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Modelling of conditions for the enantiomeric separation of β_2-adrenergic sympathicomimetics by capillary electrophoresis using cyclodextrins as chiral selectors in a polyethylene glycol gel

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Abstract

A two-factor central composite design was used to determine a mathematical model for prediction of the optimal conditions for the separation of the enantiomers of some widely used β_2-sympathicomimetic drugs (β_2-agonists) by capillary electrophoresis using cyclodextrins (CD) as a chiral selector in a polyethylene glycolgel. The effects of the chemical structure of these drugs along with the addition of polyethylene glycol to the cyclodextrin solution on the resolution of their enantiomers were studied. To allow impurity studies down to 0.1% (distomer–eutomer) a resolution of 2.5 should be warranted. Those β_2-agonists containing two hydroxylic groups in the aromatic ring structure show the highest enantiomeric separation, due to the fact that one of their enantiomers has a better geometric structure to fit into the β-cyclodextrin cavity.

Keywords: Enantiomeric separation; Cyclodextrins; Polyethylene glycol; Capillary electrophoresis; β_2-Sympathicomimetics; Optimisation; Mathematical model

1. Introduction

β_2-Sympathicomimetics (β_2-agonists) stimulate the sympathetic nervous system by reducing the tone of smooth muscle cells particularly in the lungs and the uterus. For that reason they are often used as vasodilator to decrease arterial blood pressure (oxedrine), as bronchodilator in the treatment of obstructive airway diseases (e.g. terbutaline, salbutamol), or as tocolytic agent (i.e. fenoterol, ritodrine) to avoid premature birth. Clenbuterol is a β_2-agonist that is not authorised by the European Union as a drug for human treatment but may be used for the treatment of bronchi-obstruction in cattle. When the therapeutic doses of β_2-agonists are exceeded,

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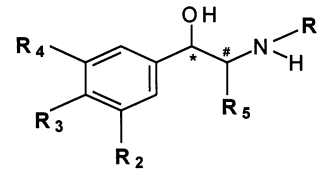
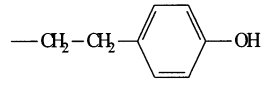
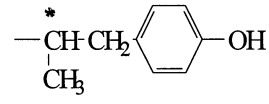
Table 1
Molecular structure of the used sympathicomimetics[a]



	Synonym	pK_a	R1	R2	R3	R4	R5
Terbutaline	Bricanyl Dracanyl	8.7 10.0 11.0	$-C(CH_3)_3$	OH	–H	–OH	–H
Salbutamol	Albuterol Ventolin	9.3 10.3	$-C(CH_3)_3$	$-CH_2OH$	–OH	–H	–H
Clenbuterol	Spiropent ventipulmin	N.A.	$-C(CH_3)_3$	–Cl	$-NH_2$	–Cl	–H
Oxedrine	Synephrine	9.3 10.2	$-CH_3$	–H	–OH	–H	–H
Isoprenaline	Isoproterenol	8.6 10.1 12.0	$-CH(CH_3)_2$	–OH	–OH	–H	–H
Ritodrine	Pre par	N.A.		–H	–OH	–H	$-CH_3$
Fenoterol	Berotec	8.5		–H	–OH	–H	$-CH_3$
	Airum	10.0					

[a] The general chiral centre is depicted with *. A possible second chiral centre (ritodrine) is depicted with an #. The second chiral centre in fenoterol in the R1 substituent is depicted with *.

these compounds act as growth promoters to improve meat-to-fat ratios in cattle. Residues of these compounds which are most abundant in liver and in meat are toxic to humans and can cause heart complications. Also the medical commission of the International Olympic Committee has listed the β_2-agonists as illicit agents for athletes when used in non-therapeutic doses.

The general molecular structure of these β_2-agonists (terbutaline, salbutamol, clenbuterol, fenoterol, ritodrine, oxedrine, and isoprenaline) along with their synonyms and their pK_a-values is given in Table 1.

These drugs are administered as a racemate, but pharmacological studies have shown that only one of the enantiomers has the desired therapeutic pharmacological effect [1]. For that reason it is of great importance that the enantiomers of such molecules can be fully baseline separated.

Basic enantiomers can be separated by capillary electrophoresis using cyclodextrins (CD) as a chiral selector [2–8]. CD's are commercially avail-

able oligosaccharides consisting of 6,7 or 8 D-(+)-glucopyranose units and they are designated as α, β, and γ-CD. Derivatization of the plain cyclodextrins leads to a large variety of CDs, all with their own selectivity in their interaction with chiral or non-chiral compounds. Derivatization of CD improves the solubility in water–methanol solutions [9].

In a recent study De Boer and Ensing have shown that the enantiomers of terbutaline can be readily separated resulting in a resolution that is satisfactory for enantiomeric impurity studies ($Rs > 2.5$) [10]. In that study the addition of polyethylene glycol 2000 (PEG 2000) showed a positive change in selectivity along with a decrease of the total amount of chiral selector necessary for one separation. In this paper it was tested if the observed phenomenon would also occur for some terbutaline-analogues. For this reason, the optimal conditions for the enantiomeric separation of the mentioned β_2-agonists were predicted by using a central composite design [11] as a chemometrical tool to generate an empirical model. The use of experimental designs in for instance pharmaceutical analysis has been well described in reviews by Corstjens et al. [12] and Altria et al. [13]. More specific was the use of a central composite design for the determination of the optimum conditions for analysis of ranitidine [14], amphetamines [15], and chlorophenols [16].

The hydroxypropyl-β-cyclodextrin (HP-β-CD) concentration and the PEG 2000 concentration (two-variable design) were chosen as the parameters for the selected design according to earlier described chiral optimisation models [17–19]. For this class of compounds HP-β-CD is the chiral selector of choice [10].

2. Experimental

2.1. Apparatus

The CE system was a Model PRINCE with a four position sample tray and a programmable injector system from Lauerlabs (Emmen, The Netherlands). Detection at 210 nm was carried out with a LAMBDA 1000 UV-VIS VWL detector (Bischoff, Leonberg, Germany). The fused-silica capillary with an outer polyimide coating (50 μm i.d., 375 μm, o.d.) was from Polymicro technologies (Phoenix, AZ). Data acquisition of CE/UV was performed by the Maclab system (ADinstruments, Castle Hill, Australia) using the Chart program (version 3.3, ADinstruments) for recording of the electropherograms. For interpretation of the electropherograms, the Peaks program (ADinstruments) was used. The vials used were 4 ml glass and were obtained from Phase Sep (Waddinxveen, The Netherlands).

2.2. Chemicals and solutions

Acetonitrile, Methanol, NaOH, and PEG 2000 all of pro analyse (p.a.) quality were obtained from Merck (Darmstadt, Germany). Hydroxypropyl-β-cyclodextrin (HP-β-CD), p.a. was obtained from Wacker-chemie GMBH (Munich, Germany). The used β_2-sympathicomimetics: terbutaline, 5-[2-[(1,1-dimethylethyl) amino]-1-hydroxyethyl]-1,3-benzenediol, salbutamol, [1]- [[(1,1-dimethylethyl) amino] methyl]-4-hydroxy-1,3-benzenedimethanol, clenbuterol, 4 - amino - 3,5-dichloro- -[[(1,1 - dimethylethyl) amino] methyl] benzenemethanol, fenoterol, 5- [1-hydroxy-2-[[2- (4-hydroxyphenyl)-1-methylethyl] amino] eth-yl]-1,3-benzenediol, ritodrine, 4- [1-hydroxy-2-[[2- (4-hydroxyphenyl) -ethyl] amino] propyl]-phenol, oxedrine, 4-[2-[(methyl amino]-1-hydroxyethyl]-phenol and isoprenaline,5-[2-[(1-methylethyl) amino]-1-hydroxyethyl]-1,2-benzenediol, were all racemates and of pharmacopoeial quality. Ritodrine and fenoterol were used in the study as mixtures of four stereoisomers. They were dissolved in a solution containing one part run-buffer and nine parts of water to a final concentration of 20 μg ml^{-1}. The CE run-buffer was prepared by dissolving sodium dihydrogen phosphate monohydrate (p.a., Merck) to a concentration of 100 mM and adjusting the pH with concentrated *ortho*-phosphoric acid (p.a. 85%,Merck) to a pH of 2.5 giving a conductivity of 7.0 mS cm^{-1}. Water was purified with a Milli-Q system (Millipore, Bedford, MA). The conductivity of the purified water was always less than 2 μS cm^{-1}.

All solutions were filtered through a membrane filter (0.45 μm) and degassed for 5 min in an ultra-sonic bath (50 kHz, Branson Europa B.V., Soest, The Netherlands), immediately prior to use.

2.3. CE conditions used for experiments

A capillary with a total length of 70 cm and an effective length of 55 cm was used. An optical viewing window with a length of 0.5 cm, obtained by burning off the polyimide coating, was aligned with the UV detection cell. The coating of the first 2 mm of the capillary was also stripped.

New capillaries were rinsed with 1 M sodium hydroxide for 10 min at 1000 mBar, with water for 10 min at 1000 mBar and with the run buffer for 10 min at 1000 mBar, respectively.

Cyclodextrins and polyethylene glycol were dissolved in the run buffer and hydrodynamically injected as a removable gel until the capillary was fully filled. The latter was monitored by UV-detection. The analytes then were electrokinetically injected in order to avoid the PEG/CD gel to be forced out. The injection (10 kV, 6 s) and separation voltage (30 kV) were ramped at 6 kV s^{-1} and took place at a constant temperature of 15°C. After each run the capillary was refilled with fresh PEG/CD gel. Because we inject the removable liquid gel directly into the capillary instead of adding it to the ground-electrolyte, the electro-osmotic flow (EOF) should be suppressed to avoid the removable gel to be forced out of the capillary. The latter is partially accomplished by the increased viscosity, but a working pH of the ground-electrolyte of $pH < 3$ will generally eliminate the EOF. This approach implies that the analyte(s) can only be introduced into the capillary by electrokinetic injection. Despite the fact that electrokinetic injection increases the sensitivity due to stacking, some precautions with respect to the amount of injected analyte(s) should be taken into account [20–23]. Especially possible vibrations that can occur during the injection process should be excluded and a constant volume in the sample should be warranted [24]. After the injection, the electrode and the capillary-end were dipped in a vial containing water. The separation was started when the ground electrode and the capillary-end were placed into the vial containing the run buffer.

3. Statistical methods for experiments

Chemometrical analysis was performed with a Design-Expert® program, version 3.05 (Stat-Ease, Minneapolis, MN). Contour-plots were produced by Matlab®: high performance numeric computation and visualisation software (Natick, MA).

4. Results and discussion

A two-factor central composite design was used to obtain data for the fitting of a second-order polynomial model that is defined as $y_{1i} = b_0 + b_1x_{1i} + b_2x_{2i} + b_{11}x_{1i}^2 + b_{22}x_{2i}^2 + b_{12}x_{1i}x_{2i} + e_{1i}$ [11] where x_1 and x_2 indicate the representative variables (quantitative factors): percentage PEG 2000 and concentration HP-β-CD, respectively. A two-factor design (i.e. x_1 and x_2) results in $2^k + (2*k) + 1 =$ nine experimental conditions. In order to estimate the experimental uncertainty, the center point of the design is measured another three times, resulting in twelve experiments for each sympathicomimetic drug that take place in random order. The window wherein the design was selected was restricted by the maximum concentrations of PEG 2000 (10%) and CD (25 mM). Larger concentrations gave rise to strong baseline fluctuations with the consequence that the efficiencies became very low or even that peaks could not be detected. The corner points of the design were selected according to previous experiments for the enantiomeric separation of terbutaline [10].

For example, the experimental conditions for the enantiomeric separation of clenbuterol were tested in random order and are presented along with the corresponding resolutions (y_{1i}) in Table 2. The coefficients of the mathematical model were obtained by matrix calculation via $b = (\mathbf{X}'\mathbf{X})^{-1}(\mathbf{X}'y)$, where $\mathbf{X}$ is a matrix containing the columns which contain the coefficients of the

Table 2
Experimental conditions for the enantiomeric seperation of clenbutorol along with the obtained resolutions and the calculated polynomial

PEG 2000 (%) = x_1	HB-β-CD (mM) = x_2	Rs
6	15	2.1
6	15	2.2
6	15	2.0
6	15	2.2
4	10	1.5
8	10	1.7
8	20	1.6
9	15	2.0
6	8	1.2
6	22	1.6
4	20	2.5
3	15	1.9

$Rs = 2.125 - 0.070x_{1i} + 0.360x_{2i} - 0.113x_{1i}^2 - 0.138x_{2i}^2 - 0.275x_{1i}x_{2i} + e_{1i}$

model parameters for each experiment. Finally the polynomial can be given as $Rs = 2.1 - 0.07x_{1i} + 0.36x_{2i} - 0.11x_{1i}^2 - 0.14x_{2i}^2 - 0.28x_{1i}x_{2i} + e_{1i}$. The adequacy of the model is summarised by the coefficient of (multiple) determination ($R^2 = 0.846$) and by the F-ratio for lack of fit ($F_{LOF} = 0.05$) [11]. Using a 95% significance level the model is probably adequate, i.e. there is no lack of fit, when the $F_{LOF} \geq 0.05$. It is important to realise that the polynomial is only valid for the tested ranges, i.e. 3–9% PEG 2000 and 8–22 mM HP-β-CD.

The results of the calculated polynomial are visualised as a contour plot in Fig. 1b. In the same manner experiments were completed for the other β_2-agonists. The resulting polynomials, the concentrations needed to warrant the aimed resolution of 2.5 or higher and the adequacy of the models are given in Table 3. As can be seen in Table 3, the calculated models for isoprenaline and oxedrine exhibit a significant lack of fit ($F_{LOF} = 0.016$ and 0.015, respectively).

For a good separation of the enantiomers of the β_2-agonists, a three point interaction or more of the hydroxyl and amino groups of one of the sympathicomimetic enantiomers with the hydroxyl groups of the cyclodextrins and a complexation of the (substituted) aromatic ring of this enantiomer with the inner hydrophobic moiety of the cyclodextrin cavity is necessary [25,26]. We try to explain the data given in Table 3 combined

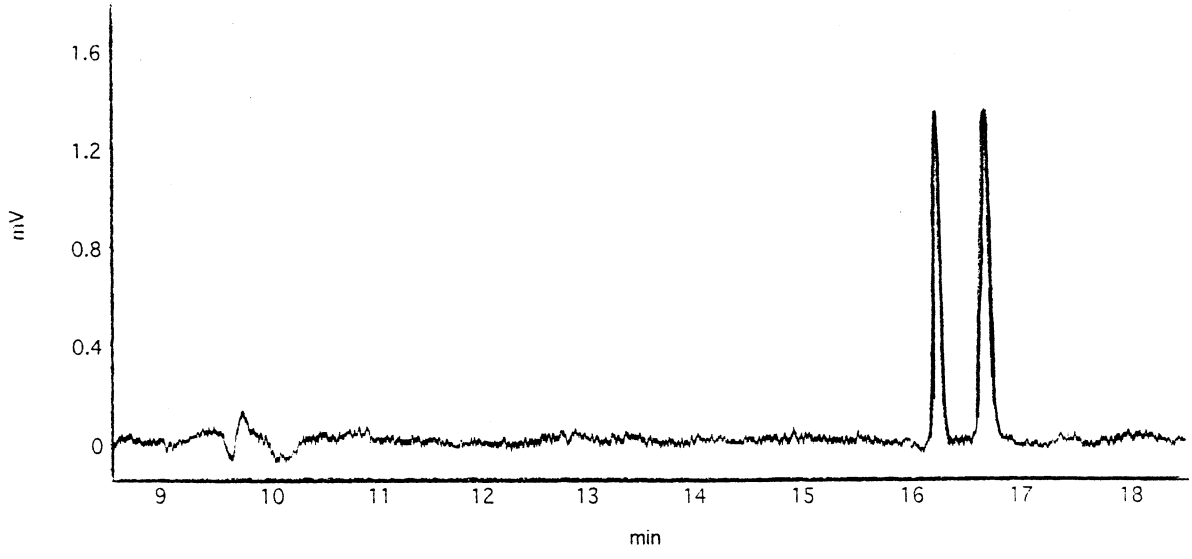


Fig. 1. The electropherogram of the enantiomeric seperation of clenbuterol using 20mM HP-β-CD dissolved in a 4% PEG2000 gel at 15°C. Electrokinetic injection, 10 KV, 6 s; seperation, 30 KV.

Table 3
The calculated polynomials with their adequacy of the β-agonists along with the conditions that will warrant a resolution of 2.5 or higher[a]

Cat	Compound	Second order polynomial	PEG 2000 (%) = x_1	HP-β-CD (Mm) = x_2	Rs
A	Terbutaline	$Rs = 3.2 + 0.073x_{1i} + 0.70x_{2i} + 0.40x_{1i}^2 - 0.027x_{2i}^2 + 0.48x_{1i}x_{2i} + e_{1i}$ $R^2 = 0.884$; F-ratio (LOF) = 0.153	5.9	10.0	2.5
A	Isoprenaline	$Rs = 1.5 + 0.17x_{1i} + 0.32x_{2I} + 0.094x_{1i}^2 - 0.032_{2i}^2 + 0.0000x_{1i}x_{2i} + e_{1i}$ $R^2 = 0.869$; F-ratio (LOF) = 0.016	9.4*	24.4*	2.5
A	Fenoterol	$Rs = 1.7 - 0.62x_{1i} - 0.41x_{2i} - 0.53x_{1i}^2 - 0.33x_{2i}^2 + 0.050x_{1i}x_{2i} + e_{1i}$ $R^2 = 0.824$; F-ratio (LOF) = 0.0676	4.7	11.7	2.0
B	Salbutamol	No separation			
C	Ritodrine	$Rs = 1.2 - 0.10x_{1i} + 0.088x_{2i} - 0.025x_{1i}^2 + 0.025x_{2i}^2 + 0.050x_{1i}x^{2i} + e_{1i}$ $R^2 = 0.592$; F-ratio (LOF) = 0.389	6.0	25*	1.5
C	Oxedrine	$Rs = 1.0 + 0.029x_{1i} + 0.16x_{2i} + 0.0234x_{1i}^2 - 0.080x_{2i}^2 - 0.064x_{1i}x_{2i} + e_{1i}$ $R^2 = 0.838$; F-ratio (LOF) = 0.0146	10.0*	15.5	1.1
D	Clenbuterol	$Rs = 2.1 - 0.07x_{1i} + 0.36x_{2i} - 0.11x_{1i}^2 - 0.14x_{2i}^2 - 0.28x_{1i}x_{2i} + e_{1i}$ $R^2 = 0.846$; F-ratio (LOF) = 0.0501	4.0	19.0	2.5

[a] Polynomials are valid for the ranges 3–9% PEG 200 (x_1) and 8–22 mM HE-β-CD (x_2).
* When the predicted concentrations are outside the tested ranges, the values are marked with an asterisk.

with the molecular structures and their pK_a values given in Table 1. Because the pK_a values of the tested compounds are practically the same and all compounds are fully protonated at the working pH 2.5, differences in resolutions can be attributed to differences in their molecular structure only.

We divided the analysed compounds in four categories. (A) compounds with two phenolic-hydroxy groups in the aromatic ring structure. (B) compounds with a phenolic hydroxy group and an aliphatic hydroxy group in the aromatic ringstructure. (C) compounds with one phenolic hydroxy group in the aromatic ringstructure. (D) other substituents in the aromatic ringstructure.

To allow enantiomeric impurity profiling down to 0.1% a resolution of 2.5 is necessary, as can be calculated from the theoretical overlap of symmetrical peaks by simple overlap studies as recently mentioned by De Boer and Ensing [10]. The resolution is defined as the extent of separation between two compounds and is formally calculated by $R = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are the migration times and w_1 and w_2 the peakwidths at the baseline of the more mobile (1) and the less mobile (2) analyte. According to Table 3 compounds belonging to category A (fenoterol, terbutaline, and isoprenaline) and to category D (clenbuterol) give the best resolution. It appears that the largest discrimination between the enantiomers is found were the β_2-mimetics contain substituents in the aromatic ringstructure and especially on the $R2$ and $R4$ position (terbutaline and clenbuterol), due to the fact that one of their enantiomers has a better geometric structure to fit into the β-cyclodextrin cavity. The electropherogram of the enantiomeric separation of clenbuterol is shown in Fig. 1. In Fig. 2(I) and Fig.

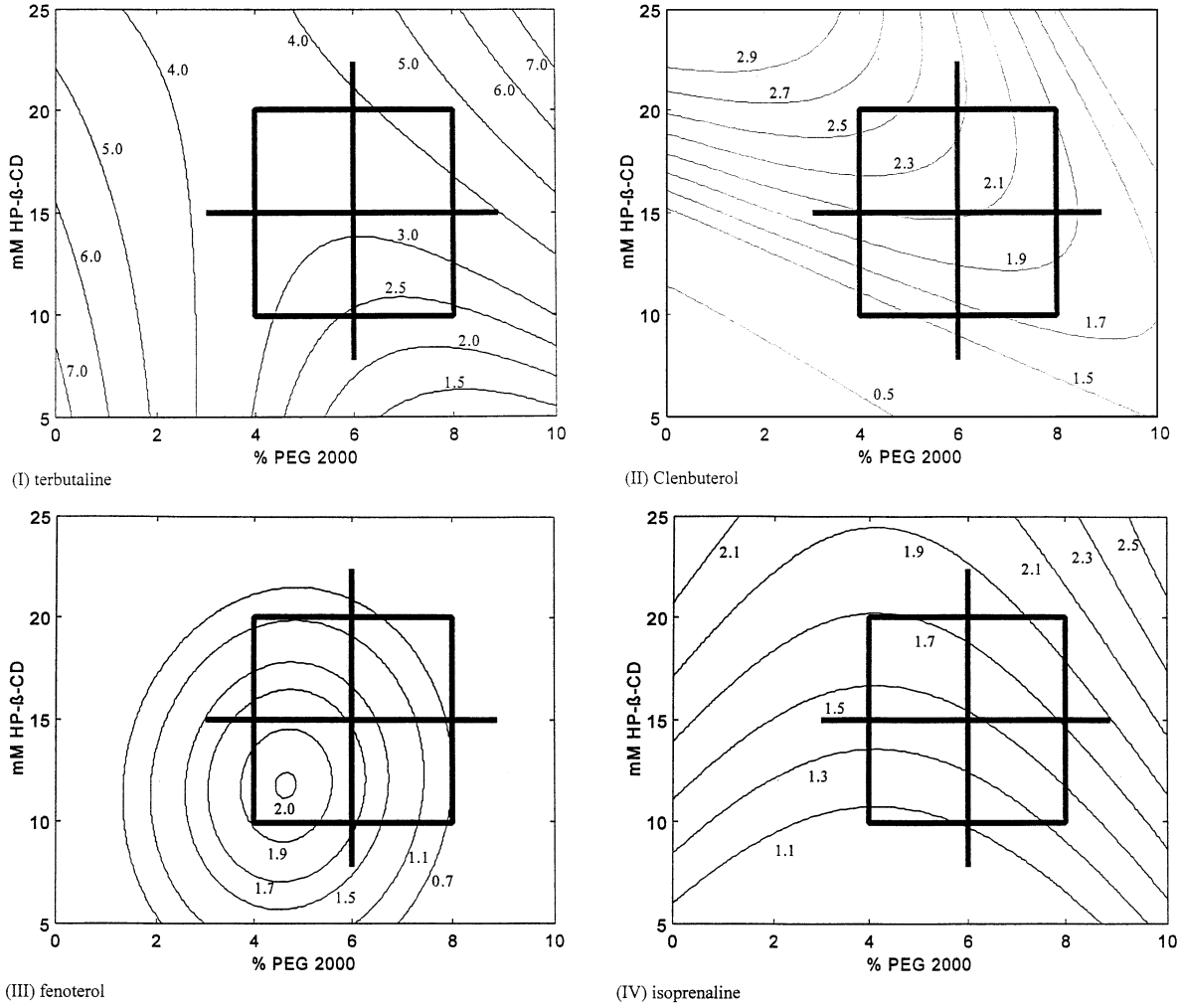


Fig. 2. The calculated contourplots of the β-agonist belonging to category A and D. (I) terbutaline; (II) clenbuterol; (III) fenoterol; and (IV) isoprenaline. The used central composite design is designated in the figure.

2(II) is shown that for clenbuterol and terbutaline, respectively the minimal resolution we were aiming for (2.5) is readily achieved within the tested design without the necessity for further optimisation.

In Fig. 2(II) can also be seen that the addition of PEG 2000, referring to the contour responding to a resolution of 2.5, results in a decrease of the necessary concentration of CD's. The same contour in Fig. 2(I) shows a different effect of the addition of PEG 2000. This phenomenon can not be explained at the moment. In both cases however should the optimal conditions for a satisfactory result (i.e. a resolution of 2.5) be achieved with the lowest concentration possible for either component.

For isoprenaline, which contains two lipophilic substituents located on $R2$ and $R3$, the minimal resolution cannot be obtained within the selected design but is still situated within the restricted

window area (10% PEG 2000 and 25 mM CD), therefore further optimisation is necessary. The latter could for instance be accomplished by using a two-factor simplex experimental design [11,12]. Fenoterol which has also the apparently essential lipophilic substituents on $R2$ and $R4$ contains also a large $R1$ substituent that appears to decrease the resolution compared to the above described compounds. Although fenoterol has two chiral centers, only one pair of enantiomers can be separated within the used maximum separation time (i.e. 30 min). A resolution of 2.5 in this case cannot be accomplished within the used restricted window area.

Apparently the methoxygroup of salbutamol on the $R2$ position, category B, prevents separation of the enantiomers because no resolution is obtained. When we compare the compounds of category C with the compounds of the other categories, we observe that for both compounds no baseline separation can be obtained.

5. Conclusion

The use of HP-β-CD as a chiral selector in a polyethylene glycol solution appears to be an adequate method to separate the enantiomers of the β_2-sympathicomimetics. The addition of PEG changes the selectivity but does not always result in an increase in resolution. In most cases addition of PEG results in a lower concentration of CD's necessary to obtain the desired minimum resolution of 2.5 necessary for the detection of impurities as low as 0.1% (distomer–eutomer).

The enantiomers of compounds containing two hydroxylic groups substituted at the aromatic ring could be readily separated, resulting in the aimed resolution, due to a higher complexation with the cyclodextrins of one of the enantiomers. The selected design within the restricted window area (10% PEG 2000 and 25 mM CD) appears to be adequate for calculation of a mathematical model for a fast optimisation of the separation. If the desired resolution is not achieved within the valid range of this two-factor composite design, further optimisation could be carried out with a two-factor simplex design. A better resolution for the compounds of category B and C can probably be achieved by using other types of cyclodextrins.

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