# Chiral Recognition in Liquid Chromatography Utilising Chargeable Cyclodextrins for Resolution of Doxazosin Enantiomers

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*ABSTRACT* The chromatographic resolution of *rac*-doxazosin using reversed-phase high performance liquid chromatography (HPLC) with the chargeable chiral mobile phase additive, carboxymethyl-β-cyclodextrin (CM-β-CD), is described. The effects of different modifiers (acetonitrile, methanol and tetrahydrofuran), pH, temperature, and cyclodextrin concentration were investigated to a) assess the key chromatographic parameters for subsequent chemometric optimisation, and b) explore the enantioselective mechanism. Assuming a 1:1 complex between each doxazosin enantiomer and CM-β-CD, studies of the relationship between the capacity factors (k′) and functions of CM-β-CD concentration indicate that the mechanisms for retention and chiral selectivity are comparable with those proposed earlier by Sybilska et al.[1] Stability constants ($K_G$) calculated for *rac*-doxazosin complexed with CM-β-CD (647 ± 55 and 594 ± 45 $M^{-1}$ for each enantiomer respectively) are significantly larger than those calculated for the barbiturates complexed with β-CD (ca. 101–108 $M^{-1}$).[1] Investigations on pH indicate an ionic or ion-pair interaction between the anionic CM-β-CD and the cationic doxazosin enantiomers.

A central composite design was used to optimise the key chromatographic parameters: pH, methanol (v/v) and CM-β-CD concentration. The Kaiser peak separation index, $P_i$, was used for the response function. The predicted response for this chiral separation has been compared with that observed experimentally and samples of the four-dimensional response surface have been assessed for their value in showing robustness. *Chirality 9:184–190, 1997.* 

*KEY WORDS:* *rac*-doxazosin; doxazosin; liquid chromatography; chiral; carboxymethyl-β-cyclodextrin; optimisation; central composite design; mobile phase additive; experimental design

Cyclodextrins (CDs) are toroidal-shaped cyclic oligosaccharides composed of six (α-CD), seven (β-CD), or eight (γ-CD) D-(+)-glucopyranose units which are bonded through α-1,4-linkages. They have a relatively hydrophobic interior enabling the formation of stereoselective inclusion complexes with a variety of molecules and ions containing a hydrophobic moiety. This inclusion process may be aided through hydrogen bonding and/or Van der Waals interactions between appropriate polar moieties of a guest molecule and secondary hydroxyl groups at the month of the cyclodextrin cavity. These have led to their extensive use as bonded chiral stationary phases (CSPs) in liquid chromatography (LC)[2–4] or as chiral mobile phase additives (CMPAs) in LC[5,6] and capillary electrophoresis (CE)[7] for the enantiomeric separation of racemic molecules.

Derivatised CDs with increased solubility relative to the native CDs have been developed for use in pharmaceutical formulations. The derivatised hydroxypropyl-β-CD (HP-β-CD) and hydroxyethyl-β-CD (HE-β-CD) have shown enhanced enantioselectivity for certain chiral entities than β-CD both in LC[8] and CE.[9] These neutral derivatised CDs, if substituted at the secondary hydroxyl groups located at the wider opening of the toroid, are thought to provide not only extra sites for hydrogen bonding but additional steric interactions as well.

Recently, a charged sulphobutyl-ether-β-cyclodextrin (SBE-β-CD) has been characterised[10] and used as a chiral mobile phase additive in LC[11] and CE,[12] enabling greater resolution at lower concentrations with reduced retention times. The application and properties of another range of ionisable CDs, carboxymethyl-β-cyclodextrins (CM-β-

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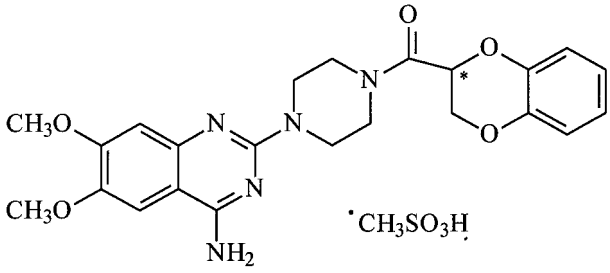


**Fig. 1.** Structure of doxazosin mesylate.

CDs), have also been studied to separate some aromatic isomers in electrokinetic chromatography[13] and used as chiral mobile phase additives in both LC[14] and CE.[15]

In this paper, we describe the use of CM-β-CD as a CMPA in LC for the resolution of *rac*-doxazosin, an $\alpha_1$-adrenoreceptor antagonist. The effects of different modifiers (acetonitrile, methanol and tetrahydrofuran), pH, temperature, and cyclodextrin concentration were investigated to a) assess the key chromatographic parameters for subsequent chemometric optimisation, and b) explore the enantioselective mechanism involved.

These key chromatographic parameters were subsequently optimised using the recently described chemometric multivariate optimisation procedure, central composite design (CCD).[11] Tentative proposals are made of the enantioselective mechanism and calculation of stability constants between this ionisable CM-β-CD and each doxazosin enantiomer based on earlier studies carried out by Sybilska et al. using native CDs as mobile phase additives.[1,16,17] The importance of controlling the pH of the aqueous buffer and thus the ionic nature of both the selector and selectand is also shown.

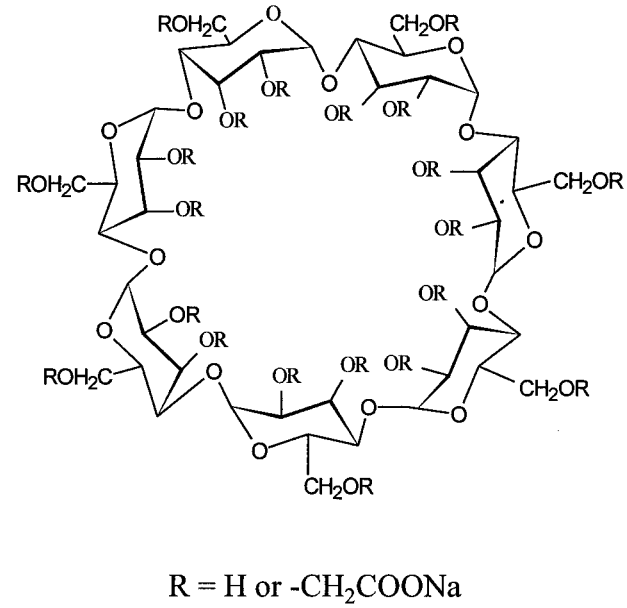
## MATERIALS AND METHODS

*Rac*-doxazosin mesylate (Fig. 1) was obtained from, and its $pK_a$ determination carried out at, Pfizer Central Research (Sandwich, Kent). CM-β-CD sodium salt (Fig. 2) was supplied and characterised† by Wacker Chemicals Ltd. (Walton-on-Thames, Surrey, UK). This represents an accepted median value for the molecular substitution pattern claimed by the manufacturer. Organic solvents (AnalaR grade) were purchased from Fisons (Loughborough, UK). Sodium dihydrogen orthophosphate dihydrate buffer was purchased from BDH (Poole, UK) and the pH adjusted to ± 0.01 with orthophosphoric acid or sodium hydroxide, as appropriate. All aqueous mobile phases were filtered using a 0.45-μm filter (Anachem, Bedfordshire, UK) and degassed by sonication under vacuum.

### *High-Performance Liquid Chromatography*

Liquid chromatography was carried out using an LKB 2150 HPLC pump at a constant flow rate of 1.0 ml/min. An LKB 2151 variable wavelength UV detector was used to measure the absorbance of *rac*-doxazosin (ca. 100 μg/ml)

† (DS = 0.76)



R = H or -$CH_2COONa$

**Fig. 2.** Structure of carboxymethyl-β-cyclodextrin sodium salt (CM-β-CD).

at 254 nm. The chromatograms were recorded on a Kipp and Zonen BD40 chart recorder and a LDC/Milton Roy C1–10B integrator. The column dimension was 150 × 3.9 mm I.D., packed with 4-μm Nova-Pak $C_8$ packing material (Waters, Milford, MA). The injection volume was 20 μl. The temperature was maintained (except where stated) at 17 ± 0.1°C using an oven (Kariba Instruments, Jones Chromatography, Hengoed, Mid Glamorgan, UK).

### *Exploratory Univariate Optimisation*

A systematic univariate optimisation was performed to evaluate the key chromatographic parameters. Mobile phases of the same total solvent strength (ST) were prepared using the Snyder approach[18] to compare the selectivities of methanol (MeOH), acetonitrile (Acn) and tetrahydrofuran (THF) as organic solvents for the chiral separation of *rac*-doxazosin using CM-β-CD as a CMPA. A range of CM-β-CD concentrations, pH and temperature were also explored during this initial univariate optimisation.

### *Implementation and Data Evaluation of the CCD*

Chromatographic parameters for the chiral separation of *rac*-doxazosin were optimised chemometrically using the Box-Wilson CCD[19] experimental design. Theoretical and practical guidelines have been discussed recently for setting up a Box-Wilson CCD applicable to any method.[11] Optimisation of the chromatographic parameters for the enantioseparation of *rac*-doxazosin using CM-β-CD as a CMPA was carried out using these guidelines. The parameter used in assessing the response criteria was the Kaiser peak separation index, $P_i$, defined as the average valley depth expressed as a ratio to the average peak height of the two enantiomeric peaks.[20] Data evaluation was carried out using a statistical software package produced by the

**TABLE 1. Experimental data show the enantioselectivity of three different organic modifiers in a standard buffer having the same solvent strength and the same CM-β-CD concentration (15 mM) for the chromatography of *rac*-doxazosin**

| Organic modifier (mL) | Aqueous[a] (mL) | $P_i$ | $R_s$ | $k'_1$ | $k'_2$ |
|---|---|---|---|---|---|
| Methanol 16.0 | 50 | 1.0 | 2.4 (0.60) | 25.1 (0.44) | 2.97 (0.35) |
| Acetonitrile 13.0 | 50 | 1.0 | 1.9 (1.8) | 20.4 (0.78) | 23.0 (0.84) |
| Tetrahydrofuran 9.25 | 50 | 0.70 (2.14) | 0.9 (3.88) | 8.8 (0.43) | 9.5 (0.43) |

[a]20 mM $NaH_2PO_4$ (pH 3.0). Values in parentheses represent RSD values as % (n = 5).

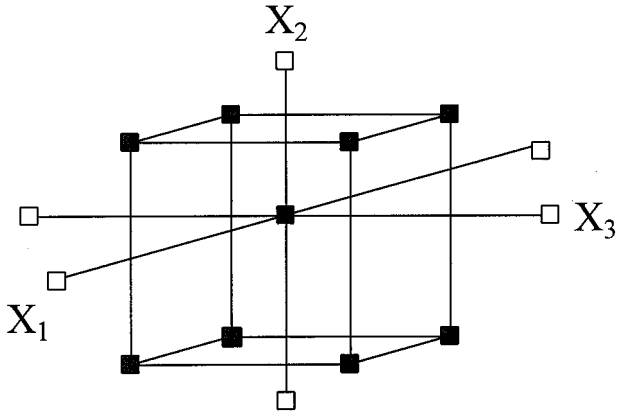



**Fig. 3.** Factors for CCD: pH ($X_1$), MeOH (v/v $X_2$), CM-β-CD concentration (mM, $X_3$).

SAS Institute (release version 6.04, SAS Software Ltd., Cary, NC, USA).

## RESULTS AND DISCUSSION

### *Exploratory Univariate Optimisation*

In order to investigate the selectivities of methanol, acetonitrile and tetrahydrofuran as potential organic modifiers, three mobile phases of nominally equal solvent strength were prepared using the Snyder model (Table 1). Enantioselectivity for *rac*-doxazosin was observed with each of the three mobile phases. Table 1 illustrates that methanol in an aqueous mobile phase [20 mM $NaH_2PO_4$ (pH 3.0) containing 15 mM CM-β-CD] was more enantioselective ($R_s$ = 2.4, $P_i$ = 1.0) than either acetonitrile ($R_s$ = 1.9, $P_i$ = 1.0) or tetrahydrofuran ($R_s$ = 0.9, $P_i$ = 0.70). Interestingly, the work of Hinze et al.[21] also showed that better resolution was obtained on a β-CD CSP for the enantiomers of dansylthreonine with methanol as organic modifier, compared with aprotic solvents (e.g., acetonitrile). The authors also observed a marked improvement in k′ and resolution ($R_s$) when the methanol content of the mobile phase was reduced as the solvent was thought to compete for the CD cavity.

CM-β-CD concentration and pH of the aqueous buffer were also found to be critical parameters for the resolution of doxazosin enantiomers. The effects of these two parameters were investigated further during the elucidation of the enantioselective mechanism. Temperature, generally an important parameter when using CD-based CSPs or CMPAs,[22] was found in this case to be less influential than organic modifier, pH or CM-β-CD concentration and was thus held constant.

**TABLE 2. Experiments required for a three-variable Central Composite Design for doxazosin enantiomers**

| Experiment no. | pH | MeOH (v/v) | CD (mM) |
|---|---|---|---|
| 1 | 3.0 | 35 | 25 |
| 2 | 3.0 | 25 | 25 |
| 3 | 3.0 | 25 | 15 |
| 4 | 3.0 | 35 | 15 |
| 5 | 5.0 | 35 | 25 |
| 6 | 5.0 | 25 | 25 |
| 7 | 5.0 | 35 | 15 |
| 8 | 5.0 | 25 | 15 |
| 9[a] | 4.0 | 30 | 20 |
| 10 | 2.3 | 30 | 20 |
| 11 | 5.7 | 30 | 20 |
| 12 | 4.0 | 30 | 28.5 |
| 13 | 4.0 | 30 | 11.4 |
| 14 | 4.0 | 21.5 | 20 |
| 15 | 4.0 | 38.5 | 20 |

[a]Ten replicate experiments of central point.

**TABLE 3. Predicted and observed experimental data for separation of doxazosin: pH ($X_1$), MeOH (v/v, $X_2$) and CM-β-CD concentration (mM, $X_3$) by CCD**

| Response | Optimum factor | Predicted | Observed |
|---|---|---|---|
| $P_i$ | $X_1$ = 4.3<br>$X_2$ = 23.7<br>$X_3$ = 15.4 | 1.0 | 0.97 |

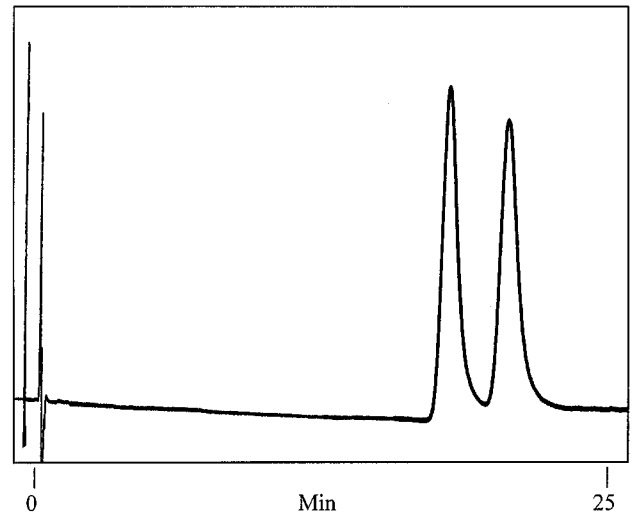



**Fig. 4.** Optimised separation of doxazosin enantiomers: methanol–20 mM $NaH_2PO_4$ (pH 4.3) containing 15.4 mM CM-β-CD (23.7:50, v/v).

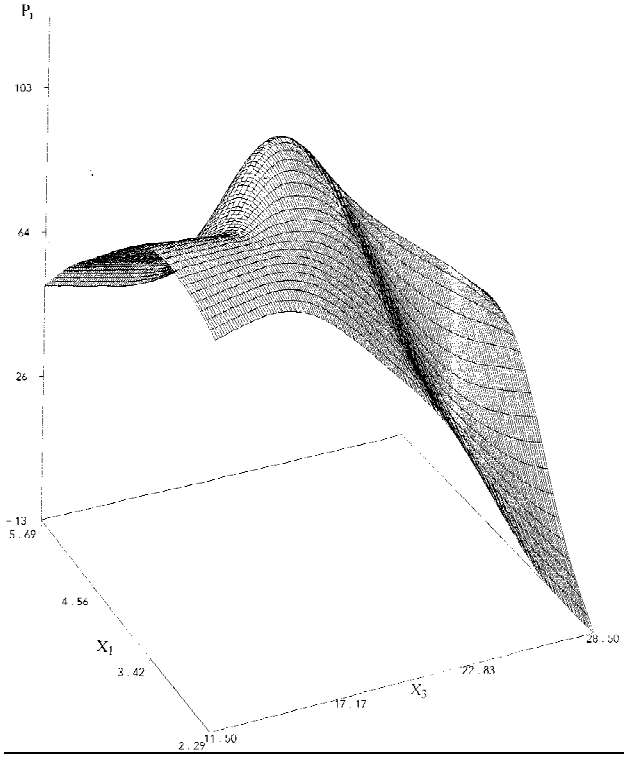



**Fig. 5.** Response surface for $P_i$ response model of doxazosin enantiomers: $X_1$ (pH) vs. $X_3$ (CM-β-CD, mM). $X_2$ (MeOH, v/v) is held constant at its optimum predicted value.

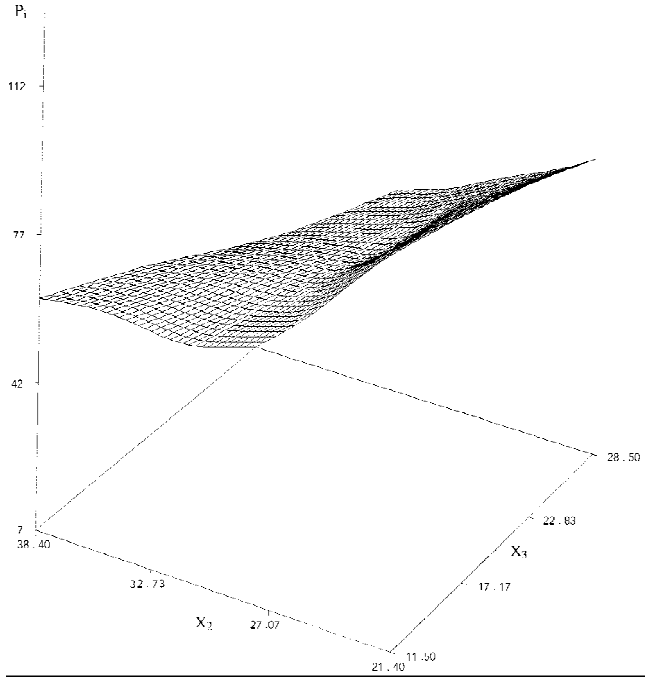



**Fig. 6.** Response surface for $P_i$ response model of doxazosin enantiomers: $X_2$ (MeOH, v/v) vs. $X_3$ (CM-β-CD, mM). $X_1$ (pH) is held constant at its optimum predicted value.

### *Optimisation Using the CCD*

After the exploratory univariate optimisation of the critical chromatographic parameters, a multivariate optimisation was implemented using the CCD. The three-factor CCD required 15 experiments and 10 centre point replicates (Fig. 3) for pH ($X_1$), methanol (v/v, $X_2$) and CM-β-CD (mM, $X_3$) as described in detail in Table 2. The factor space occupied by each parameter was defined by the exploratory univariate experiments, which thus defined the location selected for each factor point. The locations of the axial star points in factor space were then readily calculated from these factor point locations as recently described.[11] The centre point simply represents a particular set of experimental conditions at the geometrical centre of the factor space utilised. Replicates of the centre point experiment must be carried out in order to satisfactorily estimate the pure experimental uncertainty in a lack-of-fit test.

The optimum chromatographic conditions (Table 3) predicted from the CCD, methanol—20 mM $NaH_2PO_4$ (pH 4.3) containing 15.4 mM CM-β-CD (23.7:50 v/v), were examined experimentally, yielding the separation shown in Figure 4. This separation corresponds to a value of $P_i = 0.97$ (97% separation) which is in good agreement to that predicted by the statistical software ($P_i = 1.00$). As a measure of robustness, the RSD data (n = 5) for the key parameters for this separation were $P_i$ = 1.53%, $t_{R1}$ 0.38% and $t_{R2}$ 0.43%.

### *Evaluation of Response Surfaces*

The data acquired from this central composite design were analysed by SAS which generates a four-dimensional

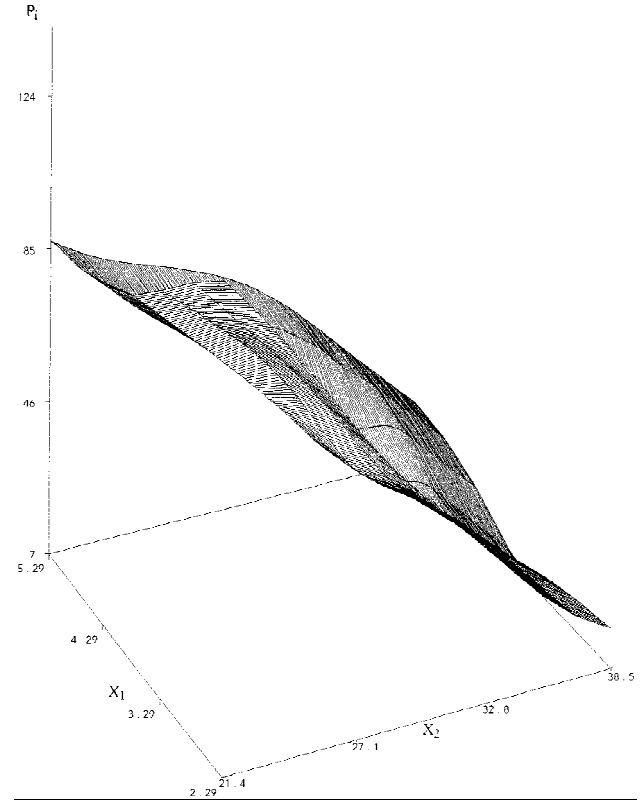



**Fig. 7.** Response surface for $P_i$ response model of doxazosin enantiomers: $X_1$ (pH) vs. $X_2$ (MeOH, v/v). $X_2$ (CM-β-CD, mM) is held constant at its optimum predicted value.

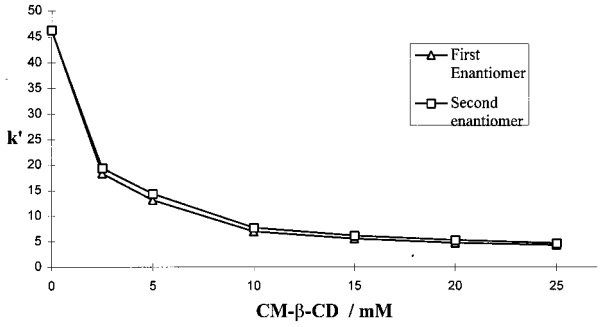



**Fig. 8.** Plot of capacity factor (k′) vs. CM-β-CD (mM) for each doxazosin enantiomer. Methanol—20 mM $NaH_2PO_4$ (pH 3.0), 35:50, v/v.

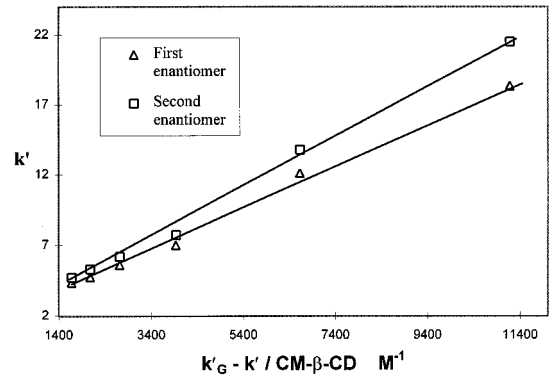



**Fig. 9.** Plot of capacity factor (k′) vs. $k'_G - k'/[CM\text{-}\beta\text{-}CD]$ for each doxazosin enantiomer. Conditions as in Figure 8.

response surface which can be readily visualised in three dimensions. The response model, $P_i$, is mapped against two experimental factors while the third is held constant at its optimum.

Figure 5 represents a three-dimensional section of the response surface generated by the $P_i$ response model for pH ($X_1$) and CM-β-CD (mM, $X_3$), while MeOH (v/v, $X_2$) was kept constant at its optimum. A clear optimum is observed for both pH and cyclodextrin concentration. This is very similar in shape to that previously reported for pH ($X_1$) and SBE-β-CD (mM, $X_3$) in LC, where Acn (v/v, $X_2$) was kept constant at its optimum for the separation of *rac*-amlodipine enantiomers.[11]

Figure 6 represents a three-dimensional section of the response surface generated by the $P_i$ response model for MeOH (v/v, $X_2$) and CM-β-CD (mM, $X_3$), while pH ($X_1$) was kept constant at its optimum. This flat profile indicates that little or no interaction is occurring between the two chromatographic parameters MeOH (v/v, $X_2$) and CM-β-CD (mM, $X_3$). The absence of interactions clearly indicates that this chromatographic system is robust and rugged, where small changes in chromatographic conditions would have little effect on the response ($P_i$).

Figure 7 represents a three-dimensional section of the response surface generated by the $P_i$ response model for pH ($X_1$) and MeOH (v/v, $X_2$), while CM-β-CD (mM, $X_3$) was kept constant at its optimum. This also exhibits a relatively flat profile, indicating little interaction between pH ($X_1$) and MeOH (v/v, $X_2$) and thus confirming a robust chromatographic system.

### *Putative Enantioselective Mechanisms*

Two models for rationalising the interactions observed between free enantiomeric solutes, a CD, and the surface of a hydrophobic stationary phase have been proposed.[1] In essence they can be summarised as follows. The first model proposes that there are for each enantiomer differences in adsorption of the solute-CD complex onto the surface of the hydrophobic stationary phase itself. The second model proposes that there are differences in the interaction of each enantiomeric solute with a layer of CD adsorbed from the mobile phase onto the surface of the hydrophobic stationary phase.

The first model involves the establishment of complexation equilibria in the bulk mobile phase solution. The second model, however, describes the formation of a dynamic chiral stationary phase (CSP). This would be expected to be comparable to the resolution mechanism that would be involved if the CD were covalently bonded to a silica surface. The present work does not allow elucidation of the enantioselective mechanism in terms of these models. Current work is in progress in this laboratory to investigate whether or not adsorption of the CD to the hydrophobic stationary phase occurs.

However, equation 1 describes a simple reversed-phase system, where the mobile phase contains a CD additive, and permits calculation of the stability constant, $K_G$, for a guest molecule-CD complex of stoichiometry 1:1 for each enantiomer:

$$k' = \frac{1}{K_G} \cdot \frac{k'_G - k'}{[CD]} + k'_{G\text{–}CD} \qquad (1)$$

where $k'_G$ and $k'_{G\text{–}CD}$ are the capacity factors of the free guest molecule and its CD complex on the stationary phase respectively. If a linear relationship exists between k′ vs. $k'_G - k'/[CD]$ this would allow the calculation of stability constants ($K_G$) for each enantiomer, and capacity factors ($k'_{G\text{–}CD}$) of these complexes on the hydrophobic stationary phase.[16]

Figures 8 and 9 show the relationship between capacity factors (k′) for each doxazosin enantiomer as a function of CM-β-CD concentration with methanol—20 mM $NaH_2PO_4$ (pH 3.0), 35:50, v/v. The decrease in k′ with increasing CM-β-CD concentration observed in Figure 8 indicates that the adsorption of the complex to the hydrophobic stationary phase is smaller than that of the corresponding free molecule:

$$k'_G > k'_{G\text{–}CD}$$

which is consistent with the work of Sybilska et al.[16]

The capacity factors calculated for the free doxazosin enantiomers ($k'_G$) were comparable with those calculated for some chiral barbiturates earlier.[11] Capacity factors calculated for the corresponding doxazosin-CM-β-CD complex ($k'_{G\text{–}CD}$) were also comparable to those calculated earlier for the barbiturate-β-CD complexes.[1] Thus the re-

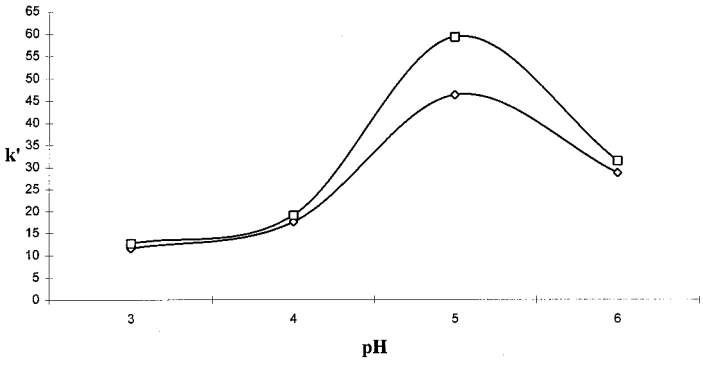



**Fig. 10.** Plot of capacity factor (k′) vs. pH for doxazosin enantiomers. Methanol–20 mM $NaH_2PO_4$ containing 15 mM CM-β-CD (15.3:50, v/v).

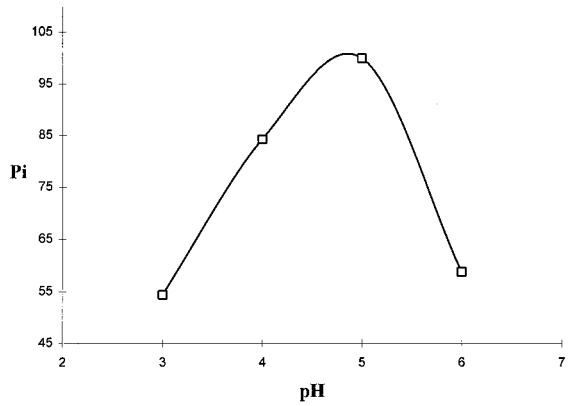



**Fig. 11.** Plot of $P_i$ vs. pH for *rac*-doxazosin. Conditions as in Figure 10.

versed-phase behaviour of doxazosin enantiomers is comparable with that of these chiral barbiturate enantiomers.

However, the stability constants ($K_G$) calculated for doxazosin complexed with CM-β-CD (647 ± 55 and 594 ± 45 $M^{-1}$ for each enantiomer respectively) are significantly larger than those calculated for the barbiturates complexed with β-CD (ca. 101–180 $M^{-1}$).[1] Additional sites for hydrogen bonding, increased steric interactions and additional ionic interactions with the anionic carboxymethyl derivative ($-CH_2COO^-$), or a combination of these three factors may be responsible for the large stability constants observed between doxazosin enantiomers and CM-β-CD.

The stability constants for each doxazosin enantiomer were calculated from the linear relationships observed in Figure 9 at pH 3.0. In the absence of any evidence for higher order association, it is suggested that no qualitative difference or deviation from linearity would be expected at higher pH values. This would not of course preclude quantitative differences being observed at higher pH values resulting from additional (or fewer) interaction leading to a greater (or lesser) degree of inclusion.

To investigate the ionic nature of this complexation process a series of mobile phases each having a different pH were prepared: methanol—20 mM $NaH_2PO_4$ containing 15 mM CM-β-CD (15:50 v/v). Figures 10 and 11 illustrate the effect of pH on the capacity factors (k′) for each doxazosin enantiomer and the Kaiser peak separation index respectively. It is quite clear that optimum enantioselectivity ($P_i$ = 1.0) is observed at pH 5.0.

CM-β-CD is thought to be partially anionic at pH 3.0 and fully anionic at all subsequent pH values.[23] *rac*-Doxazosin has a $pK_a$ of 6.93 and thus will be partially cationic at pH 6.0 and fully cationic at lower subsequent pH values. At pH 5.0 both CM-β-CD and *rac*-doxazosin are fully ionised. However, at pH 3.0 *rac*-doxazosin is fully ionised but CM-β-CD is not. Conversely at pH 6.0 CM-β-CD is fully ionised but *rac*-doxazosin is not. It is thus quite clear that there is an ionic or ion-pair interaction between the anionic CM-β-CD and the cationic doxazosin enantiomers.

The local optimum observed in Figures 10 and 11 should not be confused with the global optimum generated from the CCD. The observed enantioselectivity in Figures 10 and 11 corresponds to an overall analysis time of 70 min (k′ = 59.4), which is unacceptable. Location of the global optimum by the CCD through simultaneous multivariate optimisation of these three experimental parameters once again indicates the general utility of this approach. Notwithstanding the strong interactions between CM-β-CD and pH due to the ionic nature of the selector and selectand, the large factor space that was readily explored (Table 2) by experimental design did in fact allow the global optimum to be identified accurately and efficiently. This is an inherent strength of the CCD when applied to relatively simple systems.

## CONCLUSIONS

The CCD multivariate optimisation procedure has led to the rapid and efficient location of the optimum experimental conditions for the chiral resolution of *rac*-doxazosin in LC using the chargeable CM-β-CD chiral mobile phase additive. The statistical software package, using data from the CCD, generated three-dimensional response surfaces which clearly defined optimum experimental conditions. These response surfaces also provide valuable information on the zones of overall robustness and ruggedness for this chromatographic system.

It has been shown that stability constants for each doxazosin enantiomer and this chargeable CM-β-CD were significantly larger than those reported for a range of chiral barbiturates and β-CD. A detailed investigation was carried out to understand the nature of any ionic interactions. Optimum enantioselectivity was observed where both selectand *and* selector were *fully ionised* implying that ion-pairing was a significant factor in the overall mechanism involved. Further work utilising one- and two-dimensional NMR similar to that recently reported[24] would be required to elucidate fully this complex enantioselective mechanism.

## ACKNOWLEDGMENTS

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