. The developed method is simple, rapid and sensitive and has been used to determine tamoxifen, imipramine and their metabolites at clinically relevant levels in human urine. Before NACE determination, a solid phase extraction (SPE) procedure with a C_{18} cartridge was necessary. Real determination of these analytes in three females urines were done.

Keywords: Nonaqueous capillary electrophoresis; Tamoxifen; Imipramine; 4-Hydroxytamoxifen; Desipramine; Ruggedness; Human urine

1. Introduction

Tamoxifen (Fig. 1) is an oral nonsteroidal antiestrogen drug used in the treatment and prevention of breast cancer [1–4]. Tamoxifen's primary mechanism of action is competitive inhibition of the estrogen receptor-alpha, thereby, inhibiting growth of malignant breast cells [1,5–7]. Five years of tamoxifen administration reduces the incidence of this illness by approximately 50% [8]. Tamoxifen has a low toxicity; however, in some instances, the drug may exhibit estrogenic activity and there is evidence of increased risk for endometrial cancer in women undergoing long-term therapeutic tamoxifen administration [9].

Often, mental disorders such as depression and anxiety may arise secondary to the presence of breast cancer and in many cases, it is, therefore, necessary to provide breast cancer patients with antidepressant treatment.

Imipramine (Fig. 1) is a tricyclic antidepressant (TCA) with general pharmacological properties similar to those of structurally related tricyclic antidepressant drugs such as amitriptyline and doxepin. The mechanism of action of imipramine and other tricyclic antidepressants is not well established, but it is thought that it might be related to their action on the transmitter-uptake mechanism of monoaminergic neurons. Imipramine is well absorbed from the gastrointestinal tract. Following oral administration of 50 mg three times daily for 10 days, the mean steady-state plasma concentration was 33–85 ng mL^{-1} for imipramine and 43–109 ng mL^{-1} for desmethylimipramine, an active metabolite. Peak plasma levels are reached in 2–5 h, and plasma half-life ranges from 9 to 20 h. Approximately 86% of imipramine is bound to plasma proteins. It is excreted primarily as inactive metabolites, up to 80% in the urine and up to 20% in the feces.

Several methods have been reported for the quantitative analysis of tamoxifen and its metabolites in biological fluids and pharmaceutical formulations, mostly based on high-performance liquid chromatography (HPLC) [10,11],

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doi:10.1016/j.talanta.2004.05.059

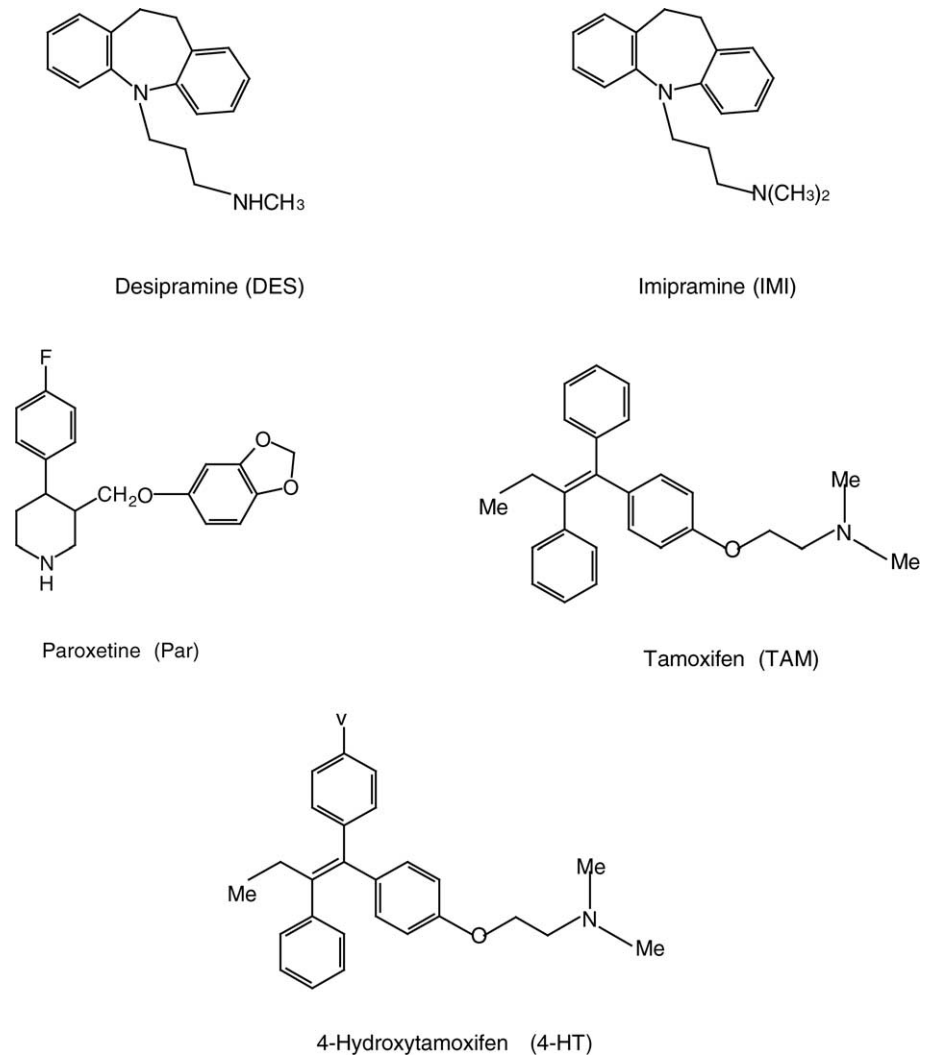

Fig. 1. Structures of the studied molecules.

nonaqueous capillary electrophoresis (CE) [12], potentiometry [13] and gas chromatography (GC) [14–18]. Other studies include the quantification of enantiomeric forms of tamoxifen by HPLC [19] and its determination in the presence of anastrozole and letrozole by GC with FID detection [20].

Imipramine and its metabolite (desipramine) has been determinate in biological fluids using techniques such as HPLC coupled with ultraviolet and electrochemical detection [21–23], GC with nitrogen–phosphorus [24] and mass spectrometry (MS) detection [25,26]. Capillary zone electrophoresis (CZE) has been used to determine imipramine and desipramine [27]. NACE was used to separate tricyclic antidepressant drugs in biological samples after at line solid phase extraction [28] and in pharmaceutical formulations and plasma samples [29].

Nevertheless, less effort have been put in the development of analytical methods that allows determination of tamoxifen along with antidepressants in biological samples. These methods could be important since it is very frequent the administration of tamoxifen and antidepressants in patients with breast cancer. Rodríguez et al. have used GC for determining tamoxifen together with two selective serotonin reuptake inhibitors (SSRIs) (fluoxetine and fluvoxamine) and one TCA (clomipramine) in urine [30], and the same authors have been proposed a NACE method for separating tamoxifen in the presence of paroxetine [31].

In this work, we have developed an analytically simple, sensitive and robust method that allows determination of tamoxifen and imipramine along with their main metabolites in urine at clinical levels by NACE. For the first time, NACE is utilized for determining a drug used in advanced breast cancer and a tricyclic antidepressants in urine.

2. Experimental section

2.1. Equipment

Analysis was performed with Beckman P/ACE System 5510 capillary electrophoresis equipment (Palo Alto) with

diode-array detection (DAD) and controlled by a Dell Dimension P133V with P/ACE station software. The 57 cm (50 to the detector) × 75 μm i.d. fused-silica separation capillary was maintained in a cartridge with a 100 μm × 800 μm detection window.

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

2.2. Chemicals

Methanol and acetonitrile (HPLC grade) was purchased from PANREAC (Madrid, Spain).

Paroxetine hydrochloride and imipramine and its metabolite were kindly supplied by GlaxoSmithKline Laboratories (Madrid, Spain) and Novartis Pharma AG (Basel, Switzerland), respectively. Tamoxifen citrate and its metabolite were supplied by TOCRIS (Bristol, UK).

Standard solutions (200 mg L^{-1}) were prepared in methanol and stored under refrigeration at 4 °C. Working standard solution were prepared daily by dilution of the stock standard solutions with methanol.

Electrophoretic separation was performed using a nonaqueous solution system composed of 80:20 (v/v) methanol/acetonitrile containing 17 mM ammonium acetate (NH_4OAc) and 1.25% acetic acid (HOAc). Before use, the electrolyte solutions were filtered through a 0.45 μm microfilter and degassed in a ultrasonic bath for 10 min.

2.3. Procedure

2.3.1. Nonaqueous CZE separation

Uncoated capillaries were used throughout. Prior to use, they were conditioned by flushing with 0.1 M NaOH for 20 min, water for 15 and 10 min with the separation electrolyte.

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 5 min with running electrolyte. The applied potential was 15 kV, average current of 15.4 μA and the capillaries were thermostated at 22 °C. The detection was performed at 211 nm. The injections of the samples were hydrodynamically for 6 s.

The running electrolyte was a nonaqueous system of 80:20 methanol–acetonitrile (v/v), containing 17 mM ammonium acetate and 1.25% acetic acid. Electrolyte solutions were prepared freshly and not used after long storage periods. Fresh running electrolyte were used to flush the capillary before each run.

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

As the electrolysis of the electrolyte solution can occur and subsequently change the electrosmotic flow (EOF), the separation electrolyte was replaced every six injections. When the capillary was not in use, it was washed with 0.1 M NaOH, water and dry-stored.

2.3.2. Extraction and preconcentration procedure

The extraction of tamoxifen, imipramine and their main metabolites from the urine samples was performed in a reverse-phase C_{18} 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).

Sample passage, different volumes (between 2 and 12.5 mL) of urine were slowly loaded into the conditioned cartridge. Once the retention step had been completed, the cartridge was washed with 8 mL of 10 mM phosphate buffer (pH 7.0), 4 mL of a 50% methanol–water solution and 0.5 mL of methanol. Finally, elution of the retained components was achieved with 2.5 mL of methanol. This extract was immediately injected into the capillary electrophoresis equipment.

Quantification was performed by evaluating the normalized area of each studied compounds versus the internal standard area (paroxetine).

2.3.3. Treatment of the urine samples

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

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

3. Results and discussion

The first experiments to separate tamoxifen, imipramine and their metabolites were carried out in aqueous buffer solutions, but due to the hydrophobic nature of tamoxifen and its metabolite (4-hydroxytamoxifen), baseline separation of the investigated compounds was not possible neither to pH basic nor acid.

An advantage of using organic solvents is the increase in selectivity compared to aqueous separations, there are two main reason for this. First, the influence on the acid–base properties of the analytes varies among the solvents for different groups. Thus, there is a possibility of separating substances that have very similar pK_a values in aqueous systems and that would not be separated in water. Second, the organic solvents show a different solvating power relative to each other and to water. Since the migration velocity of substances in the electric field depends on the radius of the solvated ions, higher selectivity results through the use of organic solvents [32–34].

3.1. Optimisation of the NACE procedure

3.1.1. Selection of BGE

Several mixtures ACN/MeOH containing 20 mM ammonium acetate and 1% acetic acid were tested for the separation of the investigated compounds in order to explore the relationship between separation selectivity of the studied compounds and the ACN/MeOH composition in the BGE. As expected, varying the methanol percentage in acetonitrile induced changes in terms of migration time and resolution due to concomitant modifications of viscosity and dielectric constant. In particular, resolution increase with the MeOH percentage, but also migration times of the studied compounds. Beyond 70% of acetonitrile in the mixture, paroxetine and 4-hydroxitamoxifen change their order of elution.

A 80:20 (v/v) MeOH–ACN solution was selected as a good compromise in terms of resolution, efficiency and separation time.

A mixture of acetic acid (HOAc) and ammonium acetate (NH_4OAc) was investigated as BGE because it is a suitable electrolyte for direct UV-detection of anions and cations in the most organic solvents. Therefore, the ionic strength of the electrolyte is depending on the concentration of NH_4OAc and HOAc added to the BGE.

In order to investigate the electrolyte effect on separation performance; first, the influence of the concentration of NH_4OAc (8–20 mM) on the migration time and resolution between peak of the investigated compounds was studied. All the electrolytes were prepared maintaining 1.0% of HOAc constant in acetonitrile–methanol, 20:80 (v/v). As expected, when the concentration of NH_4OAc increases the migration times of tamoxifen, imipramine and their metabolites and the generated electric current also increases. A 17 mM NH_4OAc concentration was selected as optimal since this value maintains good peak shape, very low generated electric current (<20 μA) and the better resolutions between peaks for all the studied compounds.

In the same way, the influence of increasing amounts of HOAc (0.5–1.5%) over resolution and migration times was studied with electrolytes that contain 17 mM of NH_4OAc in acetonitrile–methanol, 20:80 (v/v). With high values of percent HOAc in the BGE decreases the migration times of the compounds studied and increase the generated electric current and resolution between peaks. A value of 1.25% of HOAc was selected as a good compromise in terms of resolution, generated electric current and separation time for the drugs studied.

3.1.2. Selection of the voltage applied

The effect of the voltage applied from 8 to 25 kV was investigated. When the voltage increases, the migration time of all the drugs decreases, this effect is too observed in CE in aqueous buffer. A voltage of 15 kV yielded the best compromise in terms of run time, low generated electric current and resolution between peaks. Voltages higher than 23 kV generate currents higher than 25 μA. It was verified that in NACE, currents higher than 20–25 μA implicate bubble formation and solvent evaporation, and therefore, current cuts.

3.1.3. Selection of temperature

Capillary temperature control is important in CE, since changes in capillary temperature can cause variations in efficiency, migration times, electric currents and resolution between peaks. The study of this parameter on the separation was tested in the range 18–28 °C. Generally, when the temperature increases, the resistance of the buffer goes down, and as the electric field is constant, the generated electric current increase. Solute migration times increased because of changes in the viscosity the medium but the decrease in the migration times of the studied drugs at higher temperatures results in poor resolutions between all the peaks. The selected temperature was 22 °C because it provided the best resolution, run time not too long and generated electric current is lower than 20 μA.

3.1.4. Selection of the washing step

To prevent difficulties owing to adsorption of the sample components and to ensure a consistent EOF, the capillary is flushed between injections with the electrolyte during 1 min (when samples were dissolved in methanol). But, when it works with urine samples, a rinse step of 1 min of electrolyte was inadequate because the matrix is very complex and adsorption onto the capillary surface is higher than methanolic samples. In this case, it was necessary a rinse step of 3 min with electrolyte in order to obtain an unchanging EOF.

3.1.5. Summary of optimum conditions

From the studies described above, the electrophoretic conditions selected were:

- Electrolyte: 8:2 methanol–acetonitrile containing 17 mM ammonium acetate and 1.25% acetic acid;
- Voltage: 15 kV, current: 15.4 μA;
- Capillary: fused-silica (57 cm × 75 μm i.d.);
- Injection: hydrodynamic, 6 s;
- Temperature: 22 °C;
- Detection wavelength: 211 mm.

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

The optimisation of the SPE method requires a detailed study of the composition of the injection medium. Variables such washing stages using different solvents for elution of the analytes free from interferences, the percentage of acetonitrile–methanol and concentration of NH_4OAc and AcOH in the injected sample (extract) were studied.

The best results were obtained when the cartridge charged with the urine sample was washed with 8.0 mL of 10 mM phosphate buffer (pH 7.0), 4.0 mL of a 50% methanol–water solution and 0.5 mL of methanol in order to minimise interferences. Finally tamoxifen, imipramine and their mains

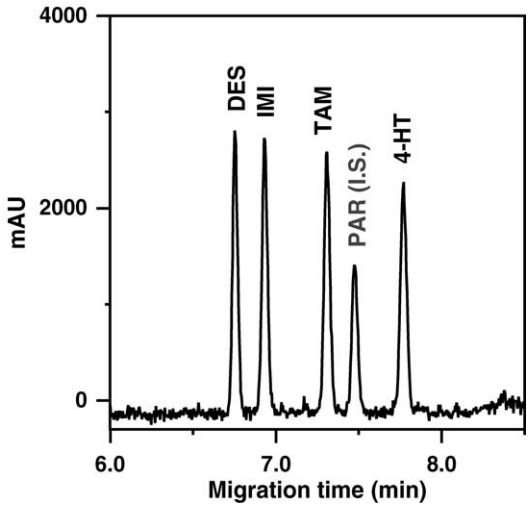

Fig. 2. Electropherogram for the extract from a urine spiked with 0.25 mg L^{-1} of tamoxifen, imipramine, their metabolites and paroxetine (IS). Operating conditions: nonaqueous system of 80:20 methanol–acetonitrile containing 17 mM ammonium acetate and 1.25% acetic acid; hydrodynamic injection, 6 s, 15 kV and 22 °C.

metabolites were eluted with 2.5 mL of methanol. The sensitivity of the preconcentration step is a function of the ratio of the initial volume of urine sample charged in the cartridge (capacity of the cartridge) and volume of extract obtained. In our work, maximal capacity of the cartridge was investigated and established in 12.5 mL, therefore, it was possible to preconcentrate five times.

Fig. 2 shows the electropherogram corresponding to the extracts from 10 mL of human urine spiked with 0.25 mg L^{-1} of each studied compounds.

3.3. Validacion of the NACE procedure

3.3.1. Linearity

The linearity of the response was examined by the injection of seven spiked urine samples after SPE treatment (n = 7). The linearity was tested over the range from 0.05 to 1.0 mg L^{-1} for each substance in the urine. In all the cases, 0.4 mg L^{-1} of paroxetine was added as internal standard. This process was repeated two different days. The first day, it was analysed of a young woman urine and the second day a mixture of four different persons (one man and three women with different ages). The results were given in terms of relative peak areas and the obtained regression lines pass through the origin in all the cases.

An analysis of variance (ANOVA) test was performed to compare the different regression lines obtained, to determine whether the data could be combined to enable estimation of appropriate quantities by use of a comprehensive regression line. F_{exp} compares the deviation between set lines with the deviations within each set from the set lines. In all the cases, the experimental value of F is lower than the theoretical value of F for different urines. For this reason, we can propose a global calibration graphs with representative slopes (Table 1).

As consequence of the previous studies, determination of all the studied drugs can be performed by direct measurement from the calibration graph.

3.3.2. Recovery

In order to test the accuracy of the proposed method, several aliquots of tamoxifen, imipramine, their metabolites and paroxetine standard solutions were added into human urine samples. These samples were analysed using the extraction, preconcentration and electrophoretic procedures described in this work. The concentration found in the test solutions were calculated by reference to the duplicate bracketing standard solutions and the recoveries obtained (upon relative peak areas measurements with regard to the internal standard) for these test solutions are shown in Table 2. As it can be seen, recoveries (mean of three values) very close to 100% were obtained in all cases.

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 nonaqueous electrophoretic procedure, eight injections of a standard of 2 mg L^{-1} of tamoxifen, imipramine their metabolites and paroxetine were carried out sequentially. This operation was repeated over 3 days. The precision of the migration times and relative peak areas were good with a R.S.D. (n = 24) between 0.20 and 0.24% for retention times and between 1.49 and 2.87% for relative peak areas for all the studied compounds.

To evaluate the precision of the overall process (extraction, preconcentration and NACE step), eight different urine samples belong to two different women, spiked with 1.0 mg L^{-1} of the drugs under study were subjected to independent triplicate analysis varying the extraction volume, therefore, varying the factor of concentration. The average recoveries obtained from the spiked urine samples were very good (near to 100%) with a standard deviation (S.D.) between 1.67 and 2.83 for all the studied drugs.

To determine the precision in the recoveries of tamoxifen, imipramine and their metabolites, a mixture of five different urine samples (taken in the morning and in the afternoon) were spiked with three different concentration (from 0.5 to 2 mg L^{-1}) of each drug. Excellent recoveries were obtained (between 96.5 ± 3.5% and 101.8 ± 4.6%) for determination of each substance in the mixture of urine samples. Similar recoveries of tamoxifen, imipramine and their metabolites were obtained for the same mixture of urine samples submitted to a frozen process. So, there are not significant differences between drugs recoveries from sanguinus urine (morning samples) or the afternoon urine nor between fresh or frozen urine samples.

3.3.4. Specificity

As in any separation technique, co-elution of peaks is possible in capillary electrophoresis; and therefore, it was

Table 1
Linearity (n = 7): LODs and LOQs for the four study drugs

	Global equation*	Coefficient of correlation	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
DES	$y = (0.672\pm0.146) + (4.210\pm0.070)x$	0.987	15	50
IMI	$y = (0.057\pm0.063) + (4.387\pm0.008)x$	0.982	11	35
TAM	$y = (0.206\pm0.524) + (5.473\pm0.009)x$	0.991	10	33
4-HT	$y = (0.489\pm1.052) + (6.225\pm0.150)x$	0.984	9	31

* Concentration (x, $mg\,L^{-1}$) vs. relative peak area (y).

Table 2
Recovery of human urine samples*

Sample	Desipramine		Imipramine		Tamoxifen		4-Hydroxytamoxifen	
	Added ($mg\,L^{-1}$)	Recovery (%)	Added ($mg\,L^{-1}$)	Recovery (%)	Added ($mg\,L^{-1}$)	Recovery (%)	Added ($mg\,L^{-1}$)	Recovery (%)
1	0.05	97.5 ± 4.0	0.05	96.2 ± 4.7	0.05	100.7 ± 3.2	0.05	99.8 ± 1.9
2	0.1	95.1 ± 4.2	0.1	100.4 ± 5.0	0.1	100.5 ± 3.6	0.1	95.5 ± 1.5
3	0.3	98.1 ± 3.8	0.3	98.1 ± 4.9	0.3	98.2 ± 3.5	0.3	101.7 ± 1.6
4	0.5	99.6 ± 4.7	0.5	94.6 ± 4.7	0.5	100.8 ± 3.6	0.5	99.9 ± 1.6
5	0.7	99.3 ± 3.6	0.7	98.4 ± 4.9	0.7	97.5 ± 3.5	0.7	97.9 ± 1.5
6	0.9	97.9 ± 4.1	0.9	96.9 ± 4.8	0.9	96.8 ± 3.4	0.9	94.4 ± 1.5
7	1.0	98.5 ± 3.9	1.0	101.2 ± 5.0	1.0	100.1 ± 3.5	1.0	95.4 ± 1.5

* Mean value ± S.D. (n = 3).

interesting and useful investigate the homogeneity or purity of the obtained peak. Specificity can also be determined by measurement of homogeneity peak. 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]:

- absorbance at two wavelengths;
- normalization and comparison of spectra from different peak sections.

Both techniques showed that the purity of the peaks corresponding to the compounds studied in urine present a high level of purity. Therefore, no interference 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 in six urine blanks. The standard deviation (S.D.) of six noise's electropherograms at tamoxifen, imipramine and their metabolites migration times using the maximal sensitivity allowed by the system was obtained. LODs was estimated by multiplying the S.D. by a factor of 3 whereas the LOQ was defined as 10 times the S.D.

The LODs and LOQs obtained, taking into account a concentration factor of 5 for all the studied compounds (from the extraction–preconcentration process), are summarised in Table 1. The LOQs were subsequently experimentally evaluated by the analysis of two different samples prepared by adding concentrations corresponding to the LOQ for each studied drug to urine samples and subjecting these samples to the analytical procedure. The obtained relative errors were less than 10% in all the cases.

3.3.6. Ruggedness/robustness

The aim of a ruggedness/robustness test may be to predict reproducibility or intermediate precision estimates [37]. Fractional factorial designs developed by Plackett and Burman [38] were used, based on balanced incomplete blocks according to procedures described by Youdner and Steiner [39].

The choice of variables and the levels at which to test them is very important if the ruggedness test is to be of value. Variables must be those which are likely to be significant in practice, and the levels must reflect the variation which are usually observed. The variables and levels selected in our study are:

A. Voltage ($12_{(-1)}$, $15_{(0)}$, $18_{(+1)}$) (kV).
B. Injection time ($5_{(-1)}$, $6_{(0)}$, $7_{(+1)}$) (s).
C. Concentration of NH_4OAc ($14_{(-1)}$, $17_{(0)}$, $20_{(+1)}$) (mM).
D. Percent acetic acid ($1.0_{(-1)}$, $1.25_{(0)}$, $1.5_{(+1)}$).
E. Percent ACN ($15_{(-1)}$, $20_{(0)}$, $25_{(+1)}$).
F. Temperature of the separation ($20_{(-1)}$, $22_{(0)}$, $24_{(+1)}$) (°C).
G. Detection wavelength ($209_{(-1)}$, $211_{(0)}$, $213_{(+1)}$) (nm).

The three values used for each variable are shown in parentheses, where level (−1) is the minimum value studied, level (0) is the optimal value for the method and level (+1) is the maximum value tested in this experiment.

The mean value of each variable is the average difference between observation made at the extreme levels and those made at the optimal level. Mean effects and standard errors (DA, DB, DC, . . .) were calculated using the procedures described by Youden and Steiner.

The ruggedness was determined from triplicate injections of a methanolic solution of 2.0 $mg\,L^{-1}$ of tamoxifen,

imipramine and their metabolites in presence of paroxetine (internal standard). Results of the effect of each factor's levels over efficacy, resolution, areas and relative peak areas were calculated.

Taking into account the deviations calculated for the different checked results when the selected operating factors were tested upon the experimental design of Plackett–Burman and the statistical treatment of Youden–Steiner, this analytical method for measuring of tamoxifen, imipramine and their metabolites has proved to be rugged to all the variations tested in this work. The validity of the Plackett–Burman design is confirmed for the purpose of ruggedness testing.

The main interaction effects over area measurement is produced by voltage and injection time; for this reason, in electrophoresis, it is recommendable to work with corrected peak areas or internal standard. The main interaction effects over resolution are produced by injection time (as it was proved in the optimisation section) and as it was expected in NACE, by the composition of the mobile phase (% of acetonitrile (DE), % of acetic acid (DD), concentration of NH_4OAc (DC)) because the electrolyte composition is the most important parameter in NACE in order to have a good resolution and the best efficacy. But ruggedness obtained in all cases (using the statistical method of Youden and Steiner) allow to use this method by different laboratories, analysts or instrument without any appreciable error.

3.4. Applications

Several urine samples of different voluntaries (patients undergoing medical treatment) were analysed in order to demonstrate the applicability of the extraction, preconcentration and NACE procedure developed.

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.

Firstly, urine of two different voluntaries undergoing medical treatment with tamoxifen were analysed. Woman A receives 20 mg day^{-1} of tamoxifen orally (2 months of treatment), and woman B takes 20 mg day^{-1} of tamoxifen orally (6 months of treatment).

The results obtained from urine samples are given in Table 3. All determination were carried out in triplicate. In order to evaluate the possible matrix effect, the method of standard addition was used for the determinations of these compounds in human urine. In all cases, the application of the *t*-test for the slopes of the calibration graphs showed no statistical differences. Consequently, there is no evidence of systematic error affecting the determination of tamoxifen and 4-hydroxytamoxifen in urine by the proposed method. Concentrations found using standard addition are shown in Table 3 and as can be seen, they coincide with those obtained by direct measurement by the proposed method. One electropherogram of this experiments under conditions optimised in this paper is shown in Fig. 3, as it can be seen no interference from the matrix was observed.

Table 3
Analysis of human urine samples*

Volunteer	Standard addition (μg L^{-1})		Direct measurement (μg L^{-1})	
	TAM	4-HT	TAM	4-HT
Woman A	52.0 ± 1.3	36.2 ± 1.8	53.1 ± 0.9	37.4 ± 1.8
Woman B	70.0 ± 1.6	84.2 ± 1.5	65.0 ± 2.1	85.3 ± 1.2

* Mean value ± S.D. (n = 3).

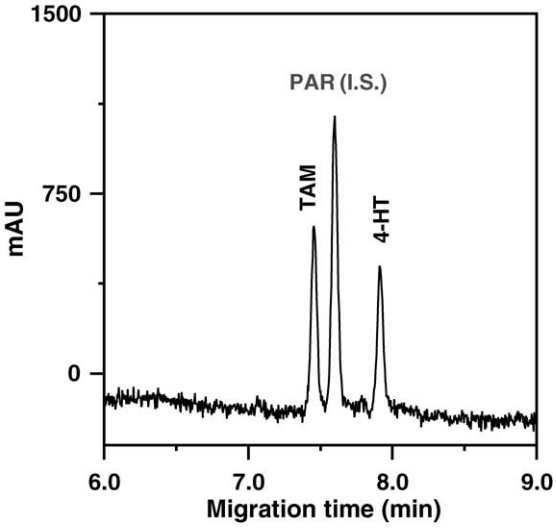

Fig. 3. Electropherogram of a urine sample from a woman on tamoxifen treatment (2 months) after 7 h of the administration of 20 mg dosage.

Also, the urine from a volunteer undergoing treatment with imipramine (25 mg day^{-1}) was analysed at different times after administration of imipramine, in order to make a pharmacokinetic study. The concentrations of imipramine and desipramine found using this method at different time intervals are shown in a typical time course of urine imipramine and desipramine levels in Fig. 4.

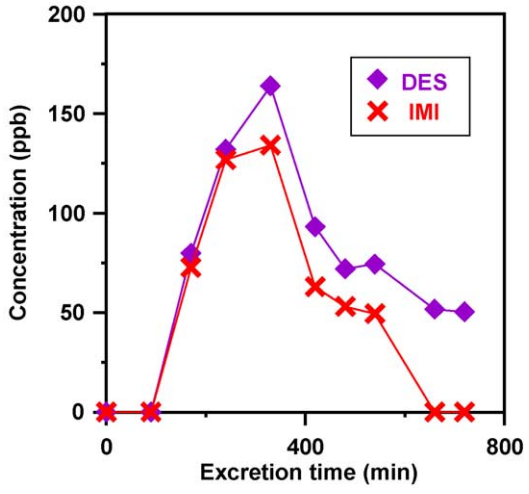

Fig. 4. Time course of urine imipramine and desipramine levels in a volunteer receiving 25 mg of the drug orally.

4. Conclusions

The electrophoretic (NACE) method has been validated for the analysis of the four compounds in human urine without any matrix interference. It has been shown that the experimental results with respect to linearity, accuracy, specificity, sensitivity, precision and ruggedness of the test validation demonstrate the reliability of the electrophoretic procedure for its intended application. Due to the breast cancer being an illness very frequent and tamoxifen can generate a depression, it is very important to have a method that enables determination of both types of substances in human urine.

Acknowledgements

The authors want to thank Novartis Pharma AG and GlaxoSmithKline Laboratories for the help in the research sample contribution of imipramine, desipramine and paroxetine. The authors are also grateful to the DGES of the Ministerio de Educación y Ciencia for the financial support (Project BQU 2001-1190).

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