Talanta 83 (2011) 1037–1049

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

The use of experimental design in the development of an HPLC–ECD method for the analysis of captopril

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ARTICLE INFO

Article history:
Received 6 October 2010
Received in revised form
10 November 2010
Accepted 10 November 2010
Available online 18 November 2010

Keywords:
High performance liquid chromatography–electrochemical detection (HPLC–ECD)
Central composite design (CCD)
Oxidative-screen
Captopril

ABSTRACT

An accurate, sensitive and specific high performance liquid chromatography–electrochemical detection (HPLC–ECD) method that was developed and validated for captopril (CPT) is presented. Separation was achieved using a Phenomenex® Luna 5 μm (C_{18}) column and a mobile phase comprised of phosphate buffer (adjusted to pH 3.0): acetonitrile in a ratio of 70:30 (v/v). Detection was accomplished using a full scan multi channel ESA Coulometric detector in the "oxidative-screen" mode with the upstream electrode (E_1) set at +600 mV and the downstream (analytical) electrode (E_2) set at +950 mV, while the potential of the guard cell was maintained at +1050 mV. The detector gain was set at 300. Experimental design using central composite design (CCD) was used to facilitate method development. Mobile phase pH, molarity and concentration of acetonitrile (ACN) were considered the critical factors to be studied to establish the retention time of CPT and cyclizine (CYC) that was used as the internal standard. Twenty experiments including centre points were undertaken and a quadratic model was derived for the retention time for CPT using the experimental data. The method was validated for linearity, accuracy, precision, limits of quantitation and detection, as per the ICH guidelines. The system was found to produce sharp and well-resolved peaks for CPT and CYC with retention times of 3.08 and 7.56 min, respectively. Linear regression analysis for the calibration curve showed a good linear relationship with a regression coefficient of 0.978 in the concentration range of 2–70 μg/mL. The linear regression equation was $y = 0.0131x + 0.0275$. The limits of detection (LOQ) and quantitation (LOD) were found to be 2.27 and 0.6 μg/mL, respectively. The method was used to analyze CPT in tablets. The wide range for linearity, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is better for the quantification of CPT than the pharmacopoeial methods.

1. Introduction

Captopril (CPT) is an orally active antihypertensive agent [1] and has been widely used for the treatment of hypertension and congestive heart failure and it acts as a potent and specific inhibitor of angiotensin converting enzyme (ACE) [2–4]. CPT is designated chemically as 1-[3-mercapto-2-(*S*)-methyl-1-oxopropyl]-*S* (L) proline [5,6] with an empirical formula of $C_9H_{15}NO_3S$ and molecular weight of 217.3 (Fig. 1) [6]. Despite the fact that CPT has two stereogenic centres, the molecule was developed and is marketed as a single enantiomer, as only one of the four possible isomers can bind with the active site of ACE [6]. It has been reported that the biological activity resides mainly in *S*-captopril while *R*-captopril possesses non-ACE inhibiting activity [7]. CPT has a pKa in the range of 2.5–3.5 and is ionized at physiological pH. The pKa and ionization of the secondary amine present in the dicarboxylate chain depends on the adjacent functional group and whether it is in the product or active form [8].

Several methods have been reported for the determination of CPT in a variety of matrices, including high performance liquid chromatography (HPLC) [9–13], capillary zone electrophoresis (CZE) [14], gas chromatography (GC) [15,16] and gas chromatography–mass spectroscopy (GC–MS) [17]. The use of electrochemical detection (ECD) for the analysis of CPT using sequential injection analysis [18] and in rat serum, liver and kidney samples [19] has also been reported.

CPT exhibits low UV absorptivity and is a relatively unstable molecule making the assay of dosage forms of CPT very difficult [20]. As a result, a pre- or post-column derivatization procedure is normally required to ensure accurate analysis of CPT resulting in an increased cost and complexity for the quantitation of CPT. However, some derivatives are either not stable [21] or their accurate measurement is hampered by the presence of excess reagent and products from side reactions that may cause inaccuracies in quantitation [22,23].

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doi:10.1016/j.talanta.2010.11.025

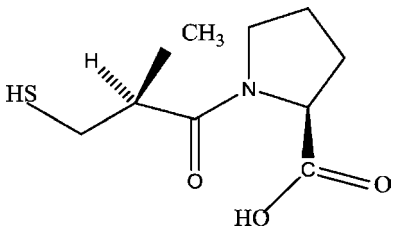

Fig. 1. Structure of CPT.

In this manuscript we report the use of HPLC with a coulometric ECD system for the quantitation of CPT. The thiol functional group of CPT can undergo electrochemical oxidation at the surface of various electrodes [24,25,18,19] and therefore coulometric detection was selected for use in these studies.

Hydrodynamic voltammetric (HDV) studies were used to determine the optimum working electrode potentials. CPT undergoes oxidation to form the dimer, captopril disulfide [26,27]. Amide hydrolysis has also been reported in aqueous solution [27] and it has been shown that oxidation of CPT is predominant in the pH range 2–5.6 and becomes an increasingly important consideration as the pH increases. Hence, a buffer pH of 3.0 was selected as the starting pH for these studies. The proposed method is a highly sensitive, simple, rapid and accurate with low detection limits for the simultaneous determination of CPT in tablets.

A response surface methodology (RSM) approach was used to identify the optimum conditions for analysis during method development. The iterative procedure used in these studies included performing experiments in the region of the best known solution, fitting a response model to the experimental data and then optimizing the estimated response model. The conventional practice of modification of a single factor at a time may result in poor optimization as other factors are maintained at constant levels that do not depict the combined effect of all the factors involved in a separation. This approach is also time consuming and requires a vast number of experiments to establish optimum levels. These limitations can be eliminated by collectively optimizing all parameters using RSM. Furthermore RSM was used to evaluate the relative significance of several other factors in the presence of complex interactions. Compared with the traditional optimization method, RSM has distinct advantages such as the use of minimum number of experiments, shorter time of operation and feasibility of generating data that may be analyzed statistically to provide valuable information on the interactions among experimental parameters. These designs are rotatable (or near rotatable) and require three levels for each factor. Diagrams of global optimum, which are more direct, were made [28].

The central composite design (CCD) statistical method approach has been applied to optimize HPLC experimental conditions, such as the resolution and time of analysis for pramipexole in tablets [29] and for the determination and optimization of voriconazole in pharmaceutical formulations [30]. CCD was used to optimize a liquid chromatographic method for the separation of six compounds [31] and for the separation of the components of a cough-syrup [32]. Moreover CCD produces response surface plots that can be evaluated to establish a desirable response which describes the experimental response for factor value variation thereby indicating the combination of factor values for an optimum response. The liquid chromatographic separation of fosinopril sodium and its degradation product, fosinoprilat was optimized using a CCD [33]. A capillary zone electrophoresis method was developed using CCD to separate trandolapril and verapamil [34]. This calculation is accomplished by use of polynomial regression undertaken according to Eq. (1):

$$Y = (\beta_0 + \varepsilon) + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i 2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (1)$$

where Y = the experimental response to be optimized, β_0 = a constant term; β_1, β_2 and β_3 = coefficients of the linear terms, β_{11}, β_{22} and β_{33} are coefficients of the quadratic terms and β_{12}, β_{13} and β_{23} are coefficients of the interaction between the factors under investigation [35].

The objective of this study was to develop an HPLC–ECD analytical method for CPT that is simple, sensitive, precise, accurate and rapid that could be applied to the quality control of CPT formulations. The method was validated according to Food and Drug Administration (FDA) [36] and International Conference on Harmonisation (ICH) [37] guidelines.

2. Experimental

2.1. Instrumentation

The modular HPLC system consisted of a Waters Model M 6000A dual piston constant flow pump (Waters Associates, Milford, MA, USA), an automated Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA, USA), a Model 5100A Coulochem dual electrode electrochemical detector with a Model 5010 analytical cell (Environmental Sciences Associates, Bedford, MA, USA) operated in the "oxidative-screen" mode. The analytical cell was preceded by a carbon filter and a Spectra-Physics Integrator Model SP4290 (San Jose, California, USA) with attenuation set at 128 was used to capture data. The mobile phase was constantly degassed using an in-line degasser Model ERC- 3000 (Erma Optical Works, Tokyo, Japan). The system included a Model 5020 guard cell (Environmental Sciences Associates) also preceded by a carbon filter (Anatech Instruments, Johannesburg, SA). The analytical column was a Phenomenex® Luna 15 cm × 4.1 mm i.d. stainless-steel, packed with 5 μm (C_{18}) material (Phenomenex®, Torrance, CA, USA). This column was preceded by an Uptight Precolumn Kit (Upchurch Scientific, Oak Harbor, WA, USA) packed with glass beads. Both the guard and analytical columns were maintained at 22 °C using a Model LC-22 temperature controller (Bioanalytical Systems, West Lafayette, IN, USA).

2.2. Chemicals and reagents

All reagents were HPLC grade. CPT was donated by Protea Chemicals (Midrand, South Africa) and the internal standard (IS), cyclizine (CYC) was donated by Aspen Pharmacare (Port Elizabeth, South Africa). As there are no solvents designed specifically for ECD use HPLC far UV grade acetonitrile (ACN) was purchased from Microsep (Port Elizabeth, South Africa). A Milli-Q Academic A10 water purification system (Millipore, Bedford, MA, USA) that consisted of an Ion–ion®-exchange cartridge and a Quantum EX-Ultrapore Organex cartridge, which was fitted with a 0.22 μm Millipak® 40 sterile filters (Millipore®) prior to use was used in-house to further purify water that was used for the preparation of buffers. Potassium hydrogen phosphate, *o*-phosphoric acid (85%) and Sodium hydroxide pellets (analytical grade) were purchased from Merck Laboratories (Merck, Wadeville, South Africa).

2.3. Preparation of stock solutions

Standard stock solutions of CPT (100 μg/mL) and 500 μg/mL IS were prepared by accurately weighing approximately 10 mg and 50 mg of CPT and CYC, respectively and then dissolving in 100 mL mobile phase. The stock solutions were sonicated for 5 min using

Table 1
Presentation of 20 experiments (Exp 1–20) with coded values for factor levels for the CCD.

Experiment(run)	Standard	Type	Mobile phase pH	Mobile phase molarity	Organic solvent concentration
	Run number		X_1	X_2	X_3
1	8	Fact	−1	−1	−1
2	9	Fact	+1	−1	−1
3	10	Fact	−1	+1	−1
4	4	Fact	+1	+1	−1
5	6	Fact	−1	−1	+1
6	1	Fact	+1	−1	+1
7	3	Fact	−1	+1	+1
8	11	Fact	+1	+1	+1
9	12	Centre	0	0	0
10	5	Centre	0	0	0
11	2	Centre	0	0	0
12	7	Centre	0	0	0
13	16	Axial	−1.682	0	0
14	15	Axial	1.682	0	0
15	13	Axial	0	−1.682	0
16	19	Axial	0	1.682	0
17	14	Axial	0	0	−1.682
18	20	Axial	0	0	1.682
19	17	Centre	0	0	0
20	18	Centre	0	0	0

a Branson B12 sonicator (Shelton, CN, USA) to aid dissolution. The stock solution was serially diluted with mobile phase to produce CPT solutions of concentration, 2, 3, 5, 10, 20, 30, 50 and 70 μg/mL. All working standards were prepared with the internal standard, CYC at a concentration of 20 μg/mL. The solutions were stable for one day when stored at room temperature (20–25 °C). The stock and standard solutions were prepared on a daily basis and stored in the dark at about 10 °C. When required, samples were protected from light using aluminium foil. All solutions were used on the day they were prepared.

2.4. Experimental design for HPLC separation optimization

Twenty experiments were conducted using the conditions described in Table 1 and the levels described in Table 2. Minimum and maximum contents of buffer pH (x_1) were fixed as 2.7 and 3.3, respectively. Likewise, minimum and maximum values for buffer molarity (x_2) were selected as 25 and 75, respectively. Organic solvent composition (x_3) was kept between 25 and 35 and the retention time for the last eluting peak was the response for these studies.

To our knowledge, not only that this is the most rapid method but also there are no reported papers based on optimization and development of ECD procedure using CPT.

The CCD approach combines a fractional factorial with incomplete block design methodology to avoid extreme vertices and to present an approximately rotatable design with three levels per factor.

The factors and ranges selected for consideration were based on previous univariate studies and chromatographic intuition. The composition of the mobile phase is defined as the volume of ACN with respect to total volume of solution for that mobile phase.

Table 2
Experimental factors and levels used in the CDC.

Factor	Level (−)	Level (0)	Level (+1)
Independent			
Buffer pH	2.7	3.0	3.3
Buffer molarity (mM)	25	50	75
Organic solvent concentration (v/v)	25	30	35
Dependent			
Y_1 = Retention time			
Y_2 = Peak symmetry			
Y_3 = Peak resolution			

The data generated were analyzed using Design Expert (Version 7.0.1, Stat-Ease Inc., Minneapolis, MN, USA) statistical software. The significance of the relevant factors was calculated using Fisher's statistical test for Analysis of Variance (ANOVA) models that were estimated and run to their first order interaction terms. ANOVA for linear regression partitions the total variation of a sample into components. These components were then used to compute an *F*-ratio that evaluates the effectiveness of the model. If the probability associated with the *F*-ratio is low, the model is considered a better statistical fit for the data. In these calculations the higher-order interaction terms were assumed not to contribute to the behaviour of the statistical model to any great extent.

2.5. Preparation of buffers

0.1M NaOH was prepared by dissolving exactly 4.0 g of sodium hydroxide pellets in a 1L volumetric flask containing HPLC grade water. Phosphate buffer (50mM) was prepared by accurately pipetting 3.4 mL of *o*-phosphoric acid (85%) into a 1L volumetric flask and making up to volume with HPLC grade water. The pH of the buffer was adjusted with 0.1 M NaOH to a pH of 3. The pH was measured with a Crison GLP 21 pH-meter (Crison Instruments, Johannesburg, South Africa) at 25 °C prior to the addition of acetonitrile.

2.6. Preparation of mobile phase

The initial mobile phase used, was comprised of 50mM phosphate buffer: acetonitrile (70:30, v/v) adjusted to pH 3.0. The mobile phase was prepared daily, degassed by sonication and filtered through a 0.45 μm Durapore® membrane HVLP filters (Millipore Corporation, Ireland) prior to use. The mobile phase was recycled throughout long term analysis.

2.7. Chromatographic conditions

The flow rate was 1.0 mL/min with a column back pressure of 120 atm. A full scan multi channel ESA Coulometric 5100A model detector was operated in the "oxidative-screen" mode with the upstream electrode (E_1) set at +600 mV and the downstream or analytical electrode (E_2) set at +950 mV, while the potential of the guard cell was set at +1050 mV. The detector gain was set at 300. The potential applied to the electrodes of the analytical and guard

cells were optimized to ensure oxidative efficiency and to reduce background noise. Under these conditions CPT and CYC eluted at approximately 3.5 min and 7.5 min, respectively.

2.8. Method validation

2.8.1. Calibration, linearity and range

The linearity of the analytical method was established by fitting calibration curve data to a least squares linear regression model using GraphPad® Prism (Version 5.01 for windows, GraphPad Software, San Diego, CA, USA) software. The CPT/CYC peak area ratio of response was plotted against concentration of CPT to generate the calibration curve. The response of six standard solutions of 2.0, 5.0, 10.0, 30.0, 50.0 and 70.0 μg/mL concentration were subject to regression analysis to establish the calibration equation and a correlation coefficient.

2.8.2. Precision

The precision of a method is usually reported as the percent relative standard deviation (% RSD) of a set of responses. Precision was represented into two categories, *viz.* repeatability (intra-day precision) and intermediate precision (inter-day precision) [38–41]. The tolerance for RSD and relative error (RE) were set at ±5% for these studies.

2.8.2.1. Repeatability or intra-day precision. Repeatability was tested by analyzing five determinations at three different concentrations, *viz.*, low, medium and high within the linearity range [41,42].

2.8.2.2. Intermediate or inter-day precision. The inter-day variability of this method was assessed over three days at three low, medium and high concentrations of CPT standard in replicates of six. The results are given in Table 7.

2.8.3. Accuracy and bias

A tolerance of 2% was set for % RSD for this parameter as this complies with the limits set by a number of pharmaceutical industries [41]. The bias is an indication of the influence of an analyst on the performance of a method. Accuracy and bias were assessed by repeat measurement of three samples of different concentration.

Accuracy was evaluated by injecting samples of three different concentrations equivalent to 80%, 100% and 120% of the intended content of active ingredient, following addition of a known amount of CPT to the sample and calculating the % recovery and RSD for each concentration.

2.8.4. Limits of quantitation and detection

Recent articles have included much discussion regarding the determination of the limits of quantitation (LOQ) and detection (LOD) values for an HPLC method [41–45]. The LOQ is also defined as the lowest amount of analyte in a sample that can be quantitatively determined with precision and accuracy under the stated experimental conditions [43,46] and the LOD is the lowest amount of an analyte in a sample that can be detected but not quantitated as an exact value [40,46].

2.8.5. Specificity

The specificity of the method was assessed by comparing chromatograms developed from the analysis of a standard solution of CPT only with that from a sample produced by dissolving commercially available tablets of CPT in mobile phase buffer. The peaks observed in the chromatograms (Fig. 3) were well resolved from the solvent front and there were no apparent peaks that interfered with that for CPT. Therefore, the method was considered specific.

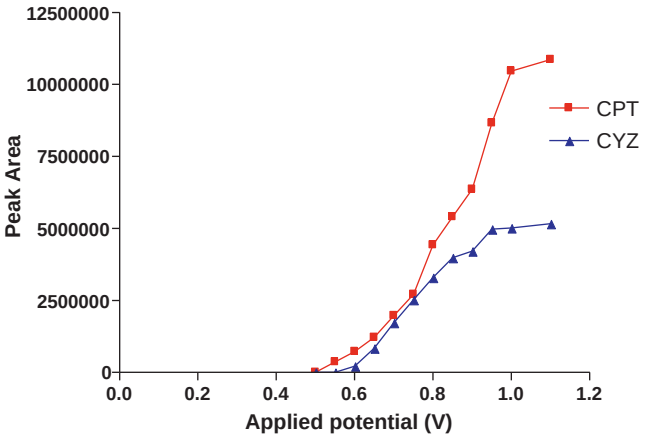

Fig. 2. Hydrodynamic voltammogram of CPT and CYC recorded in the potential range 0.0–1.2 V.

2.9. Assay

In order to establish the applicability of the method for the analysis of CPT in dosage forms, five commercially available pharmaceutical products were purchased from a local pharmacy and subjected to analysis. Twenty tablets were weighed and ground after which an accurately weighed amount of powder equivalent to 50 mg of CPT was extracted using 100 mL of mobile phase by sonication for 10 min to ensure complete dissolution. The solution was filtered into a 100 mL volumetric flask using a Millipore Millex-HV Hydrophilic PVDF 0.45 μm filter. The extract was filtered and made up to volume and mixed well.

The products that were tested were:

i. CaptoHexal® 50
ii. Merck-Captopril
iii. Zapto®-50
iv. Sandoz Captopril 50
v. Adco-Captomax 50

3. Results and discussion

The HDV for the oxidation of CPT and CYC is shown in Fig. 2. The limiting current plateau is reached at potentials >+1.1 V for CPT and at potentials >+1.0 V for CYC. Therefore, a potential of +0.9 V was selected for the detection of CPT. As can be seen, the response for CPT was sigmoid, and can be mathematically explained as a logistic function voltammogram. The porous graphite electrodes exhibited low residual current and noise. The background current was found to be <14 nA for the electrode settings selected for use.

The chosen chromatographic conditions revealed a good separation for CPT (50 μg/mL) and the internal standard, CYC (20 μg/mL) and no decomposition of CPT was observed during analysis. A typical chromatogram of the separation using a standard solution of CPT and CYZ is shown in Fig. 3.

The capacity factor calculated for this separation was within accepted values of >2 for the first peak and <1 for the second peak. The tailing factor was within the limits established in the FDA [47] guidelines. The resolution between the two peaks of interest was more than adequate for this method.

Two-dimensional contour plots are presented in Figs. 4–6 and are very useful for studying the interaction effects of the factors on the responses. The retention time for CPT decreases as the ACN (v/v) % increases (Fig. 4), when the molarity and pH of the buffer are constant. An increase in buffer molarity at constant pH and constant ACN (v/v) % results in decrease in the retention time of CPT (Fig. 5),

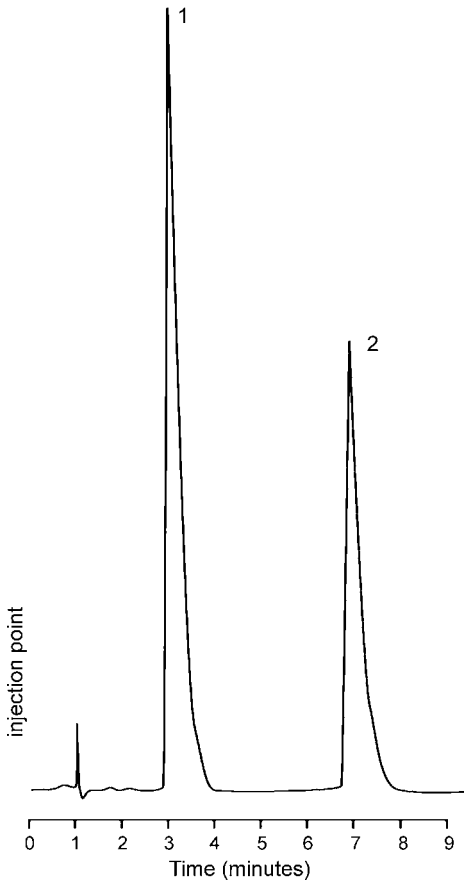

Fig. 3. Typical HPLC chromatogram of CPT (1) (50 μg/mL) in the presence of the I.S. CYC (2) (20 μg/mL).

most likely due to the increasing competition of buffer cations for silanol sites which are preferentially attached to the column. This effect is prominent when the buffer molarity is greater than 50 mM.

Silica-based analytical columns show optimum stability and performance at pH values above 2.0 [48,49]. The effect of mobile phase pH on the retention time of CPT was therefore investigated in a pH range of 2.8–3.2. As can be seen from the contour plots, an increase in buffer pH (Fig. 4, constant %, v/v ACN) did not produce any change in retention time of CPT. However, the effect of buffer molarity and buffer pH on retention time showed a non-linear relationship (Fig. 5). A nearly linear relationship of buffer molarity and ACN concentration is depicted in Fig. 6. Once again the effect of % ACN is significant. An increase in buffer molarity slightly decreased the retention time of CPT as a consequence of increasing competition of buffer cations for active silanol sites on the stationary phase. Since an increase of buffer molarity resulted in shorter retention times, a buffer of 50 mM concentration was selected for use. Retention time was considered more critical in terms of sampling and analytical run time during analysis. Buffer molarity and ACN concentration were found to be significant for the regression model assumed.

The chosen model had seven main effects and nine first-order interactions. All experiments were performed in a randomized fashion in order to minimize the effects of uncontrolled factors that may introduce bias to the response. A classical second-degree model with a cubic experimental domain was postulated. The coefficients for the second-order polynomial model were estimated by least squares regression. The equation for the Y (retention time) factor is shown in Eq. (2):

$$Y_1 = 3.54 - 0.077X_1 + 0.011X_2 - 0.26X_3 - 0.061X_1X_2 - 0.061X_1X_3 + 0.054X_2X_3 - 0.019X_1^2 + 0.00704X_2^2 + 0.076X_3^2 \quad (2)$$

The solution of the quadratic model was generated by matrix calculation with Cramer's rule and Eigen value method [50] using determinants and Eigen functions, respectively to determine the optimized conditions of chromatography. The solutions that were obtained for Y were:

1. X_1 (buffer molarity) = 51 mM,
2. X_2 (buffer pH) = 2.97
3. X_3 (% ACN) = 31.01% (v/v).

The optimized chromatographic conditions were then used for all future analytical studies. The typical chromatogram shown (Fig. 3) was obtained by using the set conditions.

The model was validated by analysis of variance (ANOVA) using Design Expert software that had been used to develop the experimental plan for RSM. The test was performed and the Fisher F-ratio was calculated. This ratio was used to measure the significance of

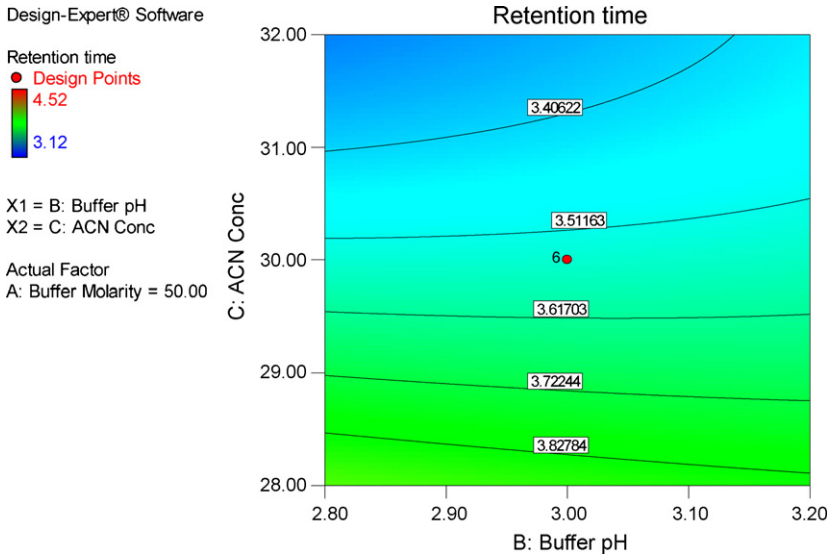

Fig. 4. Contour plot for retention time as a function of buffer pH (X-axis) and ACN concentration (Y-axis) of the mobile phase.

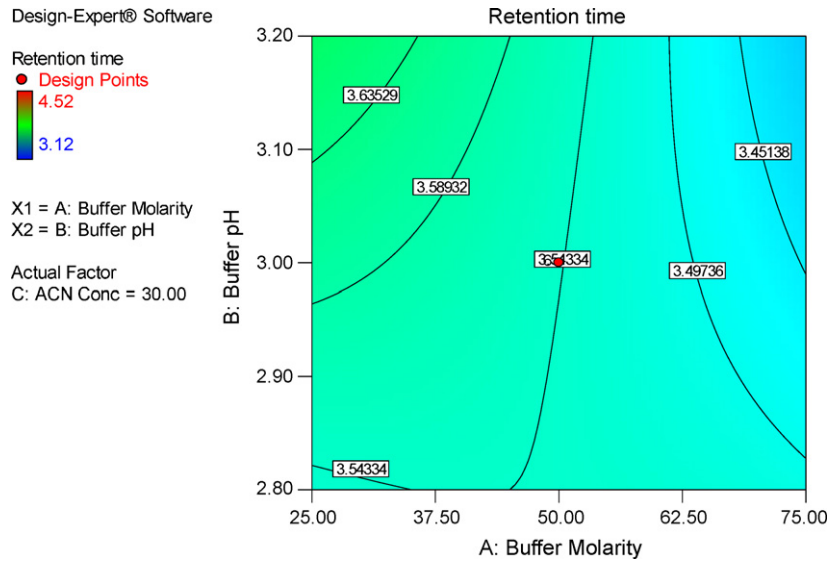

Fig. 5. Contour plot for retention time as a function of buffer molarity (*X*-axis) and buffer pH (*Y*-axis) of the mobile phase.

the model under investigation with respect to the variance of all the terms included in the error term at $p = 0.05$. In ANOVA analysis, a model that is significant is desired. The *p*-values listed in Table 3 reveal that for all responses, the cross product contribution of the model was not significant.

The Model *F*-value of 2.81 (Table 3) implies there is a 7.00% chance that a "*Model F-Value*" this large may occur due to noise. The values of "Prob > *F*" less than 0.0500 indicate that the terms in a model are significant, however in this case Prob > *F* is greater than 0.05 which indicates that the quadratic model is not significant. This means that the total contribution of the terms in the model do not have a significant effect on the response. Conversely, the effect of ACN concentration is significant. The lack of fit is not significant and this is desirable as a model that fits the data is essential for optimization studies. Since there are a number of model terms that were not significant it was necessary to perform a model reduction step in order to improve the model.

A background elimination procedure was selected as the method of choice to reduce the number of insignificant terms and the resulting ANOVA table for the reduced quadratic model is shown in Table 4. The results clearly indicate that the model is significant. To reduce a model in the presence of collinearity, backward selection is considered more robust than forward or stepwise selection. The significant model term is the concentation of ACN and this is the only variable that significantly influences the retention time of CPT ($p = 0.0016$). The lack of fit is also not significant. The R^2 value obtained is 0.5783. The predicted R^2 value is in agreement with the adjusted R^2 value. The adjusted R^2 value is particularly useful when comparing models with different numbers of terms. This comparison is however performed in the background when model reduction is undertaken. Precision was used to compare the ranges of predicted values generated at the points of the experimental design to the average prediction error. Values for the ratio >4 indicate adequate model discrimination. In this particular case the value was well above 4.

The model that has been developed can be used to predict the retention time of CPT within the limits of the experiments. The normal probability plot of the residuals and the plot of the residuals

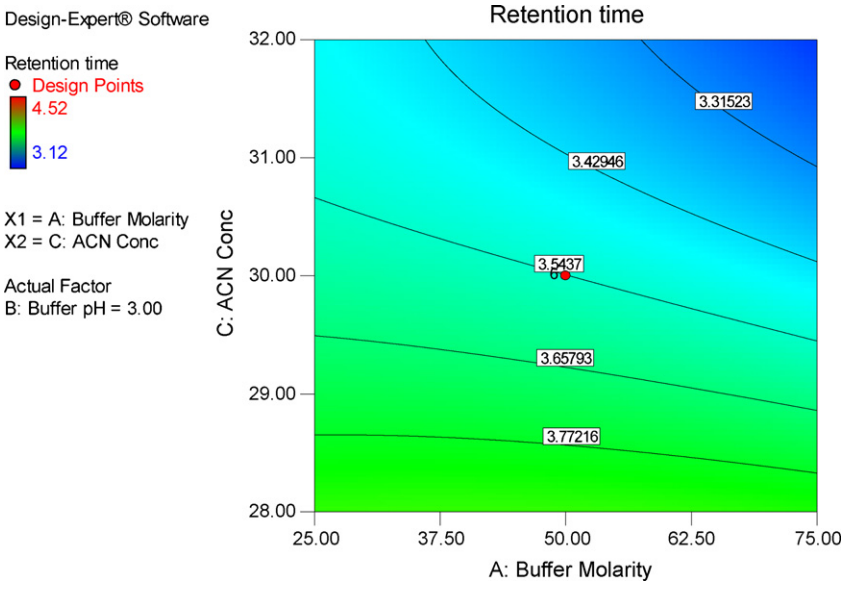

Fig. 6. Contour plot for retention time as a function of buffer molarity (*X*-axis) and ACN concentration (*Y*-axis) of the mobile phase.

Table 3
ANOVA table for response surface quadratic model for retention time.

Source	Sum of squares	df	Mean square	*F*-value	*p*-Value Prob > *F*	
Block	0.035	1	0.035			
Model	1.20	9	0.13	2.81	0.0700	Not significant
A-Buffer molarity	0.080	1	0.080	1.69	0.2257	
B-Buffer pH	1.618E-003	1	1.618E-003	0.034	0.8576	
C-ACN conc	0.94	1	0.94	19.82	0.0016	Significant
AB	0.030	1	0.030	0.63	0.4470	
AC	0.030	1	0.030	0.63	0.4470	
BC	0.023	1	0.023	0.49	0.5029	
A^2	5.459E-003	1	5.459E-003	0.12	0.7423	
B^2	7.148E-004	1	7.148E-004	0.015	0.9050	
C^2	0.083	1	0.083	1.75	0.2183	
Residual	0.43	9	0.047			
Lack of fit	0.43	5	0.085			
Pure error	0.000	4	0.000			
Cor total	1.66	19				

SD	0.22	R^2	0.7374
Mean	3.58	Adj R^2	0.4748
C.V. %	6.09	Pred R^2	−1.7108
Press	4.41	Adeq. precision	6.680

Table 4
ANOVA table for response surface model for retention time (model reduction).

Source	Sum of squares	df	Mean square	*F*-value	*p*-Value Prob > *F*	
Block	0.035	1	0.035	23.31	0.0002	
Model	0.94	1	0.94	23.31	0.0002	Significant
C-ACN conc	0.94	1	0.94			
Residual	0.69	17	0.040			
Lack of fit	0.69	13	0.053			
Pure error	0.000	4	0.000			
Cor total	1.66	19				

SD	0.20	R^2	0.5783
Mean	3.58	Adj R^2	0.5535
C.V. %	5.61	Pred R^2	0.3195
Press	1.11	Adeq. precision	11.346

versus the predicted response for both the retention time are shown in Figs. 7 and 8.

Close inspection of Fig. 7 reveals that the residuals generally fall on a straight line which indicates that the errors are normally distributed, thus supporting the fact that the model fits the data adequately. These plots are very important and are required to check the normality assumption in a fitted model. This will ensure that the model provides an adequate approximation to the opti-

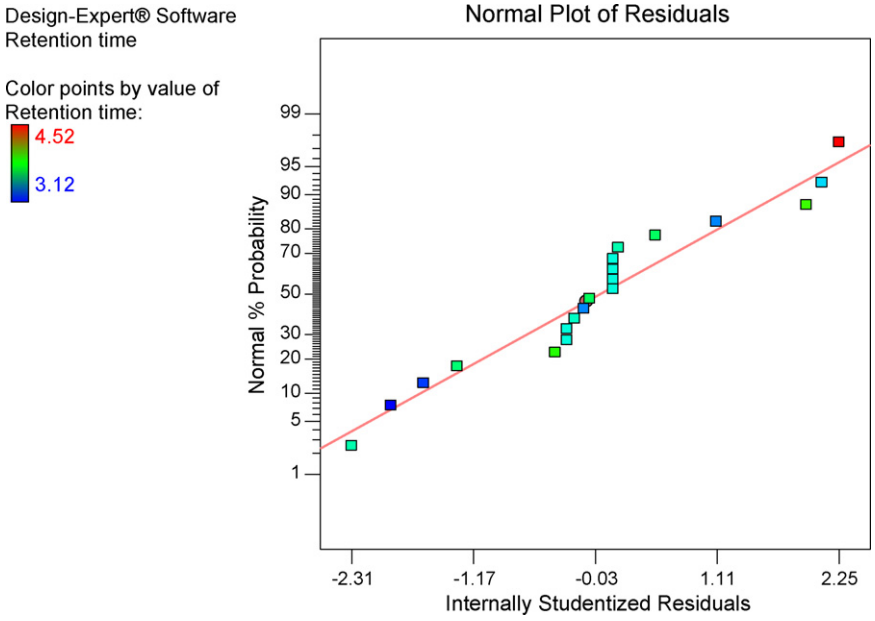

Fig. 7. Normal probability plot of residuals for retention time.

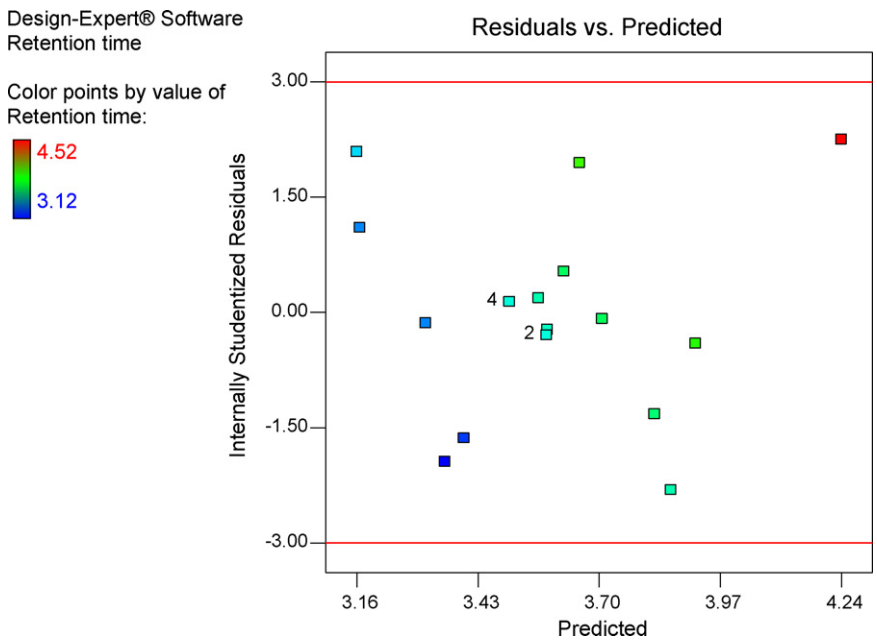

Fig. 8. Plot of residuals versus predicted response for retention time.

mization process. It is clear that there is no obvious pattern followed in the residual versus predicted response as shown in Fig. 8. The plot reveals an almost equal scatter above and below the *X*-axis, implying that the proposed model is adequate and there is no reason to suspect any violation of the independence or constant variance assumption.

To establish peak symmetry, a line was drawn through the peaks generated following analysis of samples. The line was drawn parallel to the baseline at 10% peak height. The skewness of the line was then calculated by dividing the length of the line in segments. In general, peak symmetry was improved with a decrease in pH and an increase in buffer molarity as shown in Figs. 9–11. It should be noted that the peak symmetry achieved with the optimized chromatographic conditions was 1.1 and was considered suitable for this method.

Peak resolution was significantly affected when the buffer pH was decreased and buffer molarity remained constant as depicted in Fig. 12. This result indicates that buffer pH is one of the most important parameters that can be manipulated to optimize the separation and analysis of CPT. The ionic state of CPT is influenced by pH, thus a change in pH leads to changes in migration mobility when using electrochemical detection. In the pH range investigated, the resolution of CPT was improved as the pH was increased. However since CPT degrades rapidly at pH > 3.0 further increases in pH were not considered.

The effect of buffer molarity and ACN concentration on peak resolution is depicted in Fig. 13. When using a constant concentration of ACN increasing the molarity of the buffer resulted in improved peak resolution. The concentric lines in the plot or gradient increases up to a buffer molarity of 50 mM and thereafter

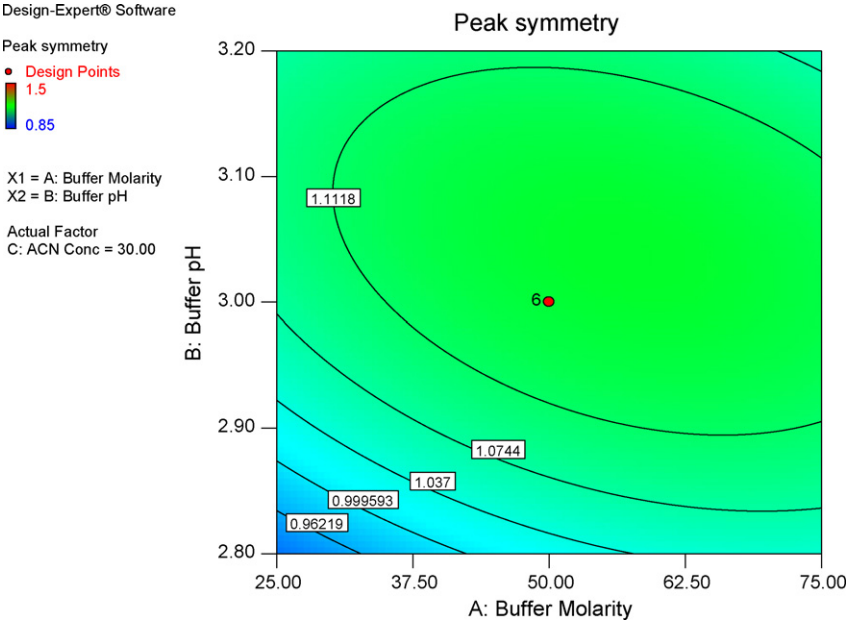

Fig. 9. Contour plot for peak symmetry as a function of buffer molarity (*X*-axis) and buffer pH (*Y*-axis).

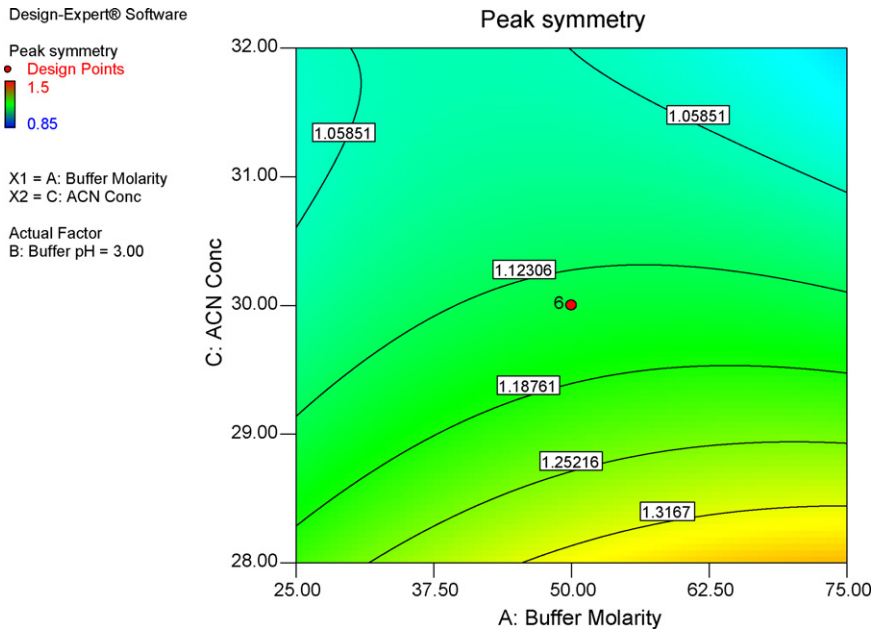

Fig. 10. Contour plot for peak symmetry as a function of buffer molarity (*X*-axis) and ACN concentration (*Y*-axis).

further increases in molarity resulted in a decrease in resolution. As shown in Fig. 14 a decrease in resolution was observed as buffers of lower pH are used in the mobile phase. This was clearly evident when mobile phases with higher ACN concentration were used. When the ACN concentration was decreased peak resolution was improved. It must be stressed that a compromise is necessary in order to develop a separation in which the peaks were adequately resolved whilst maintaining peak symmetry.

The mathematical relationship in the form of polynomial equations for the measured responses Y_2 and Y_3, are shown in Eqs. (3) and (4).

$$Y_2 = 1.14 + 0.027X_1 + 0.042X_2 - 0.14X_3 - 0.034X_1X_2 - 0.056X_1X_3 + 0.029X_2X_3 - 0.035X_1^2 - 0.078X_2^2 + 0.055X_3^2 \quad (3)$$

$$Y_3 = 1.84 - 0.0013X_1 + 0.014X_2 - 0.025X_3 - 0.069X_1X_2 - 0.019X_1X_3 + 0.044X_2X_3 - 0.12X_1^2 - 0.034X_2^2 - 0.018X_3^2 \quad (4)$$

ANOVA analysis reveals that the quadratic model was not significant ($p = 0.4373$). This suggests that the total contribution of the terms in the model did not have a significant effect on peak symmetry. Since there are a number of model terms that were not significant it was necessary to perform a model reduction step in order to obtain a linear model. A background elimination procedure was used to produce a linear model. The results following reduction revealed that the model was significant. The significant model term established was the concentration of ACN in the mobile phase and it was shown to influence the peak symmetry of CPT significantly ($p = 0.021$).

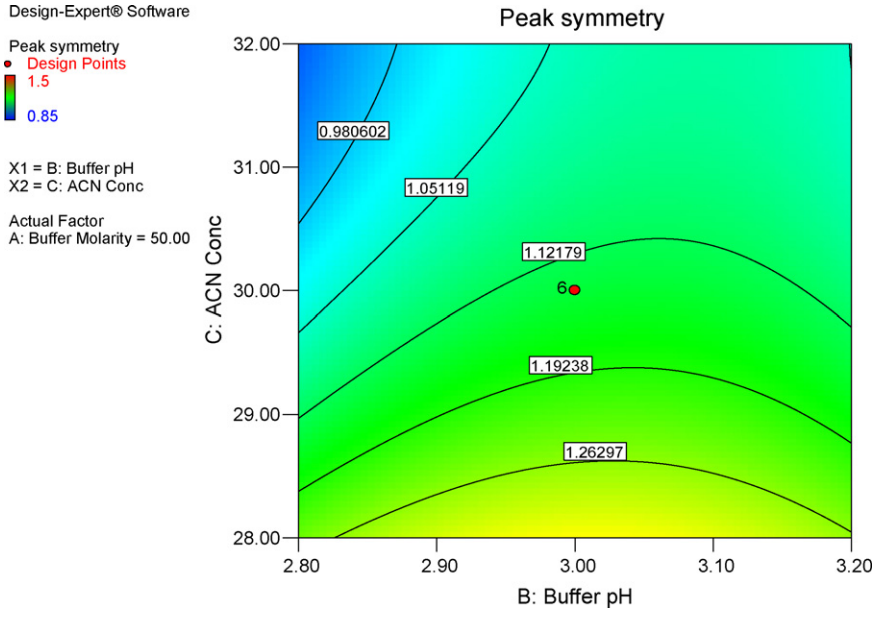

Fig. 11. Contour plot for peak symmetry as a function of buffer pH (*X*-axis) and ACN concentration (*Y*-axis).

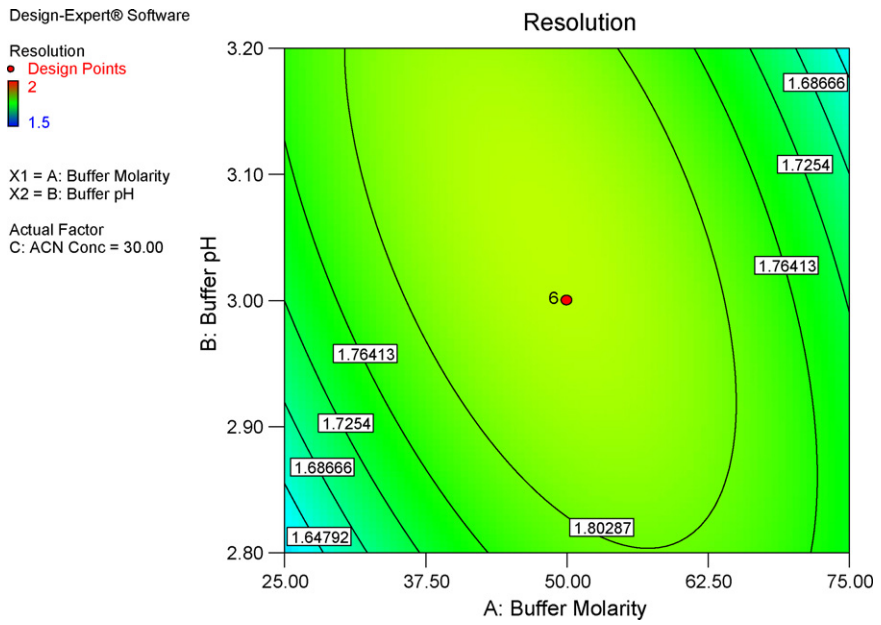

Fig. 12. Contour plot for peak resolution as a function of buffer molarity (*X*-axis) and buffer pH (*Y*-axis).

ANOVA analysis produced an R^2 value of 0.53 for Y_2 and an R^2 value of 0.64 for Y_3 ($p = 0.2048$). A model reduction step for Y_3 showed that the model was significant with the cross product of buffer molarity term being significant ($p = 0.0031$). These results are indicative of good adjustment of the models to the experimental data and indicate that these models could explain more than 50% of the response variability. The adequate precision which measures the signal-to-noise ratio was 4.2. A ratio of >4 is desirable and ensures that these models can be used to predict symmetry and peak resolution in the design space that was generated.

The contour surface plots based on the equation were generated as a function of the significant variables whilst holding the third variable constant for each response. These plots assist the prediction of different responses at any area of the experimental domain.

The contour plots show that the surface is symmetrical and peaks in the centre. The contours are concentric lines circulating about the critical points. This is not surprising as the response increases uniformly as it moves away from its minimum value. Therefore the contours should be circles around that point at which maximum occurs. In general, one looks for a minimum/maximum critical point whenever there are concentric rings for contour lines. The smallest inner circle indicates a peak. The contour lines move closer to each other as the slope of the response gets steeper. If the area has gentle gradient the contour lines are more widespread. If the area is very steep the contour lines may touch but will never cross over each other. Concentric ellipses or saddle responses indicate a region of symmetry at approximately the stationary point of for each figure.

3.1. Linearity/range

Prior to analysis of samples the analytical column was equilibrated for at least 30 min with the mobile phase. Each sample was

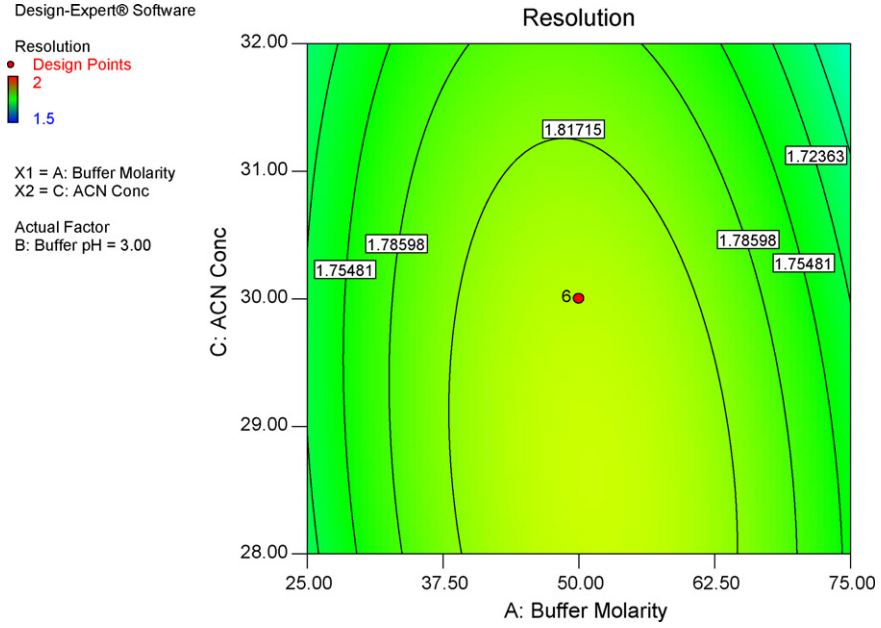

Fig. 13. Contour plot for peak resolution as a function of buffer molarity (*X*-axis) and ACN concentration (*Y*-axis).

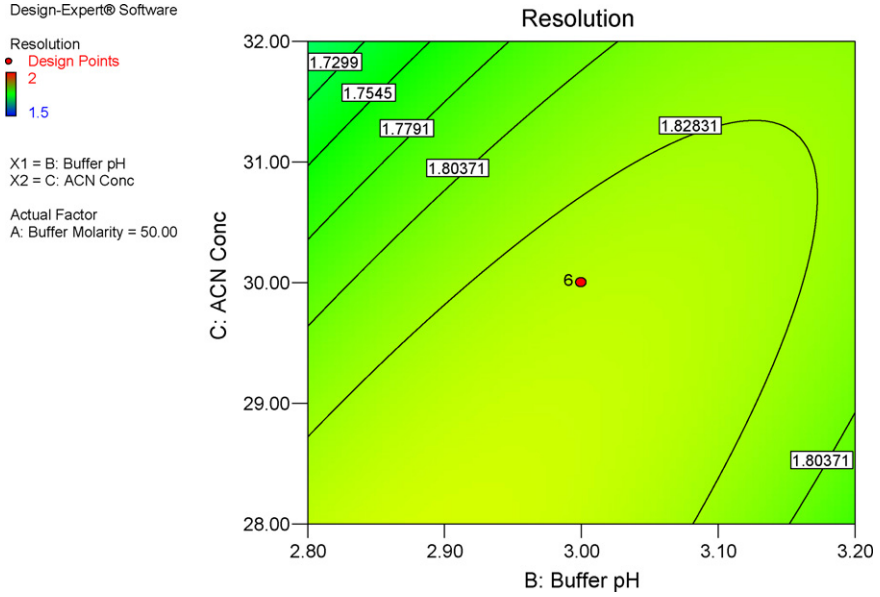

Fig. 14. Contour plot for peak resolution as a function of buffer pH (*X*-axis) and ACN concentration (*Y*-axis).

Table 5
Linearity data.

Parameter	
Concentration range (μg/mL)	2–70
Regression equation	$y = 0.0131x + 0.0275$
Correlation coefficient (R^2)	0.978
Standard error on estimation (S_e)	0.058

analyzed in replicates of five to verify the reproducibility of detector response at each concentration level. The detector responses were found to be linear over the concentration range studied and the results are summarized in Table 5. The calibration curve had a slope of 0.0131 and a *y*-intercept of 0.0275 with a correlation co-efficient of 0.978.

3.2. Precision

The intra-day precision obtained following analysis of three different standard solutions of CPT with the internal standard, CYC, and the resultant data are summarized in Table 6. The results reveal that all RSD and RE values were less than 5% and are within the limits set in our laboratory, confirming that the method is repeatable. The inter-day precision was found to be <5%, confirming that the method is precise (See Table 7).

3.3. Accuracy

The results of accuracy studies are listed in Table 8, and reveal that the greatest bias was 0.99%, indicating that no value for bias deviated by approximately more than 2.00% of the stated value. The RSD values for all the samples were less than 5% and the bias values (Table 8) were all less than 1% suggesting that the method was accurate.

Table 6
Intra-assay precision and accuracy data for CPT analysis ($n = 6$).

Concentration (μg/mL)	Calculated concentration mean ± SD	% RSD	% RE
5.00	4.92 ± 0.15	3.05	+1.60
10.00	9.89 ± 0.08	0.81	+1.10
30.11	28. 97 ± 1.12	3.87	+3.79

Table 7
Inter-day precision and accuracy data for CPT analysis.

Quality control	CPT								
	Day 1 ($n = 6$)			Day 2 ($n = 6$)			Day 3 ($n = 6$)		
Theoretical concentration (μg/mL)	5.00	10.14	30.01	5.07	10.05	30.04	5.02	10.11	30.24
Calculated concentration (μg/mL)	5.03	10.04	30.34	5.10	9.80	29.67	5.01	9.73	30.04
% RSD	0.91	0.65	1.94	0.03	0.06	0.02	0.02	0.06	0.59
% RE	−0.60	0.99	−1.10	−0.59	2.49	1.23	0.20	3.76	0.66

Table 8
Accuracy results for blinded samples.

Theoretical concentration (μg/mL)	Mean concentration determined (μg/mL) ± SD	% RSD	% Bias
5.02	5.07 ± 0.115	2.27	+0.99
9.98	10.06 ± 0.042	0.42	+0.80
30.01	30.11 ± 0.156	0.52	+0.33

3.4. Assay

The average drug content was found to be 95.65 and 99.88% of the labelled claim for all products tested. No interfering peaks were observed in the resultant chromatograms indicating that there was no interference from excipients used in the manufacture of the tablets. The results are shown in Table 9. The accuracy of the HPLC method was evaluated by the recoveries of known amounts of CPT which were added to the drug product being tested. The % recovery achieved ranged between 95.65 and 99.88% (Table 9) and the corre-

Table 9
Assay results for commercially available CPT products.

Analyt label claim (50 mg)	Amount added (mg)	Found (mg/tablet) ± SD	Recovery (%)	% RSD
CaptoHEXAL® 50	50.0	49.56 ± 0.57	99.76	1.15
MERCK-CAPTOPRIL	50.0	49.57 ± 1.03	99.78	2.08
ZAPTO-50	50.0	49.41 ± 0.47	99.46	0.95
Sandoz Captopril 50	50.0	49.44 ± 1.02	99.51	2.06
ADCO-CAPTOMAX 50	50.0	49.03 ± 1.62	98.69	3.30

sponding % RSD values were well below 5% indicating the method is accurate.

3.5. LOQ/LOD

The LOQ and LOD of the method developed for the analysis of CPT were established using a precision of ≤5.0%. By convention, the LOD was taken as 0.3 × LOQ [51]. The LOQ was found to be 2.0 μg/mL (% RSD = 2.27), and LOD based on this approach was 0.6 μg/mL.

4. Conclusions

Although HPLC methods for measuring CPT in dosage forms have been reported [52,53] this is, to our knowledge the first method in which coulometric ECD has been used. In particular the major advantage of this HPLC–ECD method over UV detection is that time-consuming pre-column derivatization procedures are eliminated. HPLC–ECD is highly selective, sensitive and is simpler than derivatization which has the potential of the reaction not going to completion. Furthermore, sample components may be absorbed by the column resulting in a low detector response.

The application of RSM in conjunction with CCD to modelling and optimizing the performance of an HPLC method has been discussed. CCD was used to design an experimental program for modelling the effects of mobile phase pH and molarity and concentration of ACN on the peak area, symmetry, resolution and retention time of CPT. Twenty experiments including centre points were conducted.

The predicted values from the model equation were found to be in good agreement with observed values and to gain a better understanding of the three variables evaluated to achieve an optimal retention time, the models were presented as 3D response surface plots. The models allow for the confident prediction of performance by interpolation of data over the range of information used to construct the response surface plots. The results reveal that the concentration of ACN has a significant effect on retention, whereas buffer molarity has less of an impact. This study demonstrates that RSM and CCD can be applied to modelling and optimizing retention time and that it is an economical method that can be used to generate a maximum amount of information in a short period of time with a small number of experiments.

It is clear that experimental design methodology is an economic approach for extracting the maximum amount of information and saves significant amounts of time in addition to the minimization of use of materials and personnel costs.

The wide range for linearity, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is better, than previously reported methods, for the quantification of CPT. In addition the method is not as complicated as those previously reported in the literature [54–56]. Furthermore slow and tedious derivatization steps and the products of possible artefactual oxidation of bases that have been reported are avoided [57,58].

Acknowledgments

The authors are grateful to the National Research Foundation (RBW), Andrew Mellon Foundation (SMK), Joint Research Committee and Rhodes University for financial support.

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