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Separation and determination of clopidogrel and its impurities by capillary electrophoresis

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ABSTRACT

Clopidogrel bisulphate, an anti-platelet drug, has been separated from its impurities, namely impurity A, B and C by capillary zone electrophoresis (CZE) using uncoated fused-silica capillary (50.0 μm internal diameter, 31.2 cm total length). Four factors affected the separation: buffer concentration, pH of the buffer, concentration of the chiral selector and the applied voltage. Optimization and robustness studies were performed with the aid of reduced central composite experimental design. The buffer used was triethylamine–phosphoric acid and the chosen chiral selector was sulphated β-cyclodextrin (SCD). The best separation was achieved by using 10 mM buffer, pH 2.3, containing 5% (mass/volume (m/v)) SCD. Reversed polarity mode was used with an applied voltage of −12 kV and the capillary temperature was maintained at 20 °C. The method was validated for quantitative determination of the drug. It offered a limit of detection (LOD) of 0.13 μg/ml, a limit of quantitation (LOQ) of 0.4 μg/ml, and a linearity range of 0.4–300 μg/ml. Commercial bulk samples were analyzed using the developed method.

1. Introduction

Clopidogrel is a potent anti-platelet and anti-thrombotic drug. It is a dihydrothienopyridine derivative pro-drug which is inactive in *vitro*. In *vivo*, it selectively and irreversibly inhibits the binding of adenosine diphosphate (ADP) to its platelet receptors [1]. Although the majority of the drug is hydrolyzed by esterase to an inactive carboxylic acid metabolite, the full anti-aggregating activity of the drug is achieved by biotransformation to 2-oxo-clopidogrel by cytochrome P450-1A. This intermediate metabolite is hydrolyzed and generates an active form which reacts as thiol reagent with the ADP receptor on platelets thus preventing the binding of ADP [2]. Clopidogrel has an absolute S-configuration at carbon 7; the corresponding R-enantiomer is totally devoid from anti-aggregating activity [3]. Both the carboxylic acid hydrolysis product and the R-enantiomer are stated by the United States Pharmacopeia to be the impurities A and C in the raw materials, besides impurity B which is a racemic mixture of isomers [4]. Fig. 1 shows the structures of clopidogrel and its impurities.

Clopidogrel (CL) has been determined in pharmaceutical preparations by spectrophotometric [5,6] and LC [7,8] methods. A stability-indicating method was developed for its determination in the presence of its degradation products under stressed conditions using TLC [9]. In plasma, a bioanalytical method was developed for its determination using LC–MS/MS [10]. CL was determined by LC in the presence of its impurities using a chiral column and its impurities were determined at low levels [11]. The carboxylic acid metabolite (impurity A) was determined in human plasma by GC–MS [12], LC with UV detection [13] and LC–MS [14]. One capillary zone electrophoresis (CZE) method was mentioned for enantioseparation of six drugs including clopidogrel, using highly sulphated γ-cyclodextrin as chiral additive, but the method did not mention any quantitative aspects for its application [15]. The aim of this manuscript is to show the capability of CZE for separation and quantitative determination of CL and its stated impurities A, B and C. No previous CZE achieved the mentioned targets.

Abbreviations: CL, clopidogrel; CD, cyclodextrin; SCD, sulphated β-cyclodextrin; HSCD, highly sulphated γ-CD; HDAS, heptakis (2,3-diacetyl-6-sulphato) β-cyclodextrin; TEA-PO_4, triethylamine/phosphoric acid solution; RCC, reduced central composite face-centered design.

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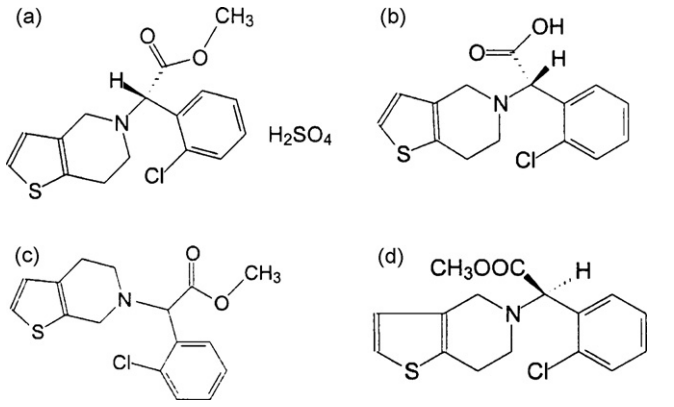

Fig. 1. Chemical structures of (a) clopidogrel (S-enantiomer), (b) impurity A (hydrolysis product), (c) impurity B (racemic mixture of isomers), and (d) impurity C (R-enantiomer).

2. Experimental

2.1. Instrumentation

The study was performed on a P/ACE MDQ instrument equipped with a photodiode-array detector from Beckman Coulter (Fullerton, CA, USA). Data acquisition was done by means of 32 Karat version 5.0 software (Fullerton, CA, USA). Uncoated fused-silica capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA). Another instrument was also used, namely SpectraPHORESIS 1000 CE instrument (Thermo Separation Products, San Jose, CA, USA) equipped with high-speed-scanning UV/VIS detector and connected to an HP 3396 Series III integrator (Agilent Technologies Company, USA). The pH measurements were performed on a Metrohm 691 pH meter (Herisau, Switzerland). To ensure consistent results, it was calibrated before each measurement with reference buffer solutions prescribed in the European Pharmacopoeia [16]. All weighing measurements were performed on a Mettler Toledo AG245 balance (Herisau, Switzerland).

2.2. Materials and reagents

Cyclodextrins (CDs) include α-cyclodextrin (α-CD) hydrate, β-cyclodextrin (β-CD) hydrate, and γ-cyclodextrin (γ-CD) hydrate purchased from Acros Organics (Geel, Belgium). Sulphated β-CD sodium salt (7–11 substitutions per molecule) (SCD) was provided by Sigma–Aldrich Chemie (Steinheim, Germany). Heptakis (2,3-diacetyl-6-sulphato) β-cyclodextrin (HDAS) was purchased from Regis Technologies (Morton Grove, IL, USA). Highly sulphated γ-CD (HSCD) was obtained from Beckman Coulter (Fullerton, CA, USA). Triethylamine–phosphoric acid solution (1:1) each 2.0 M (TEA-PO_4) and sodium dihydrogen phosphate were provided by Fluka (Steinheim, Germany). Sodium hydroxide pellets were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Hydrochloric acid p.a. was from Merck (Darmstadt, Germany). Phosphoric acid (85%) was from Acros Organics. The reference standards of clopidogrel bisulphate and impurities A, B and C were obtained from IPCA Laboratories Ltd. (Mumbai, India). Two commercial samples of clopidogrel were individually obtained from Hetero Drugs Ltd. (Hyderabad, India) and Veritech Pharmaceuticals Pvt. Ltd. (Mumbai, India). All solutions were prepared with ultrapure Milli-Q water (Millipore, Milford, MA, USA) and filtered with a 0.2-μm nylon filter (Euroscientific, Lint, Belgium) where necessary.

2.3. Standard solutions

2.3.1. Standard stock solutions of CL

Standard stock solutions were prepared by transferring 100 mg of CL powder into 20-ml volumetric flasks, and dissolving in Milli-Q water; the volume of each was completed to the mark with Milli-Q water to obtain final concentrations of 5000 μg/ml.

2.3.2. Standard stock solutions of CL R-enantiomer and impurities A and B

Standard stock solutions were prepared by transferring 5 mg of each of CL R-enantiomer (impurity C), and impurities A and B powders separately into 5-ml volumetric flasks; and dissolving in Milli-Q water; the volume of each was made up to the mark with Milli-Q water to obtain final concentrations of 1000 μg/ml.

2.3.3. Spiked standard and samples

Solutions of CL (300 μg/ml) and each of its impurities (150 μg/ml) were prepared by transferring aliquot volumes of each of the corresponding stock solutions in 1-ml Eppendorf tubes with the aid of calibrated multichannel micro-pipettes, vortexed for 30 s and the volume of each was completed quantitatively with Milli-Q water. In addition, different solution mixtures containing 50, 100 and 200 μg/ml of CL spiked with equal amounts of each of the impurities, representing 1% to 100% of CL, were similarly prepared. Solutions were then transferred into the sample vials for analysis. These solutions were used for method development and optimization of the electrophoretic conditions.

For method validation, series of CL and each of its individual impurities' solutions, having concentrations within their corresponding linearity ranges, were individually prepared.

2.4. Software

The experimental design and multivariate analysis for method optimization and robustness were performed with the support of Modde® 5.0 software (Umetrics AB, Umeå, Sweden). The optimum conditions can be predicted using an "Optimizer" option in Modde® software. The "Optimizer" uses a "Nelder Mead Simplex Method" with the fitted response functions.

2.5. Procedure

2.5.1. Electrophoretic conditions

2.5.1.1. Preliminary capillary conditioning. Before using the capillary for the first time, it was conditioned at 25 °C by rinsing with water followed by 1.0 M NaOH for 5 min and keeping it in 1.0 M NaOH for 2 h. It was then washed with 0.1 M NaOH followed by water for 5 min. Further equilibration was performed by flushing with the separation buffer for 10 min. Washing procedures were performed by applying a pressure of 20 psi.

2.5.1.2. Daily conditioning. At the beginning of each day prior to the analyses, the capillary was activated by rinsing in the following sequence: water, 0.1 M NaOH, and water for 5 min, and finally equilibrated with running buffer for 10 min, all at 25 °C. To ensure repeatability of the migration times, the first two runs were disregarded and the capillary was rinsed with the background electrolyte (BGE) for 3 min in-between runs. The inlet/outlet vials were renewed every two runs. All the washings were performed by applying a pressure of 20 psi.

2.5.1.3. Preparation of the background electrolyte (BGE). Different molar concentrations of the buffers were formed by suitable dilutions of triethylamine/phosphoric acid solution (1:1) each 2.0 M

(TEA-PO_4) with Milli-Q water and adjusted to the corresponding desired pH values by adding 1.0 M hydrochloric acid drop-wisely until these values were reached. Furthermore, sulphated β-CD (SCD) of different concentrations was dissolved in the buffer, to obtain the final composition of the BGEs.

Finally, the prepared buffers were transferred to source and destination vials using a syringe ("Becton Dickinson S.A.", Fraga, Spain) and filtered with the Teflon filters ("Whatman®", Whatman GmbH, Dassel, Germany).

2.5.2. Electrophoretic runs

The electrophoretic runs were performed using the following optimum conditions: a capillary with a total length of 31.2 cm, effective length of 20 cm, and an ID of 50 μm; a BGE either containing 6% SCD (mass/volume (m/v)) in 35 mM TEA-PO_4 adjusted to pH 1.8 using 1.0 M HCl for the intermediate method or containing 5% SCD (m/v) in 10 mM TEA-PO_4 similarly adjusted to pH 2.3 for the final method; the applied voltage was −12 kV and the capillary temperature maintained at 20 °C using liquid coolant. The samples were hydrodynamically injected for 4.0 s × 1.5 psi. On-line detection was performed at 195 nm.

Solutions of each of the CL and its impurities were separately injected and checked for their migration times and orders of elution.

3. Results and discussion

In recent years, an increasing number of studies on chiral compounds have been observed, including studies dealing with the separation of enantiomers. Many new chiral selectors have been found so far, offering a better choice of selectors for all types of pharmaceutical compounds [17].

3.1. Preliminary study of experimental conditions

The investigation started by choosing the type, concentration and pH of the buffer, while using 5% (m/v) SCD as chiral selector and the voltage applied was 10 kV in reversed polarity mode. The initial choice of SCD was based on literature recommendation for separation of chiral compounds by CZE and reversed polarity was applied [15,18,19].

3.1.1. Study of the effect of buffer type, concentration and pH

Phosphate buffers, having a concentration of 20 mM prepared from disodium hydrogen phosphate (pH 9.4) and sodium dihydrogen phosphate (pH 4.7), were tried. Due to the basic nature of the drug, precipitation of drug occurred upon using the first buffer while no separation was observed with the second one. Apparently, clopidogrel and its impurities [especially B and C (the racemic mixture of isomers and R-enantiomer)] have close pK. The change of pH will critically affect the degree of charging of the different compounds, so there will be an optimum pH where the maximum difference in charge is obtained. Generally separation of small molecules occurs at the pH where there is a maximum difference in the degree of dissociation between different molecules. This pH is the average pK value for all molecules in the sample. On the other hand, chiral separations will be dependent on either difference in the stability of the inclusion complexes formed between each enantiomer and the CD and in the mobility characteristics (depending on the charge they carry) of the host-guest complexes formed [20]. Therefore, a 20 mM triethylamine/phosphoric acid buffer was prepared by mixing equal volumes of each of 40 mM triethylamine and phosphoric acid and the pH was adjusted to 2.5 using 1.0 M HCl; good separation was obtained. For further study, different buffer concentrations ranging from 5 to 50 mM were prepared by suitable dilution of 2.0 M TEA-PO_4 (1:1) with water and adjusting the pH to different values (1.5–3.5) using 1.0 M HCl. It was observed that by increasing the buffer concentration above 45 mM, the analysis time was extensively increased (total run time 65 min) with deformation of the five peaks accompanied by noisy baseline, also the current was too much elevated (≥200 μA); while upon decreasing the concentration to 5 mM, fair baseline separation was achieved. As for the buffer pH, the resolution was improved by its increase until pH 2.5 and then resolution started to drop remarkably. Upon using a buffer having pH above 2.8, no baseline separation, peak tailing, and long migration times occurred. Although the analysis time was improved by using buffer having low pH value, it was noticed that below pH 1.8, separation was negatively affected and resolution was nearly nil.

3.1.2. Study of the effect of cyclodextrin type and concentration

Several CDs were tested, from the neutral ones α-CD, β-CD and γ-CD; to the anionic ones HDAS, HSCD, and SCD. The study was performed using 10 mM TEA-PO_4 having a pH of 2.3. With neutral CDs, no separation was obtained. The resolutions were much more encouraging with the charged CDs. Using HDAS, very poor separation accompanied by tailing of peaks occurred; while by using HSCD, separation of CL from its impurities was achieved; however impurity B (the racemic mixture of isomers) did not resolve into its components. The best separation for all investigated compounds was achieved using SCD. These observations concord with the finding that charged CDs have major advantages over their neutral relatives. One of these advantages is that they act as carrier molecules and interact with the positively charged analyte via ion pairing. This allows the resolution of analytes that only weakly interact and are poorly differentiated [20].

A wide concentration range of SCD was studied, namely 1–7% (m/v). Upon using SCD of less than 3% (m/v), no separation was obtained. Although separation started to occur at 3% (m/v), it was not a baseline separation. Noticeable decrease in the analysis time was observed upon increasing the concentration of SCD higher than 6% (m/v). Nevertheless, too high and unstable current of ≥200 μA was induced.

3.1.3. Study of the effect of applied voltage and polarity mode

The effect of voltage was studied over a range of −8 to −15 kV. Its increase above −13 kV was accompanied by a decrease in migration time, but no appreciable effect on resolution was observed; however the increased current caused baseline irregularity. Decreasing the voltage until −8 kV was associated with long analysis time of more than 30 min.

3.1.4. Study of the effect of temperature

The capillary temperature was also investigated (15–30 °C). No major effects were recorded, only slight differences in migration time and in current were observed without any effect on resolution; the baseline remained steady.

3.1.5. Choice of detection wavelength

With the aid of the photodiode-array detector, several electropherograms were run at different detection wavelengths, 195, 200 and 210 nm. The best electropherograms were obtained at 195 nm, so this wavelength was used for detection.

3.2. Optimization of experimental conditions

After the previous thorough investigation of the experimental conditions, determination of the optimum ones was performed by using a reduced central composite face-centered design (RCC) using Modde® 5.0 software. Four factors were selected for optimization,

Table 1
Factorial analysis nominal values, corresponding to (−), (0), and (+) levels of separation conditions under investigation.

Electrophoretic variables	Low value (−)	Central value (0)	High value (+)
Buffer conc., mM	5	25	45
Buffer pH	1.8	2.3	2.8
SCD%, m/v	4	5	6
Voltage, kV	−8	−10	−12

namely buffer concentration (5–45 mM), buffer pH (1.8–2.8), SCD concentration (4–6% (m/v)) and voltage (−8 to −12 kV). These factors and their ranges are summarized in Table 1. The Modde® software automatically encodes the factors as "−1, 0, +1" for mean centering and scaling. The temperature was kept at 20 °C and the capillary length of 31.2 cm (effective length 20 cm) was maintained during the whole analyses. Three responses were chosen, these were the analysis time defined as the migration time of impurity A and the resolution "Rs1" and "Rs2" of CL from the two nearest peaks, the one before and the other after the main drug peak. These peaks correspond to the racemic mixture of isomers (impurity B). The design was built to find the minimum analysis time and the maximum "Rs1" and "Rs2". Upon applying a full factorial central composite face-centered design the total number of experiments would be $2^k + 2k + n = 27$, where "k" is the number of factors and "n" is the number of central points ($n = 3$). Modde® software supports RCC for four factors with the fractional part of the design reduced from 16 to 12 runs, thus a total number of 23 experiments were performed. These experiments were carried out in duplicate and the average analysis time, "Rs1" and "Rs2" were calculated. Table 2 shows in details all conditions and results of the experiments as extracted from the software.

The R^2 and Q^2 values were calculated by the software. R^2 represents the fraction of variation explained by the model (i.e.) how well the model fits the data; a value >0.8 indicates that the model fits well with data [21]. The obtained data for responses were 0.9809, 0.9839, and 0.9954 for "Rs1", "Rs2" and analysis time, respectively. This proves the goodness of the model and the design. Meanwhile, the Q^2 values, representing the fraction of variation predicted by the model, were 0.6462, 0.7404, and 0.7971 for "Rs1", "Rs2" and analysis time, respectively; which show a high prediction ability of the model, since a value ≥0.5 indicates the validity for model prediction [21].

Table 2
Screening results for optimization of the CZE method for determination of clopidogrel and its impurities as extracted from the software.

Exp. no.	Run order	Conc.	pH	SCD	V	Rs1	Rs2	Analysis time
1	13	5	1.8	4	8	5.40	4.03	19.50
2	2	5	2.8	4	8	5.03	3.54	23.64
3	10	5	1.8	4	12	5.48	3.93	11.64
4	3	45	2.8	4	8	7.69	5.62	60.00
5	1	45	1.8	4	12	8.21	5.93	22.73
6	12	45	2.8	4	12	7.89	6.04	45.00
7	8	5	1.8	6	8	6.37	4.81	18.12
8	22	5	1.8	6	12	6.43	4.64	10.53
9	5	5	2.8	6	12	4.91	3.27	13.15
10	16	45	1.8	6	8	8.52	6.19	31.64
11	19	45	2.8	6	8	6.18	4.76	45.00
12	6	45	2.8	6	12	2.00	2.00	29.54
13	15	25	1.8	5	10	7.03	5.06	18.27
14	14	25	2.8	5	10	5.99	4.26	26.63
15	11	25	2.3	5	8	6.93	5.18	29.47
16	17	25	2.3	5	12	7.56	5.58	18.87
17	7	5	2.3	5	10	5.84	4.31	16.18
18	4	45	2.3	5	10	8.06	6.04	38.92
19	21	25	2.3	4	10	6.60	4.85	24.62
20	18	25	2.3	6	10	7.19	5.39	21.29
21	9	25	2.3	5	10	6.80	5.12	22.96
22	20	25	2.3	5	10	7.07	5.31	22.42
23	23	25	2.3	5	10	7.26	5.43	21.73

Analysis time [migration time (in min) of impurity A]; "Rs1" (resolution between clopidogrel and the first migrating isomer of impurity B); "Rs2" (resolution between clopidogrel and the second migrating isomer of impurity B; pH (buffer pH); V (voltage); Conc. (concentration of buffer in mM); SCD (percentage m/v of sulphated β-cyclodextrin).

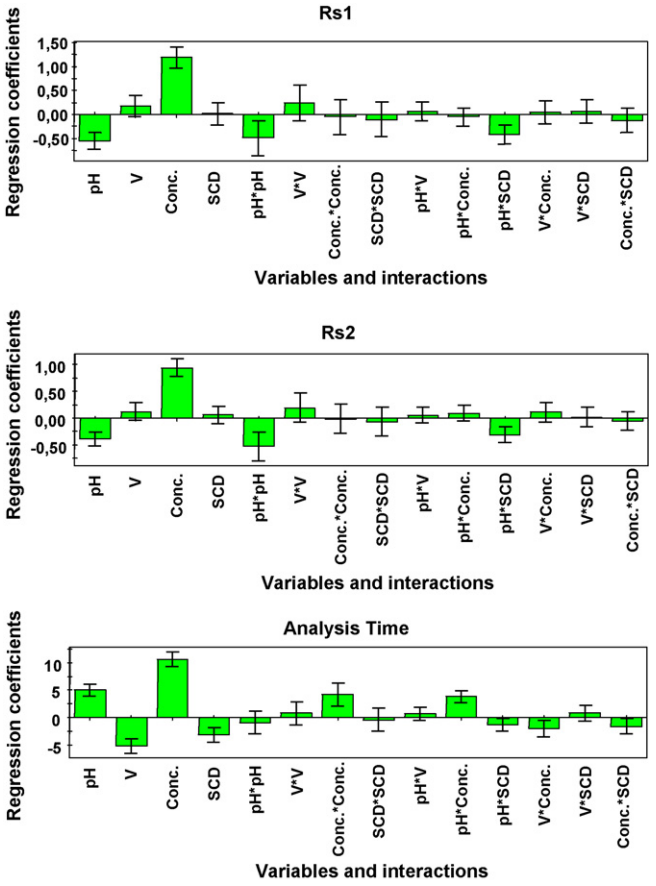

Fig. 2. Regression coefficients plots for the response variables, analysis time, resolution between clopidogrel and the first migrating isomer of impurity B ("Rs1"), and resolution between clopidogrel and the second migrating isomer of impurity B ("Rs2") (pH = buffer pH, V = voltage, Conc. = concentration of buffer in mM, SCD = percentage m/v of sulphated β-cyclodextrin).

To show the significance of different variables, the coefficients plots were displayed, in which the regression coefficients appear as bars and the confidence intervals at 95% confidence limit as error lines (Fig. 2). Bars for interaction effects and non-linear effects are also shown. The variable is considered insignificant when the error line crosses zero and greater than the regression coefficient bar. Upon examining these plots, the variables, $V \times V$ (non-linear effect), SCD × SCD, pH × V (interaction effect), $V \times$ SCD were insignificant. When the insignificant coefficients estimated by the model were deleted (such as pH × pH, $V \times V$, SCD × SCD, Conc. × Conc.), the rebuilt-model maintained the same optimum experimental conditions. The observed improvement was in the model prediction for "Rs1" and analysis time, where Q^2 turned out to be 0.7318 and 0.8570 instead of 0.6462 and 0.7910, respectively. Q^2 for "Rs2" did not show any improvement. The buffer pH was significantly effective, its increase was accompanied by decrease of resolution and increase of analysis time, however the interaction of pH with either the concentration of buffer or SCD showed that there was an optimum pH (pH 2.3) for resolution responses as observed from the

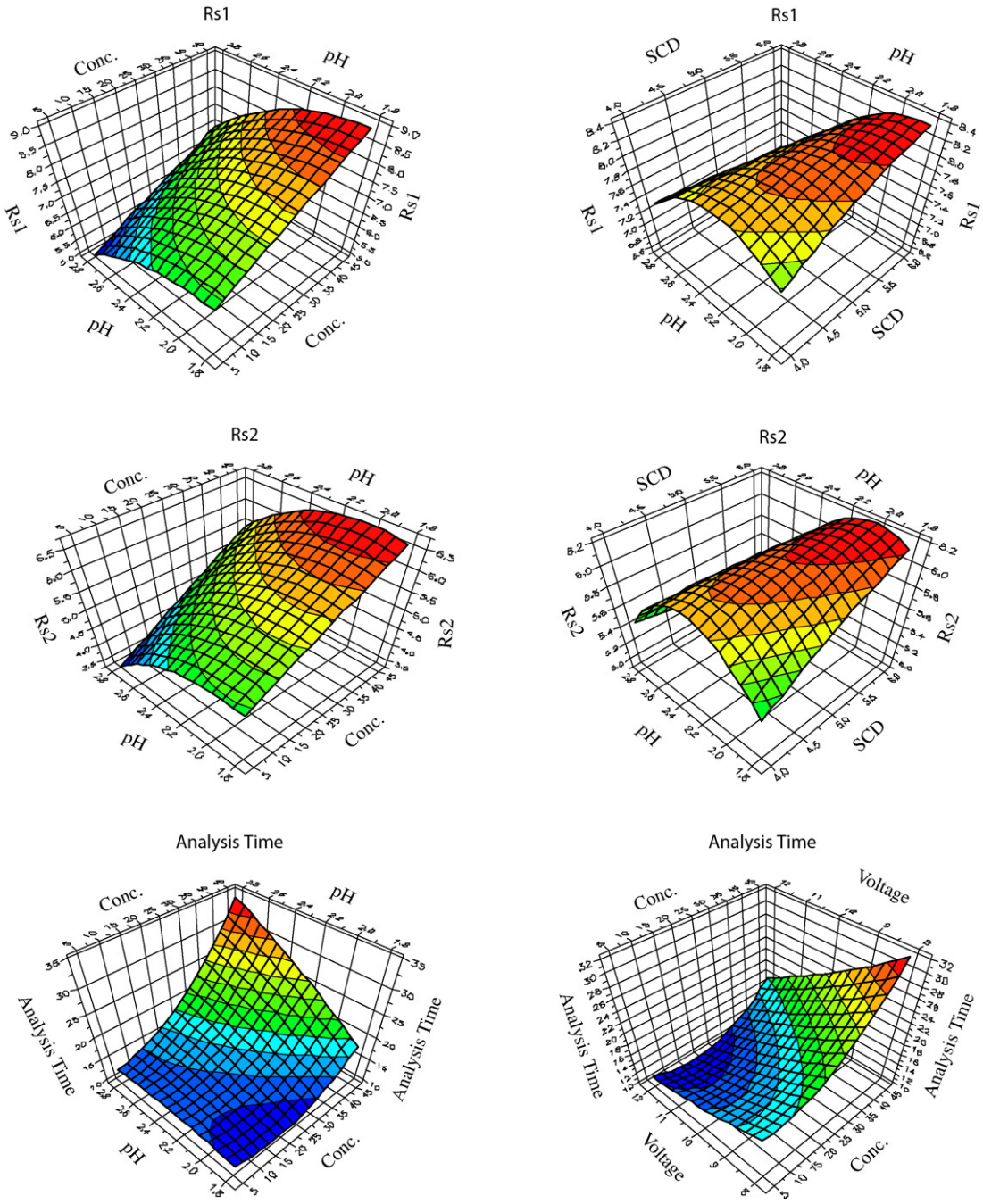

Fig. 3. Response surface plots of the response variables, analysis time, resolution between clopidogrel and the first migrating isomer of impurity B ("Rs1"), and resolution between clopidogrel and the second migrating isomer impurity B ("Rs2") as a function of the significant parameters (pH = buffer pH, Voltage, Conc. = concentration of buffer in mM, SCD = percentage m/v of sulphated β-cyclodextrin).

curvature in their surface plots (Fig. 3). The analysis time decreased with decrease of buffer pH, concentration, and with increase of voltage and SCD concentration.

The optimum conditions predicted by the design were 35 mM TEA-PO_4 buffer, pH 1.8, 6% SCD (m/v), and −12 kV, and the electropherogram obtained is shown in Fig. 4a. "Rs1", "Rs2" and analysis time were determined experimentally and found to be 7.4 ± 0.08, 5.2 ± 0.08 and 17.4 ± 0.33 min ($n = 10$), respectively. These values were comparable with the corresponding ones predicted by the model and within its validity. However, upon applying the above-mentioned conditions, a noisy baseline was obtained, due to the produced high current (172 μA). This current resulted from high buffer concentration, high voltage and low pH values. The LOQ of CL at S/N = 10 was found to be 1.25 μg/ml which in percentage relative to the nominal concentration (250.0 μg/ml) is 0.5%. Trials to enhance the sensitivity of the method were performed by raising the loading amount of CL, either by increasing the injection time or the pressure of injection, or even by dissolving the sample in BGE. These trials were unsuccessful, peak distortion and splitting were observed which may be due to reverse stacking effect. Moreover, trials to decrease the migration time of the hydrolysis product (impurity A) were also performed by applying voltage gradient after the appearance of the second isomer peak of impurity B, but it did not reach the required target.

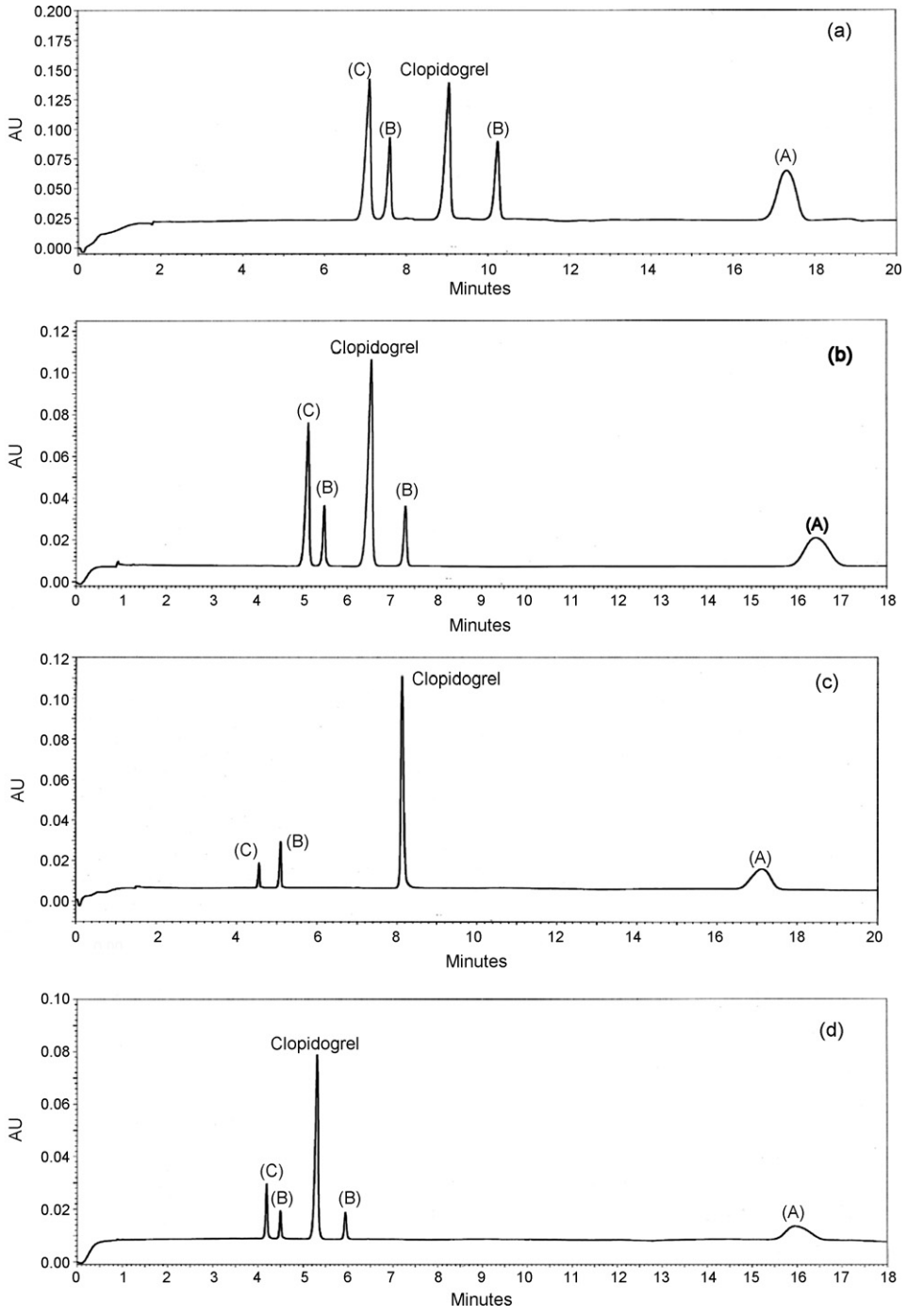

Fig. 4. Separation of clopidogrel from (A) impurity A (hydrolysis product), (B) impurity B (racemic mixture of isomers), (C) impurity C (R-enantiomer) by using an uncoated fused-silica capillary with a total length of 31.2 cm and an effective length of 20 cm (50 μm ID); the capillary temperature maintained at 20 °C; UV detection at 195 nm; sample injection: 4.0 s × 1.5 psi; an applied voltage of −12.0 kV; and a BGE containing the following: (a) 6% (m/v) SCD in 35 mM TEA-PO_4 buffer at pH 1.8 (I = 172 μA). (b) 5% (m/v) SCD in 10 mM TEA-PO_4 buffer at pH 2.3 (I = 108 μA). (c) 5% (m/v) HSCD in 10 mM TEA-PO_4 buffer at pH 2.3 (I = 118 μA). (d) 5% (m/v) SCD in 10 mM sodium phosphate buffer at pH 2.3 (I = 105 μA).

Therefore, another method was tried, where lower buffer concentration (10 mM) and SCD concentration (5% (m/v)), using the optimum pH (pH 2.3) and keeping the voltage (−12 kV) as given by the model, were chosen. These conditions gave good separation, lower current and much better baseline stability (Fig. 4b). In order to predict "Rs1", "Rs2", analysis time and current, these conditions were fed into the software after including the current as a fourth factor and rebuilding the model. The obtained results showed that the first three responses ("Rs1", "Rs2" and analysis time) were comparable with the intermediate method, while the fourth response (current) was reduced and predicted to be I = 93 μA (the experimental finding was I = 108 μA).

To check the method robustness, another experimental design by RCC was performed using narrower ranges for the factors around the chosen conditions. These ranges were buffer concentration (8–12 mM), pH (2.1–2.5), voltage (−11 to −13 kV) and SCD concentration (4–6% (m/v)). Upon examining the results of the model, no significant effects (except for the voltage on the analysis time)

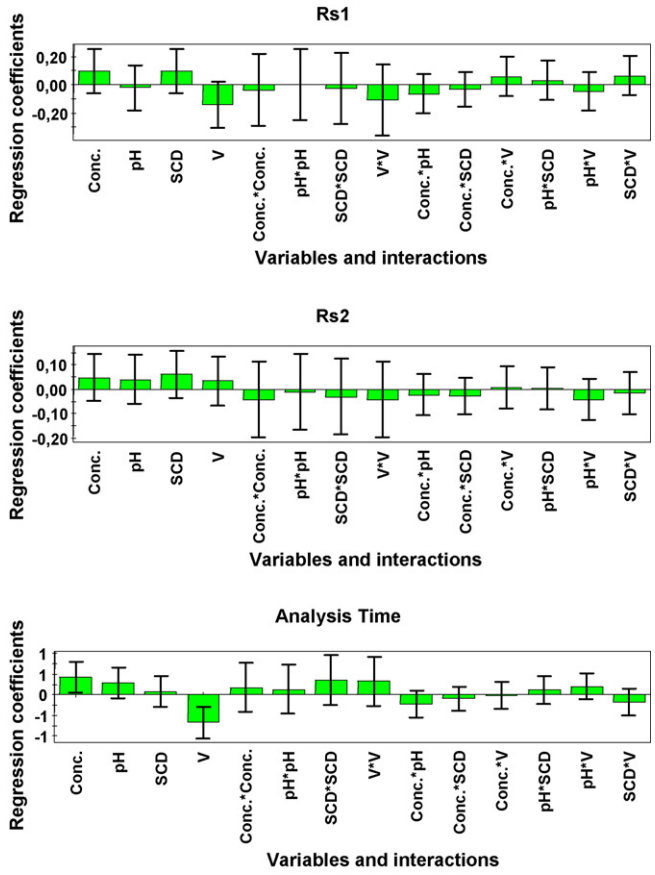

Fig. 5. Regression coefficients plots for the response variables, analysis time, resolution between clopidogrel and the first migrating isomer of impurity B ("Rs1"), and resolution between clopidogrel and the second migrating isomer of impurity B ("Rs2") (pH = buffer pH, V = voltage, Conc. = concentration of buffer in mM, SCD = percentage m/v of sulphated β-cyclodextrin).

were observed in the outcomes regarding resolutions and analysis time, as demonstrated by the coefficients plots (Fig. 5). This reveals the robustness of the method. The adapted method shows better sensitivity with slight improvement in the resolutions and analysis time. "Rs1", "Rs2" and analysis time were 9.1 ± 0.16, 6.2 ± 0.09 and 16.4 ± 0.11 min, respectively.

In preliminary studies, it was noticed that HSCD could separate CL from its impurities. To compare the separation obtained upon using SCD or HSCD, an experiment was carried out using HSCD instead of SCD (Fig. 4c), and good separation from the main peak was observed but only one peak was visible for the two isomers of the racemic mixture (impurity B).

Also, replacing TEA-PO_4 buffer by sodium phosphate buffer of the same concentration and pH 2.3 (adjusted with 100 mM phosphoric acid) did not alter the separation, and it can be used instead, which gives a good opportunity for cheaper and easier procedures (Fig. 4d). However, the resolution around the main peak was less good.

3.3. Method validation

The regression equations and all validation parameters are shown in Table 3.

3.3.1. Linearity and range

To ensure the ability of the proposed method to obtain test results which are directly proportional to the concentration of the analyte, a linear correlation was obtained between the corrected peak area (area/migration-time ratio) and concentration of CL and its impurities. The range for CL was 1.25–250.0 μg/ml (r = 0.9998) and 0.40–300.0 μg/ml (r = 0.9997) for the intermediate and final methods, respectively. The standard error of the predicted y-value $S_{y,x}$ = 1442 and 1708 for the intermediate and final methods, respectively; while $S_{y,x}$ = 376, 151 and 412 for impurities A, B and C, respectively. It can be noted that the CL sample load in the final method could be raised to 300.0 μg/ml solution (Table 3).

3.3.2. Accuracy

The accuracy of the method was tested by analyzing freshly prepared solutions of CL in triplicate at concentrations of 1.50, 50.0

Table 3
Summary of validation results.

Peak identity	Clopidogrel		Impurity A	Impurity B	Impurity C
	Intermediate method	Final method		Final method	
Linearity					
Range (μg/ml)	1.25–250	0.40–300	1.00–2.50	0.50–2.00	0.25–2.00
Correlation coefficient (r)	0.9998	0.9997	0.9984	0.9971	0.9966
Intercept (a)	−87	−103	−41	32	−51
Slope (b)	39,927	47,643	5009	1446	4244
$S_{y,x}$[a]	1442	1708	376	151	412
Accuracy (%Recovery ± R.S.D.)[b]	101.03 ± 1.95	99.45 ± 1.43	–	–	–
Repeatability					
Corrected peak area (%R.S.D.)[c]	1.9 1.7	1.3 1.6	–	–	–
Migration time (%R.S.D.)[c]	1.7 1.8	0.7 0.8	–	–	–
Sensitivity					
LOQ (μg/ml)	1.25 (10.2)[d]	0.40 (5.7)[d]	1.00 (7.1)[d]	0.50 (6.5)[e]	0.25 (8.3)[e]
LOD (μg/ml)	0.50	0.13	0.33	0.16	0.08

Intermediate method BGE: 35 mM TEA-PO_4, pH 1.8, SCD = 6% (m/v), and −12 kV. Final method BGE: 10 mM TEA-PO_4, pH 2.3, SCD = 5% (m/v), and −12 kV. Regression equation for all methods: $y = bx + a$; where "y" = the corrected peak area and "x" = concentration in μg/ml.

[a] The standard error of the predicted y-value.

[b] n = 9.

[c] n = 6.

[d] The values in parentheses represent %R.S.D. values (n = 6).

[e] n = 3.

Table 4
Composition of commercial bulk samples of clopidogrel.

Components	Content, %	
	Sample 1	Sample 2
Clopidogrel	98.09	96.86
Hydrolysis product (impurity A)	<LOQ	0.36
Racemic mixture of isomers (impurity B)	0.17	0.20
R-enantiomer (impurity C)	0.72	1.83
Unknown 1	0.19	0.28
Unknown 2	0.32	0.40

The above values were calculated on the basis of each of the respective regression equations for clopidogrel and the impurities and expressed in % (m/m) (percentage mass per mass). Sample 1: Hetero Drugs Ltd. (Hyderabad, India). Sample 2: Veritech Pharmaceuticals Pvt. Ltd. (Mumbai, India).

and 150.0 μg/ml for the intermediate method and of 5.0, 100.0 and 200.0 μg/ml for the final method. The %Recovery and R.S.D. were calculated and revealed good accuracy (Table 3).

3.3.3. Precision

The intra-assay precision was evaluated in terms of the relative standard deviation percentage (%R.S.D.) of the corrected peak areas and the migration times for CL using the proposed intermediate and final methods. Tests for area and migration time precisions, including repeatability of the corrected areas and repeatability of migration time, are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures.

Freshly prepared solutions of CL of two different concentrations each for the intermediate and final methods were injected. The %R.S.D. values for the corrected peak areas were found to be 1.9 and 1.7 for the intermediate method, while they were 1.3 and 1.6 for the final method ($n = 6$). On the other hand, for the migration times, the %R.S.D. were 1.7 and 1.8 for the intermediate method, and 0.7 and 0.8 for the final method ($n = 6$). Results are shown in Table 3.

3.3.4. Robustness

Due to the importance of robustness in analytical method development, it was thoroughly investigated to measure the capacity of the proposed method to remain unaffected by small but deliberate variations in its parameters and provides an indication of its reliability during normal usage. Therefore the experimental designs described in Section 3.2 were used to evaluate the response surface plot constructed by plotting the responses individually as a function of the most important variables.

Trials to use SpectraPHORESIS 1000 CE as an alternative to Beckman Coulter under the same optimum experimental conditions were unsuccessful; this may be due to the fact that it was impossible to work with a system in which CDs are present only at one side of the capillary. This finding has also been mentioned before [18].

3.3.5. Sensitivity

Limits of detection (LOD, determined with a S/N ratio of 3) and limits of quantitation (LOQ, determined with a S/N ratio of 10) of CL and its related impurities are presented in Table 3.

Finally, the proposed final method was used for the analysis of two commercial bulk samples. The results are shown in Table 4. Two unknown peaks were detected.

4. Conclusion

In this work a selective, cheap, robust CZE method was developed for determination of clopidogrel in the presence of its three stated impurities. Optimization and robustness studies were performed by experimental design. The separation was completed within 17 min. The obtained results prove the selectivity, repeatability, linearity and sensitivity of the method. It was used for screening and determination of clopidogrel and its impurities in two commercial bulk samples, which shows the applicability of the method for quality control.

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