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A Capillary Zone Electrophoresis Method with Multiresponse Chemometric Optimization for the Simultaneous Determination of Zofenopril Calcium and Hydrochlorothiazide in Presence of Hydrochlorothiazide Major Impurities

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Abstract

In the present decade, great importance has been focused on the development of green analytical methods (GAM) as eco-friendly techniques. Minimizing the wastes, analysis time, hazardous reagents, sample size and energy are the main important principles for development of GAM. This manuscript describes a green, novel, rapid, accurate and reliable capillary zone electrophoresis method (CZE) for the simultaneous separation and determination of zofenopril calcium (ZOF) and hydrochlorothiazide (HCT) in presence of two major impurities of HCT, namely; chlorothiazide (CT) and salamide (DSA). Uncoated fused-silica capillary (50 μm i.d. × 48.5 cm and 40 cm effective length) was used. The main factors affecting the separation were the buffer concentration, pH of the buffer and applied voltage. Optimization of the experimental conditions was performed by applying response surface methodology (RSM). The experiments were designed using central composite face-centered design (CCD). The model obtained from the design described the linear, non-linear and interaction effects of factors on the responses. The optimum conditions given by the design were running buffer of sodium borate (pH 9.15; 10 mM) and 17 kV as positive mode applied voltage. Upon applying these conditions, baseline separation for the four compounds with short analysis time of 5.0 min was achieved. UV detection was performed at 225.0 nm and the capillary temperature was maintained at 25°C. The method was validated and applied for quantitative determination of the studied drugs according to the International Conference on Harmonization (ICH) guidelines. Good linearity was obtained in the range of 10.0–100.0 μg/mL for both ZOF and HCT. As for CT and DSA (HCT impurities), linearity range was 5.0–100.0 μg/mL. The proposed method was successfully applied for the analysis of these drugs in their synthetic mixtures and in their co-formulated pharmaceutical formulations.

Introduction

The need to move towards GAM is of prime importance for sustainable development and environment preservation. From the 12 principles suggested by Anastas and Warner (1) for green chemistry, 4 may be only applicable to green analytical chemistry (GAC). On the other hand, Galuszka *et al.* (2) suggested twelve principles that

can cover all the aspects to verify GAM. It was worthwhile to apply GAC for pharmaceutical analysis as an approach for sustainable analytical procedures transfer.

Zofenopril calcium (ZOF) (Figure 1**a**) chemically designated as calcium salt of (4 S)−1-[(2 S)−3-(Benzylthio)−2-methyl-propionyl]−4-(phenylthio)-L-proline. It is an anti-hypertensive drug that belongs to the sulfhydryl angiotensin converting enzyme inhibitors, with an anti-oxidant and cardio-protective properties (3). Hydrochlorothiazide (HCT) (Figure 1**b**) is chemically designated as (6-chloro-3,4-dihydro-2 *H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide) (4). It is a diuretic drug of the thiazide class that is frequently used in anti-hypertensive formulations either alone or in combination with other drugs.

The combination of ZOF and HCT has been shown to be effective for the treatment of hypertension and has also been shown to be superior to monotherapy with either agent alone. A fixed-dose combination of ZOF (30 mg) and HCT (12.5 mg) has been approved for once-daily use in the management of mild to moderate hypertension in several countries of the EU (5).

Chlorothiazide (CT) (Figure 1**c**), 6-chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide, and Salamide (DSA) (Figure 1**d**), 4-amino-6-chlorobenzene-1,3-disulphonamide, are considered to be HCT process impurities (HCT impurity A and B) (4).

Only few methods have been described for the simultaneous determination of ZOF and HCT in pharmaceutical formulation including spectrophotometric (6, 7) and LC (8–11) methods. There is no reported method for the determination of ZOF and HCT in presence of either HCT impurities or degradation products. Furthermore, the reported methods did not consider the environmental hazards of the used chemicals or follow aspects of green chemistry application.

The present work is considered to be the first developed CZE green sustainable method for separation of the studied compounds. The developed method showed the advantages upon using a surface response modeling chemometric protocol to achieve the best separation of the studied compounds. These compounds were HCT, the co-formulated anti-hypertensive drug, ZOF and the two very closely related impurities or degradation products of HCT, namely; CT (impurity A) and DSA (impurity B). In addition, the developed method was used to determine the two drugs in their pharmaceutical formulation and in presence of the mentioned impurities. The suggested method was designed to avoid the use or production of harmful chemicals with minimum waste. It could be used for the continuous routine analysis in quality control laboratories without harming the environment.

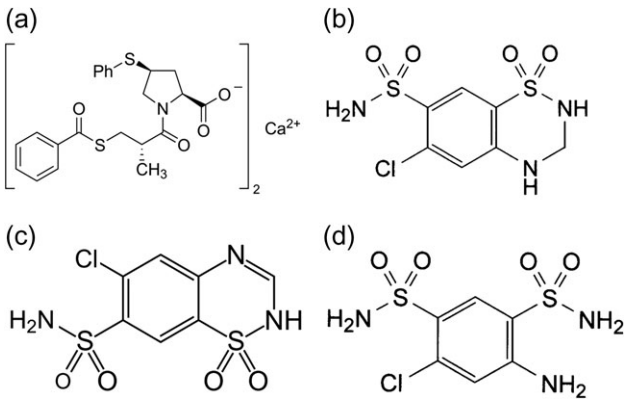

Figure 1. Chemical structures of (a) zofenopril calcium, (b) hydrochlorothiazide, (c) chlorothiazide (hydrochlorothiazide "impurity A") and (d) salamide (hydrochlorothiazide "impurity B").

Experimental

Instrumentation

The study was performed using Agilent 7100 capillary electrophoresis instrument equipped with a photodiode array detector. Data acquisition was done by Agilent ChemStation for CE system. Uncoated fused-silica capillary was obtained from Agilent Technologies (USA). The pH measurements were performed using Jenway pH meter model 3305 (UK). To ensure consistent results, it was calibrated before each measurement with reference buffer solutions.

Materials and reagents

Sodium hydroxide pellets, hydrochloric acid, phosphoric acid, sodium tetraborate decahydrate and methanol were purchased from Sigma-Aldrich, Germany. Pure sample of ZOF was purchased from A. Menarini Manufacturing Logistics and Services Srl (Firenze, Italy). Its purity was checked by the reported method (12) and was found to be 99.73 ± 1.28. HCT was kindly supplied from October Pharma (Cairo, Egypt). Its purity was found to be 99.90 ± 0.98 according to its official method (13). CT (HCT impurity A) and DSA (HCT impurity B and degradation product) were purchased from Sigma-Aldrich Chemie (Germany) with certified traceable purities of 99.80% and 99.60%, respectively. Zoprazide® tablets, Batch No. 58013 were manufactured by A. Menarini Manufacturing Logistics and Services Srl (Firenze, Italy). Each tablet is claimed to contain 30 mg ZOF and 12.5 mg HCT. Distilled-deionized water was used.

Standard solutions

Standard stock solutions of ZOF, HCT and HCT impurities A and B

Stock standard solutions of each of ZOF, HCT and HCT impurities (A and B) were prepared by accurately weighing and transferring 50.0 mg of pure samples into 50mL measuring flasks, and dissolving in methanol; the volume of each was completed to the mark with methanol to obtain final concentrations of 1.0 mg/mL.

Working standard solution and laboratory prepared samples solution mixtures

Solutions of ZOF, HCT (100.0 µg/mL) and each of its impurities (30.0 µg/mL) were prepared by transferring aliquots of each of the corresponding stock solutions into 10 mL volumetric flask, vortexed for 30 s and the volume of each was completed quantitatively with background electrolyte (BGE). Solutions were then transferred into the sample vials for analysis. These solutions were used for method development and optimization of the electrophoretic conditions. For method validation, series of ZOF, HCT and each of its individual impurities' solutions, having concentrations within their corresponding linearity ranges, were individually prepared.

In addition, different solution mixtures containing 10.0, 30.0, 50.0, 70.0 and 90.0 µg/mL of ZOF and HCT spiked with calculated amounts of each of the impurities, representing 1.0–50.0% of active drugs, were similarly prepared (figures are shown in Supporting File).

Preparation of the background electrolyte (BGE)

BGE was borate buffer without any additives. Different molar concentrations of the buffer solutions either for developing the method or running the final one, were prepared by suitable dilution of sodium tetraborate decahydrate (100 mM) with distilled-deionized water and adjusted to the corresponding desired pH values by adding 1.0 M hydrochloric acid or 1.0 M sodium hydroxide, drop-wisely, until these

values were reached. Finally, the prepared buffers were transferred to source and destination vials using a syringe and filtered with the nylon membrane filters 0.22 μm (Agela Technologies, USA).

Procedure

Capillary conditioning

Before using the capillary for the first time, the utilized protocol included conditioning by flushing with 0.1 M NaOH for 5 min, followed by flushing with 0.1 M phosphoric acid for 5 min, then washing with water for 5 min (all performed at 25°C).

While for daily conditioning, each day prior to the analyzes, the capillary was activated by rinsing in the following sequence: 0.1 M NaOH, 0.1 M phosphoric acid, each for 1 min and water for 5 min, and finally equilibrated with running buffer for 5 min. A sequence of pre-run conditioning of 0.1 M NaOH (1 min), water (3 min) and BGE (3 min), then sample injection at 50 mbar for 5 s post-run with water (5 min) was performed after each run.

Electrophoretic separation

The optimized conditions for running the experiments were found to be upon using a capillary with a total length of 48.5 cm, effective length of 40.0 cm and an ID of 50 μm; borate buffer of 10 mM concentration adjusted to pH 9.15 using 1.0 M HCl; the applied voltage was +17 kV and the capillary temperature maintained at 25°C. The samples were hydro-dynamically injected for 5.0 s × 50 mbar. The detecting wavelength was 225.0 nm. Solutions of each of the ZOF, HCT and its impurities were separately injected and checked for their migration times and orders of elution.

Application to pharmaceutical formulation

Ten Zoprazide® tablets were accurately weighed after removal of the coat and finely powdered. A weight equivalent to one tablet was transferred into 25 mL volumetric flask and shaken with 15 mL methanol followed by sonication for 15 min. The volume was completed to the mark with methanol and filtered. Of the obtained solution, 0.5 mL was diluted to 10 mL with methanol. The proposed method was applied for the analysis of the pharmaceutical formulations and the concentrations of the cited drugs were calculated.

Results

Preliminary study of experimental conditions

Before running the experimental design, one has to decide which factors affecting the electrophoretic separation and within which limits the design will be built. Thus, preliminary study of experimental conditions was carried out. The study included the effect of buffer type, concentration and pH (these studies and their related electropherograms are illustrated in Supplementary Figure S1 and S2). Upon examination of the chemical structure and pK_a of the investigated compounds, charged forms of the different compounds expected to appear at high pH values. The pH chosen allowed weak acidic compounds to be deprotonated with production of high concentration of ($RCOO^-$) with increasing of their negative charge; also it will not allow weak cations to be protonated. Thus, two types of buffers were studied; phosphate buffer and borate buffer at pH of 9.0, 9.25 and 9.5 and concentration range of 10–30 mM. No clear separation was observed upon using phosphate buffer. On the other hand, good separation of the four compounds was achieved upon using borate buffer. Acidic buffers were not tried since at lower pH charging of the compounds will be minimum that will affect their migration greatly.

The other factors investigated were the applied voltage, polarity mode and capillary temperature. Using a positive mode, separation was observed of the four studied compounds over a range of 13–17 kV. Its increase above 17 kV was accompanied by a decrease in migration time; however, the increased current caused baseline irregularity. Decreasing the voltage below 13 was associated with long analysis time.

Trials to use reverse polarity for the sake to improve separation or increase the sensitivity were of negative or no appreciable effect on resolution. No major effects were observed upon changing the capillary temperature in the range of 15–30°C, only slight differences in migration time and current were observed without any effect on resolution; the baseline remained steady. The photodiode array detector permitted running of electropherograms at several detecting wavelength, on the basis of the absorption spectra of the investigated drugs, three wavelengths 200.0, 225.0 and 270.0 nm were tried. The best electropherograms were obtained at 225.0 nm, which was chosen for detection.

Experimental design

Running the above-mentioned preliminary study showed that the buffer of choice would be borate buffer at a concentration of 30 mM and pH 9.5 while the applied voltage about 15 kV. The temperature was kept at 25°C and the capillary length of 48.5 cm (effective length 40 cm) was maintained during the whole analysis. These conditions gave good resolution of the successive peaks with analysis time of 9.41 min. (Figure 2a).

Although the separation obtained was satisfactory, yet chemometric optimization would give the real optimum conditions that consider the interaction of the effective factors. Three responses were chosen, these were the analysis time defined as the migration time of CT (HCT impurity A) and the resolutions "Rs1" and "Rs2" of ZOF from DSA (HCT impurity B) and of DSA from HCT, respectively. The obtained models would demonstrate the significant influence of the studied factors on resolution and analysis time as the responses expressing the separation process.

An optimization design was constructed using CCD as the most efficient surface response designs. CCD has been used successfully and efficiently by several researchers to optimize the experimental conditions for best HPLC and electrophoretic separation (14–17). Such a design would allow having a model between a response y and number of factors $x_1 \ldots x_n$. The outcomes of experiments are used to estimate the intercept and all coefficients of the polynomial model. At least three levels are required for each factor often denoted by the star points which are located at the center (0) and both the extreme levels (±1) of the experimental domain. Then, for the estimation of the error of the model, experimental replicates at the center are performed (18–20). In total, the number of experiments will be $N = 2^k + 2k + n$ where k is the number of factors to be optimized and n is the number of center points (replicate experiments at the center value of all factors).

For an experimental design with three factors, the model obtained would be

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + \cdots + E$$

It included, linear, quadratic and cross terms, where b is the regression coefficient and E is the experimental error. The square

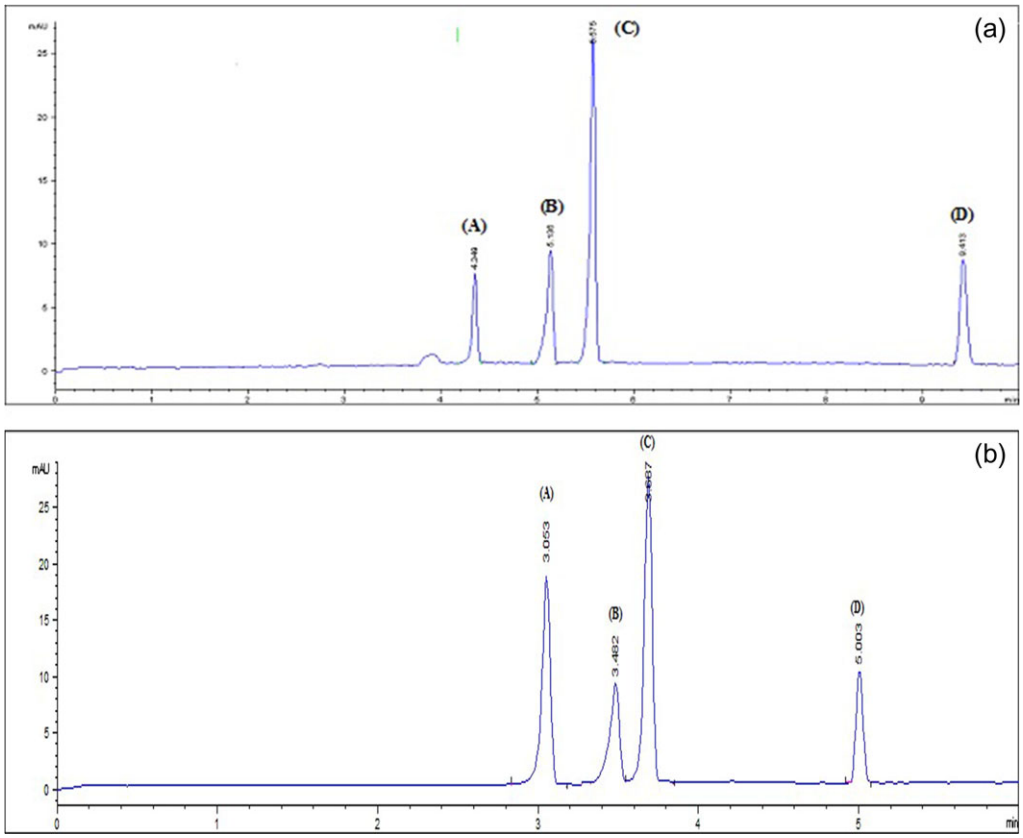

Figure 2. Electropherogram showing separation of (A) zofenopril calcium, (B) salamide (hydrochlorothiazide impurity B), (C) hydrochlorothiazide and (D) chlorothiazide (hydrochlorothiazide impurity A) using an uncoated fused-silica capillary with a total length of 48.5 cm and an effective length of 40 cm (50 μm ID); the capillary temperature maintained at 25°C; UV detection at 225 nm; sample injection: 50 mbar for 5 s; (a) an applied voltage of +15.0 kV; and a BGE of borate buffer (pH 9.50; 30 mM): ZOF (30 μg/mL); salamide (30 μg/mL); HCT (100 μg/mL) and CT (30 μg/mL). (b) an applied voltage of +17.0 kV; and a BGE of borate buffer (pH 9.15; 10 mM): ZOF (60 μg/mL); salamide (30 μg/mL); HCT (100 μg/mL) and CT (30 μg/mL).

term of each factor describes the non-linear effect on the response, while the cross term for two different factors describes their interaction on the response. CCD design is based on 2-level factorial designs, augmented with center and axial points to fit quadratic models. Face-centered CCD has three levels per each factor corresponding to (−), (0) and (+) levels. In our method, three factors are used for optimization and six center points were applied. So, the total numbers of experiments used in the design were 20 experiments. The choice of star points and levels were based on the results from the preliminary study. The nominal levels for pH were 9, 9.25 and 9.50; while for buffer concentration 10, 20 and 30 mM and finally voltage 13, 15 and 17 kV (a summary of these levels is included in Supplementary Table I). The center points are replicated to provide excellent prediction capability near the center of the factor space, the actual design runs are presented in Table I. Table I shows in details all conditions and results of the experiments as extracted from the software.

Generation of design matrix was performed between factors and responses by response surface regression analysis. ANOVA was generated to obtain the most efficient model which statistically gave *P*-value < 0.05 (ANOVA parameters are shown in Supplementary Table II). Individual coefficient of each term along with their *P*-value as extracted from the software is given in Table II.

A good fit of the data was evidenced by the good agreement between both adjusted and predicted *R*-squared values where the differences between them was <0.2. The developed three models were having a non-significant lack of fit which indicates good model, adequate signal was obtained as the signal-to-noise ratio was more than four (21) which showed good discrimination of the developed models.

As a diagnostic tool for testing the power transformation of the response data used for models development, Box Cox plots were used, untransformed raw data gave fit results for Rs1, Rs2 and analysis time as shown in Figure 3. The current power transformation for each response falls closest to the best lambda value and within the confidence interval.

The effect of each of the studied factors on the three responses individually (single factor effect) is presented in Figure 4 that are in concord with *P*-value of coefficients presented in Table II.

For Rs1, the significant model terms are A and B, while for Rs2 only B is a significant model term. On the other hand for migration time, A, B, C, AB and A^2 are significant terms. The interaction between significant model terms is presented in the surface response plot in Figure 5.

For multi-response global optimization, Derringer's desirability function was applied (22). The criteria specified were maximum resolution (Rs1 ad Rs2) and short analysis time. An optimum condition was attained as the value of the obtained desirability was reaching unity. A high desirability value (0.995) was obtained by using a set of experimental conditions; pH (A) of 9.15, buffer concentration (B) of 10 mM

Table I. Results for CCD Optimization of the CZE Method for Determination of Zofenopril, Hydrochlorothiazide and its Impurities as Extracted from the Software

Optimization		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Std.	Run	A: pH	B: Buffer conc., mM	C: Voltage, kV	Rs1 (Peaks 1 and 2)	Rs2 (Peaks 2 and 3)	Time (min)
12	1	9.25	30	15	3.19	2.53	8.499
13	2	9.25	20	13	5.29	3.03	8.222
15	3	9.25	20	15	5.43	3.22	7.290
5	4	9.00	10	17	1.52	2.01	4.794
10	5	9.50	20	15	9.61	3.59	8.832
9	6	9.00	20	15	3.34	3.37	7.820
11	7	9.25	10	15	4.85	2.1	6.034
6	8	9.50	10	17	6.4	2.18	6.235
19	9	9.25	20	15	6.45	3.94	7.403
8	10	9.50	30	17	10.57	4.92	9.971
7	11	9.00	30	17	3.6	3.83	7.162
18	12	9.25	20	15	6.26	3.91	7.335
3	13	9.00	30	13	3.95	4.35	9.422
1	14	9.00	10	13	1.58	2.25	6.183
17	15	9.25	20	15	6.88	4.33	7.918
2	16	9.50	10	13	8.05	2.93	7.934
4	17	9.50	30	13	11.39	5.65	12.768
16	18	9.25	20	15	7.18	4.66	7.885
14	19	9.25	20	17	6.84	4.32	6.935
20	20	9.25	20	15	7.52	4.74	7.986

The grey highlight runs are the replicated center points.

Table II. The Coefficients of Each of the Obtained Significant Model with Respect to the Three Responses

Response	Intercept	A[a]	B[b]	C[c]	AB	AC	BC	A^2	B^2	C^2
Rs1	5.9950	3.2030	1.0300	−0.1330	–	–	–	–	–	–
P-value		<0.0001	0.0214	0.7462	–	–	–	–	–	–
Rs2	3.5930	0.3460	0.9810	−0.0950	–	–	–	–	–	–
P-value		0.1868	0.0012	0.7099	–	–	–	–	–	–
Time	7.6129	1.0359	1.6642	−0.9432	0.3704	−0.1059	−0.2461	0.7480	−0.3115	0.0005
P-value		<0.0001	<0.0001	<0.0001	0.0387	0.5119	0.1449	0.0182	0.2679	0.9985

[a]Coefficients of pH variable.
[b]Coefficients of buffer concentration variable.
[c]Coefficients of voltage value variable.
P-value of some coefficients higher than 0.05 yet the overall developed model which has *P*-value < 0.05.

and voltage (C) of 17 kV (a figure representing Derringer's desirability function is shown in Supplementary Figure S3). For confirming the prediction efficiency of the developed models, an experiment under the optimum condition was performed, Figure 2b.

System suitability

System suitability parameters for the developed method were calculated, results are shown in Table III. The values obtained for selectivity (α) and resolution (R_s) between successive peaks were higher than 1.0 and 2.0, respectively; indicating baseline separation, while tailing factor was ≈ 1.0, indicating peak symmetry.

Method validation

Method validation was performed according to the ICH guidelines (23).

The regression equations and all validation parameters are shown in Table IV.

Linearity and range

To ensure the ability of the proposed method to obtain test results which are directly proportional to the concentration of the analyte, a linear correlation was obtained between the corrected peak areas (area/migration time ratio) and concentration of each of ZOF, HCT and its impurities A and B. Good correlations were obtained between the corrected peak area and concentration in the range of 10.0–100.0 µg/mL for both ZOF and HCT and 5.0–100.0 µg/mL for CT and DSA, with high correlation coefficient of >0.999 and variance ratios (*F*-values) with very small intercepts indicating good linearity of the calibration graphs (24) (Table IV).

The standard deviation of intercept S_a, of slope S_b and that of residuals $S_{y/x}$ is a measure of the deviation of the obtained *y*-values from the calculated ones. The smaller the $S_{y/x}$, the closer will be the points to the regression line (24). The higher the *F*-values (variance ratio), the higher the mean of squares due to regression and the lower in the mean of squares due to residuals for the same degrees of freedom. Good regression line shows high values for both (*r*) and

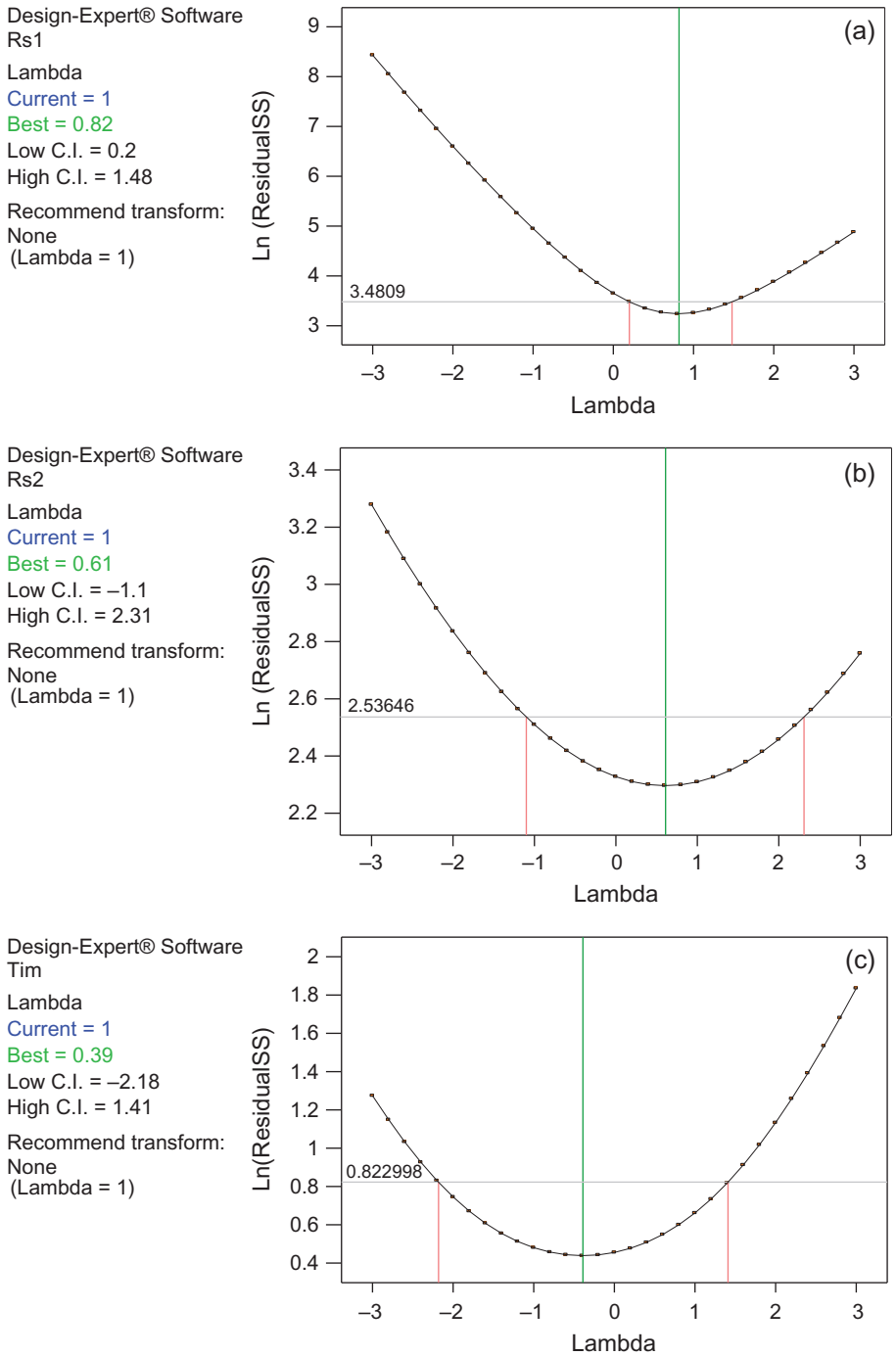

Figure 3. Box Cox plots for power transforms for the three selected responses (a) Rs1, (b) Rs2 and (c) analysis time.

(*F*) values (25). The statistical values of the regression parameters of the four studied compounds are presented in Table IV which ensures that all can be determined quantitatively with high precision.

Accuracy

The accuracy of the method was assessed by analyzing five laboratory freshly prepared solutions of ZOF, HCT and its impurities in triplicate using different concentrations of each. The %Recovery and RSD were calculated and revealed good accuracy (Table IV).

Precision

The intra-assay precision was evaluated in terms of the relative standard deviation percentage (%RSD) of the concentrations, corrected peak areas and the migration times for ZOF and HCT using the proposed method. Tests for area and migration time precisions, including repeatability of the corrected areas and repeatability of migration time, are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures.

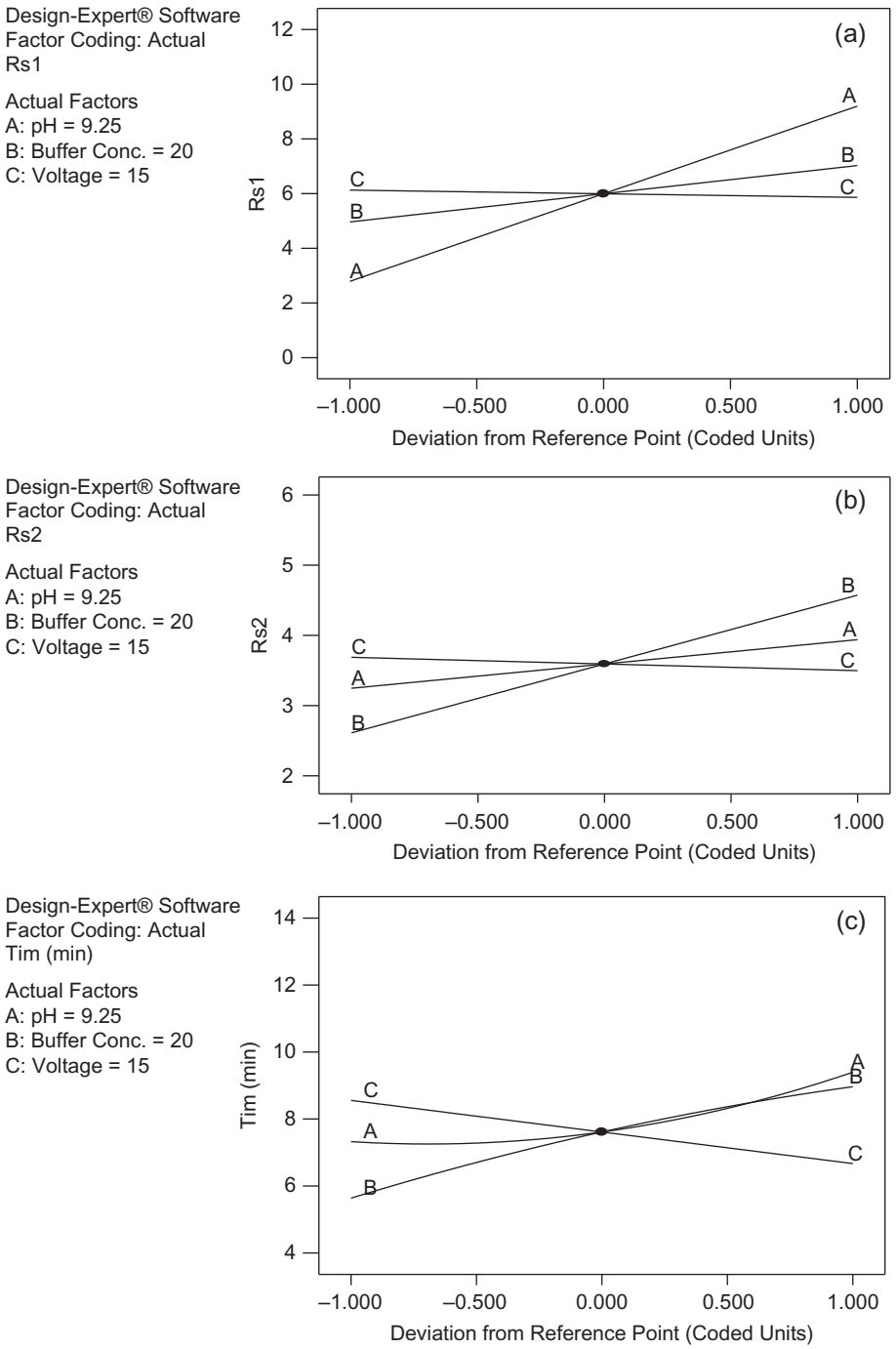

Figure 4. Single factor effect of the studied factors on resolution and analysis time: (a) Rs1, (b) Rs2 and (c) analysis time.

Freshly prepared solutions of ZOF and HCT at concentration levels of 20.0, 40.0 and 60.0 µg/mL were injected. The %RSD values for the concentrations and corrected peak areas were found to be < 2% for each drug, indicating high degree of repeatability and intermediate precision. Results are shown in Table IV.

Specificity and selectivity

Specificity and selectivity of the method were tested by analyzing five laboratory freshly prepared solutions of ZOF, HCT and its impurities in triplicate using different concentrations of each. The % Recovery and RSD were calculated revealing good specificity and selectivity (Table IV).

Limits of detection (LOD) and limits of quantitation (LOQ)

Limits of detection (LOD, determined with a S/N ratio of 3) and limits of quantitation (LOQ, determined with a S/N ratio of 10) of ZOF, HCT and its related impurities are presented in Table IV. The results revealed that the method can detect and determine HCT impurities at levels of 0.6% and 1.5% of the nominal value.

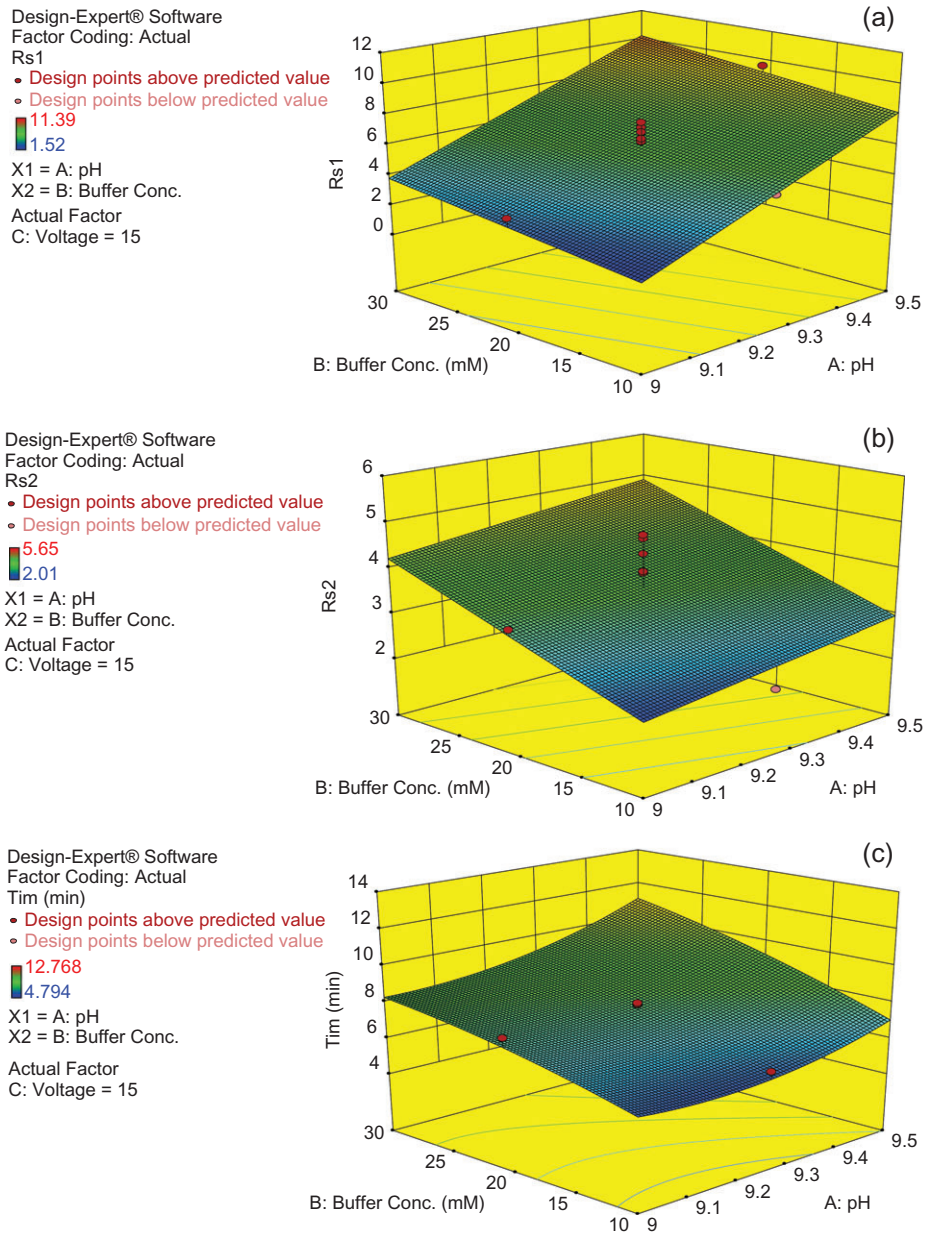

Figure 5. Response surface plots related to the significant model parameters and the studied responses; resolution and analysis time. (a) Rs1, (b) Rs2 and (c) analysis time.

Table III. System Suitability Parameters of the Proposed CZE Method

Parameter	Obtained value			
	ZOF	DSA (HCT impurity B)	HCT	CT (HCT impurity A)
Migration time (t_R) (min)	3.05	3.48	3.69	5.00
Selectivity (α)[a]	1.16	1.06	1.38	
Resolution (R_s)[a]	2.55	2.32	15.28	
Tailing factor (T)	1.02	0.92	1.17	1.00
Number of theoretical plates (N)[b]	45,740.55	50,628.63	76,561.75	17,4546.02
Number of theoretical plates per meter (TPM)[c]	94,310.41	1,04,388.94	1,57,859.27	3,59,888.71

[a]As extracted from the software.
[b]$N = 16\ (t_R/W)^2$.
[c]TPM = [1600 $(t_R/W)^2$]/L, where "L" is total capillary length in cm.

Table IV. Regression Equation[a] Parameters and Validation Results

Parameter	ZOF	HCT	CT (HCT impurity A)	DSA (HCT impurity B)
Linearity				
Range (μg/mL)	10–100	10–100	5–100	5–100
Correlation coefficient (*r*)	0.9998	0.9998	0.9997	0.9998
Intercept (*a*)	−1.9647	−1.3220	0.0493	−0.1744
SD of Intercept	0.1961	0.1537	0.1138	0.1860
Slope (b)	0.3514	0.2469	0.1759	0.2962
SD of Slope	0.0032	0.0025	0.0019	0.0031
$S_{y/x}$[b]	0.2253	0.1766	0.1357	0.2217
F (variance ratio)[b]	11834	9505.9	8768.2	9305
Significance F[b]	4.28×10^{-8}	6.635×10^{-8}	8768.2×10^{-8}	6.924×10^{-8}
Accuracy (mean %recovery ± RSD)[c]	100.38 ± 1.23	99.98 ± 1.45	100.33 ± 1.37	99.49 ± 0.96
Precision				
Repeatability[d]				
(±%RSD)	0.59	0.44	0.98	0.63
Corrected peak area (±%RSD)	1.26	1.54	0.91	0.68
Migration time (±%RSD)	0.95	1.13	0.20	0.14
Intermediate precision[d]				
(±%RSD)	1.29	1.28	1.64	1.36
Corrected peak area (±%RSD)	1.65	1.74	1.71	1.38
Migration time (±%RSD)	1.20	1.49	0.42	0.58
Specificity and selectivity	100.27 ± 1.52	100.68 ± 1.63	99.26 ± 1.38	99.15 ± 1.13
LOD (μg/mL)[e]	2.14	2.78	0.62	0.89
LOQ (μg/mL)[e]	7.14	9.23	1.65	2.96

[a]Regression equation for all methods: $y = bx + a$; where "y" is the corrected peak area and "x" is concentration in μg/mL.

[b]$S_{y/x}$ standard deviation of residuals, *F* (variance ratio) equals the mean squares owing to regression divided by the mean squares owing to residuals Significance *F*.

[c]$n = 5$.

[d]$n = 9$; at concentrations of 20, 40, 60 μg/mL; each in triplicate.

[d]Limits of detection (LOD, determined with a S/N ratio of 3) and limits of quantitation (LOQ, determined with a S/N ratio of 10).

Finally, the proposed method was successfully applied for the analysis of commercial tablets. Results obtained by the determination of the amount of ZOF and HCT in Zoprazide® tablets are shown in Table V.

The results obtained from the proposed CZE method were statistically analyzed and compared with those obtained by applying the reported method (10), showing no significant difference regarding both accuracy and precision, as the *t*-value and *F*-value were less than the theoretical ones.

The validity of the method was further confirmed by applying standard addition technique. Results were found to be 100.42% ± 0.97 and 100.20% ± 0.87 for ZOF and HCT, respectively. The accurate results obtained revealed that there was no interference from excipients to principal peaks, which clearly demonstrated the selectivity of the proposed method.

Discussion

The introduction of CZE opened a new brilliant area in pharmaceutical analysis characterized by high sensitivity, selectivity and shortening the analysis time, which is comparable to HPLC. However, the widespread application of HPLC in drug analysis probably leads to considerable contamination of environment with organic solvents, which raise questions about the toxicity/greenness of HPLC in the ecosystem (26).

To have reliable analytical results within short analysis time while miniaturization of analytical devices are important aspects of green analytical chemistry (27). The greenness of an analytical method can be assessed by its greenness profile. The profile criteria for the method was based on four key terms "PBT" (persistent, bioaccumulative and toxic), "Hazardous", "Corrosive" and "Waste" (28). "Green" principles of analytical chemistry can be most easily achieved in electrically driven separation methods due to the low consumption of solvent and minimum sample volume. A typical volume of the separation capillary in CZE is ~ 5 μL, and such volume is also required for eluent.

The determination of drug-related impurities is currently the principal role of CZE within pharmaceutical analysis and presents a challenge to both selectivity and sensitivity. The main component and structurally related impurities usually have close functional groups structures and properties that cause resolution difficulties. However, an advantage of CZE over its chromatographic counterparts is that high separation efficiencies are achievable (29).

The British Pharmacopeia (4) stated that, both CT and DSA were considered to be HCT process impurities. Also, it was reported that HCT had one primary degradation pathway which yielded 4-amino-6-chlorobenzen-1,3-disulphonamide, DSA, and formaldehyde by hydrolysis (30). CT was found to be pharmacologically less active than HCT (31). Meanwhile, HCT is fairly and rapidly absorbed from gastrointestinal tract, while CT was incompletely and variably absorbed (32).

CZE coupled with chemometric optimization can provide a complete profile of a separation process and is considered highly powerful, providing useful information of separation and factor interactions. The obtained models demonstrate a significant influence of the studied factors on resolution and analysis time as the responses expressing the separation process. The principle of experimental design is how to plan and conduct experiments at different combination of factors in order to obtain the maximum information with minimum experimental and

Table V. Assay Results for the Determination of the Studied Drugs in their Co-formulated Tablets using the Proposed and Reported Methods

Value	Proposed CZE method		Reported HPLC method[12,a]	
	ZOF	HCT	ZOF	HCT
Mean recovery% of the labeled amount ± SD[b] of Zoprazide® tablets[c]	100.72 ± 0.92	99.27 ± 1.15	99.68 ± 0.96	100.19 ± 0.82
% RSD[b]	0.91	1.16	0.96	0.82
Variance	0.85	1.32	0.92	0.67
Student's *t*-test[d]	1.76 (2.306)	1.47 (2.306)	–	–
F-value[d]	1.08 (6.39)	1.97 (6.39)	–	–

[a]Reported HPLC method using a C18 (250 × 4.6 mm, 5μm) column, mobile phase mixture of acetonitrile, methanol and 0.02 M NaH_2PO_4 buffer (adjusted to pH 7.2 with sodium hydroxide solution) in the ratio of 40:20:40 v/v/v, flow rate 1.0 mL/min and detection at 245.0 nm.

[b]Standard deviation and percentage relative standard deviation for five determinations.

[c]Labeled to contain 30 mg Zofenopril calcium and 12.5 mg hydrochlorothiazide, batch no. 58013.

[d]The values in the parenthesis are the corresponding theoretical values of t and F at $P = 0.05$ and $n = 5$.

financial efforts (33). The basic idea is to change all relevant factors, simultaneously, over a set of planned experiments to determine which of them has a significant effect on the response. There are two prime reasons for performing a design which relate a response with multiple factors. The first is optimization of conditions that result in a maximum or minimum response as appropriate. The second is to produce a detailed quantitative model (mathematical–statistical equation) that can predict, how a response relates to the different factors (18–20).

For optimization of the experimental conditions, CCD-RS was applied. Upon applying the experiments, the order of separated peaks was ZOF, DSA (HCT impurity B), HCT and CT (HCT impurity A). The results concerning the tested variables on the resolution (Rs1 and Rs2) and the analysis time were considered, where Rs1 and Rs2 are the calculated resolution between ZOF–DSA (HCT impurity B) and DSA–HCT, respectively.

A design matrix was generated between factors and responses by response surface regression analysis. ANOVA was generated to obtain the most efficient model which statistically gave P-value < 0.05. The values of signal-to-noise ratio were more than four which showed good discrimination of the developed models. Box Cox plots were used as a tool to determine the most appropriate power transformation to be applied to the response data. Derringer's desirability function was used to multi-response global optimization. In the present study, the critera specified were maximum resolution (Rs1 ad Rs2) and short analysis time. It was concluded that there were a set of coordinates, producing high desirability value (0.995), with pH (A) of 9.15, buffer concentration (B) of 10 mM and voltage (C) of 17 kV.

The developed and optimized method was subjected to method validation according to the ICH guidelines (23). Validation of the method in terms of linearity, precision, accuracy, specificity, LOD and LOQ was assessed. The LOD of HCT impurities A and B was calculated using signal-to-noise ratio. The results revealed that the method can detect and determine HCT impurities at levels of 0.6% and 1.5% of the nominal value. The obtained results for the determination of ZOF and HCT in Zoprazide® tablets indicate that the method could be successfully applied in quality control laboratories. Standard and sample solution stabilities were checked up to 24 h at room temperature, and we measured the responses on one time on each day. No degradation of ZOF or HCT was observed during this period. All the calculated validation parameters indicate that the developed and optimized method was suitable, linear, precise and accurate for the simultaneous determination of ZOF and HCT in the presence of HCT two major impurities; CT and DSA in bulk and pharmaceutical dosage forms.

Conclusion

In this work, a green, selective and robust CZE method was developed for the determination of ZOF, HCT in the presence of HCT two major impurities; CT and DSA in pharmaceutical formulation. Optimization and robustness studies were performed by experimental design. The separation was completed within 5 min. The obtained results prove the selectivity, repeatability, linearity and sensitivity of the method. It was used for screening and determination of ZOF, HCT and its impurities; CT and DSA in pharmaceutical formulation. The method has the advantage to determine HCT impurities at low levels which shows the applicability of the method for quality control.

Supplementary Data

Supplementary material is available at *Journal of Chromatographic Science* online.

References

1. Anastas, P.T., Warner, J.C.; *Green Chemistry: Theory and Practice*. Oxford University Press, Oxford, (1998).
2. Gałuszka, A., Migaszewski, Z., Namies´nik, J.; The 12 principles of green analytical chemistry and the significance mnemonic of green analytical practices; *Trends in Analytical Chemistry*, (2013); 50: 78–84.
3. Ambrosioni, E.; Defining the role of zofenopril in the management of hypertension and ischemic heart disorder; *American Journal of Cardiovasular Drugs*, (2007); 7: 17–24.
4. British Pharmacopoeia., *Her Majesty's Stationary Office*, London, (2012), Ph. Eur. monograph 0394.
5. Parati, G., Omboni, S., Malacco, E., *et al*.; Antihypertensive efficacy of zofenopril and hydrochlorothiazide combination on ambulatory blood pressure; *Blood Pressure*, (2006); 15: 7–17.
6. Carlucci, G., Di Federico, L., Iuliani, P.; Simultaneous determination of zofenopril and hydrochlorothiazide in tablets using derivative UV spectrophotometry; *Analytical Letters*, (2010); 43: 2609–2617.
7. Kurbanoglu, S., Gumustas, M., Ozkan, S.A.; Simultaneous determination and validation of some binary mixtures of antihypertensive drugs using ratio derivative spectrophotometric method; *Journal of Analytical Chemistry*, (2014); 69: 935–941.
8. Carlucci, G., Di Federico, L., Iuliani, P.; HPLC-DAD method for the simultaneous determination of zofenopril and hydrochlorothiazide in oral pharmaceutical formulations; *Journal of Separation Science*, (2010); 33: 1717–1722.
9. Aslan, S.S.; Validated RP-LC method for simultaneous determination of zofenopril and hydrochlorothiazide in pharmaceutical preparation; *Journal of Chromatographic science*, (2011); 49: 259–263.

10. Devika, G.S., Sudhakar, M., Rao, J.V.; Isocratic RP- HPLC method for simultaneous separation and estimation of zofenopril and hydrochlorthiazide in pharmaceutical dosage form; *E-Journal of Chemistry*, (2012); 9: 999–1006.
11. Kurbanoglu, S., Gumustas, M., Ozkan, S.A.; Simultaneous estimation and validation of some binary mixtures of antihypertensive drugs by RP-LC methods using two new generation silica columns; *Journal of Pharmaceutical and Biomedical Analysis*, (2013); 72: 198–201.
12. Ramesh, T, Rao, P.N., Rao, R.N.; LC–MS/MS characterization of forced degradation products of zofenopril; *Journal of Pharmaceutical and Biomedical Analysis*, (2014); 88: 609–616.
13. United States Pharmacopeia.; The United States Pharmacopeial Convention, Rockville, Maryland, USA, (2007).
14. Gomez, Y., Adams, E., Hoogmartens, J.; Analysis of purity in 19 drug product tablets containing clopidogrel: 18 copies versus the original brand; *Journal of Pharmaceutical and Biomedical Analysis*, (2004); 34: 341–348.
15. Maltampati, S., De Vulder, S., Hoogmartens, J., Schepdael, A.V.; Method development and validation for the analysis didanosine using micellar electrokinetic capillary chromatography; *Electrophoresis*, (2005); 26: 4079–4088.
16. Fayed, A.S., Weshahy, S.A., Shehata, M.A., Hassan, N.Y., Pauwels, J., Hoogmartens, J., *et al*.; Separation and determination of clopidogrel and its impurities by capillary electrophoresis; *Journal of Pharmaceutical and Biomedical Analysis*, (2009); 49: 193–200.
17. Fayez, Y.M., Hegazy, M.A.; Micellar electrokinetic chromatography (MEKC) with multiresponse chemometric optimization for the determination of hydrochlorothiazide and coformulated antihyperyensives in the presence of hydrochlorothiazide major impurity; *Journal of Chromatographic Science*, (2016); 54: 1050–1060.
18. Brereton, R.G.; *Chemometrics Data Analysis for Laboratory and Chemical Plant*. Publisher Wiley & Sons, Ltd, USA, (2003).
19. Brereton, R.G.; *Applied Chemometrics for Scientists*. Publisher Wiley & Sons, Ltd., USA, (2007).
20. Dean, A., Voss, D.; *Design and Analysis of Experiments*. Springer, New York, Inc, (1999).
21. Beg, Q.K., Sahai, V., Gupta, R.; Microbial α-amylases: a biotechnological perspective; *Process Biochemistry*, (2003); 39: 203–209.
22. Derringer, G., Suich, R.; Simultaneous optimization of several response variables; *Journal of Quality Technology*, (1980); 12: 214–219.
23. International Conference on Harmonization (ICH). of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedures: Text and Methodology Q2 (R1), (2005).
24. Miller, J.N., Miller, J.C.; *Statistics and Chemometrics for Analytical Chemistry, 4th ed*. Pearson Education Limited, England, (2000).
25. Armitage, P., Berry, G.; *Statistical methods in Medical Research*, 3rd ed. Blackwell Scientific Publications, Oxford, England, (1994).
26. Abd El-Rahman, M.K., Zazaa, H., Ibrahim, N.B., Mustafa, A.A.; Just-dip-it (Potentiometric ion-selective electrode); an innovative way of greening analytical chemistry; *ACS Sustainable Chemistry and Engineering*, (2016); 4: 3122–3132.
27. Tobiszewski, M., Mechlińska, A., Namieśnik, J.; Green analytical chemistry – theory and practice; *Chemical Society Review*, (2010); 39: 2869–2878.
28. Keith, L.H., Gron, L.U., Young, J.L.; Green analytical methodologies; *Chemical Reviews*, (2007); 107: 2695–2708.
29. Altria, K.D., Rogan, M.M.; Introduction to quantitative applications of capillary electrophoresis in pharmaceutical analysis; *Beckman Instruments, Incorporated*, (1994); 2–15. https://www.beckmancoulter.com/wsrportal/bibliography?docname=538703-CEPrimer6.pdf
30. Mollica, J.A., Rehm, C.R., Smith, J.B.; Hydrolysis of hydrochlorothiazide; *Journal of Pharmaceutical Sciences*, (1969); 58: 635–636.
31. Meltzer, E.L., Eichner, G.L., Ural, E., Kitchell, J.R.; A comparison of hydrochlorothiazide and chlorothiazide in the treatment of hypertension; *The American Journal of Cardiology*, (1959); 4: 741–744.
32. Martindale: Extra Pharmacopoeia.; *The Complete Drug References*, 34th Ed. The Pharmaceutical Press, London, UK, (2005).
33. Gemperline, P.; Practical Guide To Chemometrics, 2nd Ed., Publisher Taylor & Francis Group, Boca Raton, (2006); pp. 263–338.