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Optimisation and validation of a capillary electrophoresis method for the simultaneous determination of diazepam and otilonium bromide

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A simultaneous assay of diazepam and otilonium bromide in coated tablets by capillary zone electrophoresis (CZE) was developed. The influence of various parameters (voltage, temperature, buffer concentration and pH, ethanol percentage) on analysis time and on the theoretical plates of the two peaks was investigated by means of experimental design. A response surface study was carried out by means of a 27-run D-optimal matrix. The best background electrolyte was found to be 0.13 M, pH 2.9 Britton–Robinson buffer, containing 10% v/v ethanol. Other optimised parameters were voltage (30 kV) and temperature (30 °C). The UV detector for quantitation of otilonium bromide and diazepam was set at 280 nm and 230 nm, respectively. Procaine hydrochloride was used as internal standard and run time was less than five minutes. Validation was performed, for drug substance and drug product, according to ICH3 guidelines. For drug product the recovery for otilonium bromide and diazepam ranged from 98.3% to 101.2% and from 97.1% to 99.0%, respectively; the RSD values found for otilonium bromide and diazepam ranged from 2.4% to 3.0% and from 1.1% to 4.5%, respectively.

Introduction

Diazepam (DZ), 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is a benzodiazepine widely used as tranquilizer, antidepressant and hypnotic. Otilonium bromide (OT), diethylmethyl{2-[4-(2-*n*-octyloxybenzamido)benzoyloxy]ethyl} ammonium bromide, is a drug with myolytic activity and a combination of antimuscarinic and Ca^{2+} channel blocker properties seems to best account for its action. It acts selectively on the smooth muscle of the gastrointestinal tract by interfering with the mobilization of calcium from intra and extracellular pools and it lacks the side effects of other antimuscarinic drugs.[1–5] OT is a quaternary ammonium compound characterised by a long aliphatic chain and its chemical properties are responsible for both a poor penetration in the CNS and a prolonged binding to cell membranes.[6]

The pharmaceutical combination of OT and DZ is commercially available for the treatment of spastic pains related to neurovegetative disorders of the gastrointestinal system.[4] The combination of these two drugs is useful due to the etiopathogenetic implications of a psychosomatic nature[7–9] and is marketed in coated tablets with two different dosages: 20 or 40 mg of OT with 2 mg DZ. Zero-crossing first derivative,[3] ratio-spectra first- and second-derivative spectrophotometric methods,[1] HPLC methods[4,10] and a reflectance NIR spectroscopic method[11] have been reported for the simultaneous assay of DZ and OT.

Capillary electrophoresis has emerged as a powerful separation tool for the analysis of a wide variety of complex mixtures. Advantages of this analytical method *versus* conventional chromatographic techniques have been fully described in literature.[12–14] The characteristics of CE modality are reflected in its high separation efficiency, rapid analysis, and small sample and reagent consumption.

The purpose of this study was to develop a rapid and validated capillary electrophoretic method (CE), as an alternative to the existing ones, for the simultaneous determination of DZ and OT in coated tablets. Many variables have to be optimised when developing a CE method, such as pH and ionic strength of background electrolyte (BGE), percentage of organic modifier, voltage, temperature and so on. The obsolete step-by-step approach, although widely used, involves a large number of independent runs and does not consider the possible interactions between factors, thus a multivariate optimisation[15] was considered very useful. In particular, experimental design offers an efficient route for identifying the zone in factor space for the best resolution in CZE.[16,17] In the present study a response model that shows the relationship of each factor towards the response as well as the interactions between factors, was obtained and the factors could be optimised to give the best possible response with a relatively low number of experiments.

The proposed method was validated and successfully applied to the simultaneous determination of DZ and OT in coated tablets. This method is simple and rapid and can be used for routine analysis and as an alternative tool for quality control laboratories.

Experimental

Chemicals and solutions

All chemicals used were of analytical-reagent grade with no further purification.

Working standards of DZ and OT and tablet excipients (avicel, maize starch, magnesium stearate, silica, titanium

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bioxide, carnauba wax, talc, PVP, chlorophyll, carboxymethylcellulose sodium, methyl *p*-hydroxybenzoate and saccharose), were obtained from Menarini Pharmaceuticals (Florence, Italy). Procaine hydrochloride (PR) was purchased from Sigma (St. Louis, MO, USA) and used as internal standard. Reagent-grade water was obtained with a Milli-Q system (Millipore-waters, Milford, MA, USA) and was used to prepare all solutions. The pharmaceutical dosage form containing 20 mg otilonium bromide and 2 mg diazepam for each tablet (Spasmomen somatico 20® coated tablets, Menarini Pharmaceuticals), was purchased from the local market. pH 2.5–3.5, 0.05–0.15 M background electrolyte buffers (BGE) examined during the optimization step were prepared by mixing the adequate volume of Britton–Robinson universal buffer (0.5 M phosphoric, boric and acetic acid) and ethanol (0–15%, v/v) with water, adjusting pH with 1 M NaOH and filling up to the volume with water.

The running buffer consisted of 0.13 M Britton–Robinson buffer containing 10% (v/v) ethanol, adjusted to pH = 2.9 with 1 M NaOH. Standard stock solution of a mixture of DZ and OT, 0.1 and 1 g L^{-1}, respectively, were prepared in 20%, v/v, ethanol, first adding ethanol to achieve a faster solution of DZ and then water. Standard stock solution of internal standard PR was prepared in water at concentration of 0.16 g L^{-1}. These solutions were stored at 4 °C and used within three days. A working standard solution was prepared daily by diluting standard stock solutions with water in order to obtain the desired final concentrations.

BGE and working standard solution were filtered through 0.45 μm cellulose acetate syringe filters before use.

Equipment and capillary electrophoretic conditions

A 300 Ultrasonik bath (Ney Company, Bloomfield, USA) was used to sonicate solutions.

A Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland) was used to give an indication of the pH values (the apparent pH value when organic solvents are added to aqueous buffers). The pH meter was calibrated with pH 2 and 4 hydroalcoholic (0–15%, v/v, ethanol) buffer solutions. Instead of a classical glass electrode, an iridium–iridium oxide/calomel electrode system, previously described by one of us was used due its greater accuracy and stability in the presence of organic solvent.[18] CE experiments were carried out on a Spectra PHORESIS 1000 (Thermo Separation Products, Fremont, CA, USA) which was driven by CE software (version 3.01) operating under IBM OS/2TM (version 1.2) and contained a programmable high-speed, scanning, multiple-wavelength detector.

The fused (uncoated) silica capillaries were purchased from Supelco (Bellefonte, PA, USA) and had a total length of 44 cm (36 cm to detector), an inner diameter of 50 μm and an outer diameter of 363 μm. The detection wavelengths were 230 and 280 nm with a rise time of 0.5 s. The detection was towards the cathodic end and a detection window was created by burning off the polyimide coating on the capillary. Hydrodynamic injection was performed for 10 s. Capillary temperature was kept at 30 °C and the voltage applied was 30 kV. The standard run buffer consisted of an aqueous solution of 0.13 M Britton–Robinson universal buffer adjusted to pH = 2.9 with 1 M NaOH containing 10% (v/v) ethanol. Under these operating conditions a current of 90 μA was typically generated. At the beginning of each day, the capillary was rinsed with 0.1 M NaOH for 10 min and then with water for 10 min. To achieve high migration time repeatability and to avoid solute adsorption, the capillary was treated with 0.1 M NaOH for 2 min before each sample injection, rinsed with Milli-Q water for 2 min and conditioned with BGE for 2 min at 30 °C. Prior to each sequence, two blank injections were performed to stabilize the capillary wall surface and to allow the buffer and the sample solutions to reach a constant temperature on the autosampler tray. The plate numbers (N) were calculated according to the standard expression based on peak width at half height.[19,20]

Calibration curves

Calibration curves were obtained by plotting the concentration of each analyte/internal standard ratio *vs.* the peak area/migration time ratio of the two analytes divided by the peak area/migration time ratio of the internal standard. The curves for drug substances and drug product, were evaluated across the 80–120% range of the test concentration (DZ 0.02 g L^{-1}, OT 0.2 g L^{-1}). Procaine hydrochloride (0.03 g L^{-1}) was used as internal standard. For drug substances, five different concentrations of each analyte were prepared by diluting the standard stock solution with water. For drug product five separate weighings of synthetic mixtures of the components were used. Each solution was analysed twice.

Tablet assay

Twenty coated tablets were finely powdered and an accurately weighed portion of the powder, containing approximately 10 mg of DZ and 100 mg of OT, was transferred into a 100 mL volumetric flask. The content was diluted with 20 mL of ethanol, shaken vigorously, sonicated for 15 min and diluted to volume with water. A 2 mL portion of the liquid was filtered and transferred into a 10 mL volumetric flask, to which 2 mL of the internal standard stock solution were added. The volume was adjusted to 10 mL with water. The solution obtained was directly injected into the capillary electrophoresis system. Quantification of DZ and OT was carried out by means of drug product calibration curves. Results generated by the CZE method were compared with those obtained by a HPLC method.[4]

Experimental design

Experimental design was generated and statistical analysis of the experimental data was performed using Nemrod-W software package.[21] During the optimisation, the experiments of a 27-run D-optimal matrix were carried out in a randomised order with OT and DZ concentrations of 3.4×10^{-4} M and 8.9×10^{-5} M, respectively. An 8-run Plackett–Burman matrix was used for robustness tests. The experiments were run using a PR concentration of 1.2×10^{-4} M and OT and DZ concentrations of 3.4×10^{-4} M and 6.7×10^{-5} M, respectively.

Results and discussion

Method optimisation

Preliminary work on the electrophoretic behaviour of the two analytes using purely aqueous buffers pointed out no particular problem in the analysis of DZ and in the resolution between the two compounds, the baseline of which could be easily obtained. However, serious problems occurred in the determination of OT, in particular excessively tailed peaks, due to its quaternary ammonium nature. Thus a statistical optimisation was performed considering as responses to the analysis time (t), measured as DZ migration time, and the number of theoretical plates of OT (NOT) and DZ (NDZ). Because of the different absorbance spectra of DZ and OT, which present maxima at 230 and 280 nm, respectively, the responses concerning DZ were calculated at 230 nm, and those concerning OT at 280 nm.

A major difficulty in obtaining optimum peak shape and controlling migration times of positively charged quaternary ammonium compounds has been attributed to their propensity for adsorption on the silica capillary which leads to a reduction in efficiency.[22] Addition of modifiers to the running buffer has been used mainly to overcome this kind of problem and is an alternative to the bonded or adhered phases.[23] For example, peak tailing can be partially overcome by the inclusion of appreciable concentrations of appropriate organic solvents in the running buffer.[24] Organic solvents may affect the electrophoretic properties of the analytes two-fold: on the one hand by changing the actual mobility (that of the fully charged ion, which depends obviously on the viscosity of the solution); on the other hand by influencing the pK_a value of weak electrolytes specifically. Moreover, they affect the mobility of electroosmotic flow (EOF), determined by the zeta potential near the surface of the capillary, and the viscosity and dielectric constant of the BGE solution close to the surface.[25,26] Starting from these results, the possibility to use a non-aqueous media for the determination of DZ and OT was investigated. A preliminary study showed that ethanol was the best organic solvent for this problem, thus, its presence and its percentage in BGE, together with the concentration and the pH of Britton–Robinson buffer, used as supporting electrolyte, were investigated in order to find the optimum BGE. Other considered factors to optimise peak shape and analysis time were temperature and voltage. As concerns the experimental domain investigated, temperature (T, U_1) ranged from 20 to 40 °C; percentage of ethanol (%EtOH, U_2) from 0 to 15%; voltage (V, U_3) from 21 to 30 kV. Britton–Robinson buffer concentration (B-R conc., U_4), which had a significant effect on the analysis performance through its influence on EOF and the current produced in the capillary, was investigated from 0.05 to 0.15 M. The upper limit was fixed considering that an increase in electrolyte concentration determines an increase in baseline noise.[27] In order to reduce the negative charge on the fused silica capillary wall, and thus to minimize interactions of OT with the silica surface, only a restricted acidic pH range (pH, U_5) from 2.5 to 3.5 of Britton–Robinson buffer was considered.

Considering the number of factors and the investigated experimental domain, a response surface study seemed suitable for the resolution of the problem. In fact, response surface mapping is an effective way of locating the optimum if the data fit the chosen polynomial model. A response surface study makes it possible to determine and to draw the way in which response ranges in the investigated experimental domain. Then, the obtained response evolution map can be used in a provisional way, that is, the response can be predicted in a point where the response has not been measured, in order to find the optimum.

In this case the response surface was approximated by second-order polynomial function:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{55} x_5^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{45} x_4 x_5 + \varepsilon$$

where y represents the experimental response, x_i the independent evaluated factors, β_0 the intercept, β_i the model coefficients obtainable by multiple regression and ε the experimental error. A D-optimal design was used to select from a Doehlert design a minimum number of experiments which allowed an accurate estimate of the model coefficients to be obtained.

The number of experiments of a Doehlert design is equal to $k^2 + k + n$, where k is the number of factors studied, and n is the number of replicates at the centre of the experimental domain.[15,28] Replicates at the centre are used to determine the experimental variance. Having to study five factors, the number of experiments required by a Doehlert design is $30 + n$. In order to reduce the number of experiments, a D-optimal design was used. In particular the D-optimal design allows the best compromise between number of experiments and information quality to be obtained. This technique makes it possible, through the analysis of several quality parameters (determinant of the information matrix, inflation factors, leverage), to detect a subset of experiments from a set of candidate points that allows the best quality of information (*i.e.* the accuracy in the β coefficients estimate) to be obtained. By repeating the analysis of the quality parameters on subsets of different size, it is possible to have information on the evolution of these parameters with an increase in the number of experiments. In this way, a good compromise can be found between the quality of the information and the number of experiments to be performed.[15,28]

In this case, a good quality of information was obtained with an experimental matrix containing 23 of the original 30 experiments plus four replicates at the centre. The short analysis time usually obtained in CE allowed all 27 experiments to be performed in a day. Before statistical treatment, the number of theoretical plates of DZ and OT were transformed in their logarithms (LNOT, LNDZ), according to the Box–Cox transformation, to have a better provisional capacity of their models.[15] The experimental matrix together with the responses is reported in Table 1.

Analysis of variance (ANOVA) was used to have information about the significance and validity of the model and the regression model for each considered response was found valid and significant. In particular, ANOVA (Table 2) pointed out that the testing hypotheses about the equality of row treatment effects was to refuse, thus indicating that the change in the observed responses was due to the level change of factors.[15] In addition ANOVA allowed the validity of the model to be assessed. For this aim, the residual sum of squares was divided into two parts: pure error (calculated by means of the replicates) and lack-of-fit.[15,29] The pure error sum of squares divided by the degrees of freedom gives an unbiased estimate of the experimental variance s_1^2. The remainder of the residual sum of squares is the lack-of-fit sum of squares. Dividing this value by the degrees of freedom, another estimate of experimental variance, s_2^2, can be obtained. Fischer's test allows the two estimates of the variances, s_1^2 and s_2^2, to be compared. A ratio (s_2^2/s_1^2) much larger than 1 indicates that the estimation s_2^2 is too high and that, therefore, the model is inadequate.[15] In the present case, for the three responses, the estimations s_1^2 and s_2^2 were not considered to be significantly different, thus the model was justified. In addition, the residual analysis showed that residuals were structureless, and measured and predicted results were close.[15,30,31] It is important to underline that validation of the model during response surface study is very important since the regression model has to have provisional properties. In this case having assessed the validity of the model, the response surfaces for each considered criteria were drawn as a three-dimensional plot of two factors while keeping the others constant at their central values (Fig. 1). Response surfaces are an efficient tool to find the optimum and several criteria are simultaneously considered; they are useful to give indications about the behaviour of each response in the considered experimental domain. However, starting from these plots, the research of the global optimum is very difficult. For this type of problem multicriteria decision making, such as desirability function, can be very useful and it was used in this study to find the optimum conditions.

Fig. 1a shows how the response analysis time changes in the considered experimental domain maintaining U_1, U_4, and U_5 at their central level. From the figure, it is clear that to minimize the response, it is necessary to use a low percentage of ethanol (U_2) and to set a high voltage (U_3). For the response LNOT (Fig. 1b), there is a positive interaction between percentage of ethanol and Britton–Robinson buffer concentration (U_4), thus, to maximise this response the two factors have to be set at their

highest level. The same trend can be observed for the response LNDZ from Fig. 1c.

To reach the different goals, Derringer's desirability function was used. Each response was associated with its own partial desirability function d_i. This varied from 0 to 1, according to the closeness of the response to its target value. Using the model and the calculated coefficients, each response variable can be calculated over the experimental domain. In the same way the corresponding desirability can be calculated in all the experimental domains. The individual desirability functions were then combined, as the geometric mean, to obtain the overall desirability function (D) for the system whose maximum value could then be looked for within the domain.[15] In this case, there were three partial desirability functions: d_1, d_2, d_3, for the three responses Y_1, Y_2, Y_3, respectively, and are presented in Fig. 2. Before calculating the overall desirability function (D), the partial desirability function associated with the response LNOT, assumed double weight due to the greater number of problems of the response LNOT with respect to the other responses. From the desirability function D graph (Fig. 3), it is easy to see that there is a large zone in which D is 0, *i.e.* only limited combinations of variable levels allow the target values for all the responses to be reached. In particular, the optimum conditions to minimise analysis time and to maximise the theoretical plates of the two analytes were: temperature, 30 °C; ethanol concentration, 10% v/v; voltage, 30 kV; Britton–Robinson buffer concentration, 0.13 M, and pH 2.9.

This optimum point represented a predicted point. Thus, in order to validate the predictive ability of the hypothesised model for each response, the close agreement between predicted and measured responses was verified. Applying the optimised conditions, the confidence interval for each response at a probability level of 99% was calculated using the mean and the standard deviation obtained from replicates (t: $\bar{x}$ = 4.566 min, s = 0.116 min, n = 4; LNOT: $\bar{x}$ = 4.038, s = 0.021, n = 4; LNDZ: $\bar{x}$ = 4.91, s = 0.03, n = 4). The confidence intervals were 4.566 ± 0.339 min, 4.038 ± 0.061, 4.91 ± 0.088, for t, LNOT and LNDZ, respectively. The predicted values (t 4.841 min; LNOT 4.078; LNDZ 4.835) were inside the confidence interval for each response, thus demonstrating the predictive ability of the calculated regression models.

Method validation

The method was validated for the drug substances and drug product by means of the analysis of typical performance characteristics such as robustness, selectivity, linearity, accuracy, precision, and system suitability, according to ICH3 guidelines.[32] Injection precision in CE is generally poorer than that in HPLC due to the difficulties involved in reproducibly

Table 2 ANOVA: a, analysis time (measured as diazepam migration time); b, log of otilonium bromide number of theoretical plates; c, log of diazepam number of theoretical plates

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio
Regression	130.7381	20	6.5369	19.91[a]
Residuals	1.9694	6	0.3282	
Lack of fit	1.3125	3	0.4375	2.00[b]
Pure error	0.6570	3	0.2190	
Total	132.7075	26		
Regression	0.8436	20	0.0422	16.35[c]
Residuals	0.0155	6	0.0026	
Lack of fit	0.0138	3	0.0046	8.30[d]
Pure error	0.0017	3	0.0006	
Total	0.8591	26		
Regression	0.8644	20	0.0432	6.39[e]
Residuals	0.0406	6	0.0068	
Lack of fit	0.0159	3	0.0053	0.64[f]
Pure error	0.0248	3	0.0083	
Total	0.9050	26		

[a] 19.91 > Fcrit. = 3.87 (with 20 and 6 degrees of freedom and α = 0.05).
[b] 2.00 < Fcrit. = 9.28 (with 3 and 3 degrees of freedom and α = 0.05).
[c] 16.35 > Fcrit. = 3.87 (with 20 and 6 degrees of freedom and α = 0.05).
[d] 8.30 < Fcrit. = 9.28 (with 3 and 3 degrees of freedom and α = 0.05).
[e] 6.39 > Fcrit. = 3.87 (with 20 and 6 degrees of freedom and α = 0.05).
[f] 0.64 < Fcrit. = 9.28 (with 3 and 3 degrees of freedom and α = 0.05).

Table 1 Experimental matrix during response surface study. Factors: U_1, temperature; U_2, percentage of ethanol; U_3, voltage; U_4, Britton–Robinson buffer concentration; U_5, pH. Responses: analysis time (t, measured as diazepam migration time); Log of otilonium bromide number of theoretical plates (LNOT); log of diazepam number of theoretical plates (LNDZ)

U_1	U_2	U_3	U_4	U_5	t/min	LNDZ	LNOT
1.0000	0.0000	0.0000	0.0000	0.0000	5.936	4.828	3.894
−1.0000	0.0000	0.0000	0.0000	0.0000	7.778	4.960	3.782
−0.5000	−0.8660	0.0000	0.0000	0.0000	5.407	4.666	3.983
0.5000	−0.8660	0.0000	0.0000	0.0000	5.705	4.354	3.450
−0.5000	0.2887	0.8165	0.0000	0.0000	5.754	4.719	4.143
0.5000	−0.2887	−0.8165	0.0000	0.0000	9.625	4.782	3.568
−0.5000	0.2887	0.8165	0.0000	0.0000	5.792	4.889	4.021
0.0000	−0.5774	0.8165	0.0000	0.0000	3.860	4.731	3.793
−0.5000	−0.2887	−0.2041	−0.7906	0.0000	6.977	4.782	3.556
0.5000	−0.2887	−0.2041	−0.7906	0.0000	6.314	4.741	3.604
0.0000	0.5774	−0.2041	−0.7906	0.0000	8.770	4.918	3.882
0.0000	0.0000	0.6124	−0.7906	0.0000	5.204	4.746	3.741
−0.5000	0.2887	0.2041	0.7906	0.0000	7.900	4.997	4.309
0.0000	−0.5774	0.2041	0.7906	0.0000	4.951	4.521	3.792
0.0000	0.0000	−0.6124	0.7906	0.0000	9.040	4.853	3.836
−0.5000	−0.2887	−0.2041	−0.1581	−0.7746	5.691	5.017	3.710
0.5000	−0.2887	−0.2041	−0.1581	−0.7746	5.274	5.056	3.654
0.0000	0.5774	−0.2041	−0.1581	−0.7746	7.299	5.147	3.973
0.0000	0.0000	0.6124	−0.1581	−0.7746	4.584	5.032	3.828
0.0000	0.0000	0.0000	0.6325	−0.7746	5.403	5.139	3.839
−0.5000	0.2887	0.2041	0.1581	0.7746	10.960	5.129	4.007
0.0000	−0.5774	0.2041	0.1581	0.7746	7.337	4.694	3.850
0.0000	0.0000	−0.6124	0.1581	0.7746	14.834	4.784	3.709
0.0000	0.0000	0.0000	0.0000	0.0000	6.958	4.847	3.854
0.0000	0.0000	0.0000	0.0000	0.0000	6.787	4.667	3.823
0.0000	0.0000	0.0000	0.0000	0.0000	6.370	4.862	3.814
0.0000	0.0000	0.0000	0.0000	0.0000	7.500	4.832	3.798

injecting nL volumes of sample into the capillary. Moreover, in this case the EOF was considerably suppressed at the operating pH of 2.9, and therefore operational variation could be accentuated (*e.g.*, capillary performance or fluctuation in injection volume).[33] For this reason the internal standard method was used in order to improve precision data. Procaine hydrochloride was found to be the best internal standard (IS) having a migration time shorter than that of the two analytes. Fig. 4 shows a typical electropherogram obtained for the two drugs with IS. For the response, t, the diazepam/internal standard migration time ratio ($t_{DZ/PR}$) was used.

Robustness. It is an important aspect of method validation and can be defined as the sensitivity of a method to small changes in its settings.[34] It is essential to demonstrate method robustness prior to method transfer between laboratories to ensure successful transfer.[35–37] The aim of the test is to identify the method parameters upon which the method responses are significantly dependent and determine the ranges over which they can be varied, without unduly affecting the method performance characteristics.

In this study the robustness of the method was examined by applying a Plackett–Burman design, which is highly fraction-

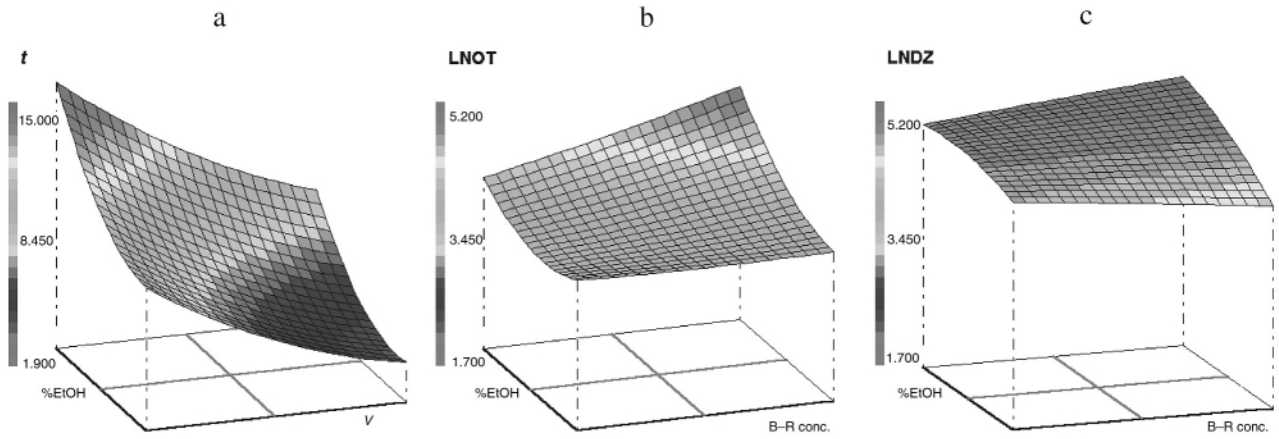

Fig. 1 (a) Analysis time response surface obtained plotting voltage (V) *vs.* percentage of ethanol (%EtOH); (b) Log of otilonium bromide number of theoretical plates (LNOT) response surface obtained plotting Britton–Robinson buffer concentration (B–R conc.) *vs.* percentage of ethanol (%EtOH); (c) Log of diazepam number of theoretical plates (LNDZ) response surface obtained plotting Britton–Robinson buffer concentration (B–R conc.) *vs.* percentage of ethanol (%EtOH).

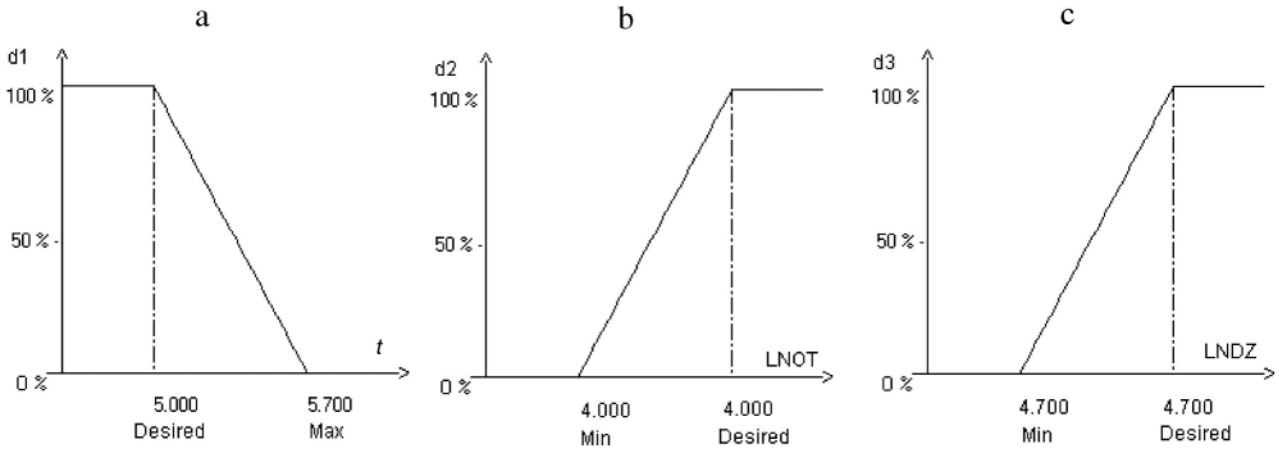

Fig. 2 Transformation of (a) analysis time (t); (b) Log of otilonium bromide number of theoretical plates LNOT; (c) Log of diazepam number of theoretical plates (LNDZ), in the individual desirability function.

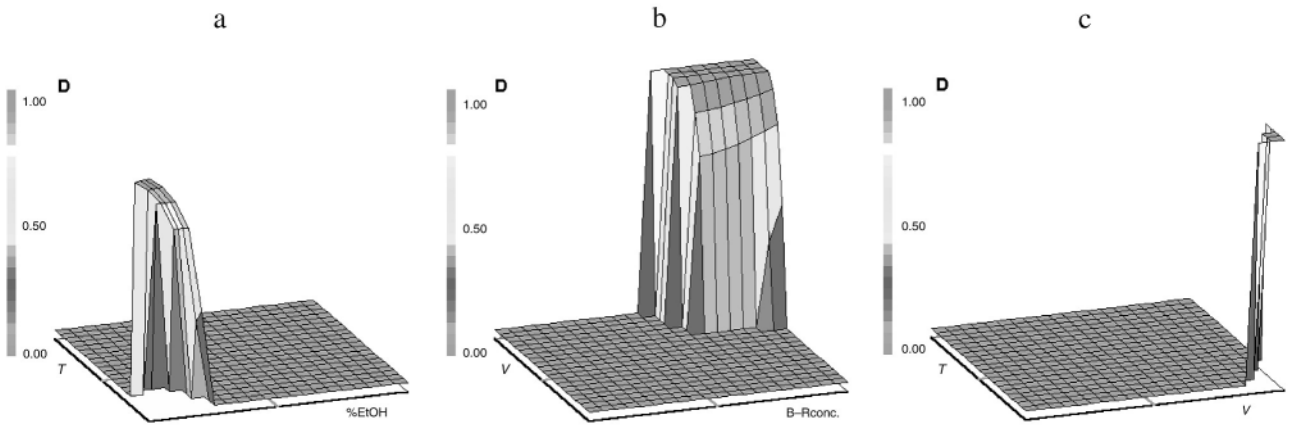

Fig. 3 Desirability function three-dimensional plot: (a) percentage of ethanol (%EtOH) *vs.* temperature (T); (b) Britton–Robinson buffer concentration (B–R conc.) *vs.* voltage (V); (c) voltage (V) *vs.* temperature (T).

ated and useful for screening purposes. The experiments were run twice to obtain an estimate of the experimental error. The factors evaluated during robustness testing were those examined during the optimisation step, but in a small experimental domain as required by this test (Table 3). The measured responses were $t_{DZ/PR}$ and the number of theoretical plates of OT and DZ, NOT and NDZ, respectively (Table 4).

By means of experimental design tools, and in particular by means of the graphic analysis of effects, the critical factors for the developed method were pointed out. Graphic analysis of effects is an experimental design tool that allows the statistically significant effects to be pointed out and the different effect of factor levels to be evaluated. The bars that exceed the reference lines, calculated on the basis of experimental error, (Fig. 5 a,b,c), correspond to the factors for which a change among the considered levels is active on the response. The analysis of results showed that the method parameters upon which the response $t_{DZ/PR}$ is significantly dependent are the ethanol concentration (U_2) and the pH (U_5), while only ethanol concentration (U_2) and pH (U_5) resulted in significance on NOT and NDZ, respectively. Thus, particular attention has to be given to the setting of these two parameters.

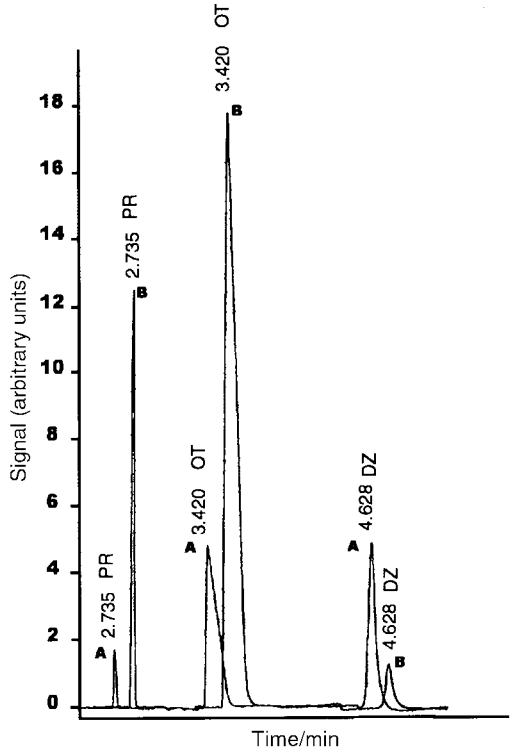

Fig. 4 Typical electropherogram obtained for otilonium bromide and diazepam with procaine hydrochloride as internal standard using the optimised conditions: 0.13 M, pH 2.9 Britton–Robinson buffer, containing 10% v/v ethanol, voltage 30 kV and temperature 30 °C. (A) UV detector set at 230 nm; (B) UV detector set at 280 nm.

Table 3 Factors and experimental domain during robustness testing

	Factor	Experimental domain
U_1	Temperature/°C	28–32
U_2	Ethanol (% v/v)	9–11
U_3	Voltage/kV	29–30
U_4	BGE concentration/M	0.12–0.14
U_5	pH	2.8–3.0

Selectivity. The selectivity of the method was assessed by looking for interfering compounds present in the dosage form. The tablet excipients were analysed according to the method described and an electropherogram absolutely free of any peaks was obtained.

Linearity and range. For drug substances and drug product, using the optimised conditions, linear relationships in the concentration range of 5.6×10^{-5}–8.3×10^{-5} M for DZ, and 2.8×10^{-4}–4.2×10^{-4} M for OT, were obtained. For drug substances, cross validated correlation coefficients (r^2_{cv}) were 0.9947 and 0.9935 for DZ and OT, respectively, with the line equations being $y_{DZ} = 11.271x - 0.2252$ and $y_{OT} = 1.5085x - 0.2809$ ($n = 5$; $k = 2$). For drug product the linear relationships found were $y_{DZ} = 7.4908x$–0.4549 and $y_{OT} = 1.4899x$–0.8546 ($n = 5$; $k = 2$) with r^2_{cv} of 0.9988 and of 0.9971 for DZ and OT, respectively.

Table 4 Robustness experimental plan. Factors: U_1, temperature; U_2, percentage of ethanol; U_3, voltage; U_4, Britton–Robinson buffer concentration; U_5, pH. Responses: diazepam/internal standard migration time ratio ($t_{DZ/PR}$); otilonium bromide number of theoretical plates (NOT); diazepam number of theoretical plates (NDZ)

U_1	U_2	U_3	U_4	U_5	$t_{DZ/PR}$	NDZ	NOT
32	11	30	0.12	3.0	1.796	79799	8128
32	11	30	0.12	3.0	1.763	84333	8204
28	11	30	0.14	2.8	1.620	116950	8260
28	11	30	0.14	2.8	1.627	122462	8110
28	9	30	0.14	3.0	1.748	82604	7998
28	9	30	0.14	3.0	1.710	87700	6918
32	9	29	0.14	3.0	1.733	78705	5495
32	9	29	0.14	3.0	1.687	87498	7943
28	11	29	0.12	3.0	1.774	86696	6998
28	11	29	0.12	3.0	1.752	85704	6934
32	9	30	0.12	2.8	1.608	110917	6592
32	9	30	0.12	2.8	1.594	109901	6427
32	11	29	0.14	2.8	1.636	126183	8511
32	11	29	0.14	2.8	1.643	115878	7889
28	9	29	0.12	2.8	1.597	123027	5848
28	9	29	0.12	2.8	1.596	121899	6053

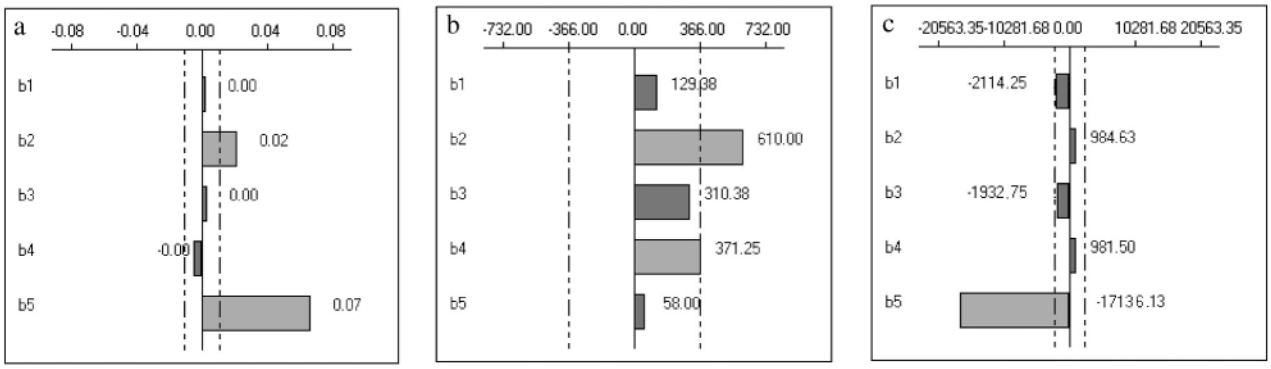

Fig. 5 Graphic analysis of effects: (a) diazepam/internal standard migration time ratio ($t_{DZ/PR}$); (b) number of theoretical plates of otilonium bromide (NOT); (c) number of theoretical plates of diazepam (NDZ).

Table 5 System suitability criteria for the CZE method for diazepam and otilonium bromide

Response	Criterion
Diazepam/internal standard migration time ratio ($t_{DZ/PR}$)	$1.594 < t_{DZ/PR} < 1.796$
Log no. of theoretical plates diazepam (LNDZ)	$4.896 < LNDZ < 5.101$
Log no. of theoretical plates otilonium bromide (LNOT)	$3.740 < LNOT < 3.930$

Accuracy and precision. These parameters, either for drug substances and drug product, were assessed, using the calibration line, on fifteen determinations over three concentration levels (five replicates each) covering the linearity range. The accuracy was measured as percentage recovery with the confidence interval. ($\alpha/2 = 0.025$) at the low, central and high concentration level of linearity range. For drug substances, the accuracy was 96.8 ± 3.9%, 98.7 ± 3.2%, 97.1 ± 1.2%, and 99.8 ± 1.7%, 99.7 ± 3.9%, 97.4 ± 3.1%, for DZ and OT, respectively.

For drug product, synthetic mixtures were used and the accuracy was 97.1 ± 1.3% , 99.0 ± 2.8%, 98.3 ± 5.5% and 98.3 ± 3.0%, 100.9 ± 3.0%, 101.2 ± 3.8%, for DZ and OT, respectively.

The investigation of precision was performed as the degree of repeatability. The considered concentration levels were those used to evaluate accuracy (five replicates each). For drug substance, the RSD values found for DZ and OT were 3.2%, 2.6%, 1.0%, and 1.4%, 3.2%, 2.6%, respectively. For drug product, the RSD values found for DZ and OT were 1.1%, 2.3%, 4.5%, and 2.5%, 2.4%, 3.0%, respectively. The values obtained show that the test procedure can be considered precise and accurate over the range studied.

In addition, the results obtained in tablets (101.5 ± 2.8 for DZ and 102.3 ± 2.1%, for OT; $n = 5$, $\alpha = 0.025$) were in good agreement with those obtained by means of an HPLC method described by one of us.[4]

System suitability criteria. Generally, suitability testing is an integral part of the analytical procedure. The tests are based on the concept that equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as such.[32] Data obtained from the robustness test were used to deduce system suitability criteria for the method as given in Table 5.

Conclusions

The CZE method discussed here is suitable for the simultaneous quantification of diazepam and otilonium bromide in a pharmaceutical dosage form. The short analysis time obtained is certainly a very attractive feature. Furthermore, the small sample solution required, provides another advantage, and the method can be considered an interesting analytical tool for the quality control of these two active ingredients. In addition, the simple experimental design strategy used, shows that a multivariate strategy is a useful alternative to univariate study for simultaneous optimisation of several chromatographic parameters.

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References

1 B. Morelli, *Fresenius' J. Anal. Chem.*, 1997, **357**, 1179.
2 S. Evangelista, A. Giachetti, B. Chapelain, G. Neliat and C. A. Maggi, *Pharmacol. Res.*, 1998, **38**, 111.
3 P. Mannucci, J. Bertini, A. Cocchini, A. Perico, F. Salvagnini and A. Triolo, *J. Pharm. Sci.*, 1992, **81**, 1175.
4 G. Santoni, L. Fabbri, P. Mura, G. Renzi, P. Gratteri and S. Pinzauti, *Int. J. Pharm.*, 1991, **71**, 1.
5 C. Signorini, S. Tosoni, R. Ballerini, M. Chinol and C. Mannucci, *Drugs Exp. Clin. Res.*, 1984, **10**, 273.
6 A. Giachetti, *Ital. J. Gastroenterol.*, 1991, **23**, 56.
7 L. Capurso, F. Del Sette, F. Ferrario and M. Tarquini, *Clin. Ter. (Rome)*, 1992, **141**, 121.
8 G. Mollica and G. Manno, *Clin. Ter. (Rome)*, 1992, **141**, 129.
9 G. Coruzzi, M. Adami, E. Poli, G. Signorini and G. Bertaccini, *Acta Bio-Med. Ateneo Parmense*, 1982, **53**, 445.
10 P. Mannucci, J. Bertini, A. Cocchini, A. Perico, F. Salvagnini and A. Triolo, *J. Pharm. Sci.*, 1993, **82**, 367.
11 P. Corti, E. Dreassi, G. Corbini, G. Ballerini and S. Gravina, *Pharm. Acta Helv.*, 1990, **65**, 189.
12 J. P. Landers, *Handbook of Capillary Electrophoresis*, CRC Press, Boca Raton, 1994.
13 N. A. Guzman, *Capillary Electrophoresis Technology*, Marcel Dekker, New York, 1993.
14 B. Chankvetadze, *Capillary Electrophoresis in Chiral Analysis*, John Wiley & Sons, Chichester, 1997.
15 G. A. Lewis, D. Mathieu and R. Phan-Tan-Luu, *Pharmaceutical Experimental Design*, Marcel Dekker, New York, 1999.
16 S. Fanali, S. Furlanetto, Z. Aturki and S. Pinzauti, *Chromatographia*, 1998, **48**, 395.
17 R. Gotti, S. Furlanetto, V. Andrisano, V. Cavrini and S. Pinzauti, *J. Chromatogr. A*, 2000, **875**, 411.
18 S. Bordi, M. Carlà, G. Papeschi and S. Pinzauti, *Anal. Chem.*, 1984, **56**, 317.
19 M Fillet, L. Fotsing, J. Bonnard and J. Crommen, *J. Pharm. Biomed. Anal.*, 1998, **18**, 799.
20 *The European Pharmacopoeia*, 3rd edn., 1996, Council of Europe, Strasbourg, France, Part 2.2.2.
21 D. Mathieu, J. Nony and R. Phan-Tan-Luu, NEMROD-W, LPRAI sarl, Marseille, France.
22 R. B. Taylor, S. Toasaksiri and R. G. Reid, *J. Chromatogr. A*, 1998, **798**, 335.
23 D. N. Heiger, *High Performance Capillary Electrophoresis–An Introduction*, Hewlett-Packard Editions, France, 1992.
24 T. Pérez-Ruiz, C. Martínez-Lozano, A. Sanz and E. Bravo, *J. Chromatogr. B*, 1997, **692**, 199.
25 K. Sarmini and E. Kenndler, *J. Chromatogr. A*, 1997, **792**, 3.
26 D. R. Ellis, M. E. Palmer, L. W. Tetler and C. Eckers, *J. Chromatogr. A*, 1998, **808**, 269.
27 K. Heinig, C. Vogt and G. Werner, *J. Chromatogr. A*, 1997, **781**, 17.
28 I. E. Frank and R. Todeschini, *The data analysis handbook*, Elsevier, Amsterdam, 1994.
29 D. L. Massart, B. G. M. Vandeginste, L. M. C. Buydens, S. De Jong, P. J. Lewi and J. Smeyers-Verbeke, *Handbook of Chemometrics and Qualimetrics: Part A*, Elsevier, Amsterdam, 1997.
30 D. C. Montgomery, *Design and analysis of experiments*, John Wiley & Sons, New York, 1997.
31 S. Furlanetto, S. Orlandini, G. Aldini, R. Gotti, E. Dreassi and S. Pinzauti, *Anal. Chim. Acta*, 2000, **413**, 229.
32 ICH Topic Q2A, Guideline on validation of analytical procedures: methodology, Nov. 1996.
33 M. A. Kelly, K. D. Altria, C. Grace and B. J. Clark, *J. Chromatogr. A*, 1998, **798**, 297.
34 *United States Pharmacopoeia 24*, United States Pharmacopoeial Convention, Rockville,2000,p. 2151.
35 M. E. Swartz and I. S. Krull, *Analytical method development and validation*, Marcel Dekker, New York, 1997.
36 Y. Zhu, C. Hoogmartens, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr. Relat. Technol.*, 1999, **22**, 1403.
37 J. A. Van Leeuwen, L. M. C. Buydens, B. G. M. Vandeginste, G. Kateman and P. J. Schoenmakers, *Chemom. Intell. Lab. Syst.*, 1991, **10**, 337.