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The use of experimental design for the development of a capillary zone electrophoresis method for the quantitation of captopril

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A capillary zone electrophoresis (CZE) method for the quantitation of captopril (CPT) using UV detection was developed. Influence of electrolyte concentration and system variables on electrophoretic separation was evaluated and a central composite design (CCD) was used to optimize the method. Variables investigated were pH, molarity, applied voltage and capillary length. The influence of sodium metabisulphite on the stability of test solutions was also investigated. The use of sodium metabisulphite prevented degradation of CPT over 24 hours. A fused uncoated silica capillary of 67.5cm total and 57.5 cm effective length was used for analysis. The applied voltage and capillary length affected the migration time of CPT significantly. A 20 mM phosphate buffer adjusted to pH 7.0 was used as running buffer and an applied voltage of 23.90 kV was suitable to effect a separation. The optimized electrophoretic conditions produced sharp, well-resolved peaks for CPT and sodium metabisulphite. Linear regression analysis of the response for CPT standards revealed the method was linear (R^2 = 0.9995) over the range 5-70 µg/mL. The limits of quantitation and detection were 5 and 1.5 µg/mL. A simple, rapid and reliable CZE method has been developed and successfully applied to the analysis of commercially available CPT products.

1. Introduction

Captopril (CPT) is an orally active angiotensin-converting enzyme (ACE) inhibitor that is used alone or in combination to manage hypertension. CPT is also used to treat cardiac conditions and diabetic neuropathy (Brunton et al. 2011; Gibbon 2012; Katzung 2009; Klein et al. 1990; Lacey et al. 1999; Reynolds 2012). Chemically, CPT is 1-(3-mercapto-2-d-methyl-1-oxopropyl)-l-proline (S, S) (Fig. 1), has an empirical formula of $C_9H_{15}NO_3S$ and a molecular weight of 217.29 (British Pharmacopeia 2011; Florey 2012).

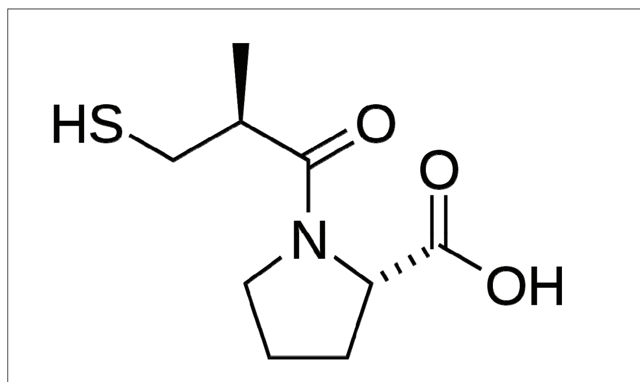

Fig. 1: Chemical structure of captopril

Several analytical methods including gas chromatography (GC) (Both and Jemal 1991; Ito et al. 1987), gas chromatography-mass spectroscopy (GC-MS) (Chik et al. 2010; Franklin et al. 1998) and high performance liquid chromatography (HPLC) using photometric (Amini et al. 1999; Bahmaei et al. 1997; Jankowski et al. 1995; Kusierek and Bald 2007), fluorimetric (Tache et al. 2002) and electrochemical (Khamanga and Walker 2011) detection have been reported for the quantitation of CPT in dosage forms and biological matrices. Capillary zone electrophoresis (CZE) is an alternative approach for the analysis of compounds due to short analysis times, high separation efficiency, relatively low cost of operation and avoidance of tedious derivatization procedures (Altria et al. 2001; Hillaert and Van den Bossche 1999; Mohammadi et al. 2004).

The assay of dosage forms that contain CPT is a challenge since CPT undergoes first order, free radical oxidative degradation at the thiol functional group to yield the dimer, captopril-disulphide (Florey 2012). CPT contains an amide link that may be hydrolysed to yield additional degradation products. Amide hydrolysis is unlikely as such degradation occurs only under forced degradation conditions and is therefore considered insignificant under normal circumstances (Florey 2012). CPT exhibits maximum stability in acidic solutions of pH < 4. The use of low oxygen headspace or nitrogen gas, increasing the CPT concentration and the addition of anti-oxidants or chelating agents have been shown to delay oxidative degradation (Florey 2012; Hillaert and Van den Bossche 1999).

Several CZE methods have been reported for the separation of lisinopril, fosinopril, cilazapril and enalapril and for the separation of complex mixtures of derivatized and underivatized thiols (Hillaert and Van den Bossche 2000; Lin et al. 1991; Prieto et al. 2001). The quantitation of CPT and its degradation products using CZE using UV and fluorescence detection as raw material and in biological matrices have been reported (Hillaert and Van den Bossche 1999; Pérez-Ruiz et al. 2006). These reports suggest a lack of a platform to facilitate visualization of the optimization of input variables graphically and do not permit elucidation of the impact of input factors on the separation and ultimately method performance. They also do not provide statistical information in respect of the contribution of each input factor on the overall separation. Therefore in order to determine the interactive effects of different input parameters and reduce the total number of experiments, a design of experiments approach using a Central Composite Design (CCD) was used to optimize this CZE method for the analysis of CPT.

Several experimental parameters including applied voltage, buffer properties and capillary length influence the separation of analytes and performance of a CZE system (Hancu et al. 2014). The influence of each of these parameters on a separation can be determined by altering one parameter at a time whilst keeping the other parameters constant. This type of approach is time consuming and tedious as many experiments must be performed for the optimisation process. Furthermore this approach excludes an evaluation of the interactive effects of different input variables. A design of experiments approach has been successfully used for the development and optimization of a HPLC method with electrochemical detection (Khamanga and Walker 2011) for CPT and bovine casein using CZE (Ortega et al. 2003).
To our knowledge this is the first report of the use of experimental design for the development of a simple, rapid, sensitive and reliable CZE method for the quantitation of CPT in pharmaceutical dosage forms.

2. Investigations, results and discussion

The key responses monitored were peak resolution and migration time of CPT of which the latter was most important as it has a major impact on the total experimental run time.
The significance and relevance of independent input factors on peak resolution and the migration time of CPT was established using ANOVA and Design Expert version 7.0.1 software (Stat-Ease Inc., Minneapolis, MN, USA). The null hypothesis was that no factor effects exist and the alternative hypothesis was that a factor effect existed. A value for Prob > F is the probability of obtaining the observed F value if the null hypothesis is true. If the two variances are similar then the ratio will approach one and it is unlikely that the independent variables have an impact on dependent responses (Babu et al. 2011; Gupta et al. 2010).

2.1. Migration time

The migration time is the time it takes for an analyte to migrate from the anode, where the sample is introduced, to the cathode and detector. The F-ratio was used to determine whether the model was significant and was set at a p = 0.05 level of significance. The resultant F-value was 10.44 suggesting that the model was significant and the data for migration time are summarised in Table 1.

Table 1: ANOVA data for Response Surface Quadratic Model for migration time

Source	Sum of Squares	df	Mean Square	F-value	p-Value Prob>F	
Model	613.50	14	43.83	10.44	<0.0001	Significant
A-pH	12.60	1	12.60	3.00	0.1051	
B-molarity	15.66	1	15.666	3.73	0.0738	
C-Voltage	346.05	1	346.05	82.47	<0.0001	
D-Capillary length	139.69	1	139.69	33.29	<0.0001	
AB	4.96	1	4.96	1.18	0.2955	
AC	0.98	1	0.98	0.23	0.6359	
AD	0.39	1	0.39	0.093	0.7649	
BC	13.25	1	13.25	3.16	0.0973	
BD	11.34	1	11.34	2.70	0.1225	
CD	10.85	1	10.85	2.59	0.1302	
A^2	17.55	1	17.55	4.18	0.0601	
B^2	1.35	1	1.35	0.32	0.5796	
C^2	10.90	1	10.90	2.60	0.1294	
D^2	5.50	1	5.50	1.31	0.2716	
Residual	58.75	144.20				
Lack of fit	58.66	9	6.52	404.61	<0.0001	Significant
Pure error	0.081	5	0.016			
Cor total	672.32	28				

It is unlikely that a Model F-Value this large could be a consequence of noise as the probability due to noise was 0.01%. Values of Prob > F that are < 0.05 indicate that the model terms are significant. The Prob > F for the entire model was < 0.0001 in this case and therefore the null hypothesis was rejected, indicating that the quadratic model was significant and that there is a factor effect with an overall contribution of terms in the model having a significant impact on the migration time of CPT. A p-value > 0.05 suggests that buffer pH and molarity had no effect on the migration time of CPT whereas the applied voltage and length of the capillary had a significant effect, in the range investigated, on the migration time of CPT.
The lack of fit for the quadratic model for migration time of CPT was significant and is undesirable as the data must fit the model. Therefore, in order to improve the model, it was necessary to consider model reduction, response transformation and presence of outliers. Model transformation was undertaken in an attempt to improve the model. The ANOVA data for the quadratic model transformed to an inverse square root model are summarized in Table 2. Following model transformation, pH, capillary length and applied voltage were established as factors that had a statistically significant impact on the migration time of CPT (p < 0.0001).

Table 2: Migration time transformation for resolution

Source	Sum of Squares	df	Mean Square	F-value	p-Value Prob>F	
Model	0.066	4	0.017	57.15	<0.0001	Significant
A-pH	*0.001354*	1	0.001354	4.66	0.0411	
B-Molarity	*0.0008509*	1	*0.0008509*	2.93	0.0998	
C-Voltage	0.049	1	0.049	168.91	<0.0001	
D-Capillary length	0.015	1	0.015	52.11	<0.0001	
Residual	0.006969	24	0.0002904			
Lack of fit	0.006957	19	0.0003662	149.74	<0.0001	Significant
Pure error	0.00001223	5	0.000002445			
Cor total	0.073	28				

The R^2 value of 0.9050 and predicted R^2 value of 0.8487 are in good agreement with the adjusted R^2 value of 0.8892. The value for Adeq Precision was used to measure the signal to noise ratio as to navigate the design space, the signal to noise ratio should be > 4. A ratio of 26.521 was calculated indicating an adequate signal suggesting that the model may be used to predict the migration time of CPT within the limits of the range studied. The experimental data was fitted to a second-order model relating migration time of CPT to the other factors and the final quadratic equation for migration time (Y_1) in terms of coded factors is shown in Eq. (1).

$$Y_1 = 11.76 - 0.72X_1 + 0.81X_2 - 4.49X_3 + 2.41X_4 - 0.56X_1X_2 - 0.25X_1X_3 + 0.16X_1X_4 - 0.91X_2X_3 + 0.84X_2X_4 - 0.82X_3X_4 + 0.81X_1^2 - 0.22X_2^2 + 0.83X_3^2 + 0.45X_4^2 \quad (1)$$

2.2. Diagnostic plots

Model adequacy was checked using normal probability plot of residuals and a plot of studentized residuals versus predicted values. The analysis of residuals was used to determine the validity and accuracy of the model. The normal probability plot of residuals depicted in Fig. 2 reveals a pattern of the residuals that is slightly S-shaped, consequently the normality assumption is loosely satisfied in this case. The plot of residuals versus predicted responses for migration time of CPT (Fig. 3) reveals no clear scatter pattern and the residuals are almost uniformly scattered above and below the central x-axis, further suggesting that the model was adequate. As such violation of independence or constant variance assumption was not suspected. Outlier points were verified by identifying any data that were scattered beyond the red limit lines of which none occurred indicating that the data is well fitted by the model.

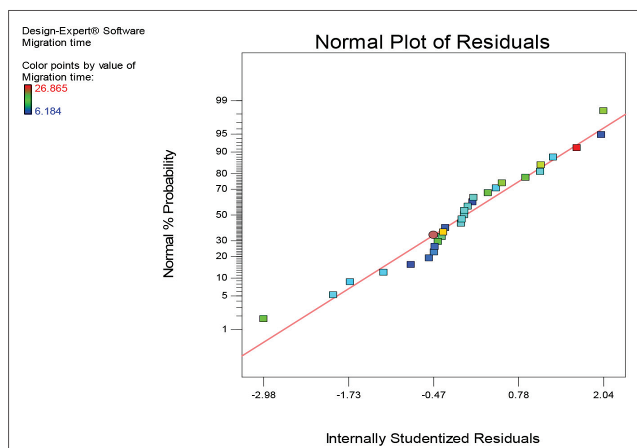

Fig. 2: Normal plot of residuals for migration time

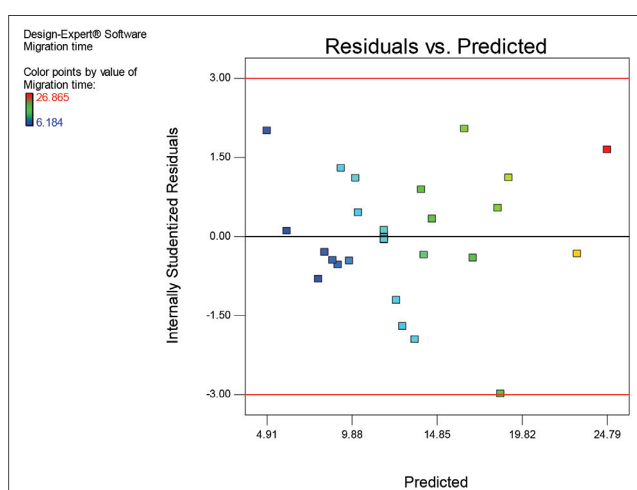

Fig. 3: Plot of residuals versus predicted values for migration time

Two dimensional contour plots were used to study the interactive effects of the input factors on the responses and these are depicted in Figs. 4 to 8.

2.3. Voltage

The velocity of the Electro-osmotic Flow (EOF) (V_{eo}) is directly proportional to the electrophoretic mobility (μ_{eo}) and the applied electric field (E) as shown in Eq. (2) and the applied electric field is the quotient of voltage s = and capillary length Eq. (3).

$$V_{eo} = \mu_{eo} E \quad \textbf{(2)}$$

$$E = \text{Voltage/Length of capillary} \quad \textbf{(3)}$$

Increasing the applied voltage and therefore the electric field, results in an increase in EOF with a consequent decrease in the migration time of CPT that was observed with an increase in the applied voltage when length of capillary length, buffer molarity and pH were constant (Figs. 4 and 5). The use of extremely high voltages during analysis is undesirable as Joule heating occurs and the generation of high current negatively impacts peak resolution as a result of band broadening (Kim et al. 2001; Xu 1996). It was therefore necessary to select an applied voltage that was sufficiently high to produce a rapid separation but that did not cause Joule heating. An applied voltage of 23.9 kV generated a sufficiently low current and was therefore selected as the optimum voltage for this separation

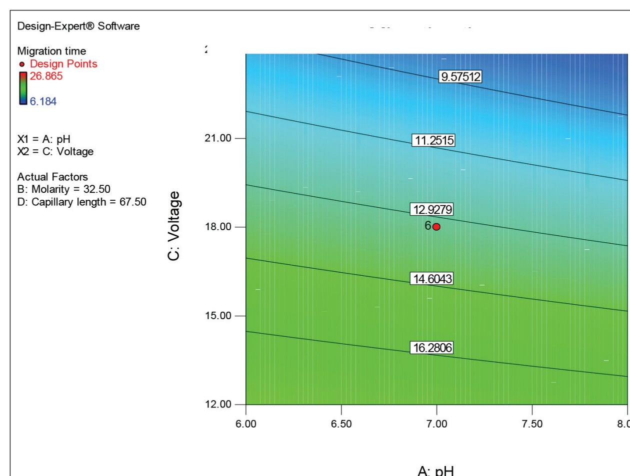

Fig 4: Contour plot depicting the impact of buffer pH and voltage in migration time

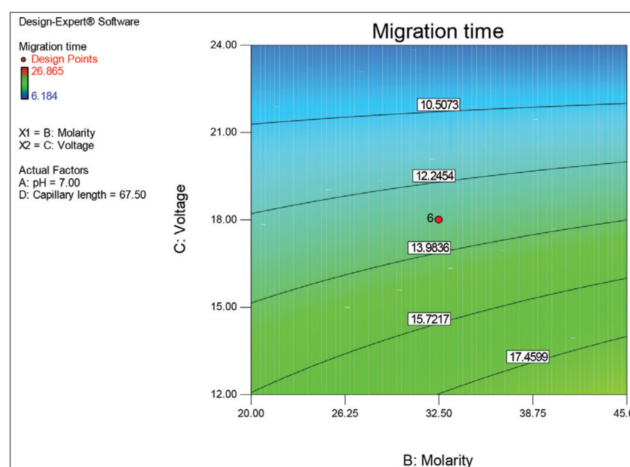

Fig. 5: Contour plot depicting the impact of buffer molarity and voltage on migration time

It is clear that increasing the pH whilst maintaining the voltage constant has no impact on the migration time and when maintaining the pH constant, increasing the voltage had an impact on migration time. When maintaining the voltage at a constant level, increasing the molarity appeared to have a slight impact on the migration time whereas increasing the voltage resulted in a decrease in migration time when the molarity was constant.

2.4. Buffer molarity

Buffer molarity in the range 20 – 45 mM was investigated. High buffer concentrations were avoided as they induce Joule heating and generate high background current (Prieto et al. 2001). An increase in buffer molarity with constant buffer pH, applied voltage and capillary length resulted in an increase in the migration time of CPT (Fig. 6). This is likely due to a decrease in electro-osmosis that occurs as a consequence of the collapse of the electric double layer (EDL) when buffer concentrations are increased (Xu 1996). Analyte adsorption onto the capillary wall may occur when low buffer concentrations are used (Prieto et al. 2001) and it was necessary to select a buffer concentration that did not compromise the quality of the separation by causing adsorption or Joule heating.

2.5. Buffer pH

CPT is amphoteric with an isoelectric point of approximately 6.8. At pH values < 6.8 CPT is positively charged and migrates towards the cathode and at pH > 6.8, it is negatively charged. In the latter case CPT still migrates towards the cathode since the EOF is greater than electrophoretic mobility. At a pH of 6.8, CPT possesses no net

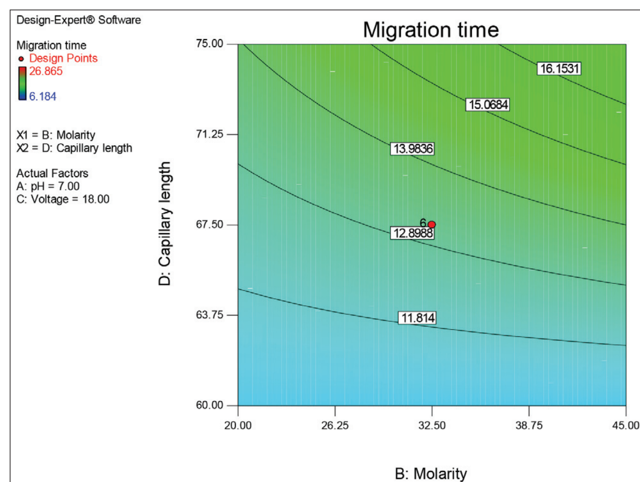

Fig. 6: Contour plot depicting the impact of buffer molarity and capillary length on migration time

charge and therefore exhibits no electrophoretic mobility however the EOF causes CPT to migrate towards the cathode.

The pH of the surrounding solution may also affect the nature of the charge on the wall of the capillary wall. At low pH values, electro-osmosis is reduced as a result of the protonation of SiO^- to form SiOH which results in a decrease in the Zeta potential of the system. The Zeta potential is directly proportional to the electrophoretic mobility Eq. (4) (Xu 1996) and therefore the use of buffers of low pH value is undesirable despite the stability of CPT, as it results in long migration times.

$$\mu e = \varepsilon \zeta / (4\tilde{O} \eta) \quad \textbf{(4)}$$

where, μ_{eo} = electrophoretic mobility, ε = dielectric constant, ζ = zeta potential, η = viscosity of the medium

Consequently, sodium metabisulfate was included in sample solutions to ensure the stability of CPT without compromising the migration time. The optimum pH was in the range pH 6 to 8 and increasing the buffer pH (Fig. 7) with low molarity resulted in short migration times with the optimum pH established as pH 7.0. At this pH good peak shape and acceptable resolution were observed and the migration time for CPT was < 6 min.

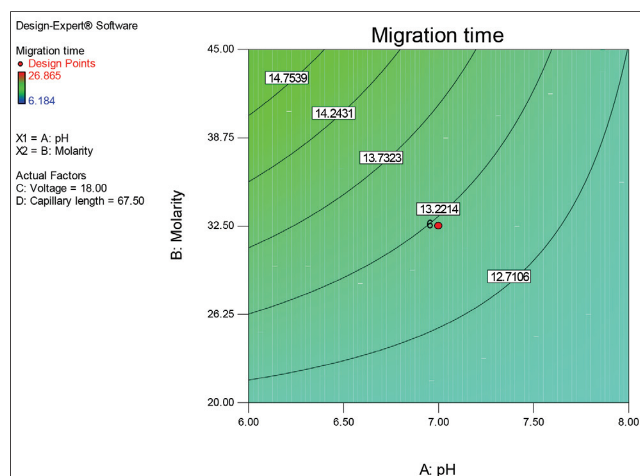

Fig. 7: Contour plot depicting the impact of buffer pH and molarity on migration time

2.6. *Capillary length*

The effect of different capillary length on migration time of CPT and peak resolution was evaluated. During a CZE separation, an electric field (E) is applied to a separation resulting in mixtures of analytes separating into discreet zones of pure analyte as a consequence of their apparent mobility (Lin et al. 1991; Xu 1996). The apparent migration time of an analyte is a function of the distance from the injection point to the detector and the apparent velocity (Xu 1996).

It was found that increasing capillary length whilst maintaining constant voltage, buffer pH and buffer molarity resulted in an increase in the migration time of CPT (Fig. 8) which is a direct result of an increase in the length of the migration path.

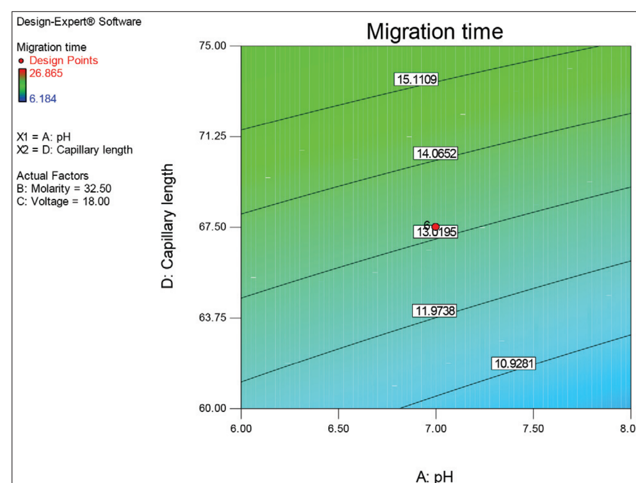

Fig. 8: Contour plot depicting the impact of buffer pH and molarity on migration time

The experimental conditions identified as the optimum conditions for the CZE analysis of CPT are summarized in Table 3 and were used for all future analyses. This combination of factors produced adequate separation of CPT, the internal standard and sodium metabisulfate (Fig. 9) and peak resolution was suitable for the quantitative analysis of CPT.

Table 3: Optimum electrophoretic conditions

Factor	Optimum conditions
pH	pH 7.0
Molarity	20mM freshly prepared phosphate buffer
Voltage	23.90kV (Ramp 6kV/sec)
Capillary	Total length - 67.5cm, Effective length - 57.5cm

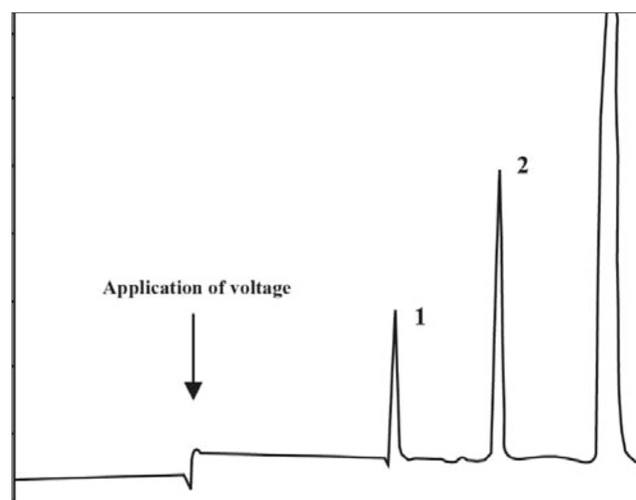

Fig. 9: Typical electropherogram of a standard solution of IS (1) and CPT (2) using a 20 mM phosphate buffer, pH 7.0, applied voltage 23.9 kV, capillary length 67.5 cm (57.5 cm effective length) x 75 μm I.D at 214 nm.

2.7. Method validation

2.7.1. Calibration, linearity and range

Calibration curves were constructed on each day of validation and were found to be linear over the entire range 5-70 µg/mL. A typical calibration curve over the concentration range 5-70 µg/mL exhibited an equation for the line $y = 0.0356x + 0.0078$ with an R^2 of 0.9995.

2.7.2. Precision and accuracy

The method was validated with respect to repeatability and inter-day precision. Nine replicate samples of concentrations 15 µg/mL, 45.50 µg/mL and 65.50 µg/mL were prepared and analysed on the same day (intra-day precision). The same three sample concentrations were also analysed on three consecutive days and the data obtained was used to establish the inter-day precision of the method. The experimental results were expressed as % RSD of the peak height ratio and were found to range between 2.13-4.01 % for intra-day precision and 0.65-4.04 % for inter-day precision. Interpolation of replicate peak height ratio of the three accuracy standard samples, from the relevant calibration curve was used to establish the accuracy of the method. The results summarized in Table 4 demonstrate the accuracy of the method.

Table 4: Accuracy data for CZE analysis of CPT (n=3)

Theoretical concentration µg/mL	Actual concentration µg/mL	Accuracy ± SD	% RE
15.5	15.46	99.74 ±1.30	+0.53
45.5	44.67	98.18 ± 1.51	+1.81
65.5	67.09	102.43 ±2 .00	-2.42

2.7.3. Assay

The analytical method was applied to the analysis of commercial products containing CPT. The data collected and summarized in Table 5 reveal that the average CPT content for all products was between 96.19 and 101.77 % of label claim declared by the pharmaceutical companies. There was no interference from the tablet excipients, running buffer or impurities as no interfering peaks were observed on the electropherograms when monitored at 214 nm.

Table 5: Assay data following analysis of commercially available dosage forms

Product	Amount found mg	% Recovery	% RSD
Zapto® - 50	48.10	96.19	2.46
Sandoz Captopril 50	50.89	101.77	0.75
Adco-Captomax 50	49.65	99.29	2.58
Mylan Captopril 50	49.61	99.23	2.19

2.7.4. LOQ/LOD

The LOQ was determined by establishing the lowest concentration of CPT that produced a value of % RSD which was ≥ 5%. The LOQ was 5 µg/mL and using the conventional practice the LOD was taken as one third of the LOQ and was 1.5 µg/mL.

2.7.5. Method development and validation summary

The CZE method reported has the necessary linearity, accuracy, precision, and sensitivity for the analysis of CPT in pharmaceutical formulations. The LOQ and LOD were in the µg/mL range. The use of RSM and a CCD reduced the number of experiments and the time required to develop the analytical method and has been successfully applied to modelling the experimental data and optimization of method. The influence of buffer pH and molarity, applied voltage and capillary length on peak resolution and the migration time of CPT were evaluated. CZE offers several advantages over conventional HPLC methods including a short analysis time, low cost of operation as the capillaries used for CZE are significantly cheaper than the columns used in HPLC. In addition, the proposed method avoids the inclusion of tedious and lengthy derivatization procedures and the use of toxic expensive organic solvents is avoided. The method developed is the first reported CZE method that has been optimized using CCD for the analysis of CPT and is rapid and has been successfully used to analyse CPT in dosage forms and may be suitable for routine analysis of CPT in dosage forms.

3. Experimental

3.1. Instrumentation

CZE was performed using a PrinCE (4 tray) Electrophoresis System Model 0500-001 (Prince Technologies, Emmen, Netherlands). The analyses were performed at an ambient temperature of 22 °C and detection achieved with a Model 0206 Linear UV/Vis-206 Multiple Wavelength Detector (Linear Instruments Corporation, Reno, Nevada, USA) set at 214 nm. The detector output was interfaced via a SATIN® box to Waters Empower® Chromatographic Software (Waters Chromatography Division, Milford, MA, USA) that was used to collect and evaluate all chromatographic data. To avoid siphoning effects, a fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of small (50 µm) internal diameter (i.d.) and an outer diameter (o.d.) of 360 µm was used. The pH of solutions was monitored using a Crison GLP21 pH-meter (Crison Instruments, Johannesburg, South Africa).

3.2. Chemicals and reagents

All chemicals and reagents were at least of analytical grade and were used without additional purification. CPT was donated by Protea Chemicals (Midrand, South Africa). Anhydrous theophylline was used as an internal standard (IS) and was donated by Aspen Pharmacare (Port Elizabeth, South Africa). Sodium hydroxide pellets and 85 % v/v *o*-phosphoric acid were purchased from Merck Laboratories (Merck, Wadeville, South Africa).

HPLC grade water for the preparation of buffer solutions was purified using 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 fitted with a 0.22 µm Millipak® 40 sterile filter (Millipore, Bedford, MA, USA). All sample solutions were filtered using a Millex HV® Hydrophilic PDVF, 0.45µm filter purchased from the same source.

3.3. Capillary conditioning

Prior to analysis, a small section of the polyimide coating, of each capillary used for the separation, was removed by exposing that area to a flame of low heat to create a window for detection. The charred coating on the capillary was removed with an ethanol dampened soft tissue. The clear uncovered portion of the capillary was aligned to the UV source on the detector block. Each new capillary was conditioned by flushing with 0.1 M NaOH for 30 minutes, 1 M NaOH for 60 min and HPLC grade water for 15 min. To ensure the presence of an optimal charge density on the capillary wall between consecutive runs, the capillary was conditioned and regenerated by rinsing it with HPLC grade water for 1min, followed by 0.1M NaOH for 2 min and with running buffer for 4 min. Capillary conditioning was facilitated by the application of a constant external pressure of 300 K Pa with nitrogen.

3.4. Preparation of stock solutions

Standard stock solutions of CPT (100 µg/mL) and IS (100 µg/mL) in buffer solution were prepared on a daily basis. Approximately 10mg of CPT and IS were accurately weighed and transferred into separate 100 mL A-grade volumetric flasks and made up to volume with buffer solution. To aid dissolution, the stock solutions were sonicated for 10 min using a Branson B12 sonicator (Shelton, CN, USA). Serial dilution of the CPT stock solution with buffer yielded solutions of 10, 20, 40, 50 and 70 µg/mL. All samples were protected from light, using aluminium foil, and stored at 4 °C for a maximum period of 24 h.

3.5. Preparation of running buffer

20 mM phosphate buffer solutions (pH 7.0) were freshly prepared on each day of analysis by pipetting 1.36 mL of 85% w/w ortho-phosphoric acid into a 1 L A-grade volumetric flask and making up to volume with HPLC grade water. A 0.1 M NaOH solution was prepared by dissolving 0.4 g of sodium hydroxide pellets in 100 mL of HPLC grade water and was used to adjust the pH of the buffer solutions to pH 7.0. The running buffer was filtered through a 0.45 µm hydrophilic PVDF filter membrane prior to transfer into the inlet and outlet vials located in the instrument.

3.6. Stability of sample solutions

The effect of the presence of an anti-oxidant sodium metabisulphite on the stability of sample solutions prior to analysis was studied. The rationale for undertaking such studies was based on information that indicate oxidation of CPT yields captopril disulphite and therefore 0.2% m/v sodium metabisulphite was included to offer protection from oxidative degradation of CPT over a 24 h period.

3.7. Central composite design

The use of experimental design during method development is a technique for establishing the impact of several independent factors alone or in combination on a set of responses being monitored in an experimental setting (Ahmadi et al. 2005). Following a preliminary study of the influence of electrolyte and system variables on the CZE separation of CPT, a CCD approach was used to optimize the method. The number of experiments required for a CCD approach includes a standard 2^k factorial with the origin located in the centre, 2k points fixed axially (star) from the centre and replicate tests at the centre of the design, where k is the number of variables, so as to facilitate generation of quadratic terms. The independent variables optimized for the CZE separation were buffer pH (x_1) and molarity (x_2), applied voltage (x_3) and capillary length (x_4). Migration time (Y_1) and peak resolution (Y_2) were the dependent responses monitored. Therefore a 2^4 + star study with 16 corners, 8 axial and 6 centre points was undertaken after establishing the range of values for the input variables to be studied. The factors were coded to lie at factorial, centre or axial points. A 2^4 randomized full factorial design in which four factors were evaluated at two levels viz., -1 and +1 with six replicates was at the centre point to estimate experimental error. Design Expert (Version 7.0.1, Stat-Ease Inc., Minneapolis, MN, USA) statistical software was used to analyse the data generated from thirty experiments. Analysis of variance (ANOVA) models were used to analyse the data and calculate the significance and relevance of the critical factors in that model. The upper and lower limits selected for the independent factors are summarised in Table 6 and the experimental conditions used are listed in Table 7. All experiments were performed in a random order to avoid systematic error and the introduction of bias.

Table 6: Translation of coded levels used for the CCD 24 + star design

Independent factors	High value (-1)	Centre (0)	Low value (+1)
Buffer pH	6.0	7.0	8.0
Buffer molarity (mM)	20.0	32.5	45.0
Applied voltage (kV)	12.0	18.0	24.0
Capillary length (cm)	60.0	67.5	75.0

3.8. Electrophoretic conditions

Freshly prepared 20 mM phosphate buffer solution of pH 7 and a constant applied voltage of 23.90 kV for the running buffer and applied voltage was used for all analyses and a current of 35 μA was observed under these conditions. All samples were injected, using a hydrodynamic process, into a fused silica capillary of 67.5 cm total and 57.5 cm effective length using an injection pressure of 50 mbar for 6 s. Hydrodynamic injection was preferred to electrokinetic injection to avoid the introduction of bias due to differences in electrophoretic mobility that can occur when electrokinetic injection is used. The electrophoretic conditions selected were found to produce a good separation between CPT, the IS and sodium metabisulphite.

Table 7: CCD coded values for factor levels investigated

Experiment (Run)	Type	x_1	x_2	x_3	x_4
1	Axial	0	2	0	0
2	Axial	0	-2	0	0
3	Centre	0	0	0	0
4	Centre	0	0	0	0
5	Fact	1	1	1	-1
6	Fact	-1	-1	1	-1
7	Fact	1	1	1	1
8	Fact	1	-1	-1	1
9	Axial	0	0	2	0
10	Fact	1	-1	-1	-1
11	Centre	0	0	0	0
12	Axial	2	0	0	0
13	Fact	-1	-1	1	1
14	Fact	-1	1	-1	-1
15	Fact	-1	1	-1	1
16	Fact	-1	-1	-1	-1
17	Fact	1	1	-1	-1
18	Fact	-1	-1	-1	1
19	Centre	0	0	0	0
20	Fact	-1	1	1	1
21	Axial	0	0	0	-2
22	Axial	-2	0	0	0
23	Fact	1	1	-1	1
24	Fact	-1	1	1	-1
25	Fact	1	-1	1	-1
26	Axial	0	0	0	2
27	Centre	0	0	0	0
28	Centre	0	0	0	0
29	Axial	0	0	-2	0
30	Fact	1	-1	1	1

3.9. Method validation

3.9.1. Calibration, linearity and range

Calibration curves for peak height ratio of the CPT and response were constructed from five standards on each day of validation and were used to establish the linearity of the analytical method over the concentration range 10-70 μg/mL. The calibration standards were prepared by appropriate dilution of the stock solution with running buffer, followed by the addition of IS. Least squares linear regression analysis was used to determine the regression equation and correlation coefficient and test sample concentrations were elucidated by interpolation.

3.9.2. Accuracy and precision

The accuracy and precision of the method was established by conducting intra and inter-day studies. The repeatability of the method was determined by injecting replicate samples at three different concentration levels and calculating the percent relative standard deviation (% RSD) for the responses. The accuracy of the method was determined by comparing the experimental results to the actual concentration.

3.9.3. Limits of quantitation (LOQ) and detection (LOD)

The lowest amount of analyte in a sample that can be quantified with adequate precision and accuracy is known as the LOQ. The LOD is the lowest amount of analyte in a sample that can be detected but not quantitatively determined to a correct value (ICH 2005).

3.9.4. Specificity

The specificity of a method can be determined by comparing electro-pherograms generated following the analysis of pure CPT with those following analysis of commercially available formulations dissolved in buffer.

3.9.5. Assay

Four commercially available CPT products were purchased from a local pharmacy and were used to determine the applicability of the method the quantitation of CPT in pharmaceutical formulations. The products tested were Zapto®-50, Sandoz Captopril 50, Adco-Captomax 50 and Mylan Captopril 50 were purchased from a local pharmacy. For each of the four products, the label claim state that each tablet contained 50 mg CPT. In order to assay, twenty tablets were weighed and pulverized to a fine powder using a mortar and pestle. An amount of powder equivalent to the weight of one tablet was quantitatively transferred to a 100 mL A-grade volumetric flask and was made up to volume with running buffer. In order to ensure complete extraction and dissolution of CPT, the mixture was sonicated for 15 min prior to filtration using a Millipore Millex HV® Hydrophilic PDVF 0.45 μm filter. The solution was diluted with running buffer and a suitable amount of the IS added to achieve a final concentration of CPT of 50 μg/mL. The samples were then analyzed using the CZE method.

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