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Multicriteria Optimization Methodology in Stability-Indicating Method Development of Cilazapril and Hydrochlorothiazide

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Abstract

Multicriteria optimization methodology was applied in development of UHPLC-UV–MS method for separation of cilazapril, hydrochlorothiazide and their degradation products. This method is also applicable for analysis of cilazapril, hydrochlorothiazide and their degradation products in combined tablet formulation. Prior to method optimization forced degradation studies were conducted. Cilazapril and hydrochlorothiazide were subjected to acidic (0.1, 0.5 and 1.0 M HCl), basic (0.1, 0.5 and 1.0 M NaOH), thermal (70°C), oxidative (3–30% H_2O_2) degradation and photodegradation (day light). Cilazapril appeared to be unstable toward acid and base and resulted in formation of cilazaprilat. Hydrochlorothiazide significantly degraded after acid, base and thermal hydrolysis and formed degradation product was 4-amino-6-chlorobenzene-1.3-disulfonamide. For both substances, after oxidative degradation unknown products have arisen. Initial percentage of acetonitrile in mobile phase, final percentage of acetonitrile in mobile phase, time of gradient elution and column temperature were defined as variables to be optimized toward two chromatographic responses by means of central composite design and Derringer's desirability function. The satisfactory chromatographic analysis was achieved on *Kinetex* C18 (2.6 μm, 50 × 2.1 mm) column with temperature set at 25°C. The final mobile phase consisted of acetonitrile and 20 mM ammonium formate buffer (pH adjusted to 8.5). The flow rate of the mobile phase was 400 μL min^{-1} and it was pumped in a gradient elution mode.

Introduction

Hydrochlorothiazide (Figure 1A) is thiazide diuretic which is chemically known as 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. Its pharmacological effects include inhibition of the sodium and chloride reabsorption from renal tubules and enhancement of potassium secretion. Hydrochlorothiazide thus increases secretion of sodium and water. It is used for treatment of hypertension, edema associated with heart failure and kidney and hepatic disorders. It is also used for treatment of edema followed by premenstrual syndrome, treatment of diabetes insipidus, for prevention of water retention caused by corticosteroids and prevention of renal calculus formation in patients with hypercalciuria (1).

Cilazapril (Figure 1B) is angiotensin-converting enzyme which is chemically known as (1*S*, 9*S*)-9-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6Hpyridazino[1,2-a] [1,2] diazepine-1-carboxylic acid monohydrate. Cilazapril is pro drug of cilazaprilat (diacid) which is also active metabolite. Cilazapril is rapidly absorbed and metabolized in liver to more polar cilazaprilat. Cilazapril is used for treatment of hypertension, heart failure and myocardial infarction (1).

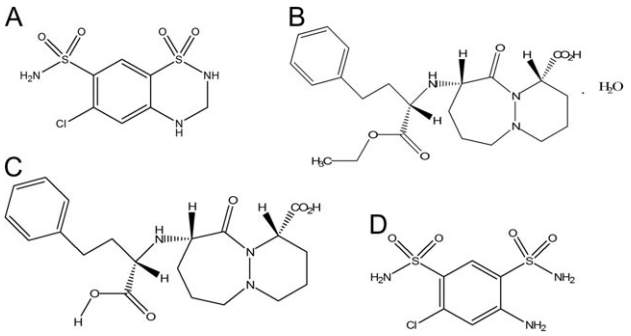

Figure 1. Structures of hydrochlorothiazide (A), cilazapril (B), cilazaprilat (C) and hydrochlorothiazide impurity B (D).

Cilazapril and hydrochlorothiazide can be used as monotherapy, but in combination they have synergistic antihypertensive effect which results in a higher percentage of patients responding satisfactorily. Fixed combinations of these two drugs with different mechanism are used in order to apply minimal doses which will minimize clinical and metabolic side effects (2). Forced degradation studies are complementary part of drug development strategy being undertaken for identification of degradation products, to elucidate the degradation pathway and intrinsic stability of the drug. These studies are conducted under more severe conditions then those used for accelerated stability testing. Influence of environmental stress factors like pH, temperature, humidity, oxygen and light are evaluated. Investigation of degradation products formed under stress conditions is useful in development and validation of suitable stability-indicating analytical procedures and it is also necessary due to changes in toxicity, bioavailability or therapeutic effects of the dosage form (3–5).

Experimental design as a chemometric tool represents the methodology of planning and conducting experiments in order to gather as much information as possible from minimal number of experiments (6). The main aim of experimental design is examining influence of every factor or method parameter as well as the possible factor interactions and to optimize these factors after conducting series of experiments. Central composite design (CCD) is response surface design which is commonly used in optimization and robustness assessment of analytical methods (7). CCD consists of full factorial design and star design. The total number of experiments is defined by relation $N = 2^k + 2k + p$, where k represents the number of factors to be tested, p represents the replicates in central point. Star points in the design are at the distance equal to $+\alpha$ or $-\alpha$ from the experimental domain center, while the value α depends on experiment criteria. Face centered CCD is created when the value of α is set to 1. Repeats in central point serve for precise assessment of pure experimental error, to stabilize variation of predicted response and to measure the adequacy of the models (8–11).

When a single response is being analyzed, the model analysis indicates the areas in the design region where the process is likely to give desirable results, which is a relatively easy task. Local optima corresponding to only one objective can be avoided by taking into account the whole spectra of objectives. The purpose of multi-objective optimization (MOOP), also called multicriteria decision-making approach, is to obtain optimality on the basis of compromises among the various objectives. This methodology allows the identification of the best solution by optimizing several dependent properties simultaneously. The advantage of MOOP approach is creating a local optimal solution through a group of different objectives which may contribute in a positive or a negative way to the final solution (12). Desirability functions are the most widely used in this research area. This approach is based on the idea that a quality of the process that has multiple quality characteristics, with one of them outside of some "desired" limits, is completely unacceptable. The method finds operating conditions x that provide the "most desired" response values (13, 14).

The literature search revealed numerous methods for cilazapril and hydrochlorothiazide determinations individually and in combined pharmaceutical dosage forms. Various methods had been described such as spectrophotometric (15–17), liquid chromatographic (18–21) and capillary electrophorethic (22). Chromatographic methods with amperometric and photometric detection for rapid determinations of cilazapril and cilazaprilat in urine were also reported (23–26). RP-TLC determination of lipophilicity of cilazapril and hydrochlorothiazide was also described by using cellulose layers and three linear solvent systems (27). Enzyme immunoassay had been used for cilazapril determinations directly with serum or plasma samples without pretreatment (28).

But to date, no information of stability-indicating method for cilazapril and hydrochlorothiazide is available in the literature. Concerning this, comprehensive UHPLC-UV–MS study of cilazapril and hydrochlorothiazide degradation under stress conditions and development and validation of UHPLC method has been reported. Cilazapril, hydrochlorothiazide and their degradation products showed different physical–chemical properties such as solubility, lipophilicity, absorption and polarity. Therefore, it was difficult task to conduct their simultaneous separation in one chromatographic run. The novelty of this work includes the evaluation of cilazapril and hydrochlorothiazide degradation pathway and chromatographic behavior of two active substances and their degradation products toward various variables in order to define the optimal chromatographic conditions using experimental design and multiresponse optimization methodology. These conditions enabled separation of all compounds within the shortest possible analysis run time.

Materials and methods

Chemicals and reagents

The standard substances of cilazapril, hydrochlorothiazide, cilazaprilat and impurity B of hydrochlorothiazide were obtained from Sigma-Aldrich (Taufkirchen, Germany). Acetonitrile HPLC-gradient grade was purchased from J.T. Baker (Deventer, the Netherlands). All other used reagents were of analytical grade. Ammonium formate was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ammonium hydroxide was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Purified water was obtained using Simplicity 185 Water purification system, Millipore (Billerica, Massachusetts, USA). Sodium hydroxide and hydrochloride acid were purchased from Zorka Pharma (Šabac, Serbia), while 30% (v/v) hydrogen peroxide solution was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The Prilazid plus® film tablets containing 5 mg of cilazapril and 12.5 mg of hydrochlorothiazide (Galenika a. d., Belgrade, Serbia) were purchased from a local drugstore. Syringe driven filter units (LLG-nylon syringe filter 0.45 µm, LLG Labware, Meckenheim, Germany) were used for sample solution filtration.

Chromatographic conditions and equipment

Chromatographic analysis was performed on Thermo Scientific UHPLC system (Thermo Fisher Scientific Inc., Waltham, USA)

which is equipped with *Accela* photodiode array (PDA) detector, *Accela* auto sampler, *Accela* quaternary pump, column thermostat, degasser and *TSQ Quantum Access Max* triple quadrupole mass spectrometer. ChromQuest software, version 5.0 (Thermo Fisher Scientific Inc., Waltham, USA) was used for data acquisition and processing. Chromatographic separation was done by using Kinetex C18 (2.6 µm, 50 mm × 2.1 mm) column (Phenomenex Inc., Torrance, USA). Electrospray ionization technique (ESI) was used and data were acquired by means of Xcalibur version 1.3 software (Thermo Fisher Scientific Inc., Waltham, USA). Tuning the mass spectrometer by means of optimizing the ESI source and collision cell was established in ESI-negative mode conditions with cilazapril and hydrochlorothiazide solutions at concentration of 1 µg mL^{-1}. Both standard solutions were infused with the syringe pump at a flow rate of 5 µL min^{-1} in mobile phase composition defined as a center point of CCD. The mass spectrometer operated in full scan mode with mass to charge ratio (*m/z*) in the range of 100–2,000. Spray needle voltage was set at 4,000 V. Nitrogen was used as a sheath and auxiliary gas. Sheath gas pressure was 10 psi and auxiliary gas pressure was 55 psi. Vaporizer temperature was 354°C and capillary temperature was 200°C. Argon was used as the collision gas with a pressure of 2 mTorr.

Optimal chromatographic conditions included mobile phase which consisted of acetonitrile (mobile phase A) and buffer solution of 20 mM ammonium formate (pH = 8.5) (mobile phase B). The mobile phase was pumped at 400 µL min^{-1} flow rate in a gradient elution mode: at 0 minute 5% acetonitrile and 95% of buffer solution, at 15 minute 35% acetonitrile and 65% buffer solution, at 16 minute 5% acetonitrile and 95% of buffer solution. The detection performed using PDA detector was done at 250 nm. The chosen temperature of the column was 25°C.

Design-Expert 7.0 software (Stat-Ease Inc., Minneapolis, USA) was used in order to generate experimental design and to analyze obtained responses. *Marvin Sketch* software (Chem Axon Ltd., Budapest, Hungary) was used to calculate the molecular descriptors, log *P* and pK_a values.

Experimental

Preparation of mobile phase, standard and sample solutions

Standard stock solutions at concentrations of 1 mg mL^{-1} for both cilazapril and hydrochlorothiazide were prepared separately. Stock solutions were prepared by dissolving 25 mg of standard substances in the mixture of acetonitrile–water (50:50, v/v) in 25 mL volumetric flasks. The solutions were dissolved in the ultrasonic bath for 5 minutes and volumetric flasks were filled to volume with mixture of acetonitrile–water (50:50, v/v). Working concentration for both cilazapril and hydrochlorothiazide solutions was 100 µg mL^{-1}. This concentration was obtained after dilution of stock solutions with the mixture of acetonitrile –20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v). Further dilution to concentration of 1 µg mL^{-1} was achieved using the same mixture.

The 20 mM ammonium formate buffer solution was prepared and its pH value was adjusted to 8.5 with ammonium hydroxide. Before the analysis, mobile phases (buffer solution and acetonitrile) were filtered by means of glass vacuum system *Sartorius Stedim Biotech* (Göttingen, Germany) through nylon filter membrane 47 mm 0.45 µm (Agilent Technologies, Santa Clara, USA). Mobile phase was prepared freshly on the day required in the amounts that is sufficient for daily use. This ensured that the mobile phase stayed unchanged or that buffer pH was unaffected by prolonged storage and that there was no microbial growth that could affect chromatography.

Working standard solutions used for forced degradation studies were prepared from stock solutions and contained cilazapril and hydrochlorothiazide in concentration of 100 µg mL^{-1}, separately. Working sample solution contained cilazapril and hydrochlorothiazide in concentrations of 100 and 250 µg mL^{-1}, respectively. These concentrations were obtained after mixing 1 mL of stock solution with 1 mL of stress agent and filling to volume of 10 mL with the mixture of acetonitrile –20 mM ammonium formate buffer pH = 8.5 (20:80, v/v) after appropriate time of stress testing. Acid hydrolysis was performed by mixing 1 mL of cilazapril or hydrochlorothiazide stock standard solutions separately in 10-mL volumetric flasks with 1 mL of 0.1, 0.5 and 1.0 M HCl solution, respectively. Alkaline hydrolysis was performed separately by mixing 1 mL of cilazapril or hydrochlorothiazide stock standard solutions in 10-mL volumetric flasks with 1 mL of 0.1 M, 0.5 M and 1.0 M NaOH, respectively. These mixtures were kept at room temperature and at temperature of 70°C for 3 and prolonged to 6 hours if no degradation was observed. Afterwards, the solutions were filled to volume with mixture of acetonitrile –20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v). Oxidative forced degradation study was performed by mixing 1 mL of cilazapril or hydrochlorothiazide stock standard solutions separately in 10-mL volumetric flasks with 1 mL of 3% (v/v), 10% (v/v) and 30% (v/v) H_2O_2 aqueous solutions, respectively. These mixtures were kept at room temperature for 3 and 6 hours, respectively. Finally, volumetric flasks were filled to volume with mixture of acetonitrile –20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v). Thermal degradation was examined with 1 mL of stock sample solutions of cilazapril or hydrochlorothiazide separately which was exposed to 70°C for 3 and 6 hours, respectively. Afterwards, the solutions were filled to volume with mixture of acetonitrile –20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v). Photodegradation was performed with 1 mL of stock standard solutions of cilazapril or hydrochlorothiazide separately which were exposed to the daily sunlight for a period of 2 weeks, respectively. Afterwards, the solutions were filled to volume with mixture of acetonitrile –20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v). Photodegradation was investigated in the similar manner (time of exposure to daily sunlight) with solid samples of both cilazapril and hydrochlorothiazide which were used afterwards for preparation of working solutions in the same concentrations as mentioned above.

The sample solution for analysis of cilazapril and hydrochlorothiazide in tablets was prepared from *Prilazid plus*® film tablets. The film from those tablets was previously removed. The quantity of 20 tablets was accurately weighted, finely powdered and the amount of powder equivalent to double average tablet mass was transferred into 10 mL volumetric flask adding the mixture of acetonitrile–water (50:50, v/v). The solution was dissolved in the ultrasonic bath for 20 minutes and the mixture was filled to volume of 10 mL with the same solvent. The solution was filtered through syringe driven filter units 0.45 µm prior the injection. The obtained concentrations of cilazapril and hydrochlorothiazide in this stock sample solution were 1 and 2.5 mg mL^{-1}, respectively.

Forced degradation studies of combined dosage form of cilazapril and hydrochlorthiazide in tablets were performed in the same way as forced degradation studies on standard substances by means of harmonization of concentration levels, diluents and stress conditions as previously described.

During forced degradation studies, control samples were prepared and used for proper interpretation of drug stability properties for every stress condition. Besides, standard stock solutions of cilazapril and hydrochlorothiazide were kept in the dark and refrigerated during the whole study and the stress samples were prepared freshly when needed for every investigated stress condition.

For method validation purposes, standard stock solutions of cilazapril and hydrochlorothiazide were prepared in concentrations of 1 mg mL^{-1} and standard stock solutions of cilazaprilat and hydrochlorothiazide impurity B were prepared in concentrations 200 μg mL^{-1}, separately by dissolving standard substances in the mixture of acetonitrile–water (50:50, v/v). Further dilutions of standard stock solutions were done with the mixture of acetonitrile –20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v) in order to prepare working solutions with concentrations in the following ranges: 44.00–132.00 μg mL^{-1} for cilazapril, 110.00–330.00 μg mL^{-1} for hydrochlorothiazide, 0.11–1.32 μg mL^{-1} for hydrochlorothiazide impurity B and 0.044–0.528 μg mL^{-1} for cilazaprilat. The quantitative analysis of cilazaprilat was done using methodology of standard addition. Prior to completion to volume, cilazaprilat standard substance was added to investigated solutions. The stock sample solution prepared from *Prilazid plus*® film tablets containing 1 mg mL^{-1} of cilazapril and 2.5 mg mL^{-1} of hydrochlorothiazide, which preparation was described in relation to forced degradation studies was also used for method validation purposes.

Stability of all standard solutions was inspected by comparison of chromatograms of freshly prepared solutions with those that were stored in refrigerator and/or on room temperature for 24 hours. No statistically significant differences in obtained results indicated that stability of standard solutions was not compromised.

Results

Forced degradation study

Forced degradation study included appropriate stress conditions in accordance with the ICH regulatory guidelines (3). Various approaches were also found in the literature describing practical aspects of development of stability-indicating methods (5, 29–35).

For stability testing, cilazapril and hydrochlorothiazide were exposed to stress conditions in solution state. Stress testing included investigation of the thermal stability, acidic and alkaline hydrolytic stability as well as stability toward oxidative agents. Control samples were prepared for comparison with the stressed samples. For every stress condition, it was necessary to prepare at least four samples, which refers to the solution of active pharmaceutical ingredient (API) stored under ambient conditions, blank solution containing stress agents, zero time sample containing the drug stored under ambient conditions and analyzed immediately after preparation and drug solution submitted to the stress conditions. Comparisons of these results gave the proper assessment of changes.

The percentage of drug degradation was calculated as a ratio of peak area of cilazapril and hydrochlorothiazide under final stress condition versus peak area of these substances in the solution stored under ambient conditions from the chromatograms recorded under optimal chromatographic conditions. The confirmation of peak identity in all stress samples was done with the assistance of mass spectrometry. Mass spectra of standard substances and stress samples were recorded in the negative ESI mode. Degradation products were identified based on their retention times and mass to charge ratios (*m/z*) in mass spectrum. The obtained *m/z* value of 415.99 matched with that of cilazapril standard substance and *m/z* value of 295.6 matched with that of hydrochlorothiazide standard substance. Obtained percentage of degradation and obtained degradation products of cilazapril and hydrochlorothiazide formed under each stress condition were presented in Table I. The representative chromatograms presented in Figures 2–5 enabled insight into the composition of cilazapril and hydrochlorothiazide stress solutions, respectively.

Chromatographic method development

Preliminary testing indicated which factors had influence on the chromatographic behavior of the investigated substances. Acetonitrile was selected as organic modifier because investigated substances showed the best peak symmetry by using this solvent. Among buffers compatible with mass spectrometer, ammonium formate was selected and ammonium hydroxide was used for pH adjustment. It was decided to work with 20 mM ammonium formate buffer solution which pH value was set at 8.5. Because of the differences in solubility, lipophilicity and polarity between investigated substances, it was decided to use gradient elution mode in chromatographic method development. Initial percentage of acetonitrile, final percentage of acetonitrile, time of gradient elution mode and temperature of the column were optimized with the assistance of CCD. The domains

Table I. Stress Conditions, Obtained Degradation Products and Percentage of Degradation for Cilazapril and Hydrochlorothiazide in Defined Time of Exposure

Stress condition	Time of exposure	Mass balance (%)	Degradation level (%)	Degradation product
Cilazapril				
Acid hydrolysis, 1.0 M HCl 70°C	6 hours	98.43	10.04	Cilazaprilat
Base hydrolysis, 0.1 M NaOH	30 minutes	99.29	15.65	Cilazaprilat
Thermal degradation, 70°C	6 hours	100.00	No degradation	
Oxidation, 15% (v/v) H_2O_2	15 minutes	98.25	15.00	DPC_1 (5.94%) DPC_2 (3.98%) DPC_3 (5.08%)
Photodegradation (day light)	2 weeks	99.50	No degradation	
Hydrochlorothiazide				
Acid hydrolysis, 1.0 M HCl 70°C	1 hour	98.51	10.52	Hydrochlorothiazide impurity B (DPH_1)
Base hydrolysis, 1.0 M NaOH 70°C	3 hours	98.51	6.27	Hydrochlorothiazide impurity B (DPH_1)
Thermal degradation, 70°C	3 hours	98.78	8.64	Hydrochlorothiazide impurity B (DPH_1)
Oxidation, 30% (v/v) H_2O_2	3 hours	97.56	20.00	DPH_2
Photodegradation (day light)	2 weeks	99.21	No degradation	

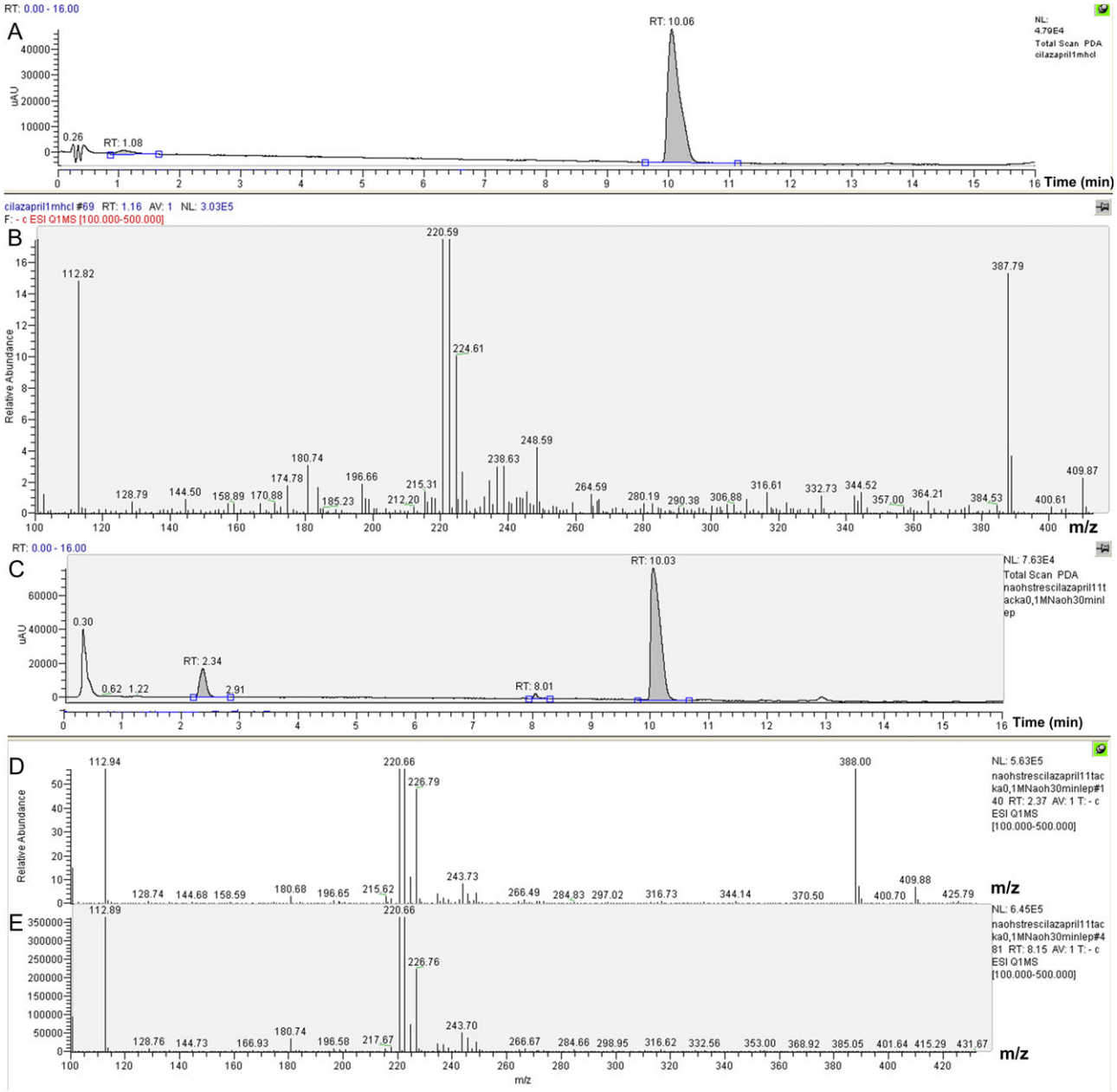

Figure 2. The representative chromatograms of cilazapril acid degradation products with PDA detector (A) and mass spectrum (B), cilazapril base degradation products with PDA detector (C) and mass spectrum (D, E).

of investigated variables for CCD are presented in Table II, whereas the plan of the performed experiments and corresponding responses are presented in Table III. Experimental design included 30 experiments varying four factors. All experiments were performed randomly with six repeats in central point, in order to provide a precise estimation of an experimental error, adequacy of the model (lack of fit) and the statistical significance of influence of each variable.

Investigated responses were retention factors of cilazapril and hydrochlorothiazide degradation products, DPC_3 and DPH_1, respectively, as the first and the last eluting substances. Mathematical model was built for each response. *Design Expert 7.0.0.* software proposed presented response surface quadratic models for both responses:

$$k_{DPH_1} = 1.17 - 0.41x_1 + 0.076x_2 - 0.033x_3 - 0.29x_4 + 0.045x_1x_2 + 0.069x_1x_3 + 0.18x_1x_4 + 0.046x_2x_3 - 0.020x_2x_4 - 0.024x_3x_4 + 0.27x_1^2 - 3.105E^{-003}x_2^2 - 0.13x_3^2 + 0.094x_4^2$$

$$k_{DPC_3} = 36.17 - 2.51x_1 - 6.43x_2 + 3.59x_3 - 0.92x_4 + 0.013x_1x_2 - 0.48x_1x_3 - 0.12x_1x_4 - 0.91x_2x_3 - 0.090x_2x_4 - 0.43x_3x_4 - 0.28x_1^2 + 1.55x_2^2 - 0.30x_3^2 + 0.058x_4^2$$

Here, k_{DPH_1} denotes the retention factor of DPH_1 degradation product, k_{DPC_3} denotes retention factor of degradation product

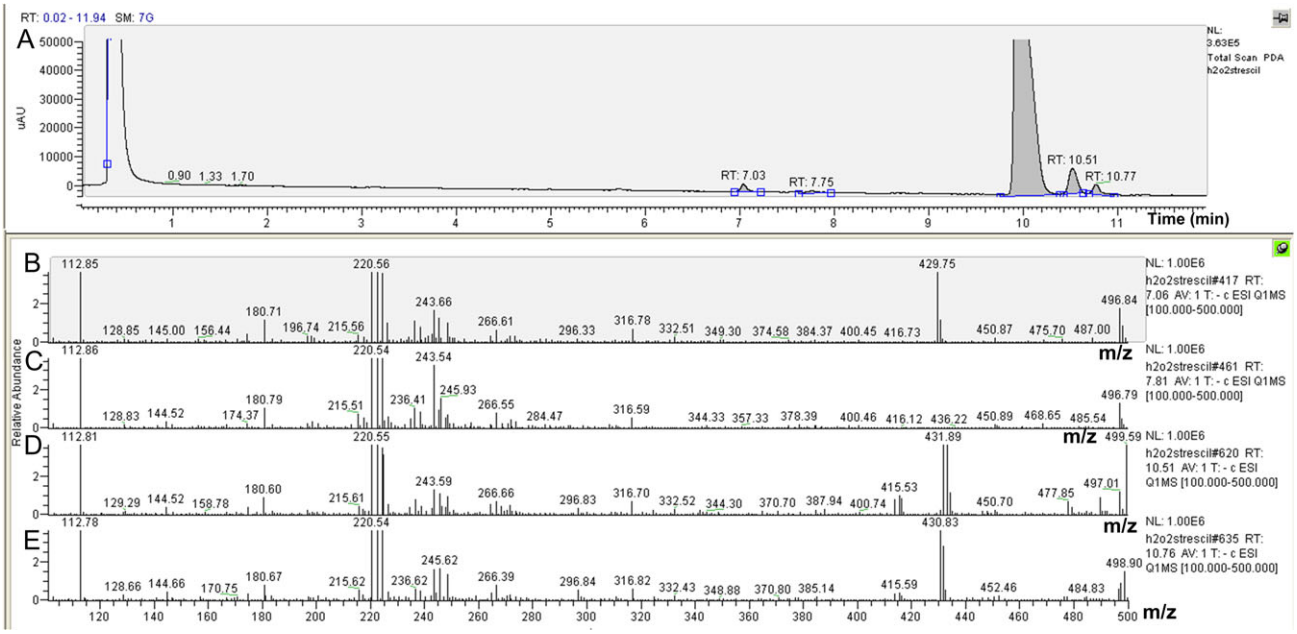

Figure 3. The representative chromatograms of cilazapril oxidative degradation products with PDA detector (A) and mass spectrum (B, C, D, E).

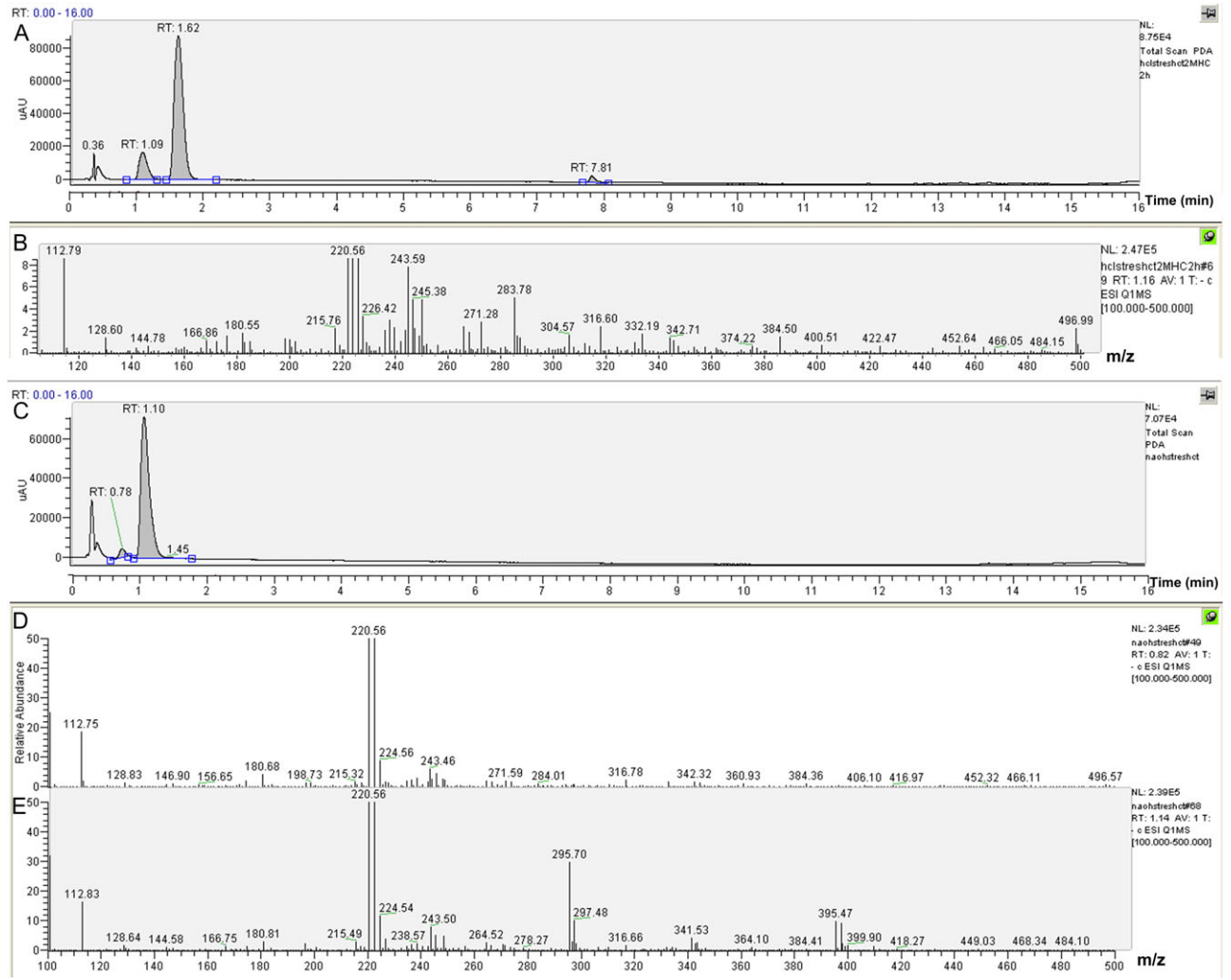

Figure 4. The representative chromatograms of hydrochlorothiazide acid degradation products with PDA detector (A) and mass spectrum (B), hydrochlorothiazide base degradation products with PDA detector (C) and mass spectrum (D), mass spectrum of hydrochlorothiazide (E).

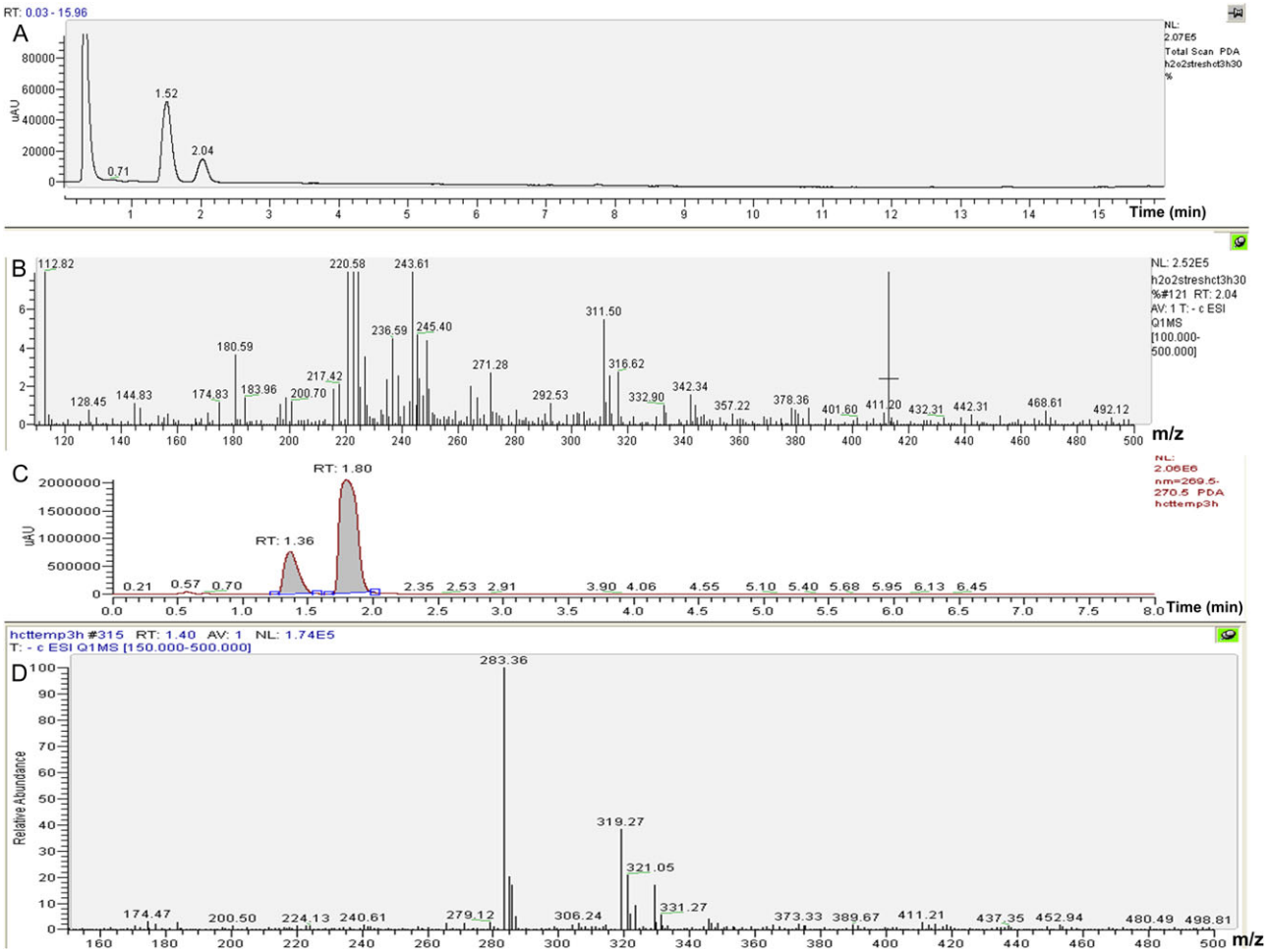

Figure 5. The representative chromatograms of hydrochlorothiazide oxidative degradation products with PDA detector (A) and mass spectrum (B), hydrochlorothiazide thermal degradation products with PDA detector (C) and mass spectrum (D).

DPC_3 while variables x_1, x_2, x_3 and x_4 denote initial percentage of acetonitrile, final percentage of acetonitrile in the gradient elution program, time of the gradient elution and temperature of the column, respectively.

The regression lack of fit for mathematical models of both substances was determined performing *F*-test. *F* values were obtained and then its corresponding *P*-values were calculated. If *P*-values were above 0.05 it would indicate absence of lack of fit and consequently these models could be accepted as good predictive ones (36). In our study, obtained *P*-values were $P_{kDPH1} = 0.5445$ and $P_{kDPC3} = 0.5830$.

In order to achieve the best chromatographic performance of the developed method, it was needed to select the experimental conditions that enable the satisfactory retention for all investigated substances as well as to achieve this in the shortest possible time. Therefore, two optimization goals were defined (Table IV). The first goal included the maximization of the retention factor of DPH_1 degradation product in order to avoid its possible non-retention behavior. The second goal was to minimize the retention factor of degradation product DPC_3 as the last eluting substance. As these two goals were in contradiction, MOOP was done by means of desirability functions. The mathematical models for both k_{DPH_1} and k_{DPC_3} were used for calculation of predicted responses $\hat{Y}_i$ and the individual desirability functions d_i afterwards. Different desirability functions can be used depending on whether the response has to be maximized, minimized or assigned a target value. Desirability functions proposed by Derringer and Suich were used in this study (37).

Table II. Investigated Variables and their Domains Included in CCD

Variables	Domains of investigated variables		
	−1	0	+1
(A) Initial percentage of acetonitrile (%)	5	7.5	10
(B) Final percentage of acetonitrile (%)	25	30	35
(C) Time of gradient elution mode (minutes)	15	17.5	20
(D) Temperature of the column (°C)	25	32.5	40

In case of the maximization of the response k_{DPH_1}, its individual desirability function was defined in the following equation:

$$d_i = \begin{cases} 0 & \text{if} \quad \hat{Y}_i \le L_i \\ \left[\frac{\hat{Y}_i - L_i}{T_i - L_i}\right]^S & \text{if} \quad L_i < \hat{Y}_i < T_i \\ 1 & \text{if} \quad \hat{Y}_i \ge T_i = U_i \end{cases} \qquad (1)$$

Table III. The Plan of the Experiments of CCD Design and Investigated Responses

Experiment no.	Variables				Responses	
	A	B	C	D	k_{DPH1}	k_{DPC3}
1	−1	−1	1	1	1.027	50.138
2	−1	−1	−1	−1	2.416	41.722
3	1	−1	1	1	0.805	43.833
4	0	−1	0	0	0.944	44.502
5	0	0	1	0	1.166	40.000
6	0	0	0	0	1.300	36.123
7	1	−1	1	−1	0.944	46.250
8	0	0	0	0	0.900	35.900
9	0	0	0	−1	1.611	37.194
10	0	0	0	1	0.944	35.916
11	0	0	0	0	1.000	35.000
12	−1	0	0	0	1.694	38.611
13	1	−1	−1	1	0.888	36.250
14	1	1	−1	1	0.805	25.638
15	0	0	−1	0	0.944	32.388
16	1	1	1	−1	1.222	32.250
17	−1	1	−1	1	1.472	29.750
18	1	0	0	0	1.222	33.833
19	−1	1	1	1	1.342	34.868
20	−1	1	1	−1	2.250	37.888
21	0	0	0	0	1.277	36.583
22	0	1	0	0	1.416	31.583
23	−1	−1	1	−1	2.222	51.972
24	−1	−1	−1	1	1.611	40.638
25	1	1	1	1	1.000	28.472
26	0	0	0	0	1.300	35.400
27	1	−1	−1	−1	0.861	37.805
28	0	0	0	0	1.127	36.000
29	1	1	−1	−1	1.277	26.472
30	−1	1	−1	−1	2.305	30.444

In this equation, L_i, U_i and T_i were assigned to be the lower, upper and target values, respectively, desired for the response $\hat{Y}_i$, with $L_i \le T_i \le U_i$. In this way, T_i was interpreted as a large enough value for the response, which can be U_i.

In case of the minimization of the response k_{DPC_3}, the following equation was used:

$$d_i = \begin{cases} 1 & \text{if} \quad \hat{Y}_i \le T_i = L_i \\ \left[\frac{\hat{Y}_i - U_i}{T_i - U_i}\right]^S & \text{if} \quad U_i < \hat{Y}_i < T_i \\ 0 & \text{if} \quad \hat{Y}_i \ge U_i \end{cases} \qquad (2)$$

Here, T_i denotes a small enough value for the response, which can be L_i.

The exponent s in both equations was used to determine how important is to hit the target value T_i. For $s = 1$, the desirability function would increase linearly toward T_i, larger s value would indicate that it is very desirable that the value of $\hat{Y}_i$ is close to T_i or increase rapidly above L_i and small values of s would indicate that almost any value of $\hat{Y}_i$ above L_i and below U_i are acceptable or if having values of $\hat{Y}_i$ considerably above L_i are not of critical importance. In this study, the need to reach satisfactory retention factors of all substances was the same and therefore the values of s exponent was assigned to be one. The lower and upper bounds and defined goals used for construction of Derringer's desirability function have been presented in Table IV.

Table IV. Set Criteria for Construction of Derringer's Desirability Function of Investigated Responses

Response	Goal	Lower limit	Upper limit
k_{DPH1}	In range	2.40	2.50
k_{DPC3}	To minimize	25.64	51.97

For each predicted response $\hat{Y}_i$, calculated desirability functions d_i values were in the range between 0 and 1. The desirability function with $d_i = 0$ represented a completely undesirable value of $\hat{Y}_i$ and $d_i = 1$ represented a completely desirable response value.

The individual desirability functions were further combined using the geometric mean in equation (3) with k denoting the number of responses. In such way, the overall desirability D was estimated:

$$D = \left(d_1^{r_1} x d_2^{r_2} x .. \, x d_k^{r_k}\right)^{\frac{1}{k}} \qquad (3)$$

The values (r_k) describing the importance for satisfying proposed goals for individually responses were defined to be the same for all investigated responses and in this study, all r_k values were assigned to be 1. Value D gave the overall assessment of the desirability for the combined response levels. It was noticed that the range of D values fell in the interval [0, 1] and increased as the balance of the properties became more favorable. For any response $d_i = 0$, the overall desirability was $D = 0$. Desirability maximum was located at the levels of the independent variables that simultaneously produced the maximum desirability.

Based on the optimization goals defined in this study (Table IV), the software *Design-Expert 7.0.0* offered the global optimal conditions. The proposed optimal values for all investigated variables have been presented in Figure 6. Optimal chromatographic conditions included initial percentage of acetonitrile to be 5%, final percentage of acetonitrile 35%, time of gradient elution mode 15.38 minutes during which percentage of acetonitrile would linearly grow and temperature of the column should be set to 25°C. The level of satisfaction of each goal (individually desirability functions) and both goals at the same time (over all desirability function) has been presented in Figure 7. Further 3-D response surface of overall desirability function has been presented in Figure 8.

Discussion

Forced degradation study

The intensity of stress agents and the duration of exposure periods were chosen on the basis of total substance degradation. The specified stress conditions were selected in order to obtain 5–20% degradation of active substance (32). Degradation level below 5% was considered as not significant because very small amount of degradation products was generated and degradation profile could not be completely and reliably defined. On the other hand, degradation above 20% is useless since such high levels of degradation lead to secondary and tertiary degradation product formation that might not be observed under appropriate storage conditions and during the shelf-life of dosage form (5, 36, 37).

The study on individual molecules was performed primarily in order to be able to correctly interpret the drug degradation process

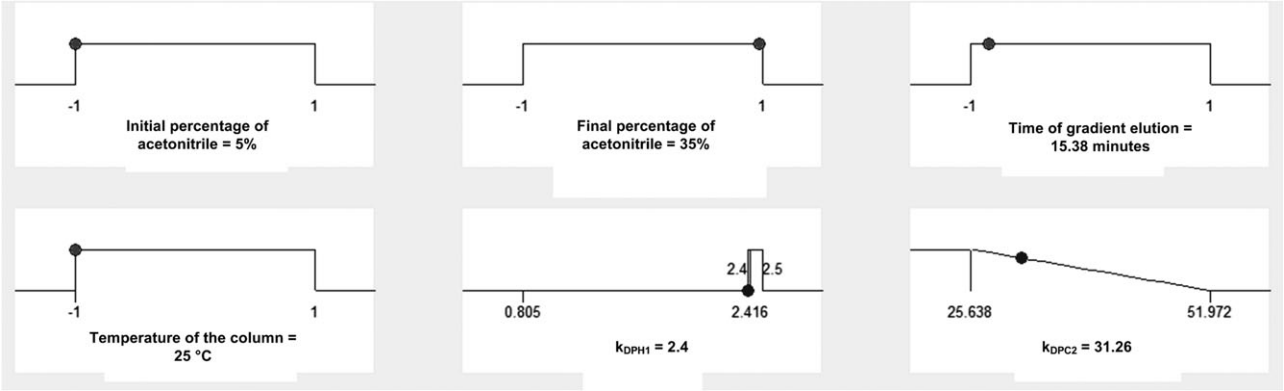

Figure 6. Graphically presented ramps of the optimal chromatographic conditions on global chromatographic optimum.

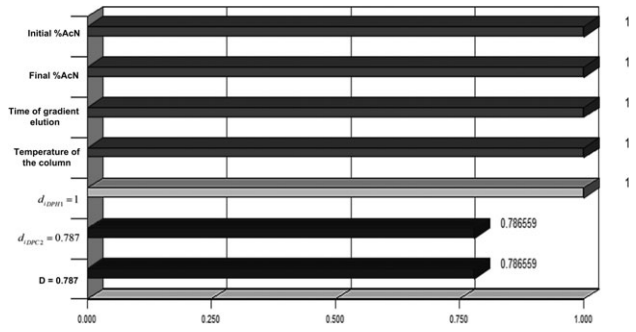

Figure 7. Representative Pareto chart of level of desirability for individual and overall desirability function.

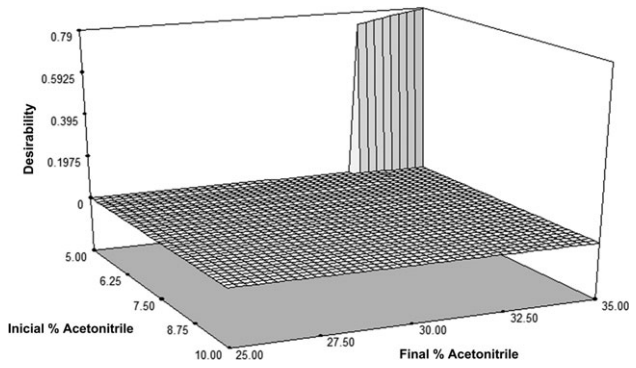

Figure 8. Response surface plot of global desirability function D for the optimization of initial % ACN and final % CAN.

and evaluate the impact of drug formulation and the presence of two drugs in the mixture. Consistence of the MS spectra recorded by the mass spectrometric detector and the UV absorption spectra recorded by the photodiode array detector that had been monitored through the whole forced degradation study, enabled the clear understanding of the sum result of degradation processes in all investigated stress samples. Mass balance values calculated using data from chromatograms of each stress sample were very close to 100% in all cases (Table I), which proved that there were no undetected peaks or degradation products that have not been taken under consideration.

Firstly, the degradation of cilazapril will be discussed. As could be seen from the Table I, under the influence of 1.0 M HCl at 70°C in 6 hours cilazapril had been degraded in amount of 10.04%. The influence of 0.1 M HCl at room temperature was firstly investigated and degradation of cilazapril was beyond 5%. To induce degradation of the substance the strength of the acid was increased to 0.5 M and then to 1.0 M HCl. Still the degradation was not above 5% after 3 days. For this reason, the temperature was raised to 70°C and after 6 hours the degradation of 10.04% was achieved. The degradation process resulted in formation of cilazaprilat which appeared to be the only degradation product (Figure 1C). This result was in accordance with theoretical knowledge since cilazapril chemically represents an ester and it was expected to hydrolyze under the influence of the acid and largely hydrolyze under the influence of base. The representative chromatograms of acid degradation have been presented in Figure 2A and B. Figure 2A presents the chromatogram obtained with PDA detector set at 250 nm. Only one peak of degradation product has been noticed at retention time of 1.08 min. To identify this peak, the mass spectrum was obtained at this retention time and it was confirmed that the *m/z* value of this degradation product was 387.79 (Figure 2B). This *m/z* value corresponds to cilazaprilat as it was expected.

Further, under the influence of 0.1 M NaOH at room temperature in 30 minutes cilazapril had been degraded in amount of 15.65%. This was expected as described above. The representative chromatograms have been presented in Figure 2C–E. PDA chromatogram obtained at 250 nm has been presented in Figure 2C. Two peaks of degradation products have been noticed. One peak has retention time of 2.34 minutes and the other has been noticed at 8.01 minutes. Using mass spectrometer, it was confirmed that peak at 2.34 minutes corresponded to cilazaprilat. Mass spectrum obtained at this retention time and only *m/z* value was 388.0 which correspond to cilazaprilat has been presented in Figure 2D. In Figure 2E, it could be seen that all *m/z* values correspond to background and this peak was the consequent of gradient flow of the mobile phase. It could be concluded that degradation of cilazapril under basic conditions resulted in formation of one degradation product and this degradation product was identified as cilazaprilat. It was noticed that the same degradation product appeared at two different retention times. This was the consequence of the differences in pH values that existed in investigated samples since 1.0 M HCl was added in one sample and 0.1 M NaOH in the other.

Next step in the investigation was to obtain data concerning oxidative degradation of the drug. The drug was exposed to H_2O_2 as

oxidative agent. After exposure to 30% (v/v) H_2O_2 solution the degradation was above 20% and the exposure was repeated with 15% H_2O_2. After 15 minutes, the achieved degradation was 15.00% and obtained degradation products have been presented in Figure 3A–E. PDA chromatogram and three degradation products can be noticed at retention times 7.03, 10.51 and 10.77 minutes in Figure 3A. Mass spectrum of first degradation product which retention time was 7.03 minutes has been presented in Figure 3B. The *m/z* which corresponds to this degradation product is 429.75. One more peak at retention time 7.75 minutes was observed (Figure 3A) but this peak was confirmed to be the background noise (Figure 3C). Further in Figure 3D, it could be noticed that *m/z* value that corresponds to degradation product which retention time is 10.51 minutes is 431.89 and the last degradation product at 10.77 minutes has *m/z* 430.83 (Figure 3E). It could be concluded that three different degradation products have been formed under the influence of oxidation agent. These degradation products were denoted as DPC_1 (7.03 minutes), DPC_2 (10.51 minutes) and DPC_3 (10.77 minutes). The changes in cilazapril molecule due to oxidation could be proposed according to the *m/z* values and fragmentation pattern obtained from LC–MS-MS analyses, but further elucidation of chemical structures of degradation products is planned for future investigation.

Cilazapril showed to be stable to high temperature since no degradation was observed after exposure to 70°C for 6 hours. Cilazapril showed to be stable toward photodegradation since no significant degradation was observed after exposure to the daily sunlight for a period of 2 weeks. The same results were observed in the experiments with cilazapril solid substance exposed to daily light as well as with cilazapril in solution state.

Secondly, the degradation of hydrochlorothiazide will be discussed. It could be seen from the data in Table I that hydrochlorothiazide appeared to be unstable under all investigated conditions, except photodegradation. The addition of 1.0 M HCl at 70°C in 1 hour resulted in 10.52% degradation of active pharmaceutical substance. The influence of 0.1 M, 0.5 M and 1.0 M HCl at room temperature resulted in no degradation and consequently it was decided to increase temperature to 70°C. The degradation resulted in formation of one degradation product eluting at 1.09 minutes as can be seen in Figure 4A. The identity of the peak was confirmed with mass spectrum presented in Figure 4B. This degradation product has the *m/z* 283.78 which matched with m/z value of the known hydrochlorothiazide impurity B (Figure 1D) listed in the European Pharmacopoeia (38). It was expected from available literature and confirmed under these experimental conditions that hydrochlorothiazide will degrade to hydrochlorothiazide impurity B (39). This impurity was further denoted as DPH_1. The peak seen at 7.81 was confirmed to be the background noise.

Under the influence of 1.0 M NaOH at room temperature no degradation was observed for 3 days. For this reason, the temperature was increased to 70°C and in 3 hours degradation was increased to 6.27%. One degradation product was noticed eluting at 0.78 minutes (Figure 4C). This degradation product was also identified as DPH_1 (Figure 4D). Difference in retention times of DPH_1 in both samples was due to different pH values in these samples. In one 1.0 M HCl was added and in another 1.0 M NaOH. Also, the difference in retention times of hydrochlorothiazide after acid and base hydrolysis could be noticed. The identity of the drug was confirmed using mass spectra and it could be seen in Figure 4E where *m/z* value of 295.7 confirmed that both peaks belonged to hydrochlorothiazide.

Next step in the investigation was to obtain data concerning oxidative degradation of the drug. The drug was exposed to H_2O_2 as oxidative agent. After exposure of the drug to 30% (v/v) H_2O_2 solution in 3 hours, it resulted in the degradation of 20.00%. Influence of oxidative agent resulted in formation of one degradation product. It could be seen in Figure 5A that retention time of this degradation product was 2.04 and its *m/z* value was 311.5 (Figure 5B). The identity of this degradation product is not known and it was denoted as DPH_2.

Sensitivity of hydrochlorothiazide to thermal degradation was investigated. The drug degraded 8.64% in 3 hours and the degradation resulted in formation of one degradation product (Figure 5C). The obtained degradation product was identified as DPH_1 (Figure 5D). The exposure of hydrochlorothiazide solid substance and in solution state to daily sunlight for a period of 2 weeks did not lead to any significant degradation. Therefore, it was concluded that the drug is stable toward photodegradation.

Experiments that involved combination dosage form of cilazapril and hydrochlorothiazide in tablets were performed in order to prove the applicability of the method to the real time situations and to asses if excipients of the formulation could demonstrate additional impact on the formation of degradation products. After these experiments, it was confirmed that the same degradation processes could be noticed as for individual molecules.

Chromatographic method development and validation

Prior to experiments, log *P* and pK_a values were calculated for both cilazapril and hydrochlorothiazide. The log *P* for cilazapril was 1.95 and for hydrochlorothiazide log *P* was 0.16. These different values obviously indicated the presence of high differences in polarities between these substances. This was confirmed experimentally as much higher percentage of acetonitrile was needed for elution of cilazapril comparing to hydrochlorothiazide. It was not possible to select the appropriate amount of acetonitrile for isocratic elution of both substances in a reasonable run time. For this reason, it was decided to use gradient elution mode. Further, pK_a values and pH dependence of ionic and non-ionic forms plotted for both substances indicated that cilazapril exists in anionic form when pH value of drug solution is in the range between 3 and 6, and in dianionic form when pH value is above 6. On the other hand, hydrochlorothiazide exists in molecular form when pH value of the solution is equal or lower than 8.5.

It was further decided to work with 20 mM ammonium formate buffer solution with pH value set at 8.5. This decision was made based on the peak symmetries of investigated substances. Among both substances, hydrochlorothiazide had a peak tailing which jeopardized its integration. This problem was solved with mobile phase composition consisted of acetonitrile and 20 mM ammonium formate buffer solution whose pH was adjusted to 8.5.

Photodiode array detector is being used during forced degradation studies because of its ability to perform simultaneous detection of analyte on different wavelengths in order to have insight in the formation of degradation products with different absorption properties in comparison to parent drug. In this study, UV spectra of cilazapril and hydrochlorothiazide and of all their degradation products were recorded in order to differentiate them by their spectral characteristics. After the analysis of absorption spectra, it was concluded that all compounds could be detected on three wavelengths used for detection of stress samples (215, 250 and 270 nm). Finally, during stability-indicating method development, it was

decided to perform detection on single wavelength of 250 nm because all compounds showed to have good absorption on this wavelength (40–42).

Peak purity calculations that photodiode array detector provides, were done in order to ensure the "purity" of the chromatographic peak, the absence of co-elution and in such way to prove the selectivity of the method. Peak purity index which was greater than 990 confirmed homogenous and pure peaks of cilazapril, hydrochlorothiazide and their degradation products in all analysed samples. Consistence of the MS spectra recorded by the mass spectrometric detector that had been monitored through the whole forced degradation study also proved the absence of co-elution.

DPH_1 was the first eluting substance and the most polar one. Consequently, this impurity showed non-retention behavior for most of experimental conditions. For this reason, one of the optimization goals was retention factor of DPH_1 to be >2. Only in this way it could be expected to have satisfactory retention behavior and good separation of this compound from the peak of the mobile phase. Besides this goal, it was also needed to optimize method in order to avoid unnecessarily long chromatographic run time. For this reason, second optimization goal was to minimize the retention time of the last eluting compound (DPC_3). As these two goals were in contradiction, it was necessary to find the best compromise between them. It also has to be noted that in case that DPH_1 had retention factor above 2, no resolution between neighboring peaks was jeopardized and there was no need for implementation of any additional optimization goals.

After conducting the experiments according to the CCD experimental plan the obtained data was processed with the assistance of the *Design-Expert 7.0.0* software. The software indicated that global optimal conditions may include initial percentage of acetonitrile set at 5%, final percentage of acetonitrile 35%, time of gradient elution mode of 15.38 minutes during which percentage of acetonitrile would linearly grow and temperature of the column to be set at 25°C. This experimental conditions have completely satisfied first goal of MOOP concerning (k_{DPH1}) and obtained individual desirability function had maximal value ($d_{i\,DPH1} = 1$). But, in case of the second response, (k_{DPC3}), the optimization goal was not completely satisfied since the individual desirability function was $d_{i\,DPC2} = 0.787$. Based on the fact that these chromatographic conditions enabled satisfactory retention of the first compound and as lowest possible chromatographic run time, these results were accepted as the optimal ones. Graphically presented ramps of the optimal chromatographic conditions and lower and upper bounds have been presented in Figure 6 and the level of desirability for individual and overall desirability functions has been presented in Figure 7.

The response surface plot of global desirability function $D = f$ (initial% ACN, final% ACN) when temperature of the column was set at 25°C and time of gradient elution was 15.38 minutes have been presented in Figure 8. The highest value of global desirability function was obtained with lowest percentage of acetonitrile in initial step and with the highest percentage of acetonitrile at the end point of the gradient elution. But the response surface indicated that the proposed optimal conditions would not contrive a robust method. Small changes of experimental conditions would lead to drastic changes of global function and consequently of investigated responses. These facts were not of further consideration as the satisfactory retention would be obtained with small changes and the initial and final percentage of acetonitrile are mechanically controlled and could not be changed in such extent to influence the chromatographic method. Because of this, the proposed experimental conditions were selected as the optimal ones. Beside all previous discussion of the influence of chromatographic parameters on retention factors of all investigated substances described in relation to chromatographic method development, some of instrumental parameters usually involved in method robustness testing were also considered. It was concluded that the change of detection wavelength in the range of ±2 nm or mobile phase flow rate in the range of ±0.1 mL min^{-1} did not significantly alter the chromatographic behavior of investigated substances.

The system suitability parameters determined by analyzing the chromatograms recorded on optimal chromatographic conditions have been presented in Table V.

The developed method was subjected to final method validation as per ICH guidelines (43). The validation procedure was performed for both active substances and for two known impurities described in European pharmacopoeia (38), cilazaprilat and hydrochlorothiazide impurity B. Limits of detection and quantification (LOQ) were determined experimentally for impurities. Linear least squares regression was applied and reported correlation coefficients indicated acceptable linearity of all calibration curves since they were greater than 0.99 and 0.98 in cases of APIs and impurities, respectively (44). Intra-assay precision was demonstrated for six replicate analyses of sample solutions prepared in order to attain concentrations of 88 mg mL^{-1} for cilazapril and 220 mg mL^{-1} for hydrochlorothiazide. Since the range of measured areas of cilazaprilat chromatographic peaks used for construction of calibration curve was very narrow, the methodology of standard addition was used for its quantification (45). In such was proper estimation of its amount and reliable measurements were enabled. Obtained values of relative standard deviations (RSD) met the acceptance criteria. In case of API, RSD should not exceed 2% and in case of impurities

Table V. System Suitability Parameters of the Developed Method

System suitability parameter	Retention time (min)	Relative retention time[a]	Resolution factor, Rs	Number of theoretical plates	Asymmetry factor
Cilazapril	10.84	1	$Rs_{DPC1/Cilazapril} = 13.58$	3,881	1.08
Cilazaprilat	3.62	0.34	$Rs_{Cilazaprilat/DPH2} = 4.26$	3,719	1.11
DPC_1	7.92	0.73	$Rs_{Cilazaprilat/DPC1} = 11.94$	3,976	1.07
DPC_2	11.22	1.04	$Rs_{DPC2/Cilazapril} = 1.95$	3,766	1.15
DPC_3	11.39	1.05	$Rs_{DPC2/DPC3} = 1.36$	3,253	1.18
Hydrochlorothiazide	1.81	1		3,590	1.12
DPH_1	1.37	0.76	$Rs_{Hydrochlorothiazide/DPH1} = 1.32$	4,119	1.13
DPH_2	2.15	1.19	$Rs_{Hydrochlorothiazide/DPH2} = 1.62$	3,285	1.16

[a]Measured in relation to the retention time of appropriate parent drug.

Table VI. Results of Method Validation

Validation parameter	Cilazapril	Hydrochlorothiazide	Cilazaprilat	DPH_1
Linearity				
Calibration equation ($y = ax + b$)	$34{,}622.5x - 380{,}849.0$	$87{,}820.5x - 71{,}720.0$	$54{,}520.2x - 82{,}820.2$	$553{,}401.7x - 6{,}152.4$
Correlation coefficient, r	0.9985	0.9958	0.9955	0.9943
Assay in tablets (%)[a]	102.75	98.91	0.49	0.09
Intra-assay precision				
RSD (%)	1.57	1.70	3.99	4.75
Accuracy[b]	98.11	99.23	103.38	91.17
Recovery value (%)	101.79	99.05	96.21	102.85
	101.90	101.18	99.36	100.01

[a]According to the specification, the assay of both cilazapril and hydrochlorothiazide should be 95–105% of label claim and the MAC of both cilazaprilat and hydrochlorothiazide impurity B is 0.5%.

[b]Concentration levels investigated were 80%, 100% and 120% of label claim for cilazapril and hydrochlorothiazide and concentration equal to LOQ, MAC and 120% of MAC for cilazaprilat and hydrochlorothiazide impurity B.

present in maximum allowed content (MAC) between 0.5 and 1.0% RSD should not exceed 10% (44). The *Recovery* values obtained for the method accuracy investigations were within ±2% for both APIs and within ±10% for impurities (44). Results of method validation have been summarized in Table VI.

Conclusion

CCD and Derringer's desirability function were applied for optimization of chromatographic variables toward various responses in order to define stability-indicating UHPLC-UV–MS method for determination of cilazapril, hydrochlorothiazide and its degradation products. Forced degradation studies were carried out in order to assess which degradation products of cilazapril and hydrochlorothiazide should be involved in development of proposed method. The main advantage of this method is evaluation of chromatographic behavior of cilazapril, hydrochlorothiazide and their degradation products.

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