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Multivariate optimisation of a cyclodextrin-assisted-capillary zone electrophoretic method for the separation of torasemide and its metabolites

Urtzi Akesolo, Lorena González, Rosa María Jiménez, Rosa María Alonso*
Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

Abstract

In this work, a rapid cyclodextrin-assisted capillary electrophoretic method is developed for the separation of the diuretic torasemide and three of its metabolites. Both fractional factorial and central composite designs were employed to optimise the separation method. The factors studied were pH, concentration of methyl-β-cyclodextrin, concentration of the background electrolyte and percentage of acetonitrile as organic modifier. Monitored response was a composite quality response (Q^*) which balanced conflicting normalized responses, such as resolution and migration time. Optimal separation of the four studied compounds was achieved in less than 6.5 min, using an electrolyte of 60 m*M* borate buffer with no organic modifier and 25 m*M* methyl-β-cyclodextrin concentration adjusted to pH 8.0 at a potential of 30 kV. Detection wavelength and temperature were 197 nm and 20 °C respectively. This work means a significant improvement with regard to a previous separation method for these compounds developed in our laboratory.

Keywords: Experimental design; Factorial design; Central composite design; Background electrolyte composition; Optimisation; Torasemide; Diuretics

1. Introduction

Torasemide (1-isopropyl-3-{[4-(3′-methyl-phenyl-amino)pyridine]-3-sulfonyl}urea) is a high-ceiling loop diuretic considered to be the the most active derivative of the pyridine sulfonylurea diuretics. Torasemide combines the long duration of action of the thiazides with the features of a loop diuretic [1]. It acts in the ascending limb of the loop of Henle by inhibiting tubular reabsorption of sodium and chloride and interacting with the sodium/chloride/potassium cotransport system [2,3]. Torasemide has usually no side effect, its action profile being similar to that of Furosemide. Nevertheless, torasemide has a longer elimination half-life, longer duration of action, longer bioavailability and it is more potent compared to this diuretic, either in oral or intravenous administration [4]. Compared to the thiazide diuretics (the ones usually regarded as first line agents in antihypertensive therapy), torasemide has been shown to be as effective as them, whilst lacking the adverse metabolic and kaliuretic effects associated with these compounds [3].

The metabolic profile of torasemide differs greatly

*Corresponding author. Tel.: +34-94-601-2686; fax: +34-94-464-8500.
E-mail address: qapalror@lg.ehu.es (R. María Alonso).

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from the one of other loop diuretics. Torasemide undergoes extensive hepatic metabolism by hydroxilation and oxidation, resulting mainly in metabolite 1 (1-isopropyl-3-{[4-(3′-hydroxymethyl-phenylamino)-pyridine]-3-sulfonyl}urea), metabolite 3 (1-isopropyl-3-{[4-(4′-hydroxy-3′-methyl-phenylamino)pyridine]-3-sulfonyl}urea), and metabolite 5 (1-isopropyl-3-{[4-(3′-carboxy-phenylamino)pyridine]-3-sulfonyl}-urea) metabolites, the first two being active as well. M1, M3 and M5 will stand for metabolite 1, metabolite 3 and metabolite 5, respectively, from here onwards. Formulae of these compounds are collected in Fig. 1.

Torasemide is excreted unchanged in urine in a 20% percentage of the administered dose. Approximately 10% is excreted as M1, 44% as M5 and about 2% as M3. This extensive metabolisation accounts for some of the differences between torasemide pharmacokinetics and response when compared with other high ceiling diuretics [2,3].

The use of capillary electrophoresis for the analysis of drugs and pharmaceutical formulations has greatly increased in the last few years [5]. Step by step methodology involving a high number of independent runs is being replaced by multivariate optimisation methodologies, such as experimental design approaches. The suitability of this kind of approach has been reported for chiral separations [6–9], separations of compounds of the same family [10,11] and separation of a compound and its degradation products [12].

Experimental design approaches simultaneously vary several variables, instead of studying each one separately. Monitoring of the response with regard to the experimental matrix leads to a response model in which the relationship of each variable (i.e. factor) towards the response as well as interactions between factors are shown.

Determination and screening of torasemide has not been widely covered in literature. The first developed methods for the determination of these compounds consisted of a reversed-phase chromatographic sepa-

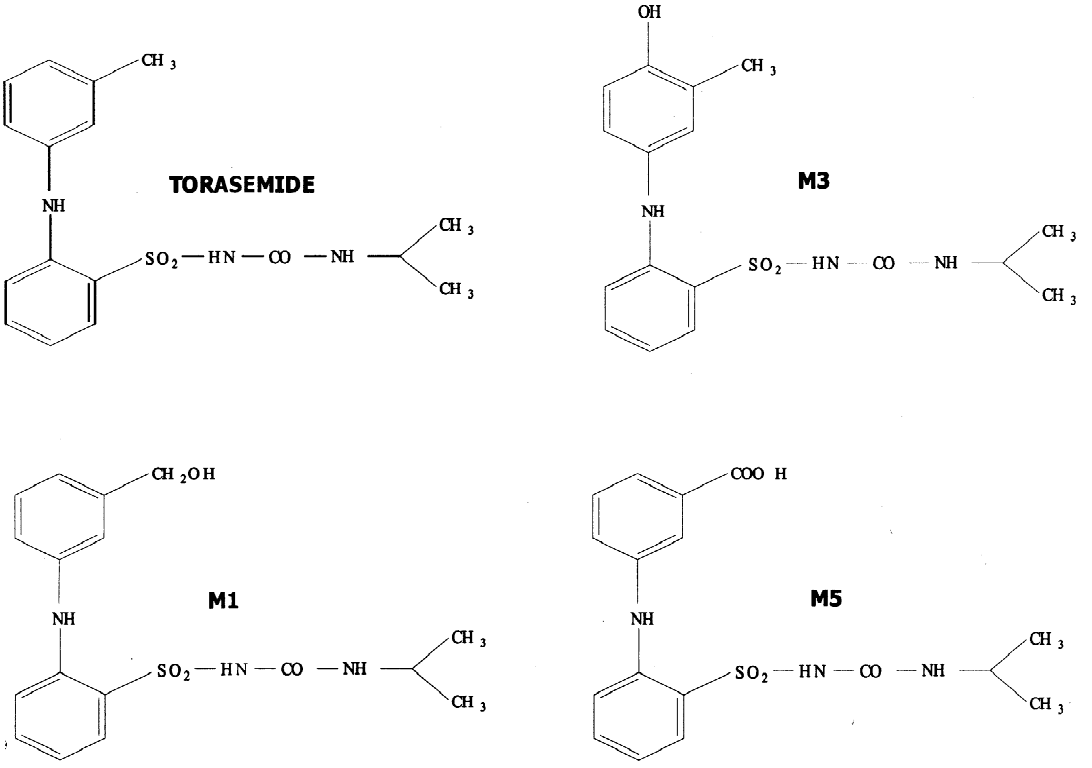

Fig. 1. Chemical formulae of torasemide, M1, M3 and M5.

ration with photometric detection [13–17]. Later, two different voltammetric analytical methods were developed in our laboratory [18,19] and then a chromatographic method with amperometric detection was reported for the determination of the parent drug [20]. Finally, a gas chromatography–mass spectrometry analysis [21], and two more recent chromatographic screenings [22,23] were developed.

The aim of this work was to improve the separation of torasemide and its major metabolites by capillary zone electrophoresis using an experimental design approach and selective additives. Previous effort was made in our laboratory to achieve this aim, results having been published last year [24]. However, separation was not optimal, since there was a critical pair of compounds for which resolution values were poor. The main problem to achieve good selectivity in capillary zone electrophoresis was that the four considered compounds were very similar in their chemical structure and molecular mass, and they co-migrated through the capillary.

In order to overcome this fact, it was decided to employ selective additives, such as cyclodextrins (CDs). CDs are cyclic oligomers of several D (+)-glucopyranose units. Depending on the number of monomers of the molecule they are classified as α, β or γ-CD. Considering the size of the analytes to be separated, it was decided to employ the medium-sized CD, those containing seven monomer units (β-CD). Modified CDs present several advantages compared to the natural ones, such as higher solubility and better stabilization of the inclusion complexes they form [25]. In this study two modified CDs were employed: hydroxypropyl-β-CD and methyl-β-CD.

Both fractional factorial and central composite designs were used in the optimisation of the separation. The former was employed in a screening study to disregard the non-affecting variables, the latter being used to optimise the conditions for the separation of torasemide and its metabolites.

2. Experimental

2.1. Chemicals and solutions

Torasemide and its metabolites M1, M3 and M5 were kindly supplied by Roche (Basel, Switzerland). Acetonitrile (ACN) was Romil Super Purity Solvent grade (Harvehill, UK) and borate was of analytical grade and was supplied by Merck (Darmstadt, Germany). Hydroxypropyl-β-CD and methyl-β-CD were purchased from Wacker-Chemie. Water was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA, USA).

Stock solutions of torasemide and M5 (1000 μg/ml), M1 and M3 (500 μg/ml) were prepared in methanol and kept in amber glass volumetric flasks. These stock solutions were stored in dark under refrigeration to avoid possible decomposition. Working solutions were also prepared in amber glass volumetric flasks by appropriate dilution with water just before use. Running electrolytes were prepared by diluting the 0.5 *M* borate stock buffer and adding ACN to give the desired organic modifier percentage. pH was adjusted by adding drops of NaOH conc.

2.2. Apparatus and electrophoretic conditions

This work was performed on a Hewlett-Packard HP ^{3D}CE Capillary Electrophoresis System (Waldbronn, Germany) equipped with a diode array detector. The sample tray was refrigerated at 20 °C with a Selecta Frigiterm-10 external bath (Barcelona, Spain). The fused-silica capillaries were 58.5 cm×50 μm I.D.×375 μm O.D., obtained from Composite Metal Services (Worcester, UK) with the detection window at 50 cm. The samples were introduced hydrodynamically for 8 s at 50 mbar injection pressure and the capillary temperature was set at 20±0.1 °C. The running electrolyte consisted of a 60 m*M* borate buffer with no organic modifier at pH 8. Applied potential was 30 kV and detection wavelength was 197 nm.

pH of solutions was measured with a Radiometer Copenhagen PHM84 pH meter (Bargsvaerd, Denmark) using a Crison glass-combined electrode model 5209 (Barcelona, Spain) equipped with a reference system Ag/AgCl and electrolyte KCl 3*M* saturated AgCl.

2.3. Capillary conditioning

The capillary was conditioned every day with an

initial wash cycle consisting of 1 *M* NaOH for 15 min, deionized water for 10 min and running electrolyte for 5 min. Between injections, the capillary was washed with 0.1 *M* NaOH for 2 min, deionized water for 1 min and running electrolyte for 3 min. The separation buffer was refreshed after a few runs. Daily after finishing the experiments, the capillary was washed with 1 *M* NaOH for 10 min and deionized water for 10 min and purged with air for 3 min.

3. Results and discussion

3.1. Factors affecting separation: experimental designs

Optimisation of the separation method was made by means of two different experimental designs: a fractional factorial design (FFD) to evaluate which of the considered variables were significant factors, and a central composite design (CCD) to optimise these factors in the previously selected experimental domain.

CD type and concentration, background electrolyte (BGE) concentration, pH and percentage of organic modifier were thought to have the highest influence on CD assisted capillary electrophoretic methods [26–33]. Although there are some additional variables that can also influence the separation, electrolyte variables are the key variables regarding the optimisation of method selectivity [34]. Applied voltage and injection volume were kept constant in the experimental designs (25 kV, 5 s, 50 mbar). Afterwards, these variables were optimised once the optimal analysis conditions had been reached.

Some research works deal with the type of CD as a factor in the experimental design [6]. Concentration and temperature levels can be easily set since there is a direct linear relationship between numerical values. However, establishing levels for CD nature or type is somehow more complicated. Thus, we decided to carry out some previous runs in order to select the CD to be studied [7]. From these runs, methyl-β-CD was chosen as electrolyte selector for the multivariate designs, its results being better than those achieved for hydroxypropyl-β-CD.

To some extent, it could seem strange to use an organic modifier when a selective additive such as a CD is being used. However, according to the theory of Wren [30], the addition of an organic modifier can greatly enhance or decrease the association between the analytes and the CD. When the CD concentration is at or below its optimum for resolution, adding an organic modifier results in a decrease of resolution. The opposite effect occurs when the CD concentration is higher than the optimal one. These effects can be explained considering the changes in the complexation constants between the compounds depending on its concentration and the presence or absence of organic modifier.

3.2. Screening design: fractional factorial design

The experimental variables considered in the FFD for the separation of torasemide and its metabolites were pH (x_1), concentration of methyl-β-CD (x_2), concentration of the borate buffer (x_3) and percentage of ACN as organic modifier (x_4). In the previous work dealing with separation of torasemide and its metabolites developed at our laboratories, methanol had been used as organic modifier. Since it has no significant effect, we selected another organic modifier such as ACN to check its influence on separation.

When applying experimental design methodologies, it is advisable to keep the number of variables as low as possible in order to avoid very complex response models and large variability [35]. Thus, the 2^4 experiments needed to complete a whole factorial design were reduced by introducing a confounding and running the so-called fractional factorial design. This design confounds some main effects with interactions and interactions among themselves, resulting in a smaller set of experiments. Nevertheless, it is able to identify the influence of each parameter as well as first-order interactions between factors. Fractional factorial design involves 2^{k-p} experiments, where k is the number of the factors studied and p accounts for the degree of fractionality of the fractional factorial design ($p < k$) [36]. In this case, the main effect estimate for factor %ACN was confounded with the estimate of the interaction effect for pH, CD concentration and BGE concentration as shown in Eq. (1). Number of runs was then reduced from 16 to 8.

$$\%\ \mathrm{ACN} = \mathrm{pH} \cdot \text{concentration of methyl-β-CD} \cdot \text{concentration of borate buffer} \quad (1)$$

The design generator $I = 1234$ was defined in this way as pH, CD and BGE concentration and was expected to be a non-significant interaction.

A two-level fractional factorial design involving 8 runs and 3 replicates of the centre point was carried out.

In order to obtain a global response reflecting criteria of different and conflicting nature, a composite quality response Q^* which balanced different responses was defined, having previously been reported elsewhere [37]. The composite Q^* response considered both the relative total resolution ($R_{s_{tot}}$) and analysis time (t_m). These are the two main factors affecting separation quality in CE and have been also considered by other authors [8,12,33,38,39].

Resolution (R_s) between peaks is usually defined according to Eq. (2).

$$R_s = 1.177 \cdot \frac{t_j - t_i}{w_{0.5j} + w_{0.5_i}} \quad (2)$$

where t_j and t_i and $w_{(0.5)j}$ and $w_{(0.5)i}$ are the migration time and the peak widths at half height of two successive peaks, peak i being the first one and peak j the last one.

In order to reduce the number of monitored responses and considering the separation mechanism of capillary electrophoresis, a relative total resolution ($R_{s_{tot}}$) was calculated by measuring the resolution between the first (1) and the last (n) migrating peaks and dividing the result by $(n-1)$, where n is the number of analytes to be separated [10]. Formula for relative total resolution is given in Eq. (3).

$$R_{s_{tot}} = 1.177 \cdot \frac{\dfrac{t_{m_n} - t_{m_1}}{w_{0.5_n} + w_{0.5_1}}}{n-1} \quad (3)$$

In order to define the Q^* quality response, both t_m and $R_{s_{tot}}$ were normalized. The shortest t_m and the highest $R_{s_{tot}}$ value (the more desirable situation) were given the value 1 (maximum), while the longest t_m and the lowest $R_{s_{tot}}$ values (the unwanted one) were given the value 0 (minimum). Linear interpolation allowed us to calculate the normalised values for the remaining t_m and $R_{s_{tot}}$ values. Normalised values were called t_m^* and $R^*_{s_{tot}}$. The latter was considered to be a more important response regarding the optimisation of the separation of the considered analytes. Thus, it was given double weight compared to migration time as shown in Eq. (4), where n accounts for the number of analytes to be separated.

$$Q^* = \frac{t_m^* + 2R^*_{s_{tot}}}{n-1} \quad (4)$$

Table 1 shows the studied factors and the selected experimental levels, and Table 2 the FFD matrix along with the response values for each run (t_m, $R_{s_{tot}}$, t^*_m, $R^*_{s_{tot}}$ and Q^*).

The data analysis of the results for torasemide and its metabolites was performed using the non-linear regression analysis program NLREG [40]. Responses (Y) were taken as a function of the considered variables (x_i) using polynomials of different degree depending on the experimental design followed. The general polynomial function is:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_{ij} \beta_{ij} x_i x_j \quad (5)$$

where Y is Q^*, x_i are the variables considered for the

Table 1
Factors and levels of the 2^{4-1} factorial fractional design

Factor		Level		
		−1	0	+1
x_1	pH	8	9	10
x_2	Concentration of methyl-β-cyclodextrin (mM)	5	15	25
x_3	Concentration of borate buffer (mM)	20	50	80
x_4	Acetonitrile (%)	0	7.5	15

Table 2
The 2^{4-1} factorial fractional design. Analytical responses, normalized responses and combined quality response (*)

Trial	x_1	x_2	x_3	x_4	t_m	$R_{s_{tot}}$	t^*_m	$R^*_{s_{tot}}$	Q^*
1	10.02	25	80	15	14.49	25.50	0	1.00	0.67
2	7.98	25	80	0	8.25	22.57	0.80	0.80	0.80
3	10.00	5	80	0	13.96	14.52	0.07	0.26	0.20
4	8.03	5	80	15	11.76	20.31	0.35	0.65	0.55
5	10.03	25	20	0	6.63	16.16	1.00	0.27	0.58
6	8.00	25	20	15	11.43	18.84	0.39	0.55	0.50
7	10.01	5	20	15	7.07	14.57	0.94	0.26	0.49
8	8.14	5	20	0	10.74	10.67	0.48	0	0.16
9	9.03	15	50	7.5	8.38	13.76	0.78	0.21	0.40
10	9.01	15	50	7.5	8.05	12.71	0.82	0.14	0.36
11	9.03	15	50	7.5	8.16	13.08	0.81	0.16	0.38

optimisation of the response and β_i and β_{ij} are the numerical parameters to be calculated. The estimation of the parameters (β_i and β_{ij}) was achieved by the minimisation of the square sum of errors (U) as given by the equation

$$U = \sum_{i}^{n} (Y_{\text{exp}} - Y_{\text{calc}})^2 \quad (6)$$

where n is the number of experiments, Y_{exp} is the experimental response and Y_{calc} is the response calculated by the program based on the proposed regression model.

The analysis of the output was based on the evaluation of the *prob*(t) parameter associated with each β_i parameter, since *prob*(t) indicates the probability of β_i being zero. Those parameters whose probability of being zero was greater than 10%, i.e., *prob*(t) > 0.1, were systematically eliminated.

The most general function allowed for the FFD of the studied analytes considering the design generator $I = 1234$ is shown in Eq. (7).

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_2 x_3 + \beta_{23} x_2 x_3 \quad (7)$$

where Y stands for Q^*, x_1 is the pH value, x_2 is the CD concentration (mM), x_3 is the BGE concentration (mM) and x_4 is the percentage of ACN (%). Model parameters were limited as reflected in Eq. (7) considering the set of aliases {1 = 234, 2 = 134, 3 = 124, 12 = 34, 13 = 24, 23 = 14}.

Different regressions were assayed corresponding with the most general function allowed for this design. The choice criteria was the best fitting to the regression (percentage of variance explained). The final equation obtained for response Q^* is Eq. (8):

$$Y = -1.461 + 0.180 x_1 + 0.014 x_2 + 0.036 x_3 - 0.004 x_1 x_3 \quad (8)$$

Percentage of variance explained was 86%.

According to these parameters, percentage of ACN had no single or interaction influence on the separation of the four studied compounds (there is no parameter involving x_4), so it was disregarded for the following optimisation design. pH, concentration of methyl-β-CD and concentration of the background electrolyte appeared as main factors.

There was only one interaction parameter, which involved pH and concentration of the background electrolyte. Thus, it corroborated the influence of these factors on CE separation of torasemide and metabolites. Q^* representation vs. each of the mentioned three factors showed that there was no general trend except for the second factor, the concentration of CD. Highest Q^* values were always reached at the highest level of CD concentration.

Considering this fact and the positive β parameter of the resulting equation, it was decided to set this factor at its highest value (25 mM) to face the optimisation design.

3.3. Optimisation design: central composite design

Optimisation of the separation of the four studied compounds was carried out by means of a CCD. A

Table 3
Factors and levels of the central composite design

Factor		Levels				
		$-(2^{1/2})$	−1	0	+1	$+2^{1/2}$
x_1	pH	7.6	8	9	10	10.4
x_2	Concentration of borate buffer (m*M*)	8	20	50	80	92

Table 4
The $2^2+2\cdot2+3$ central composite design. Analytical responses, normalized responses and combined quality response (Q^*)

Trial	x_1		x_2		t_m	$R_{s_{tot}}$	t^*_m	$R^*_{s_{tot}}$	Q^*
1	+1	9.06	+1	80	9.68	17.15	0.58	0.58	0.58
2	−1	8.03	+1	80	14.08	23.26	0	1.00	0.67
3	+1	9.98	−1	20	6.44	12.23	1.00	0.23	0.49
4	−1	8.06	−1	20	9.24	20.14	0.63	0.78	0.73
5	$+2^{1/2}$	10.45	0	50	9.02	20.05	0.66	0.78	0.74
6	$-(2^{1/2})$	7.61	0	50	9.79	18.76	0.56	0.69	0.64
7	0	9.03	$+2^{1/2}$	92	8.66	18.05	0.71	0.64	0.66
8	0	9.06	$-(2^{1/2})$	8	7.47	8.89	0.86	0	0.29
9	0	8.96	0	50	7.34	16.33	0.88	0.52	0.64
10	0	9.01	0	50	7.39	17.74	0.88	0.62	0.70
12	0	8.98	0	50	7.42	18.46	0.87	0.67	0.73

CCD is a second-degree design enabling modeling of the nonlinear effect of variables. It was first proposed by Box and Wilson and consists of a full factorial design plus a star design. That way, each factor is studied at five different levels ($-\alpha$, −1, 0, +1, $+\alpha$). In order to maintain the highest symmetry possible, $-\alpha$ and $+\alpha$ levels were located at $-(2^{1/2})$ and $+2^{1/2}$ respectively.

From the four proposed variables for the FFD, two had been already fixed (percentage of ACN (0%) and concentration of methyl-β-CD (25 m*M*)). Thus, the central composite design was a $2^2+2\cdot2+k$ design, k being the number of replicates of the centre point ($k=3$).

Table 3 shows the two studied factors and the considered levels for each of them.

The regression model proposed for this design is given in Eq. (9):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_1 x_1^2 + \beta_2 x_2^2 \quad (9)$$

with Y being the studied response (Q^*) and x_1 and x_2 pH and concentration of the BGE respectively.

The data collected in Table 4 were analysed by NLREG. From the output data the following equation that best fit to the experimental data could be deduced (Eq. (10)). Model choice criteria were the same as in FFD.

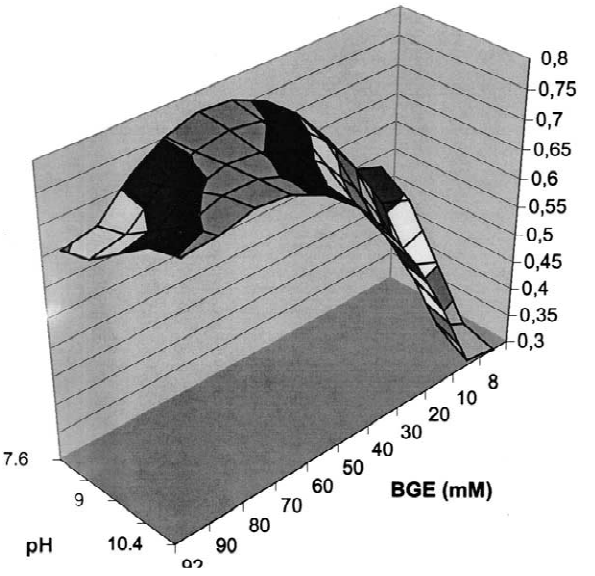

Fig. 2. Response surface for quality response Q^*.

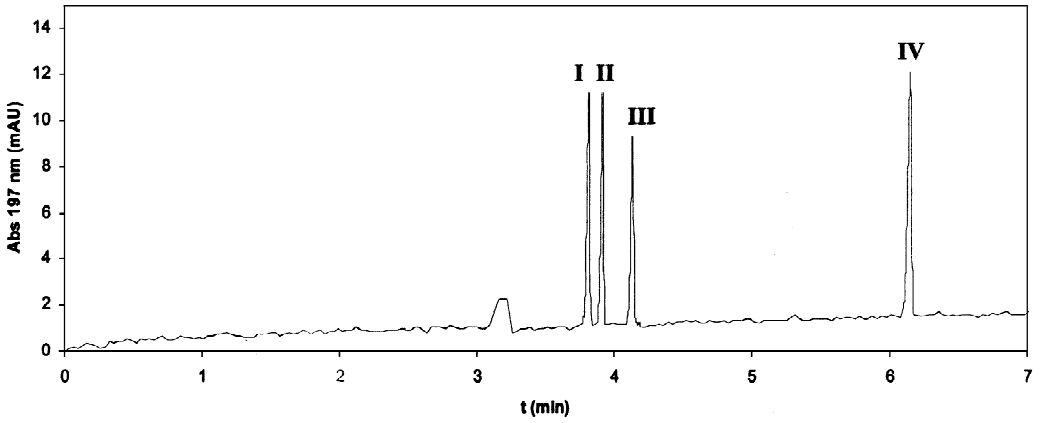

Fig. 3. Electropherogram of a standard solution of the studied four compounds at the optimal conditions (10 μg/ml each). I, M3: II, torasemide; III, M1 and IV, M5. See Section 3.3 for experimental conditions.

$$Y = 1.2058 - 0.1003\,x_1 + 0.0015\,x_1\,x_2 - 0.0001\,x_2^2 \quad (10)$$

Fig. 2 shows the three-dimensional plot for the response Q^* vs. pH and concentration of BGE. It is clearly shown that highest Q^* values are reached at BGE concentration = 50–70 m*M* and pH = 7.6–8.4. Analysis of the data led to the optimal analysis conditions: pH 8.0 and borate concentration 60 m*M*.

Once the optimal electrolyte had been chosen, instrumental variables were optimised. Applied voltage was selected according to Ohm's plot, and injection time was studied with regard to the area and height vs. injection time plots. From these representations, 30 kV and 8 s, 50 mbar were selected as optimal values for these variables. Analysis wavelength was determined according to the spectra of the analyte mixture in the optimised conditions.

Fig. 3 shows an electropherogram of a standard solution of the studied four compounds at the optimal conditions (10 μg/ml each).

4. Conclusions

The developed CD assisted CZE method has proved suitable for the separation of torasemide and its metabolites (M1, M3 and M5). Some aspects of the results confirm a previous research work developed for these compounds in our laboratory, but significant enhancement on resolution and analysis time, both of them being critical factors on the separation of a mixture of analytes, was shown.

In this study, optimisation of the proposed separation is attempted by means of a systematic multivariate procedure. Use of experimental designs provides a great deal of information about the effect of each factor while performing the minimum number of experiments. The studied response was a composite quality response, Q^*, which considered normalized relative total resolution and migration time values. This way, conflicting quality responses could be optimised at the same time with minimum experimental effort.

A four-factor considering FFD enabled the selection of critical factors on response. From the ones initially selected (pH, CD concentration, BGE concentration and ACN%), the last one was disregarded as a critical factor and the CD concentration could be fixed for the optimisation CCD design.

Response surface modelling from the two-factor considering CCD design allowed selection of the optimal analysis conditions for the separation of the four studied compounds.

Optimal separation of torasemide and its metabolites was reached with a mixed 60 m*M* borate buffer with no organic modifier at pH 8.0. Applied voltage and temperature were 30 kV and 20 °C respectively. The fused-silica capillary was 58.5 cm (50 cm effective length) × 50 μm I.D. Complete separation

of the four compounds was reached within 6.5 min. Resolution values and migration time were remarkably better compared to the ones of the previous separation study.

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