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Received September 24, 2008
Revised November 12, 2008
Accepted November 20, 2008

Research Article

Pitfalls and success of experimental design in the development of a mixed MEKC method for the analysis of budesonide and its impurities

A mixed MEKC method for the analysis of budesonide and its related substances is presented. The micelles were formed from sodium cholate (CHOL) and 3-(*N*,*N*-dimethylmyristylammonio)propanesulfonate (MAPS). A multivariate optimisation was carried out with the aim of obtaining a baseline separation of all compounds. The influence of voltage, borate concentration, cholate concentration, MAPS concentration and pH was evaluated on the responses, corresponding to critical resolution values. Problems with the investigated experimental design were encountered due to the complexity of the separation process. As a consequence, a first design was not sufficient to reach the optimal conditions, but was needed in order to obtain the necessary information to successfully plan a second in-depth study by means of response surface methodology. The optimal conditions were as follows: capillary total and effective lengths of 48.5 and 40.0 cm, respectively, with 50 μm id; 70 mM borate buffer (pH 8.8) containing 65 mM CHOL and 10 mM MAPS; temperature 20°C and voltage 16 kV. Separation of all the compounds, including *R*- and *S*-epimers of budesonide, was obtained in a reasonable time. Validation of the method was performed for both drug substances and drug product.

Keywords:
Budesonide / Experimental design / Impurities / Mixed MEKC / Validation
DOI 10.1002/elps.200800626

1 Introduction

Budesonide (BD, an epimeric mixture of the α- and β-propylic forms of 16α,17α-butylidenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione) is a corticosteroid with mainly glucocorticoid activity. It is used for the management of asthma and various skin disorders, for the prophylaxis and treatment of rhinitis and for the management of inflammatory bowel and collagenous colitis [1]. According to the information provided by the drug producer, AstraZeneca (Lund, Sweden), four main budesonide impurities (I_1, I_2, I_3, I_4) may be found in the drug substance (DS). Their chemical structures are shown in Fig. 1. I_2, I_3 and I_4 are epimeric mixtures of the α- and β-propylic forms.

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Abbreviations: AN, *p*-anisaldehyde**; BD,** budesonide**; CHOL,** sodium cholate**; DP,** drug product**; DS,** drug substance**; I_1,** budesonide impurity 1**; ICH,** International Conference on Harmonisation**; MAPS,** 3-(*N*,*N*-dimethylmyristylammonio)-propanesulfonate**; MMEKC,** mixed MEKC**; USP,** United States Pharmacopeia

Several methods have been described for the analysis of BD in combined dosage form [2, 3], serum or plasma [4–10] and in bronchoalveolar lavage [11]. Analysis of BD epimers has been performed both in dosage form [12, 13] and in plasma [14–16]. An HPLC method can be found in European Pharmacopoeia [17] for the assay of BD and its related substances and it has recently been applied for the estimation of BD tablets impurities [18]. Another HPLC method has been developed for the determination of epimers, impurities and total content of BD [19]. To the best of our knowledge, no CE method has been reported, neither for BD assay in dosage form nor for BD impurity profiling. Thus, the aim of this study is to develop a CE method for the simultaneous determination of BD and its related impurities, which could have application for pharmaceutical quality control.

Several reports have been dedicated to corticosteroids analysis by CE, considering different test mixtures. For the separation of this type of compounds, MEKC was successful [20–25] and additives such as cyclodextrins were also effective [26, 27]. Mixed MEKC (MMEKC) [28–36] and microemulsion electrokinetic chromatography methods [21, 37] have also been developed for this purpose.

In this study, MMEKC was found to be the most suitable for the target compounds on the basis of selectivity. Many factors critically influenced the analysis; thus the system was deeply investigated using an experimental design strategy [38, 39]. In general, the experimental design

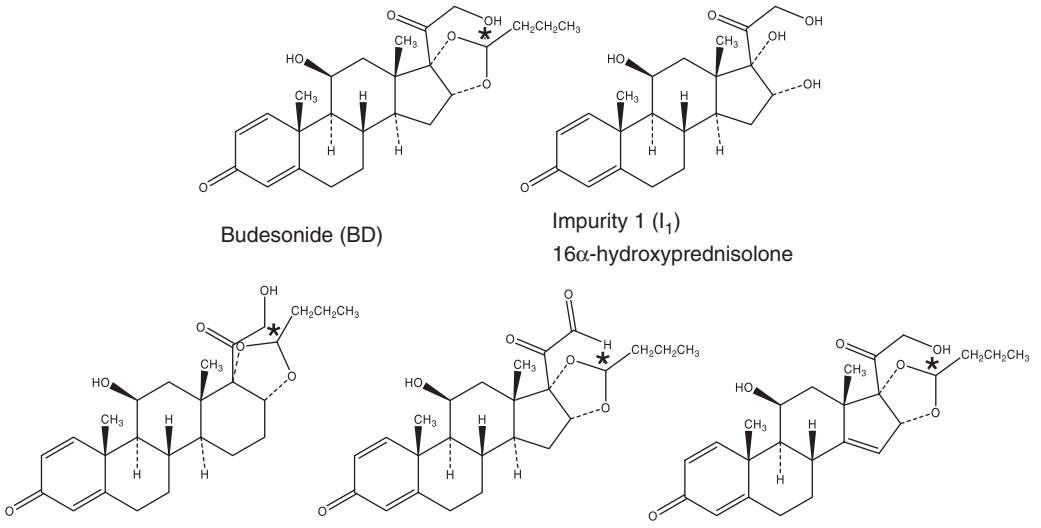

Figure 1. Structural formulas of BD and its related impurities.

approach starts with the correct formulation of the problem, stating the aims of investigation (*i.e.* maximisation of resolution, minimisation of analysis time), the variables involved in the optimisation process and the experiments to carry out. The latter are defined by an experimental matrix chosen in function of the desired information. Screening matrices allow information about dominating factors and correct solution regions to be obtained, whereas response surface study allows a map of response variation to be drawn.

However, experimental design is not free from hazards. For example, at the beginning of CE method development, in some experiments peak splitting and inversion in migration order can occur and the response cannot be univocally measured, with the consequence that statistical treatment of the responses is not possible. Another important point is the proper selection of the investigation domain that can require preliminary experiments.

In the present work, the experimental design strategy involved a first set of experiments necessary to obtain preliminary information on the system, and a second set necessary to optimise the system. Several problems encountered at the beginning of the work were overcome and good results were obtained in a limited number of experiments. The final optimal conditions were found by means of desirability function [39], and the method was validated for drug substances and drug product (DP) according to the International Conference on Harmonisation (ICH) guidelines [40].

2 Materials and methods

2.1 Chemicals and reagents

Sodium borate was from BDH Laboratory Supplies (Poole, UK). Methanol (HPLC grade), sodium cholate (CHOL) and 3-(*N*,*N*-dimethylmyristylammonio)propanesulfonate (MAPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The reference standards of BD and its related substances (coded I_1, I_2, I_3 and I_4 depending on the final order of migration under the optimised conditions) and controlled-release capsule excipients were kindly donated by AstraZeneca. Entocir® controlled-release capsules (labelled to contain 3 mg of BD, acetyl tri-*n*-butyl citrate, antifoam M, ethylcellulose aqueous dispersion, methacrylic acid–ethyl acrylate copolymer (1:1) dispersion (30%), polysorbate 80, sugar spheres, talc) were purchased locally in pharmacies. *p*-Anisaldehyde (AN), employed as an internal standard, was from Sigma-Aldrich. Ultrapure water used throughout the study was provided by a Simplicity 185 system (Millipore, Billerica, MA, USA) after an electrodeionisation treatment using an Elix system (Millipore).

2.2 Solutions and sample preparation

The standard stock solutions of BD (30 mg/mL), AN (internal standard, 40 mg/mL) and BD impurities (1 mg/mL) were prepared in methanol. The sample mixture was prepared daily by adding the appropriate volume of each of the stock solutions directly in a vial and diluting to 500 μL with methanol. All these solutions were stored at 4°C and used within 1 wk.

In the optimisation phase, the test concentration used in the first experimental design was 0.03 mg/mL for both BD and its impurities. The test solution used in the second experimental design was constituted by 10 mg/mL BD, 100 μg/mL impurities (corresponding to 1% w/w with respect to the main component) and 4 mg/mL AN. The BGEs examined during the optimisation step were prepared by weighing the adequate amount of borate and adjusting pH with 1 M HCl. An accurately weighed amount of CHOL (CMC ≈ 13–15 mM) [41] and MAPS (CMC ≈ 0.1–0.4 mM)

(http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/T7763/) was then added to the buffer.

With regard to sample preparation, the contents from 40 capsules were weighed and crushed to fine powder and the equivalent of 100 mg BD, accurately weighed, was transferred for the analysis into a 25 mL beaker. The content was diluted with 10 mL methanol solution containing 4 mg/mL internal standard, shaken vigorously, sonicated for 10 min and shaken again. An aliquot of 1 mL of the mixture was then centrifuged and 500 μL of the supernatant were introduced into a vial for the analysis, thus obtaining a final BD test concentration of about 10 mg/mL.

2.3 Apparatus and operating conditions

A 300 Ultrasonik ultrasonic bath (Ney Company, Bloomfield, USA) was used to sonicate solutions and a Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland) was used to measure pH. A microcentrifuge 5415 D (Eppendorf, Hamburg, Germany) was employed to centrifuge samples.

CE analyses were performed with an Agilent Technologies ^{3D}CE system (Agilent Technologies, Waldbronn, Germany), which was driven by ^{3D}CE ChemStation software (Rev. A.09.01, Agilent Technologies) and had a UV–visible DAD and an air thermostating system. The separations were carried out in an uncoated fused-silica capillary (50 μm id and 375 μm od, Composite Metal Services, Ilkley, UK), which was 48.5 cm long (40.0 cm to the detector). The detection window was built-in by burning off the polyimide coating on the capillary. Detection wavelength was 245 nm. The injection time of both the sample and the subsequent buffer plug was 1 s at 50 mbar. Temperature was 20°C and voltage was 16 kV, with a rise time of 0.20 min. The optimum BGE was 70 mM borate (pH 8.8) with 65 mM CHOL and 10 mM MAPS. Applying these experimental conditions, a generated current of about 110 μA was observed.

Each new capillary was initially conditioned with 1 M NaOH and water for 5 min each. Between two runs, the capillary was rinsed with methanol (2 min), 1 M NaOH (1 min), 0.1 M NaOH (1 min), water (1 min) and run buffer (4 min). Owing to the particularly high ionic strength of the buffer, in order to maintain good system performances, particular care had to be taken to instrument maintenance and cleaning, with special attention to electrodes and pre-punchers.

2.4 Calibration curves

The curves were obtained by preparing five different concentrations of each analyte and analysing the solutions twice, using 4 mg/mL AN as the internal standard. For the calibration curve of BD, which corresponded to two separated peaks in the electropherogram related to the two epimers of BD, BD_1 and BD_2, the sum of BD_1 and BD_2 corrected areas/internal standard corrected area ratio was plotted *versus* BD/internal standard concentration ratio. For the calibration curve of BD-related substances, each analyte/internal standard peak area ratio was plotted *versus* each analyte/internal standard concentration ratio.

2.5 Calculations and software

In the first experimental design, the concentration of all the compounds was kept low and equal to 0.03 mg/mL; the obtained peaks were symmetric and had comparable areas and widths. Thus, resolution values R were calculated by the usual United States Pharmacopeia (USP) method [42]:

$$R = 2\frac{t_b - t_a}{w_b + w_a} \quad (1)$$

where t_b and t_a are the migration times, and w_b and w_a the peak base widths of adjacent peak pairs, respectively.

In the second experimental design, due to the different concentrations of the analytes (BD, 10 mg/mL; BD impurities, 100 μg/mL), resolution values between I_4 and the previous peak BD_1 (R_1) or the following peak BD_2 (R_2) were calculated using the statistical method as the involved peaks were significantly different for area and width [43, 44]:

$$R = \frac{M1_b - M1_a}{w_{Sb} + w_{Sa}} \quad (2)$$

where $M1_a$ and $M1_b$ are the mean migration times for adjacent peak pairs (first statistical moment), w_{Sb} and w_{Sa} are the widths derived from statistical moments= $\sqrt{(M2)}$, where $M2$, the second statistical moment, is the peak variance, which is a measure of lateral spreading. The detailed information for this calculation method is summarised in the software manual [43].

The experimental design software used to set-up designs and to perform ANOVA was NEMROD-W [45].

3. Results and discussion

BD and its related substances are all neutral compounds in aqueous solution; moreover, they have very similar structures based on the steroidal skeleton. Thus, different pseudostationary phases were initially examined in order to reach a baseline separation of the compounds. Both MEKC and microemulsion electrokinetic chromatography operative modes were taken into consideration, testing the addition of different surfactants, organic additives and cyclodextrins. In these preliminary experiments, the concentration of BD was kept low and similar to that of the impurities (0.03 mg/mL) to obtain clear information on the behaviour of the system. From these first runs it was clear that the main problems were the critical separation of BD and I_4, which differ for only a double bond, and the detection of I_3, which showed a lower absorbance than the other impurities. In some experimental conditions, this impurity presented such a low efficiency that it could not even be detected.

From these exploratory trials MMEKC was found to be the most suitable for the target compounds. By combining

surfactants with different structural properties, solute–micelle interactions can be manipulated in order to elicit the desired separation. As a consequence, different solubilisation characteristics may be obtained, which can be exploited to tune selectivity [46]. On the basis of selectivity for the pair BD/I_4 and detectability/peak shape of I_3, the best pseudostationary phase was identified in a mixed micelle system composed of the anionic surfactant CHOL and the zwitterionic MAPS in sodium borate buffer. By using these surfactants, not only the impurities but also the two epimers of BD (BD_1 and BD_2) could be separated. No problems were evidenced for the separation of I_1, I_2 and I_3, but the real analytical challenge was still represented by the separation of I_4, whose peak fell just between the two peaks of the BD epimers. For quantitative determination, AN was selected as a suitable internal standard.

3.1 Preliminary application of a multivariate approach

Having to deal with different factors, an optimisation based on a multivariate strategy is useful and preferred for its several well-known advantages such as fewer trials, large number of factors studied, detection of interaction between factors, detection of optima, optimisation of the results and model-building from the results [47].

The limited number of factors we had to deal with made it possible to directly apply response surface methodology and the experimental domain for the factors was: X_1, voltage (V), 15–25 kV; X_2, buffer concentration (borate conc.), 30–70 mM; X_3, CHOL concentration (CHOL conc.), 40–80 mM; X_4, MAPS concentration (MAPS conc.), 5–15 mM; X_5, pH 8.2–9.2. At the centre of this experimental range, the migration order was I_1, I_2, I_3, BD_1, I_4, BD_2. The critical resolution values were mainly represented by R_1 (resolution between BD_1 and I_4) and R_2 (resolution between I_4 and BD_2), which were to be maximised, calculated according to the USP method [42]. A Doehlert design was initially chosen to estimate the coefficients of a quadratic model describing the relationship between the factors and the response:

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

where y represents the experimental response, x_i the independent evaluated factors, β_0 the intercept, β_{ij} the model coefficients obtainable by multiple regression and ε the experimental error.

For $k = 5$ variables, Doehlert design required $k^2+k+n = 33$ runs for $n = 3$ replicates at the centre of the domain. The number of experiments was deemed too high and was reduced by means of D-optimal design [39], leading to a matrix that involved 27 experiments, including three runs at the centre. However, this first experimental design led to different practical problems involving planning the experiments and/or data treatment: (i) choice of the test concentration of the compounds; (ii) detection of impurity I_3 and (iii) inversion in the migration order of I_1 and I_2.

The first problem was the selection of the test concentration of the analytes to be used when running the design. In fact, usually response surface study is carried out at the final test concentration of the compounds in order to reproduce the final conditions of a real routine analysis. In this case, we dealt with a pharmaceutical quality control problem where the separation of the compounds should be assured, keeping the related substances at 1% w/w of the main compound. The test solution for the analysis was constituted by 10 mg/mL BD and 100 μg/mL impurities. In this condition, the BD peak would present a much greater area than the others, with the consequence of presumably covering other impurities with similar migration time, in particular, I_4. Instead, to understand the system under study, it is necessary to know the separation pattern and the migration behaviour of the compounds exactly. Therefore, the concentration of all the compounds was kept low and equal to 0.03 mg/mL, in order to obtain an electropherogram where all the peaks had comparable heights and widths. The drawback of this choice is that it was not possible to transfer the prediction of the calculated model to the final test concentration of the compounds and thus to find the optimum. In fact, the resolution values achieved in this step could not be maintained increasing the concentration of BD up to the test concentration of 10 mg/mL.

With regard to the detection of I_3, this compound, as mentioned previously, showed a lower absorbance than the other related substances. Furthermore, from the results of D-optimal design, some experimental conditions led to the partial or complete separation of the two epimers, further reducing the height and thus the detectability of the related peak(s).

Finally, again from the results of D-optimal, the variations in the composition of BGE could lead to the overlap or inversion in the migration order of I_1 and I_2, meaning that the response related to the resolution between these two peaks could not be univocally measured, and if necessary it could not be treated statistically.

As a consequence, this first experimental design failed in giving sufficient information to directly find the optimum. In fact, BD concentration was more than 300 times lower than the final test concentration, and the variability in I_3 peak splitting and migration order of I_1 and I_2 could not be predicted and/or kept under control. Nevertheless, as for the responses R_1 and R_2, measured without problems in all experiments, data treatment by ANOVA [38] evidenced that the postulated model was significant and valid. The response surfaces were not drawn, but the advantage of statistically planning experiments lay in the possibility of applying desirability function [39], fixing a minimum value

of 1.5 for R_1 and R_2 to be reached. Desirability function made it possible to select some potential experimental points, which could fulfil the requirements for R_1 and R_2. These points were experimentally tested using the final test concentrations in order to check the maintenance of a sufficient selectivity for the pairs BD_1/I_4, I_4/BD_2 and I_1/I_2 and to verify that I_3 was not split in two peaks, with the aim of choosing the most suitable as starting point for a second response surface study carried out at the final test concentrations. The best examined point constituted the centre of the new experimental domain to be investigated. Thus, this second experimental design could focus on the aspect related to resolution between peaks BD_1/I_4 and I_4/BD_2, as in the new experimental domain the encountered problems related to migration order of I_1 and I_2, and detectability of I_3 had already been overcome.

3.2 Response surface methodology

The most important variations for the new domain were the decrease in voltage to expand the migration window, the increase in borate conc. and CHOL conc. and the reduction in the domain for MAPS conc. and pH: X_1, *V*, 13–17 kV; X_2, borate conc., 60–80 mM; X_3, CHOL conc., 60–80 mM; X_4, MAPS conc., 6–12 mM; X_5, pH 8.2–8.8.

The considered responses were the critical resolutions R_1 (BD_1/I_4) and R_2 (I_4/BD_2), calculated according to the statistical method [43] due to the different characteristics of the involved peaks. In fact, applying the test concentration values for BD (10 mg/mL) and related substances (100 μg/mL), the obtained peaks for BD epimers and I_4 were very different for width and symmetry. Using the statistical method instead of the USP formula, it is possible to obtain a more reliable value for resolution [44]. The other resolutions were not considered because no problem was pointed out.

A new Doehlert design for five factors was planned to estimate the coefficients of a quadratic model. No reduction in the number of experiments was made in order to have the maximum possible information. The 34-runs experimental plan with the measured responses is reported in Table 1. In some runs, an almost complete overlap of two adjacent peaks made it difficult to exactly determine the real resolution value and for these experiments an arbitrary resolution value of 0.01 was introduced in order to fulfil the software requirements. Both the calculated regression models, one for each considered response, were found valid and significant by means of ANOVA [38].

R_1 and R_2 response surfaces, involving the factors for which significant interactions were detected, were graphically represented by plotting two variables at a time and setting the other three at the central value of the experimental range (Figs. 2 and 3). With a multivariate approach, the investigation of the obtained response surfaces allows a good electrophoretic description of the system to be achieved. Obviously, with a univariate strategy, it is difficult to find interactions and the correct solution can be missed. In this case, the factor voltage was critical for all responses; moreover, strong interactions were given by buffer concentration with different factors.

R_1 response surfaces are represented in Fig. 2. The positive interaction between voltage and buffer concentration can be noted in Fig. 2A. Figure 2B points out a negative interaction between *V* and CHOL conc., whereas a positive interaction is evidenced in Fig. 2C for voltage and MAPS concentration. Finally, the strong positive interaction shown in Fig. 2D pointed out that low levels or high levels of both borate conc. and MAPS conc. were required to maximise R_1.

As concerns R_2, the response surfaces are represented in Fig. 3 and can be similarly examined in order to point out significant interactions between the factors and the zones of the experimental domain leading to the best results. In particular, there was a negative interaction for MAPS conc. and pH (Fig. 3f), but a quadratic effect of both factors was also present and so the response was maximised at the centre of the experimental domain.

Starting from the single response surfaces, the search for global optimum was very difficult due to the number of factors and responses involved. Thus, the Derringer desirability function was used [39] in order to find the optimum conditions, fixing for both the responses a target value of 1.5. The total desirability function *D* was then obtained by calculating the geometric mean of the partial desirability functions

$$(D = \sqrt{d_1 d_2}).$$

Its representation is reported in Fig. 4, where *D* is shown for two factors at a time, setting the others at their optimised value. Only a small set of values for *V*/pH (Fig. 4A) and for CHOL conc./MAPS conc. (Fig. 4C) led to maximum desirability, whereas a larger number of borate conc./CHOL conc. combination values (Fig. 4B) led to the desired values of the responses. The selected optimum was: *V*, 16 kV; borate conc., 70 mM; CHOL conc., 65 mM; MAPS conc., 10 mM; pH 8.8.

Applying these conditions, a complete separation of the analytes was obtained in a reasonable time with a generated current of about 110 μA. A typical electropherogram of the mixture is shown in Fig. 5. The predictivity of each considered model was verified by evaluating if the measured values for the responses fell within the confidence intervals, centred on the predicted values [39]. The experimental values found were included in the confidence limits; thus good agreement between predicted and observed responses was verified.

3.3 Validation

The developed method was validated following ICH guidelines [40], for both DS and DP, in order to demonstrate that the method was suitable for its intended purpose. Unless stated otherwise, a test mixture of 10 mg/mL BD and

Table 1. Doehlert design experimental plan and responses

Exp. no.	*V* (kV)	Borate conc. (mM)	CHOL conc. (mM)	MAPS conc. (mM)	pH	R_1	R_2
1	17	70	70	9	8.5	1.27	2.12
2	13	70	70	9	8.5	3.30	0.01
3	16	80	70	9	8.5	2.41	1.94
4	14	60	70	9	8.5	4.76	0.01
5	16	60	70	9	8.5	1.91	1.83
6	14	80	70	9	8.5	2.49	2.32
7	16	73	80	9	8.5	1.31	2.51
8	14	67	60	9	8.5	1.84	1.88
9	16	67	60	9	8.5	1.52	1.91
10	15	77	60	9	8.5	1.70	0.01
11	14	73	80	9	8.5	2.95	1.16
12	15	63	80	9	8.5	3.77	0.01
13	16	73	72	12	8.5	2.07	1.91
14	14	67	68	6	8.5	4.64	0.01
15	16	67	68	6	8.5	1.05	1.92
16	15	77	68	6	8.5	0.01	0.01
17	15	70	77	6	8.5	2.65	0.01
18	14	73	72	12	8.5	1.73	1.88
19	15	63	72	12	8.5	1.07	0.01
20	15	70	63	12	8.5	1.00	0.01
21	16	73	72	10	8.8	1.43	1.73
22	14	67	68	8	8.2	2.64	0.01
23	16	67	68	8	8.2	0.01	0.01
24	15	77	68	8	8.2	2.09	1.93
25	15	70	77	8	8.2	2.09	0.01
26	15	70	70	12	8.2	0.90	1.89
27	14	73	72	10	8.8	1.88	0.01
28	15	63	72	10	8.8	1.81	0.01
29	15	70	63	10	8.8	1.66	1.90
30	15	70	70	6	8.8	1.19	2.04
31	15	70	70	9	8.5	1.60	1.43
32	15	70	70	9	8.5	1.84	1.80
33	15	70	70	9	8.5	2.20	1.84
34	15	70	70	9	8.5	1.62	1.46

100 μg/mL for each of the related substances (corresponding to 1% w/w with respect to the main component) was used. Validation of the DP was carried out adding analyte reference standards to synthetic mixtures of the excipients.

3.3.1 Selectivity

The mixture was separately spiked with each of the compounds in order to identify the peaks. The baseline separation of the analytes was verified calculating the resolution values ($n = 4$, $\alpha/2 = 0.025$): R_1 (BD_1/I_4), 1.62 ± 0.16; R_2 (I_4/BD_2), 1.72 ± 0.20. No interference in the electropherogram was found for the presence of excipients.

3.3.2 Robustness

Robustness test quantifies the insensitivity of the results for a method transfer to another laboratory or instrument [48]. In this study, robustness was evaluated by means of a multivariate strategy [49, 50], considering the same variables examined in the optimisation step, with the addition of temperature, and their effect on R_1 and R_2. The small experimental range, symmetrically chosen around the optimal conditions, was: X_1, temperature (T), 19–21°C; X_2, V, 15–17 kV; X_3, borate conc., 68–72 mM; X_4, pH 8.7–8.9; X_5, CHOL conc., 63–67 mM; X_6, MAPS conc., 9–11 mM. When evaluating a small interval, as in robustness testing, curvature or interaction effects can be neglected [48]. Thus, in this case a Plackett–Burman matrix that allows examining six factors by eight experiments was used in order to calculate the coefficients of the linear model $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5$, where b_i are the estimates of the real coefficients β_i. The experimental plan and the measured responses are shown in Table 2.

Graphic analysis of effects [50, 51] was used to identify the critical factors, after introducing the experimental variance ($n = 4$) for each response (Fig. 6). Figure 6A indicates that none of the factors had a significant effect on R_1. Instead, for R_2, represented in Fig. 6B, borate conc. (X_3) and CHOL conc. (X_5) were identified as critical para-

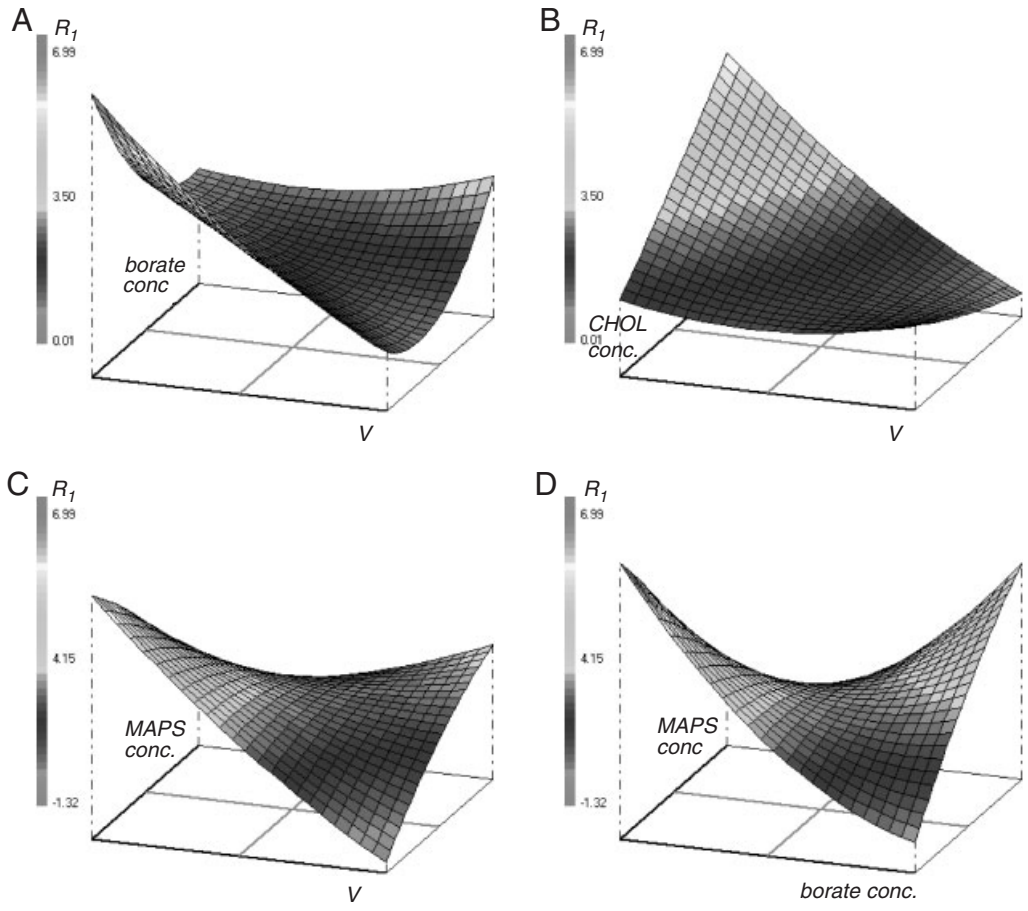

Figure 2. . R_1 response surfaces obtained by plotting: (A) voltage *versus* buffer concentration; (B) voltage *versus* cholate concentration; (C) voltage *versus* MAPS concentration; (D) buffer concentration *versus* MAPS concentration.

meters, having a positive effect on R_2. As a consequence, it was necessary to include a precautionary statement about carefully controlling these two factors when preparing the BGE.

3.3.3 Migration time and peak area precision

The repeatability (within-day precision) of the method in terms of migration times and corrected peak area ratios was evaluated carrying out six replicate injections of the test mixture. The same mixture was analysed over 3 days ($n = 18$) to assess between-day precision. The within-day RSD values for corrected area ratios were in the range of 0.3–0.7% for BD and in the range of 1.3–4.2% for BD-related substances, whereas the between-day RSD values were 0.8% for BD and ranged from 2.3 to 3.8% for the impurities. For analysis time, measured as BD_2 migration time, within-day RSD ranged from 1.3 to 5.7%, whereas between-day RSD was 8.4%.

3.3.4 LODs and LOQs

LOD and LOQ values for the impurities were evaluated from *S*/*N*, equal to 3:1 and 10:1, respectively. The values of LOD were: I_1, 20 μg/mL; I_2, 20 μg/mL; I_3, 30 μg/mL; I_4, 20 μg/mL. The values of LOQ were: I_1, 30 μg/mL; I_2, 30 μg/mL; I_3, 40 μg/mL; I_4, 30 μg/mL. LOQ was verified calculating the corrected area ratio RSD obtained performing eight replicate injections at LOQ concentration for both DS and DP, and the values ranged from 4.9 to 7.0%.

3.3.5 Linearity, accuracy and precision

The linearity range for BD was 8–12 mg/mL, corresponding to 80–120% of the test concentration. The linearity equation was $y = 4.245x - 2.791$ ($R^2 = 0.998$) for DS and $y = 3.923x - 2.025$ ($R^2 = 0.997$) for DP, where y is the sum of BD_1 and BD_2 corrected areas/internal standard corrected area ratios. For the impurities, linearity was evaluated in the range from the respective LOQ values to 2% w/w with respect to the main component, and values of R^2 ranged from 0.995 to 0.998.

Accuracy and precision were evaluated at three concentration levels, each with three replicates, covering the linearity range, for both DS and DP. The recovery values ranged from 99.0 ± 2.7 to 101.4 ± 0.4% for BD and from 98.1 ± 9.0 to 104.8 ± 14.0% for BD-related substances. The maximum RSD values were 1.1% for BD and 5.4% for BD-related substances.

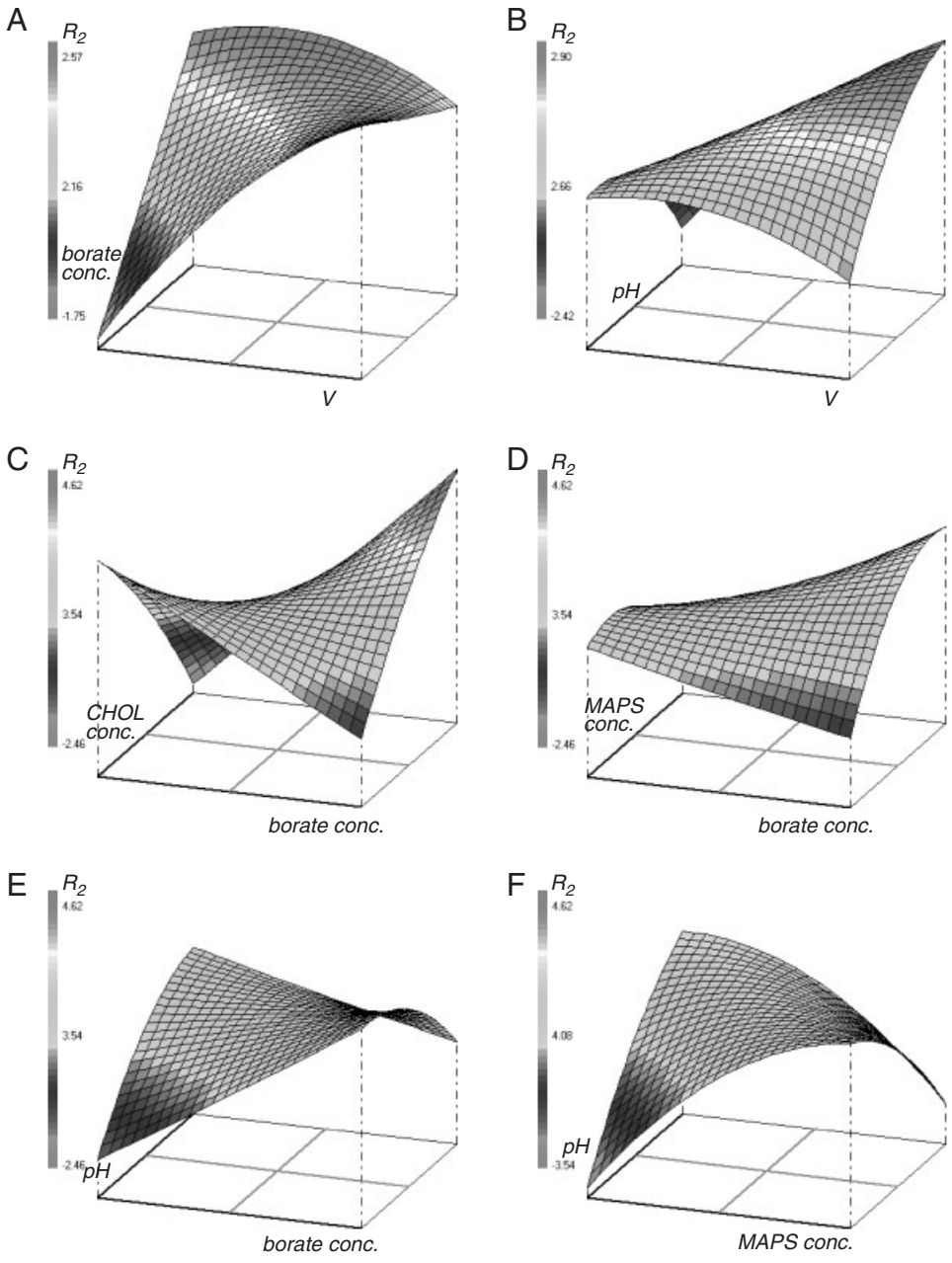

Figure 3. R_2 response surfaces obtained by plotting: (A) voltage *versus* buffer concentration; (B) voltage *versus* pH; (C) buffer concentration *versus* cholate concentration; (D) buffer concentration *versus* MAPS concentration; (E) buffer concentration *versus* pH; (F) MAPS concentration *versus* pH.

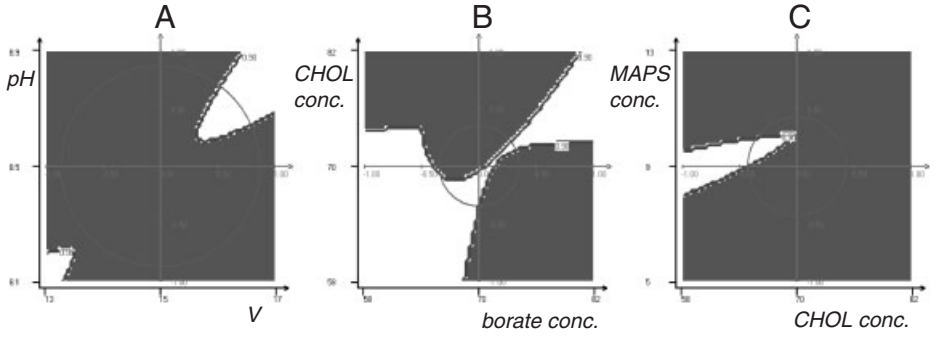

Figure 4. Desirability function bidimensional plots obtained by plotting: (A) voltage *versus* pH; (B) buffer concentration *versus* cholate concentration; (C) cholate concentration *versus* MAPS concentration.

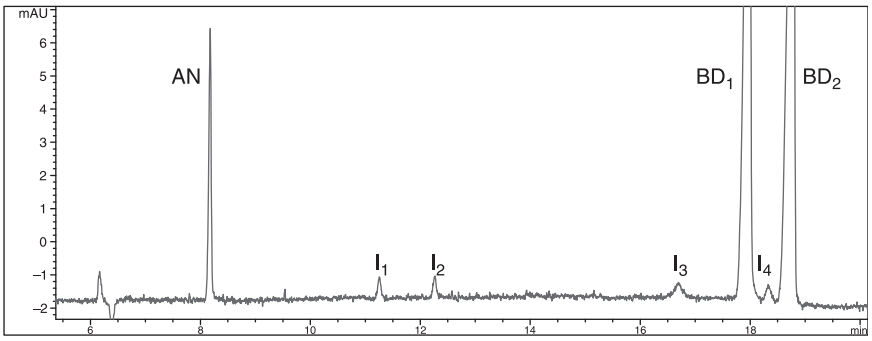

Figure 5. Electropherogram showing BD and its related impurities under the optimal conditions: voltage, 16 kV; BGE: pH 8.8, 70 mM borate buffer with CHOL 65 mM and MAPS 10 mM; temperature, 20°C. Concentrations: 10 mg/mL BD, 100 μg/mL impurities, 4 mg/mL AN. Hydrodynamic injection: 50 mbar, 1 s. Detection wavelength: 245 nm.

Table 2. Eight-run Plackett–Burman experimental plan and responses for robustness testing

Exp. no.	*T* (°C)	*V* (kV)	Borate conc. (mM)	pH	CHOL conc. (mM)	MAPS conc. (mM)	R_1	R_2
1	21	17	72	8.7	67	9	1.99	2.09
2	19	17	72	8.9	63	11	1.26	1.76
3	19	15	72	8.9	67	9	1.31	2.49
4	21	15	68	8.9	67	11	2.11	1.96
5	19	17	68	8.7	67	11	1.17	1.59
6	21	15	72	8.7	63	11	1.29	1.74
7	21	17	68	8.9	63	9	1.17	1.56
8	19	15	68	8.7	63	9	2.47	1.67

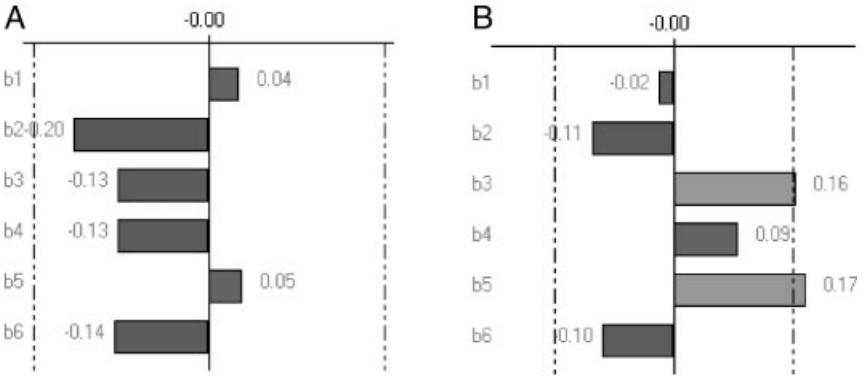

Figure 6. Robustness testing graphic analysis of effects: (A) response R_1; (B) response R_2. Coefficients of the linear model: b_1, *T*; b_2, *V*; b_3, borate conc.; b_4, pH; b_5, CHOL conc.; b_6, MAPS conc.

3.3.6 System suitability test

The critical resolution values R_1 and R_2 were chosen as performance parameters [49, 52]. The lowest and the highest resolution values observed during system precision study over 3 days defined the requirements that this system must meet prior to analysis: $1.38 < R_1 < 2.07$, $1.45 < R_2 < 1.98$. In fact, system precision is suitable to determine system suitability limits as it provides valuable information about the variability of the analytical system [49].

3.4 Application and comparison with other methods

Applying the method to the analysis of a real sample of Entocir® capsules ($n = 4$, $\alpha/2 = 0.025$), the content was in agreement with the declared composition: recovery 98.4 ± 1.8%, RSD 1.1%. No BD impurity was detected.

The developed method was then critically compared with an analogous HPLC one [18], involving the separation of ten impurities including degradation products in a stressed sample of BD. In this study, the BD isomers were not baseline separated; thus selectivity was not fully reached. On the other hand, all the impurities could be quantified at lower levels than the present method (from 0.018 to 0.33%). The drawback of the CE method is surely related to the values of LOQ for the impurities (about 0.3%), which unfortunately exceed those required by ICH (0.1%). In fact, as well-known, poor sensitivity is often a real issue in CE using DAD. In this case, LOQ values were improved for I_1, I_2 and I_3 by increasing injection time or volume, but problems arose for $BD_1/I_4/BD_2$

resolution values. Another possibility was the use of a higher test concentration of BD; however, in addition to the mentioned overlapping, other problems were evidenced concerning difficulties in sample preparation due to the low amount of BD in the real sample of Entocir® (3 mg in a 350 mg capsule). Thus, for I_1, I_2 and I_3, the required LOQ values could be reached, whereas for I_4, it was not possible to fully satisfy ICH requirements. Finally, the major advantage of the developed method was surely related to the aspect of a very simple method requiring very small amounts of solvents, thus playing a role in green chemistry.

4 Concluding remarks

An MMEKC method for the determination of BD and its four related impurities was developed. The use of a mixed micelle system composed of CHOL and MAPS allowed the necessary selectivity to be obtained. Owing to the lack of knowledge about the mechanism of solute–micelle interactions and the difficulty of separating I_4 from the two epimers of BD, the system was investigated in depth using a multivariate strategy. The combination of a real complex sample to be analysed and the large number of possible interactions between factors influencing the analysis in CE constituted a valuable field in order to examine the real problems researchers have to face when using experimental design strategies. Although several problems were encountered, method development was neither time-consuming nor inefficient because experiment planning was suitable to cover the experimental space and to obtain the desired final optimisation.

In conclusion, it should be underlined that in any case, also in the presence of complex systems, the single most critical piece of equipment is the researcher's own brain, whereas experimental design speeds up and improves the optimisation step, allowing an analytical method to be developed in an elegant way.

The authors gratefully acknowledge AstraZeneca (Lund, Sweden) for the gift of BD and its related substances reference samples.

The authors have declared no conflict of interest.

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