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Abstract: A fast and selective capillary electrophoresis method has been developed for the simultaneous determination of the antihypertensive drugs captopril and hydrochlorothiazide and their related impurities in a combined dosage form. Method development was carried out implementing each step of Quality by Design workflow, the new paradigm of quality outlined in International Conference on Harmonisation Guidelines. Captopril is characterized by the lack of a strong chromophore and contains a proline-similar moiety, which gives rise to the presence of interconverting cis-trans isomers and leads to the possible interference between electrophoretic migration and reaction of isomerization. The scouting phase was dedicated to the investigation of several operative modes in order to overcome detection and isomerization issues. The best performances were obtained with sodium cholate-based micellar electrokinetic chromatography with the addition of n-butanol and γ-cyclodextrin. Critical quality attributes were represented by the critical resolution values and by analysis time. Critical process parameters were defined as temperature, voltage, concentration and pH of borate buffer, concentration of sodium cholate, n-butanol and γ-cyclodextrin. Screening experimental design was applied for investigating knowledge space. Response surface methodology pointed out several significant interaction effects, and with Monte-Carlo simulations led to map out the design space at a selected probability level. Robustness testing was carried out and a control strategy based on system suitability tests was defined. The selected working conditions gave a complete separation of the analytes in less than three minutes. The method was validated and applied to the analysis of a real sample of coformulation tablets.

Opposed Reviewers:

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DIPARTIMENTO
DI CHIMICA
"UGO SCHIFF"

Sesto Fiorentino, May 2nd 2016

Dear Editor,
Herewith enclosed you will find the manuscript entitled "**Quality by Design meets combined dosage forms: fast determination of captopril, hydrochlorothiazide and their related substances by a cyclodextrin- and solvent-modified micellar electrokinetic chromatography method**", which is intended for publication in ***Talanta***.

In this manuscript, a fast and selective capillary electrophoresis (CE) method has been developed for the simultaneous determination of the antihypertensive drugs captopril and hydrochlorothiazide and their related impurities in a combined dosage form. Method development was carried out by Quality by Design (QbD) approach.
The novelty of the present study was to apply QbD principles to the development of a separation method for the assay of coformulated drugs and their impurities. Another novelty was represented by setting up a method able to determine captopril, its main degradation product captopril disulphide, hydrochlorothiazide and its impurities chlorothiazide, salamide and hydrochlorothiazide dimer. No CE method has been reported yet for the simultaneous analysis of these compounds, while previous chromatographic methods involved a lower number of impurities.
Method scouting made it possible to overcome detection and cis-trans isomerization issues by selecting a suitable separation system made by sodium cholate-based micellar electrokinetic chromatography with the addition of *n*-butanol and γ-cyclodextrin. The separation system was deeply investigated through multivariate techniques, which were used both in the screening phase and in response surface methodology (RSM). The use of experimental design led to the significant benefits of overcoming the challenges due to the high number of critical process parameters and of modeling of critical quality attributes, represented by critical resolution values and analysis time. RSM and Monte-Carlo simulations led to map out the design space at a selected probability level. The proposed method enables the simultaneous determination of the two active compounds and their related impurities within 3 min.

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Best regards,

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NOVELTY STATEMENT

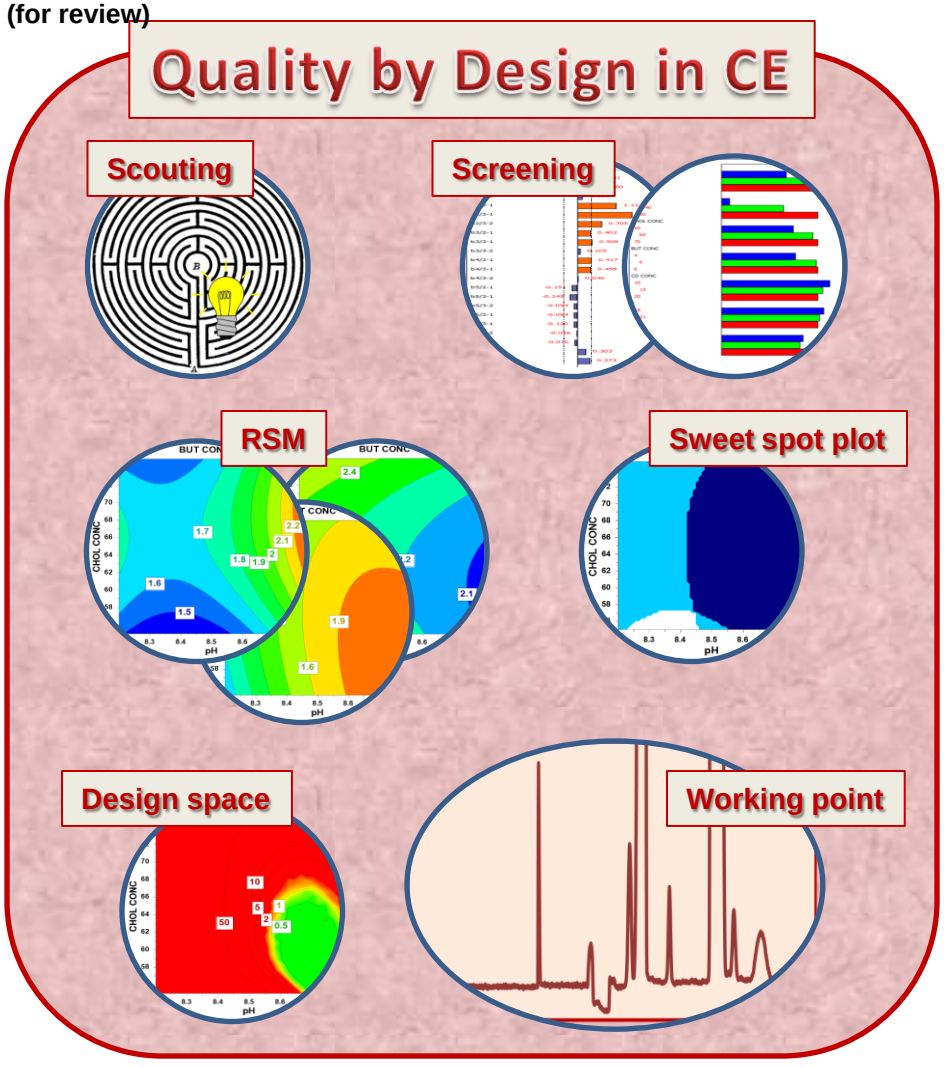
- The novelty of the present study was to apply Quality by Design (QbD) approach to the development of a separation method for the assay of coformulated drugs and their impurities. The selected method was capillary electrophoresis and the coformulated drugs were captopril and hydrochlorothiazide. QbD consisted in a systematic and proactive approach making it possible to obtain a great increase of knowledge and thus to overcome detection, isomerization and selectivity issues.

- Another novelty was represented by setting up a method able to determine captopril, its main degradation product captopril disulphide, hydrochlorothiazide and its impurities chlorothiazide, salamide and hydrochlorothiazide dimer. No capillary electrophoresis method has been reported yet for the simultaneous analysis of these compounds, while previous chromatographic methods involved a lower number of impurities.

HIGHLIGHTS

- A CE method for the assay of a coformulation was set up by Quality by Design.
- Captopril, hydrochlorothiazide and their impurities were the analytes of interest.
- The scouting phase allowed detection and isomerization issues to be overcome.
- Solvent- and cyclodextrin- modified MEKC was the selected operative mode.
- Probability maps enabled the definition of the design space.

*Graphical Abstract (for review)



Pasquini et al., Graphical Abstract

Quality by Design meets combined dosage forms: fast determination of captopril, hydrochlorothiazide and their related substances by a cyclodextrin- and solvent-modified micellar electrokinetic chromatography method

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Abstract

A fast and selective capillary electrophoresis method has been developed for the simultaneous determination of the antihypertensive drugs captopril and hydrochlorothiazide and their related impurities in a combined dosage form. Method development was carried out implementing each step of Quality by Design workflow, the new paradigm of quality outlined in International Conference on Harmonisation Guidelines. Captopril is characterized by the lack of a strong chromophore and contains a proline-similar moiety, which gives rise to the presence of interconverting cis-trans isomers and leads to the possible interference between electrophoretic migration and reaction of isomerization. The scouting phase was dedicated to the investigation of several operative modes in order to overcome detection and isomerization issues. The best performances were obtained with sodium cholate-based micellar electrokinetic chromatography with the addition of *n*-butanol and γ-cyclodextrin. Critical quality attributes were represented by the critical resolution values and by analysis time. Critical process parameters were defined as temperature, voltage, concentration and pH of borate buffer, concentration of sodium cholate, *n*-butanol and γ-cyclodextrin. Screening experimental design was applied for investigating knowledge space. Response surface methodology pointed out several significant interaction effects, and with Monte-Carlo simulations led to map out the design space at a selected probability level. Robustness testing was carried out and a control strategy based on system suitability tests was defined. The selected working conditions gave a complete separation of the analytes in less than three minutes. The method was validated and applied to the analysis of a real sample of coformulation tablets.

Keywords: Capillary Electrophoresis; Cis-trans isomerization; Combined dosage form; Experimental Design; Impurities; Quality by Design

1. Introduction

The control of impurities is a fundamental part and a critical analytical issue of the quality control of drug products. The development of suitable separation analytical methods for impurity assay is characterized by the need of adequate sensitivity and selectivity [1], which can only be reached by a comprehensive investigation of the analytical system. Recently, the new concept of Quality by Design (QbD) [2-4] has found fertile ground in this field, exploiting its great potentiality [5-9]. The QbD approach can be summarized as a systematic, risk-based and proactive strategy to analytical development, which gives a significant support for achieving a sound comprehension of the effects of the critical process parameters (CPPs) on analytical performances, represented by critical quality attributes (CQAs). Design of Experiments (DoE) [10] is a key aspect of QbD, having the role of carrying out the screening phase and the response surface study, and finally of enabling the definition of the multidimensional region of successful operating ranges for the CPPs, namely, the design space (DS). Among the analytical methods applied for impurity assay of pharmaceuticals, HPLC is the main method recommended by different pharmacopeias, but capillary electromigration methods represent a significant alternative. This is especially due to the inherent flexibility for the various available operative modes and to the orthogonality with respect to chromatographic principles, besides other well-known advantages including lower costs and higher eco-compatibility [1,11].

The aim of this study was to set up a fast and simple capillary electrophoresis (CE) method for the simultaneous determination of captopril (CPT), hydrochlorothiazide (HCT) and their related substances in the combined dosage form, following QbD tenets. CPT is a thiol-containing angiotensin-converting enzyme inhibitor used in the management of hypertension, in heart failure, following myocardial infarction and in diabetic nephropathy. HCT is a thiazide diuretic used in the treatment of hypertension, oedema associated with heart failure and with renal and hepatic disorders [12]. CPT and HCT are successfully used in combination to increase the efficacy of treatment in hypertension patients, as blood pressure control is important for the management or prevention of cardiovascular diseases and their complications [13].

The structural formulas of the two drugs and of the related impurities considered in this paper are shown in Fig. 1. According to the European Pharmacopeia [14], HCT specified impurities are reported as I_A, I_B and I_C. I_A and I_C are two main process impurities, while the primary HCT degradation pathway yields I_B and formaldehyde by hydrolysis [15]. CPT may present several impurities, but in aqueous solution undergoes spontaneous oxidative degradation at the sulphydryl group, generating the dimer captopril disulphide (I_{DIS}), which represents the main degradation product [16,17]. In general, CPT determination is very challenging mainly due to the aliphatic structure that lacks any strong chromophore, and to the vulnerability to oxidation of the sulphydryl group. Furthermore, CPT exists as an equilibrium

mixture of cis and trans isomers which are formed by rotation around the proline amide bond [18,19]. The cis-trans isomerization of CPT can be observed during chromatographic or electrophoretic separation, since the isomerization occurs on the time order of minutes. This chemical behavior can give rise to peak splitting or to a typical pattern constituted by a plateau between the two conformer peaks [20,21].

Several methods have already been reported for the simultaneous determination of CPT and HCT in combined dosage forms or in biological fluids, including spectrophotometry [22], voltammetry [23], batch injection analysis with amperometric detection [24], HPLC [25-28], CE [29]. Stability-indicating chromatographic methods have been presented for the analysis of CPT [30,31] or HCT alone [32-34], or for HCT in combination with other drugs [15, 35-41]. Stability-indicating CE methods have been used for the determination of CPT and its degradation products 3-mercapto-2-metyl propanoic acid and I_{DIS} [42] or I_{DIS} and captopril-sulphonate [43], as well as for the quality control of HCT alone considering I_A and I_B [44], or HCT in combination with other drugs [45,46]. Quantification of I_{DIS} as a degradation product has been also performed using near-infrared spectroscopy and chemometrics [16]. Only two HPLC methods have been developed for the simultaneous determination of CPT, HCT and their impurities, represented by I_A, I_B and I_{DIS} [47] or I_B and I_{DIS} [48]. To the best of our knowledge, no CE method has been reported yet for the simultaneous analysis of CPT, HCT and their impurities.

In this study, the CE method development was carried out implementing each step of analytical QbD approach [3]. A large part of the experiments of the scouting phase was dedicated to the selection among different pseudostationary phases based on micelles, with or without additives, in order to overcome CPT detection and isomerization issues. Sodium cholate-based micellar electrokinetic chromatography (MEKC) with the addition of *n*-butanol and γ-cyclodextrin was selected as suitable separation system. The knowledge gained from the screening phase was utilized to fix some of the method CPPs at a definite level and to plan the further investigation by response surface methodology (RSM) [10]. The assurance of meeting the predefined accepted levels for the CQAs was provided by RSM and Monte-Carlo simulations by means of calculated probability maps [49], within which the DS was established. Finally, the method was validated following International Conference on Harmonisation (ICH) guidelines [50] and applied to a real sample of coformulation tablets.

2. Materials and methods

2.1. Chemicals and reagents

The reference standards of CPT, HCT, their impurities and metformin hydrochloride (MET), used as internal standard, were kindly supplied by A. Menarini Industrie Farmaceutiche Riunite (Florence, Italy), as well as all the

coated tablets excipients: microcrystalline cellulose, pregelatinized corn starch, lactose monohydrate, magnesium stearate, stearic acid, Sunset Yellow FCF (E110). Acediur® tablets (A. Menarini Industrie Farmaceutiche Riunite), labeled to contain 50 mg CPT and 25 mg HCT, were purchased in local pharmacies. Boric acid, sodium tetraborate decahydrate (borax), 86.1% phosphoric acid, acetic acid, tris(hydroxymethyl)aminomethane, methanol, *n*-butanol (HPLC grade), sodium dodecyl sulphate, sodium cholate, sodium deoxycholate, sodium taurocholate, sodium taurodeoxycholate, 3-(*N*,*N*-dimethylmyristylammonio)propanesulfonate (MAPS), polyoxyethylene (23) lauryl ether (Brij 35), all the cyclodextrins (CDs) tested with the corresponding degree of substitution (D.S.), i.e. α-CD, γ-CD, methyl-β-cyclodextrin (D.S. 1.5-2.1), (2-hydroxypropyl)-α-CD (D.S. 0.6), (2-hydroxypropyl)-β-CD (D.S. 0.6), (2-hydroxypropyl)-γ-CD (D.S. 0.6), (2-hydroxyethyl)-β-CD (D.S. 0.7), sulfated-β-CD sodium salt (D.S. 12-15) and all the other chemicals used were from Sigma-Aldrich (St. Louis, MO, USA). The water used in the experiments was purified by Elix and Simplicity 185 systems (Millipore, Billerica, MA, USA).

2.2. Solutions, pseudostationary phases, sample preparation

Standard stock solutions of CPT and HCT (10 mg ml^{-1}), CPT and HCT impurities (1 mg ml^{-1}) and MET (1 mg ml^{-1}) were prepared in methanol and were stored at 4 °C for 1 week. Working standard solutions were daily prepared. Running buffer solutions were prepared by adjusting the pH value of a proper volume of the corresponding 0.5M acid or mixture of acids by NaOH (or HCl in the case of borax buffer) and by dilution up to the desired concentration. Britton-Robinson universal buffer was made by a mixture of phosphoric, acetic and boric acid.

Pseudostationary phases made by micelles and mixed micelles were prepared by weighting the proper amount of the surfactant or the two surfactants to which appropriate volume of buffer was added. Other additives (CDs, organic solvents) were directly added to the mixtures containing the pseudostationary phases in order to obtain the final background electrolyte (BGE).

Twenty tablets were weighted and crushed to a fine powder. Then, an amount of the powder equivalent to about 100 mg CPT and 50 mg HCT, accurately weighed, was transferred in a beaker to which 10 mL of methanol were added, thus obtaining a concentration of about 10 mg ml^{-1} CPT and 5 mg ml^{-1} HCT. One milliliter of the mixture was stirred, sonicated and centrifuged for 10 min each. After centrifugation, 200 μL of the surnatant were diluted in a vial up to 500 μL by adding 50 μl of internal standard stock solution and 250 μl of water in order to achieve a test concentration equal to about 4 mg ml^{-1} for CPT and to about 2 mg ml^{-1} for HCT, with a MET concentration equal to 0.1 mg ml^{-1}.

2.3. Capillary electrophoresis equipment and analysis

Electrophoretic experiments were carried out using an Agilent Technologies ^{3D}CE System (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) with the detection wavelength set at 220 nm. Instrument control, data acquisition, peak processing were performed using the Rev.A.09.01 software (Agilent Technologies). CE separations were carried out in a fused-silica capillary (50 μm inner diameter, 375 μm outer diameter, total length 33.0 cm) with a detection window at 24.5 cm created by removing the polyimide coating. New capillaries were flushed with 1M NaOH for 5 min, followed by 0.1M NaOH and water for 5 min each. Before every run, the capillaries were conditioned by flushing with methanol for 2 min, 0.1 M NaOH for 2 min, water for 1 min and BGE for 3 min. Hydrodynamic injections of samples were made at the cathodic end using a pressure of 50 mbar for 10 s, followed by a BGE plug (50 mbar for 10 s). The selected working conditions (with the related range defining the DS) were the following: BGE, 100 mM borate buffer pH 8.55 (8.48-8.62), 64 mM (60-68 mM) sodium cholate, 6.1 %v/v (5.4-6.8 %v/v) *n*-butanol, 12 mM (11-13 mM) γ-CD; voltage, 27 kV (26-28 kV), temperature, 21°C. Applying these conditions, the measured current was about 85 μA.

2.4. Calculations and softwares

The peak corrected area (area/migration time) ratios of the analyte to internal standard were plotted against the corresponding analyte concentration to obtain the calibration graphs. Ten samples were prepared, two for each of five different concentration values, and were analyzed using MET as internal standard at a concentration value of 0.1 mg ml^{-1}. The CPT and the HCT regression curves were calculated in the range 60-120% with respect to the corresponding test concentration, corresponding to 2.4-4.8 mg mL^{-1} for CPT and 1.2-2.4 mg mL^{-1} for HCT. The regression curves for the impurities were from the respective limit of quantitation (LOQ) to 1% with respect to the test concentration of the corresponding main compound: I_{DIS}, 0.0040-0.0400 mg ml^{-1}; I_A, 0.0020-0.0200 mg ml^{-1}; I_B, 0.0006-0.0200 mg ml^{-1}; I_C, 0.0016-0.0200 mg ml^{-1}.

Nemrod-W [51] software was used for generating the symmetric screening matrix and the Plackett-Burman designs. MODDE 10 [52] software was employed for generating Central Composite Face Centered Design (FCD) design and to draw risk of failure maps by Monte-Carlo simulations. The runs of the experimental plans were carried out in a randomized order with a test solution containing 4 mg ml^{-1} CPT, 2 mg ml^{-1} HCT and 0.0400 mg ml^{-1} I_{DIS}, 0.0200 mg ml^{-1} I_A, I_B and I_C, namely corresponding to 1% with respect to the related main compound.

3. Results and discussion

3.1. Analytical target profile, method scouting, critical quality attributes

The analytical target profile of the CE method was to get the baseline separation of the six analytes in a short analysis time and to achieve an accurate determination of the compounds in the pharmaceutical dosage form. The validation requirements as described in ICH Guideline Q2(R1) [50] and in detail reported in Ref. [6] should be fulfilled, and in particular LOQ values for the impurities should be 0.1% or lower with respect to the related main compound. From the first experimental runs, the main analytical issues were evidenced and were mainly attributable to: i) presence of analytes with ionizable moieties presenting different acid-base properties; ii) low UV absorbance value and efficiency of I_{DIS}; iii) possible peak broadening or splitting of CPT due to the interference between electrophoretic migration and cis-trans reaction of isomerization. In particular, this interference can depend on several factors including the chemical nature of the system (BGE, electro-osmotic flow, electrophoretic mobility of the molecules), effective length of the capillary, applied electric field and temperature [53]. In this study, the possibility of using high temperature for avoiding peak splitting was discarded and ambient temperature was selected in order to provide more practical experimental conditions.

Hence, a large part of the experiments of the scouting phase was intended to the selection of a suitable operative mode enabling to overcome separation, detection and isomerization issues. In the scouting phase the concentration of all the compounds was kept low and equal to 0.06 mg ml^{-1}, in order to obtain clear information on the separation pattern and the migration behavior of the compounds. Capillary zone electrophoresis (CZE) with plain buffers as well as MEKC with different pseudostationary phases, with or without additives, were examined in order to find an useful separation system.

The CZE experiments were run with a Britton-Robinson buffer in order to be able to evaluate the effect of different pH values in a wide range (from 5.0 to 11.0) and to determine the safe zone of pH useful to avoid the peak splitting of the CPT isomers and to enable the simultaneous determination of all the compounds. The best results were obtained using an alkaline BGE with a pH value included in the range 8.00-8.80. At higher pH values the individual peaks of the separated conformers of CPT were connected by the typical plateau due to interference [20,21], confirming that pH value is essential to avoid peak splitting [54]. Lower pH values were detrimental for the analysis of HCT and HCT impurities, which present basic characteristics, leading to a decrease in selectivity. The use of other buffers at different concentration values (20-100 mM), including borate, phosphate, phosphate-borate, tris(hydroxymethyl)aminomethane, led to similar results in terms of operative range for pH. Within these buffers, a 100 mM borate system pH 8.10 was selected as basis for evaluating the addition of different pseudostationary phases.

The use of sodium dodecyl sulphate-based MEKC did not lead to good results, especially for a scarce efficiency of the peaks related to basic compounds. The use of the biliar salts mentioned in Sec. 2.1. and especially of sodium cholate was decisive for ameliorating the peak separation pattern. Sodium cholate-based MEKC was also further tuned

by addition of MAPS and Brij 35, but the use of mixed micelles was not useful. Hence, the addition of different CDs, reported in Sec. 2.1., was evaluated. It was found that the use of γ-CD was fundamental for obtaining a great increase in I_{DIS} peak efficiency, also producing a decrease in analysis time and an increase in selectivity. By adding *n*-butanol to this system, further beneficial effects on I_{DIS} peak efficiency were observed, confirming that this organic modifier can exploit an active role in controlling the partitioning of the analytes, with a great potential for ameliorating the electrophoretic pattern [6,55]. Hence, the sodium cholate-based MEKC operative mode with the addition of γ-CD and *n*-butanol enabled to overcome all the main analytical issues which had been evidenced in the preliminary experiments, and was selected for further optimization steps. With this separative system, the migration order of the compounds was the following: MET (internal standard), I_B, HCT, I_C, CPT, I_A, I_{DIS}, with the resolution values indicated by Rs_1 (I_B/HCT), Rs_2 (HCT/I_C), Rs_3 (I_C/CPT), Rs_4 (CPT/I_A), Rs_5 (I_A/I_{DIS}). The responses which were selected as CQAs were critical resolution values Rs_1 and Rs_4, whose desired values were both set as ≥1.5, and analysis time (*t*), for which the accepted value was set as ≤3 min.

3.2. Critical process parameters and screening of knowledge space

In the selected separative system, the separation mechanism was based on the different mobilities of the solutes, their partitioning in the sodium cholate micelles and on host-guest inclusion complexation in the cyclodextrin, with the consequence of a high number of experimental factors which could affect one or more CQAs. In order to ensure that the final method is reliable and meets the requirements for the CQAs, a risk analysis with a fishbone diagram (not shown) [56] was used to identify the variables which further needed an in-depth study by DoE. Seven CPPs were identified and were investigated by a multivariate screening procedure [57]. The CPPs were both related to the BGE composition, *e.g.* borate concentration (*B CONC*), pH of the buffer (*pH*), sodium cholate concentration (*CHOL CONC*), *n*-butanol concentration (*BUT CONC*), γ-cyclodextrin concentration (*CD CONC*), and related to instrumental settings, *e.g.* temperature (*T*) and voltage (*V*). For all the CPPs the experimental domain was selected on the basis of the scouting experiments and was split into three levels, as shown in Supplementary Table S1. In particular, pH was investigated in a small domain (8.00-8.80), which had been identified in the scouting phase as the only suitable range useful to overcome the evidenced critical analytical issues.

The effect of the change of levels of the CPPs was studied by the symmetric screening matrix 3^7//16 reported in Supplementary Table S2. Among the measured CQAs, for Rs_1 and Rs_4 a considerable number of experiments with a complete overlap of the peaks was reported, evidencing the particular criticism for selectivity responses; on the other hand, all the values obtained for *t* were lower than 5 min. Graphical analysis of effects, shown in Supplementary Figure S1, allowed the effect of the change of levels to be visualized. The first kind of plot (Supplementary Fig. S1a-c)

highlights the amplitude of the difference between the effects of two selected levels, which is related to the bars' length, and makes it possible to point out which are the changes of levels leading to a significant change on a CQA. The second kind of plot is reported in Supplementary Fig. S1d-f and visualizes bars whose length is related to the value of the response; the longer the bar, the higher the response. The graphs showed that Rs_1 was increased by medium-high levels of *B CONC*, *CHOL CONC* and *BUT CONC* and high levels of pH; for Rs_4, the preferred settings were high level for pH, medium level for *CHOL CONC*, medium level for *BUT CONC*, while the maximization of this response was obtained at low levels of *CD CONC*. For both Rs_1 and Rs_4, the change of levels of pH was undoubtedly of outmost importance, confirming the important role of this factor. As regards *t*, the only factor which exerted a significant effect was voltage. The information from the two types of plots was matched, mediating the best values and ranges of the CPPs with respect to the different CQAs. Hence, the final outcome of the screening phase consisted in fixing *B CONC* and *T* at their central value (100 mM and 21 °C, respectively) and in defining a new experimental domain for the other five CPPs to be focused on by RSM (as reported in Supplementary Table S1).

3.3. Response surface methodology and design space

A five-factors FCD was employed for building a second order quadratic model for the selected CQAs [10]. Each CPP was studied at three levels, covering the new experimental domain, with the star points located at the center of each face of the factorial space, with $\alpha=\pm1$. Twenty-nine experiments were required, including three replicates at the center point, and the experimental plan with the measured responses is shown in Supplementary Table S3. The calculated models were edited by removing some of the interaction or quadratic terms which did not exert a significant effect on the response, in order to ameliorate values of coefficient of determination R^2 and coefficient of predicted variation Q^2. The following final values were achieved: Rs_1, $Q^2=0.923$, $R^2=0.983$; Rs_4, $Q^2=0.821$, $R^2=0.963$; *t*, $Q^2=0.840$, $R^2=0.976$. The ANOVA demonstrated that the regression models for Rs_1 and Rs_4 were statistically valid and significant, while the model for *t* was observed to be not valid due to the small experimental variance.

Isoresponse surfaces for Rs_1 (Fig. 2a-c), Rs_4 (Fig. 2d-f) and *t* (Fig. 2g-i) were obtained by plotting *CHOL CONC* *vs.* pH at three values of *BUT CONC*, corresponding to 5 %v/v (Fig. 3a,d and g), 6 %v/v (Fig. 3b,e and h) and 7 %v/v (Fig. c,f and i), while maintaining *CD CONC* and *V* at their central values. As for Rs_1, the higher values were achieved for high values of pH, which showed very important effects, both linear and quadratic. *CHOL CONC* exerted another important quadratic effect on Rs_1. Also for Rs_4, pH showed very important linear and quadratic effects, with the best zone for increasing this CPP represented by high values of pH and low values of *CHOL CONC*. Significant quadratic effects on *t* were observed for *BUT CONC*, *CD CONC* and *V*, with the latter, as expected, showing the most important

linear effect as well. For all the three CQAs, several significant interactions were observed, among which it was evidenced a notable negative one between *CHOL CONC* and *CD CONC* in the case of Rs_4.

The sweet spot plots, reported in Supplementary Fig. S2, were drawn maintaining *CD CONC* and *V* at their central values. These graphs made it possible to highlight by different colours the areas where one (white), two (pale blue) or three (dark blue) predicted CQAs fulfilled the requirements. The plots confirmed that it is necessary to set the pH value at medium-high levels, but wide zones leading to required CQAs values were obtained for all the values of *BUT CONC*.

In order to take into account the concept of probability, risk of failure maps were drawn (Fig. 3a-c) by MODDE software [52], on the basis of the calculated models and of Monte-Carlo simulations for risk analysis [49]. Monte-Carlo simulations were performed to propagate the error in prediction of the models from the factors to the response, giving access to the distributions of the responses for each operating condition of the RSM [58]. The set-point was the following: pH, 8.55; *CHOL CONC*, 64 mM; *BUT CONC*, 6.1 %v/v; *CD CONC*, 12 mM; *V*, 27 kV. The DS was calculated from this set-point by using a search function [52] that expands the possible factor ranges to the largest possible range where all the response predictions are still within the specifications at a probability level of $\pi \geq 99\%$, and corresponded to the following intervals: pH, 8.48-8.62; *CHOL CONC*, 60-68 mM; *BUT CONC*, 5.4-6.8 %v/v; *CD CONC*, 11-13 mM; *V*, 26-28 kV (Supplementary Table S1). In Fig. 3, the DS is highlighted in green and is included in the line corresponding to 1% risk of failure. In contrast to what was observed in the sweet spot plots, the DS cannot be calculated when setting *BUT CONC* at 7 %v/v (Fig. 3c). Furthermore, especially for the BGE modifiers, the DS interval resulted to be quite narrow. The ranges could be in theory widened by selecting a lower level for probability; anyway, it was deemed reasonable to keep a low risk of failure, mainly due to the criticism of the responses related to selectivity. The lower and the higher limits of the DS range for each CPP were selected as the -1 and +1 levels of a Plackett-Burman design [10], which was used for validating the DS and verifying that the observed values for the CQAs were within the selected specifications. The working conditions corresponded to the set-point used for calculating the DS and made it possible to achieve the complete separation of the analytes in less than 3 min (Fig. 4).

3.4. Robustness testing and control strategy

For robustness testing, the 8-run Plackett-Burman design shown in Supplementary Table S4 was employed in order to calculate the main effects of small variations of all the seven CPPs considered in the screening phase, *e.g. B CONC* (98-102 mM), *T* (20-22 °C), pH (8.45-8.65), *CHOL CONC* (62-66 mM), *BUT CONC* (5.6-6.6 %v/v), *CD CONC*, 11-13 mM, *V*, 26-28 kV. The graphic analysis of effects is shown in Supplementary Fig. S3 and revealed that pH was the only factor which exerted a significant effect on Rs_1, while only *V* was influent on *T*. These findings

emphasized once again the importance of carefully setting the proper value of pH when preparing the BGE. For planning the control strategy of the method [2], system suitability criteria were defined from system repeatability studies, picking out the upper and the lower measured values for the CQAs. These values constituted the limits of system suitability intervals, defined as the following: $1.71<Rs_1<1.94$, $1.73<Rs_4<2.02$, $2.29 \text{ min} < t < 2.42 \text{ min}$.

3.5. Validation and application to real samples

Validation was carried out to assure the suitability of the method for the quality control of the coformulated dosage form. The method was validated according to the ICH guidelines [50] with respect to selectivity, linearity and range, limit of detection and quantitation, accuracy and precision, and the related data are reported in Supplementary Information.

After method validation, Acediur® tablets containing 50 mg CPT and 25 mg HCT were analysed and a typical electropherogram is shown in Supplementary Fig. S4. The percentage of claimed amount of the drugs was: CPT, 98.2±2.4% with a RSD of 1.5%; HCT, 98.7±1.9% with a RSD of 1.2% (n=4, $\alpha/2$=0.025). As for the impurities, I_A was not detected, while the other impurities were detected at concentration values lower than the respective LOQ.

4. Conclusions

For the first time in the literature, QbD principles have been followed in the development of a method for the simultaneous determination of two drugs and their impurities in a combined dosage form, represented by a coformulation of captopril and hydrochlorothiazide. The implementation of QbD in drug analysis involves a thorough understanding of separation methods, enabling to ensure the quality of analytical data and the quality of pharmaceutical products as final outcome. In this study, method scouting made it possible to overcome the detection and cis-trans isomerization issues by selecting a suitable separation system made by sodium cholate micelles with the addition of *n*-butanol and γ-cyclodextrin. This separation system was deeply investigated through multivariate techniques, used both in the screening phase and in response surface methodology, leading to the significant benefits of overcoming the challenges due to the large number of critical process parameters and of modeling of critical quality attributes. The design space was identified as the multidimensional zone where the values for critical resolutions and analysis time fulfilled the requirements with a selected probability. The proposed method enables the simultaneous determination of the two active compounds and their related impurities within 3 min, confirming the successful binomium between QbD methodology and CE as a very powerful means for impurity profiling.

Acknowledgements

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Figure Captions

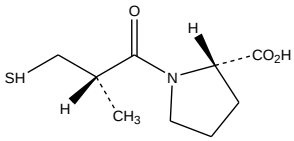
Fig. 1. Structural formulas, nomenclature and CAS of the analytes and of the internal standard.

Fig. 2. Isoresponse surfaces drawn for Rs_1 (a,b,c), Rs_4 (d,e,f), t (g,h,i) by plotting sodium cholate concentration vs. pH at different values of *n*-butanol concentration: 5 %v/v (a,d,g), 6 %v/v (b,e,h) and 7 %v/v (c,f,i). γ-cyclodextrin concentration, 12 mM; voltage, 26 kV.

Fig. 3. Risk of failure maps obtained by plotting pH vs. sodium cholate concentration at different values of *n*-butanol concentration: 5 %v/v (a), 6 %v/v (b) and 7% v/v (c). The design space is the green zone included in the line corresponding to 1% risk of failure.

Fig. 4. Electropherogram in the working conditions of a solution 4 mg ml^{-1} CPT, 2 mg ml^{-1} HCT, 0.04 mg ml^{-1} I_{DIS}, 0.02 mg ml^{-1} I_A,I_B and I_C, 0.1 mg ml^{-1} MET. BGE, 100 mM borate buffer pH 8.55, 64 mM sodium cholate, 6.1 %v/v *n*-butanol, 12 mM γ-cyclodextrin; voltage, 27 kV; temperature, 21°C. Capillary length, 33.0 cm; hydrodynamic injection, 50 mbar for 10 s; detection wavelength, 220 nm. Symbols as in Fig. 1.

Figure



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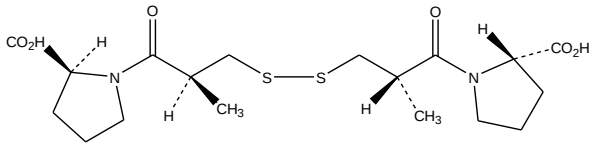
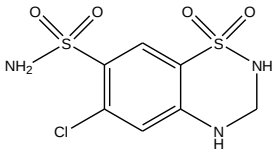
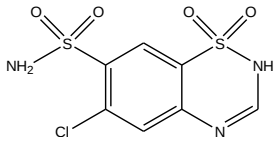
pyrrolidine-2-carboxylic acid
CAS 62571-86-2



Captopril disulfide (I_{DIS})
1,1'-[disulfanediylbis[(2*S*)-2-methyl-1-oxopropane-3,1-diyl]]bis[(2*S*)-pyrrolidine-2-carboxylic] acid
CAS 64806-05-09



Hydrochlorothiazide (HCT)
6-Chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide
CAS 58-93-5



Chlorothiazide (I_A)
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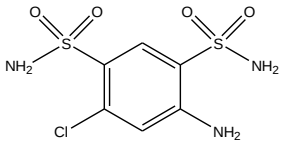
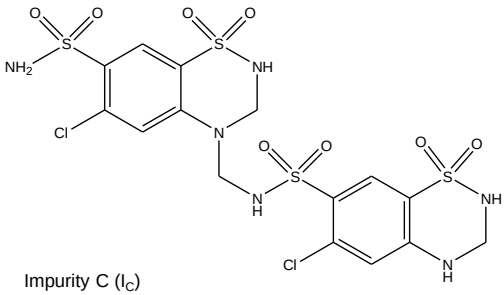
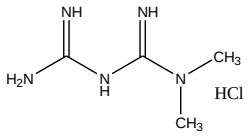
CAS 58-94-6



Salamide (I_B)
4-Amino-6-chlorobenzene-1,3-disulfonamide
CAS 121-30-2



Impurity C (I_C)
6-Chloro-*N*-[(6-chloro-7-sulfamoyl-2,3-dihydro-4*H*-1,2,4-benzothiadiazin-4-yl 1,1-dioxide)methyl]-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide
CAS 402824-96-8



Metformin hydrochloride (MET)
1,1-Dimethylbiguanide hydrochloride
CAS 1115-70-4
(internal standard)

Fig. 1, B. Pasquini et al.

Figure

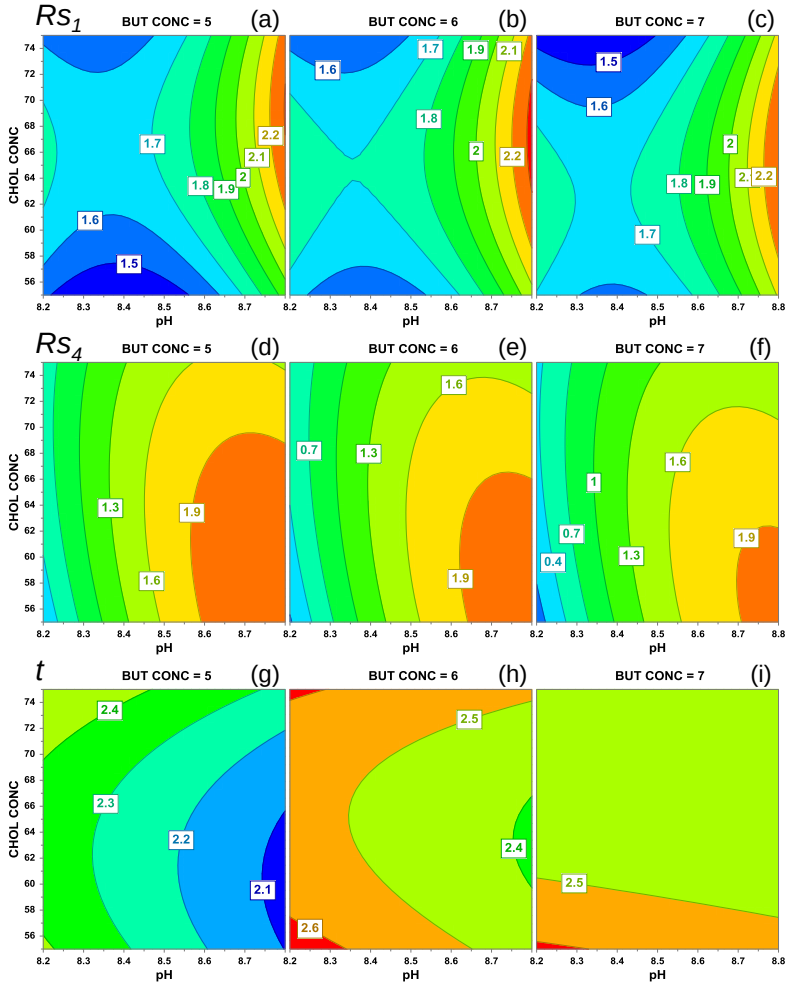


Fig. 2, B. Pasquini et al.

Figure

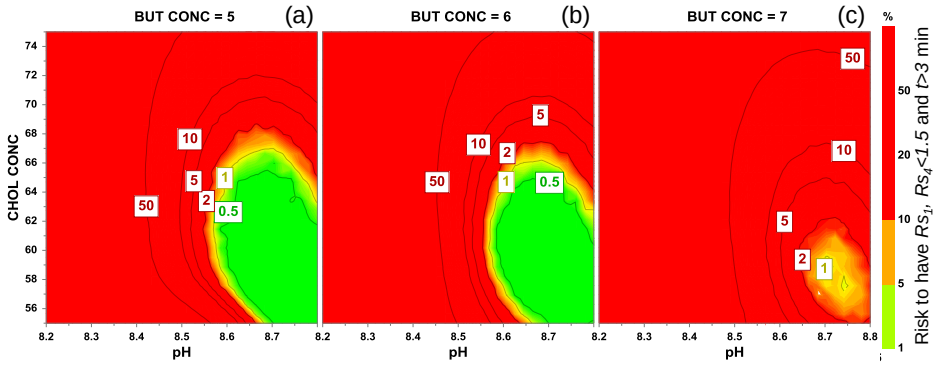


Fig. 3, B. Pasquini et al.

Figure

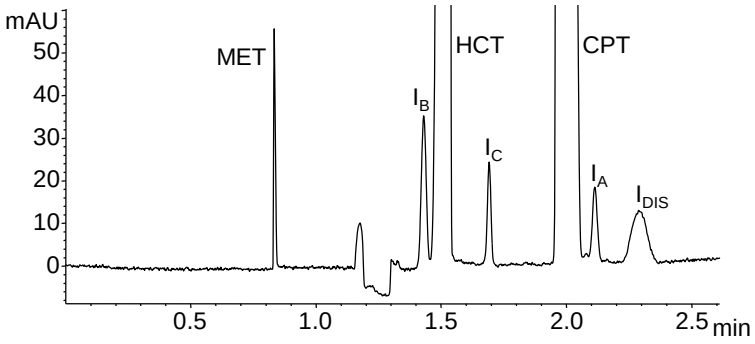


Fig. 4, B. Pasquini et al.

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Sesto Fiorentino, May 2nd 2016

Dear Editor,

Herewith you will find a list of suggested potential referees for the manuscript entitled "**Quality by Design meets combined dosage forms: fast determination of captopril, hydrochlorothiazide and their related substances by a cyclodextrin- and solvent-modified micellar electrokinetic chromatography method**", which is intended for publication in ***Talanta***:

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