Juana Rodríguez-Flores
Ana M. Contento Salcedo
Maria J. Villaseñor Llerena
Lorena Muñoz Fernández

Department of Analytical Chemistry and Foods Technology, University of Castilla-La Mancha, Ciudad Real, Spain

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Research Article

Micellar electrokinetic chromatographic screening of letrozole and its metabolite in human urine: Validation and robustness/ruggedness evaluation

A simple, rapid, and sensitive method has been proposed and validated to directly quantify letrozole (LE) and its metabolite, bis-4-cyanophenylmethanol (ME) in urine samples (without any additional treatment) by micellar electrokinetic capillary chromatography (MEKC). In an effort to improve the selectivity and sensitivity of the method, the chemical and instrumental parameters were optimized. The best conditions were: 70 mM borate buffer (pH 9.2) and 40 mM SDS as BGE, 25 kV and 20°C as working voltage and temperature, respectively, with hydrodynamic injection for 6 s. The reliability of the proposed method was also proved by means of a validation procedure based on precision, accuracy, linearity, LOD (15 μg/L for both of them) and LOQ studies. Moreover, an innovatory experimental and statistical design approach, upon a Plackett-Burman fractional factorial model, which involves the simultaneous evaluation of the global robustness and ruggedness effects, was applied. As it has been already stated, the proposed method has been successfully used to directly quantify both compounds in human urine samples, without any additional treatment, but the previously reached LOD and LOQ values can be improved by applying an SPE preconcentration procedure, also developed and optimized by the authors in this work. Real determinations of these analytes in clinical urines of a patient under LE treatment were performed, too.

Keywords:
Breast cancer / Letrozole / Micellar electrokinetic capillary electrophoresis / Human urine
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1 Introduction

Aromatase (estrogen synthetase) is an enzyme that catalyses various steps in the conversion of androgens into estrogens. Letrozole (LE) is a potent and selective nonsteroidal aromatase inhibitor that limits, *e.g.*, the conversion of adrenally, which could generate androstenedione and testosterone that would be transformed into estrone and estradiol, respectively, in peripheral tissues, as well as in tumors. It also significantly decreases the estradiol concentration in serum for postmenopausal women. Besides, it provides a powerful treatment for postmenopausal women with hormone-sensitive breast cancer [1], by actuating on estrogens-dependent tumors cell death. The recommended therapeutic dose for LE is 2.5 mg/day. The major metabolic elimination route of LE allows to bis-4-cyanophenylmethanol (its main metabolite; ME) (Fig. 1). LE and its ME are excreted unchanged and their apparent elimination half-life time in plasma have been reported within approximately two days (Novartis Pharmaceutical Canada, http://www.pharma.ca.novartis.com/downloads/e/femara_scrip_e.pdf).

Recently, new positive experiences about treatment of epilepsy and male infertility have been reported [2, 3] (Schust, D. J., Aromatase inhibitors in infertility, http://www.veritasmedicine.com/archives.cfm?did=23&itemid=2713&mode=2). In the previous literature, LE is freely offered as an "anti-aging drug" too (Biogenesis Laboratories, http://www.biogenesis.co.za/pi-femara.asp (online 14.12.2004)). Femara may be used "unofficially" (in about 1.25 mg) once or twice *per* week by men who suffer a de-

Correspondence: Dr. Juana Rodríguez-Flores, Department of Analytical Chemistry and Foods Technology, University of Castilla-La Mancha, E-13071 Ciudad Real, Spain
E-mail: juana.rflores@uclm.es
Fax: +34-926-295318

Abbreviations: LE, letrozole, **ME,** bis-4-cyanophenylmethanol (letrozole's main metabolite)

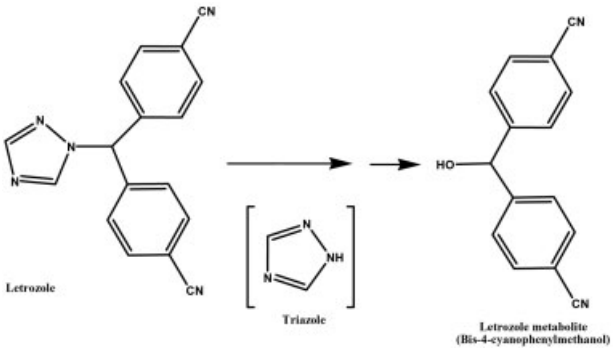

Figure 1. Chemical structures and main metabolic pathway of LE and its ME.

crease in their estrogen levels and they want to increase their testosterone levels. Until now, just two methods have been reported for LE and ME determination. Marfil *et al.* [4] published an HPLC method with a previous fully automated liquid–solid extraction and fluorescence detection, which offers high sensitivity for the quantization of LE in plasma and urine, but not for its ME in any of them. Mareck *et al.* [5] reported a gas chromatographic/mass spectrometric method for their determination, too; but this method required a complex and long treatment of the urine with different steps, *e.g.*, extraction, incubation of the sample during 1 h, dryness evaporation, adding of organic solvent and finally a derivatization step [5]. Our objective is to propose a fast and simple method for a reliable quantification of LE and its ME on human urine directly without sample treatment. However, the lacking of interferences has been also proved by a selectivity/specificity study. The evaluation of method's reliability to quantify LE and its ME at clinical levels has been also performed in terms of a validation and of an integral robustness/ruggedness study. Moreover, the authors have also optimized an SPE preconcentration procedure to determine both analytes in urine at lower levels just if necessary.

2 Materials and methods

2.1 Reagents and solutions

All solvents and reagents were of analytical grade unless indicated otherwise. All the solutions were prepared with deionized water (Milli-Q quality). LE and its ME were supplied by Novartis laboratories (Spain). Standard stock solutions of analytes were prepared by dissolving the appropriate amounts of pure substances in 100 mL of ethanol–water 50:50 v/v to give a final concentration of 100 mg/L. The resulting solutions were stored at 4°C. Electrophoretic separation was driven using a borate buffer solution (pH 9.2, 70 mM) and 40 mM SDS as BGE. This BGE was daily prepared and the separation vials sets were changed every six runs. The buffer solution was prepared by dissolving the appropriate boric acid amount in deionized water and then by adding NaOH to reach a 9.2 pH value. All these reagents were from Panreac (Barcelona, Spain).

2.2 Apparatus

A Beckman P/ACE 5510 CE system (Palo Alto, CA, USA), fitted with a DAD was used for data acquisition. The system was controlled by a Dell Dimension P133V card with a P/ACE station software for data treatment. Separations were carried out on a 57 cm total length (50 cm effective) × 75 μm id × 375 μm od fused-silica capillary, placed in a cartridge with a 800 × 75 μm detection window. The extraction and preconcentration processes were achieved using device developed in our laboratories. This apparatus consisted of a Supelco (Bellefonte, PA, USA) water manifold coupled to a Millipore XF 5423050 vacuum pump. The SPE step was performed using C_{18} cartridges (500 mg) obtained from Waters (Milford, MA, USA). pH measurements were performed using a Crison model 2002 pH meter with a combined glass electrode (Alella, Barcelona). Urine centrifugation was carried out with a Selecta apparatus (Abrera, Barcelona).

2.3 Urine sample treatment

Fresh human urine samples were obtained from different healthy volunteers, whereas clinical urine samples were provided by a woman (58 years old), who has been submitted to LE treatment for three years. Extraction of LE and its ME from the biological samples was performed with the next procedure after a centrifugation step (5000 rpm, 5 min, 20°C) and they were subsequently introduced into the CE equipment.

2.4 Extraction-preconcentration procedure

Extraction of LE and its ME from the urine samples was performed in an RP C_{18} cartridge (Waters Sep-Pak Plus). Every step of this SPE procedure (nature and volume of organic and aqueous solvents in washing stages, sample volume, elution volume, final volume of extracts, *etc.*) was evaluated in an effort to achieve a complete extraction. Owing to the high level of complexity of the urine matrix, were the analytes under investigation were present at very low levels this SPE procedure was also necessary as a preconcentration and cleaning stage. The cartridge was conditioned prior to use with methanol (5 mL) followed by a phosphate buffer solution (5 mL, 10 mM, pH 7.0). After that, different volumes of urine (between 2 and 8 mL) were loaded into the conditioned cartridge. Once the retention step had been completed, the cartridge was washed with phosphate buffer (8 mL, 10 mM, pH 7.0) and consecutively with a methanol/water solution (1.5 mL, 20:80 v/v). Then, LE and ME were eluted with methanol (1.5 mL). Later on, this methanolic extract was evaporated to dryness by a

nitrogen stream and finally, it was reconstituted with Milli-Q water (1 mL) and then transferred to the appropriate vials to be injected into the CE equipment.

2.5 Operating conditions

Prior to the first use, the capillary was conditioned by consecutive flushings with 0.1 M NaOH for 20 min, deionized water for 10 min, and finally electroconditioning with the BGE solution for 10 min. Between consecutive separations the capillary was rinsed with 0.1 M NaOH for 2 min and the separation buffer for 5 min in this order to avoid adsorption processes in the internal surface of the capillary. Prior to overnight storage the capillary was washed with 0.1 M NaOH and water for consecutive periods of 20 min, followed by a drying stage with air for 10 min. Different vials of electrolytes were used for rising and separating operations in order to maintain a constant electrolyte level on the anodic side. The set of separation vials was changed after every six separation runs. Injection of the samples was performed by hydrodynamic mode for 6 s and maintaining the anodic vial at 8°C in order to avoid sample degradation. Separations were performed at 25 kV for 13 min at 20°C. Under these conditions the current was 85.4 μA. Duplicate injections of the solutions were performed and average peak areas were used for the quantization.

3 Results and discussion

3.1 Optimization of the separation conditions

In the pH range 2.0–12.0 a baseline separation of the studied compounds by CZE was not observed. For this reason we tried this separation by micellar electrokinetic capillary chromatography (MEKC) and using SDS as micellar additive. From the pH study in this new BGE, a 9.2 pH value was selected since it provided the best resolution between peaks by MEKC. The selection of buffer concentrations was performed by preparing a set of five borate buffer solutions at concentrations ranging from 20 to 80 mM (pH 9.2, 40 mM SDS, 25 kV, 20°C). Their effects on migration times and resolution between peaks were investigated, showing that an increasing in buffer concentration led to higher migration times for both compounds. Thus, a buffer concentration of 70 mM was selected as a compromise solution among good peak shape, low current, and baseline resolution. The effect of SDS concentration on migration times and resolution between peaks was researched over the range of 20–60 mM (pH 9.2, 70 mM borate buffer, 25 KV, 25°C). Our results reveal that the SDS concentration has a big influence on the mobility of the two compounds. As expected, when the micelle numbers increased, lower mobilities and longer migration times were obtained for both compounds. So, a 40 mM SDS concentration was selected as optimal value. The effect of varying the voltage from 15 to 30 kV was checked under the conditions already chosen. A 25 kV voltage value was selected as the best compromise in terms of run time, generated current, and efficiency in the separation. The effect of temperature on the separation was tested between 18 and 25°C. As expected, an increase of temperature resulted in an increase of the EOF that allows a decrease of migration times due to the electrolyte viscosity decreasing. A 20°C cartridge temperature was selected as suitable with regard to resolution, run time, and generated current (85.4 μA).

In order to improve the sensitivity of the analysis for LE and ME on human urine, the injection time was varied between 2 and 9 s. As it was expected again, an increase in injection times led to bigger peak areas for both compounds, but from 6 s value a loss of resolution between peaks was observed. For this reason the optimal chosen value was 6 s. The used pressure for injection step was always 0.5 psi (1 psi = 6894.8 Pa).

From the performed studies, we can conclude the following conditions as the most suitable ones for an efficient separation of the studied compounds: (i) Capillary: 57 cm fused-silica capillary (50 cm effective length) × 75 μm inner diameter and an 800 × 75 μm detection window. (ii) BGE: 70 mM borate buffer, pH 9.2, 40 mM SDS. (iii) Injection: hydrodynamic, 6 s, 0.5 psi (1 psi = 6894.76 Pa). (iv) Temperature: 20°C. (v) Voltage: 25 kV. (vi) Detection wavelength: 203 nm.

3.2 Reliability of the method

In order to asses the reliability of our MEKC, its analytical performance characteristics were evaluated on spiked urine from healthy volunteers.

3.2.1 Stability of solutions

The stock (ethanol 1/1, 100 mg/L) and diluted (water, 5 mg/L) solutions of LE and ME were stored at 4°C and were found to be stable for at least one month and 7 days, respectively. This stability study was performed by spectrophotometric measurements.

3.2.2 Precision

The precision of the proposed method for determining LE and its ME was investigated in repeatability and intermediate precision terms for migration times and peak areas of both compounds in accordance with the ICH (International Conference on Harmonization) criteria. The results were expressed as RSD. To perform repeatability studies, seven injections of a spiked urine standard solution containing LE and ME (1 mg/L) were carried out sequentially. The RSD values were lower than 0.1 and 0.34% for LE and ME, respectively, for migration times, and lower than 4.0 and 4.8% for peak areas. With regard to intermediate pre-

cision, this operation was repeated on different days. RSD values lower than 0.82% (LE) and 0.78% (ME) were obtained for migration times whereas for peak areas these values were lower than 5.42% (LE) and 4.86% (ME). Comparison of the two sets of data with the aim of detecting random errors was carried out by applying the Snedecor *F*-test on these RSD values. Significant differences were not found in any case at a confidence level of 95% and seven freedom degrees.

3.2.3 Linearity in the working concentration range

The linearity of the proposed method was checked by injecting urine solutions spiked with the drugs at concentrations ranging from 0.05 to 1.5 mg/L for both compounds. The calibration was determined from triplicate injections at eight different concentration levels for every compound. The satisfactory linear regression equations and their regression coefficients (Table 1) could indicate the linearity of LE and ME responses over the studied concentration range and that these lines pass through the origin.

However, according to the Analytical Methods Committee (AMC) [6], a regression coefficient close to unity is not necessarily the outcome of a linear relationship and, as a consequence, the lack of fit should be checked in order to confirm linearity. This test was carried out by plotting the residuals (distances of the experimental points from the fitted regression lines) *versus* concentration. If there is no lack of fit (*i.e.*, the calibration is inherently linear), the plot will look like a random sample from a normal distribution with zero mean. This is the situation observed. This test was applied to our calibration graphs and so the linear nature of the relationship was thus confirmed.

3.2.4 Accuracy

In order to test the accuracy of the proposed method, several aliquots of LE and ME solutions were added into human urine samples. These samples were analyzed using the proposed electrophoretic procedure. As it can be observed in Table 2, recoveries between 96 and 105.2% were obtained in all cases. Recoveries were calculated *versus* external standards with lower and upper concentrations for each sample. The use of a photodiode detector allowed us to confirm the identity of LE and ME peaks in urine samples not only by their migration times, but also by the overlay of the UV–Vis spectra of the peak urine samples with that one of a standard solution.

3.2.5 LODs and LOQs

The LODs and LOQs were estimated in the usual way. The LOD was obtained as the concentration of the drug corresponding to a peak three times higher than baseline noise level. The LOQ was calculated as three times the LOD. The reached LOD and LOQ values for both compounds are shown in Table 1. The LOQs were subsequently validated by the analysis of five different blank urine samples spiked with amounts of each compound corresponding to their respective LOQs. The relative errors obtained in this verification were lower than 10% in all cases.

Table 1. Statistical parameters of the MEKC method

	LE	ME
Calibration equation	$Y = 11\,759.12X + 436.73$	$Y = 13\,328.72X + 202.71$
R^2	0.9912	0.9923
Working concentration range (mg/L)	0.05–1.5	0.05–1.5
SD slope	530.3	623.26
SD intercept	396.1	465.5
LOD (μg/L)	15	15
LOQ (μg/L)	45.1	45.1

Table 2. Accuracy of the proposed MEKC method

Sample	ME		LE	
	Added (mg/L)	Recoveries (%)	Added (mg/L)	Recoveries (%)
1	0.1	102.3	0.1	104.8
2	0.25	104.0	0.25	96.0
3	0.5	99.0	0.5	104.0
4	0.7	104.0	0.7	105.2
5	1.2	100.5	1.2	101.6

3.2.6 Specificity

Comigration of peaks is also possible in MEKC as in any other separation technique. It was therefore useful to investigate the homogeneity of the separated peaks. The techniques used to assess the purity of LE and ME peaks in the clinical human urine samples were reported previously [7] and involved: (i) normalization and comparison of spectra from different peak sections, and (ii) comparison of absorbance at two different wavelengths. Both techniques proved the purity of all of the peaks and even interferences from the urine matrix can be ruled out. Representative electropherograms are shown in Fig. 2 for drug-free human urine and for LE and ME spiked urine. The compounds were well separated from urine endogenous components and the lacking of interferences was proved at the retention times of studied drugs.

Furthermore, several urine samples were spiked with others drugs commonly used in patients with breast cancer like several antidepressants (venlafaxine, fluvoxamine, fluoxetine, and clomipramine), anticancerigenic (5-fluoro-

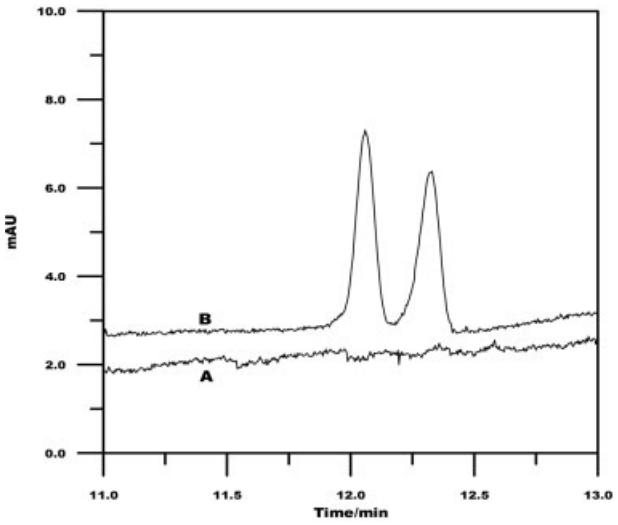

Figure 2. (A) Blank of urine. (B) MEKC electropherogram of a urine sample spiked with 1 mg/L of LE and its ME. Operating conditions: hydrodynamic injection (6 s, 0.5 psi); separation: 70 mM (pH 9.2) borate buffer and 40 mM SDS, 25 kV as voltage of separation, 20°C as capillary temperature and detection at 203 nm.

acile, tamoxifen, anastrozole, methotrexate) and anti-inflammatory (ibuprofene). Once again, interferences were not observed in these cases.

3.2.7 Integral robustness–ruggedness evaluation

The United States Pharmacopeia (UPS) defines ruggedness as "the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of normal test conditions such as different days, several reagent lots, different lots, different instruments, various laboratories, different elapsed assay times…" where all of these factors are external to the written analytical method. The robustness of a method is defined by both the USP and ICH Tripartite guidelines as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal use [8]". Ruggedness can therefore be regarded as a measure of the absence of external influences on the test results, whereas robustness measures the lack of internal influences on these results. In this work we have tested the influence of variations in both internal and external parameters of the method (*e.g.*, pH and ionic strength of buffer, SDS concentration, voltage, capillary temperature, different days for analysis, *etc.*), whose influence has been studied at different levels. The Plackett–Burman fractional factorial model, which is based on balanced incomplete blocks, was employed to evaluate this aspect of the method. For statistical reasons (concerning effects on interpretation), designs with fewer than eight experiments are not used, while those with more than 24 experiments were considered unpractical [9, 10]. To date, this model has been usually satisfactorily applied just in the evaluation of robustness. A novel Plackett–Burman design that involves the evaluation of both robustness and ruggedness effects (11 factors and 12 experiments, $N = 12$) is presented in Table 3 (http://www.locumusa.com/pdf/general/article01.pdf). The choice of variables (factors) and the levels at which they are tested is very important for a reliable robustness/ruggedness test. In our case, the variables selected as factors have been instrumental and chemical parameters that are significant in the performance of the proposed method. The selection about the levels of these factors should reflect slight variations which could be usually observed. The external (ruggedness) and internal (robustness) factors (A–K) selected for our model are

Table 3. Experimental design for the whole robustness–ruggedness evaluation using the Plackett–Burman model

Number of experiments	External/internal changes or variations (11)										
	Selected factors										
	A	B	C	D	E	F	G	H	I	J	K
1	+	+	−	+	+	+	−	−	−	+	−
2	−	+	+	−	+	+	+	−	−	−	+
3	+	−	+	+	−	+	+	+	−	−	−
4	−	+	−	+	+	−	+	+	+	−	−
5	−	−	+	−	+	+	−	+	+	+	−
6	−	−	−	+	−	+	+	−	+	+	+
7	+	−	−	−	+	−	+	+	−	+	+
8	+	+	−	−	−	+	−	+	+	−	+
9	+	+	+	−	−	−	+	−	+	+	−
10	−	+	+	+	−	−	−	+	−	+	+
11	+	−	+	+	+	−	−	−	+	−	+
12	−	−	−	−	−	−	−	−	−	−	−

−,+: Levels for the factors.

Table 4. Variables selected as factors and values chosen as levels

Factors	External/internal	Optimal	Level (–)	Level (+)
A. Different days	External	–	2	1
B. Different buffers	External	–	2	1
C. Different patients	External	–	2	1
D. pH	Internal	9.2	9.0	9.4
E. [SDS]	Internal	40 mM	38	42
F. [Buffer]	Internal	70 mM	65	75
G. Voltage	Internal	25 kV	20	30
H. Washing buffer volume	Internal	5 min	4	6
I. Injection time	Internal	6 s	5	7
J. λ detection	Internal	203 nm	201	205
K. Temperature	Internal	20°C	18	22

Table 5. Results corresponding to the ranked effects *vs. M* values

	ME			LE	
	Migration time	Area	R_s	Migration time	Area
Equation	$Y = 1.14X + 0.18$	$Y = 2.1E^{-4}X + 0.24$	$Y = 5.99X - 0.07$	$Y = 1.006X + 0.45$	$Y = 2.7E^{-4}X + 0.34$
R^2	0.98	0.93	0.97	0.97	0.92
Residual mean square	0.025	0.086	0.031	0.038	0.082

presented in Table 4, which also shows the (+) and (−) levels for every factor, that are, respectively, upper and lower values with regard to the optimal one in the procedure.

The effects of varying the levels on the most critical electrophoretic responses of the method were investigated.The ranked effects for every selected factor on a specific electrophoretic response were calculated by simple addition of its (−) and (+) assay test results, upon the design shown in Table 3. The total result so obtained for every factor was divided by half the number of samples. The *M* values are statistic constants for any given design table with a number of 11 elements, which are the factors in our case [11]. Finally, the obtained ranked effects for the 11 selected factors were plotted (on the *x*-axis, in an increasing order) against the *M* values (on the *y*-axis) for each critical electrophoretic response. The results from this plot must be near to a straight line. If a value lies outside this straight line, it can be concluded that the method is not rugged/or robust at this point (as classified by its corresponding factor). However, if the results from the plot form a (nearly) straight line, it can be concluded that the analytical method is rugged and robust over the conditions tested in the run design. The robustness/ruggedness evaluation was performed in our case by carrying out triplicate injections of spiked urine samples containing 1.0 mg/L of LE and ME. The results of the levels variations effects for the 11 factors on resolution between peaks, migration times, and peak areas were calculated and they are shown in Table 5. As an example, Fig. 3 shows the plot corresponding to the ranked effects of the 11 selected factors *versus M* values for the resolution between peaks. It can be seen from this plot that all the points lie on a straight line and, therefore, our analytical method can be considered robust and rugged with regard to this electrophoretic response.

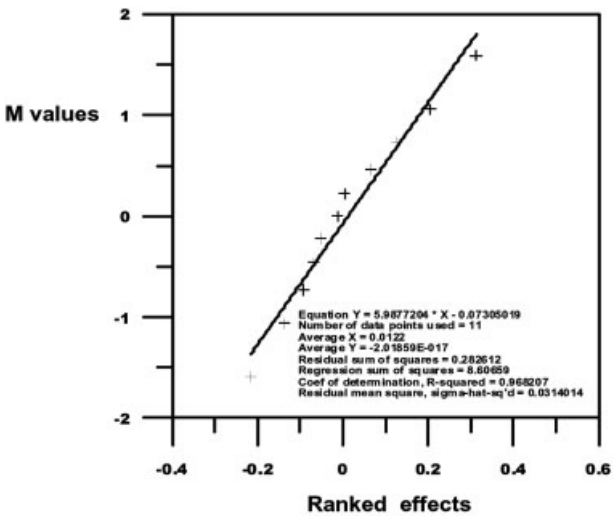

Figure 3. Plot corresponding to the ranked effects of the 11 selected factors *vs. M* values for resolution between peaks.

From the analysis of this study, we concluded that the main interaction effects over LE and ME peak areas and resolution electrophoretic responses were produced by injection time (I factor) and temperature (K factor) whereas for the retention times response the influence of different days (A

factor) and urine samples of different patients (C factor) were the most significant. So, in general terms, the described robustness/ruggedness test showed that our electrophoretic method is enough robust and rugged for the critical electrophoretic responses, being assessed for all the variations tested in this study.

3.3 Applications

Several urine samples from a woman (58 years) old undergoing medical treatment for three years were analyzed in order to demonstrate the applicability of the proposed method. The use of a photodiode detector allowed us to confirm the identities of the peaks, not just by their migration times, but also by overlaying the UV–Vis spectra from the samples with that from a standard one. Direct urine from this volunteer undergoing treatment with LE (2.5 mg/day) was analyzed at 4 h after its administration. The determination was carried out in duplicate and the concentration of LE and its ME found was 150 and 63 μg/L, respectively.

3.3.1 SPE and recovery studies

Until now, all analyses have been performed on human urine samples which had not been submitted to any special treatment. But in an effort to obtain more sensitivity for the quantization of LE and its ME and to reach some kind of pharmacokinetic information, a new study was performed using SPE as a preconcentration step prior to electrophoretic determination. C_{18} cartridges were used to accomplish this step. As already explained, variables such as organic solvent, washing stages using different solvents, volume and ratio of organic solvent/water, *etc.*, were studied in order to elute our analytes free from interferences. The best results were obtained when the cartridges charged with the urine samples were previously washed with a phosphate buffer solution (8 mL, 10 mM, pH 7.0) and a methanol–water solution (1.5 mL, 20%). LE and its ME were eluted with 1.5 mL of methanol and finally this extract was evaporated to dryness and reconstituted with 1 mL of water. The maximal capacity of the used cartridge was checked and established at 8 mL of urine, therefore, it was possible to preconcentrate eight times (from 8 mL of urine sample an extract of 1 mL was obtained). Several aliquots of LE and its ME standard solutions were added into human urine samples. These samples were analyzed using the SPE and the electrophoretic procedures described in this work and recoveries between 95.0 and 97.2% were obtained. The urine from a volunteer undergoing treatment with LE (2.5 mg/day) was analyzed at different times after its administration in order to obtain pharmacokinetic information. All the determinations were carried out in duplicate. The urinary excretion profile of LE and its ME is presented in Fig. 4. One of the SPE electropherograms obtained under our optimized conditions is shown in Fig. 5.

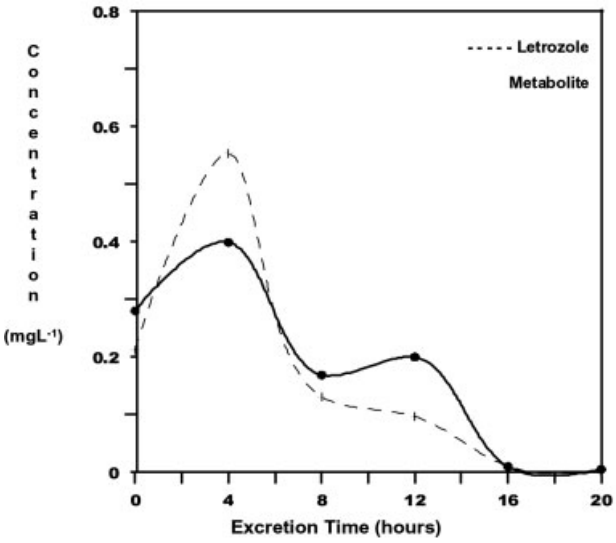

Figure 4. Urinary excretion profile of LE and its ME in a patient under 2.5 mg/day LE treatment.

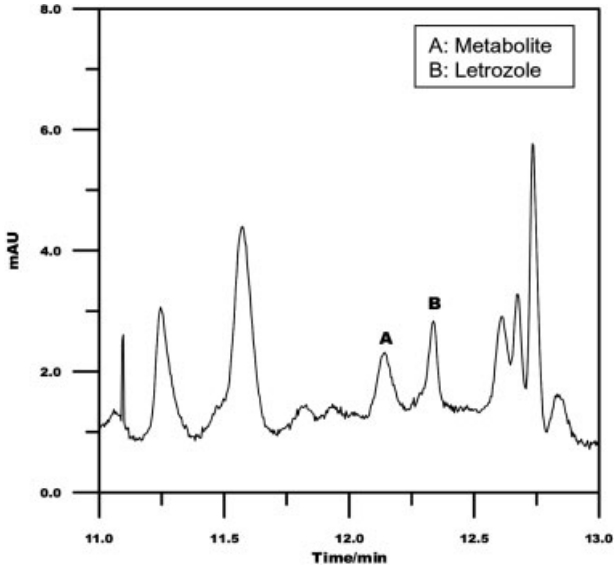

Figure 5. SPE electropherogram of a urine sample from a patient under LE treatment at 4 h after daily doses administration (2.5 mg). Operating conditions: hydrodynamic injection (6 s, 0.5 psi); separation: 70 mM (pH 9.2) borate buffer and 40 mM SDS, 25 kV as voltage of separation, 20°C as capillary temperature and detection at 203 nm.

4 Concluding remarks

In this work, a rapid, easy, robust, and sensitive method has been developed for the simultaneous determination of LE and its ME in direct human urine samples using MEKC for the first time. The electrophoretic method has been validated for the analysis of the two compounds in human urine di-

rectly without any matrix interference. It has been shown that the experimental results with respect to linearity, recovery, precision, sensitivity, and ruggedness of the test validation prove the reliability of the electrophoretic procedure for the quantization of the studied compounds at clinically relevant concentrations.

The MEKC mode complies with the requirements of clinical and/or forensic analysis in terms of reproducibility, accuracy, and robustness, and it is also useful for routine analysis. Additionally, it offers advantages such as handling simplicity, flexibility and economy.

The authors have declared no conflict of interest.

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