J. Iran. Chem. Soc., Vol. 5, No. 4, December 2008, pp. 553-558.

JOURNAL OF THE
Iranian
Chemical Society

Enantiomeric Separation of Racemic Clenbuterol by Capillary Zone Electrophoresis

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(Received 12 April 2007, Accepted 16 November 2007)

A method for capillary electrophoretic enantiomeric separation of a racemic clenbuterol has been established with hydroxypropyl-β-cyclodextrin as the chiral selector. General equations and data analysis are presented to relate mobility to the equilibrium constants in simple binding equilibria and used to determine binding constants and thermodynamic parameters for host-guest complexation of clenbuterol enantiomers with hydroxypropyl-β-cyclodextrin as a selector. The effects of β-cyclodextrin type and concentration, buffer type, concentration and pH, as well as separation voltage and capillary temperature were investigated in detail. A maximal resolution of 6.78 was obtained. The binding constants of the host-guest complex of clenbuterol enantiomers with hydroxypropyl-β-cyclodextrin, $K_{R\text{-}CD}$ and $K_{S\text{-}CD}$ are 22.50 and 43.09 l mol^{-1}, respectively.

Keywords: Electrophoretic enantiomeric separation, Clenbuterol enantiomers, Hydroxypropyl-β-cyclodextrin, Binding constants, Thermodynamic parameters

INTRODUCTION

Clenbuterol, a β-adrenergic agonist, is a sympathomimetic drug with potent β_2-adrenoceptor stimulating properties. Used for the treatment of pulmonary diseases, it is also applied in veterinary medicine as a tocolytic agent. Due to lipolytic and anabolic effects at higher doses, clenbuterol has been illegally applied as a growth promoter in the meat-producing industry and as a doping agent in sports. The (-)-*R*-enantiomer is reportedly responsible for the mimetic effect on β_2-receptors, while the (+)-*S*-enantiomer has a blocking effect on the β_1-receptors [1-4].

High performance liquid chromatography (HPLC) [1,2] and capillary zone electrophoresis (CZE) [3,4] were commonly used to resolve the enantiomers of clenbuterol.

Compared to HPLC, CZE has recently emerged as a promising analytical technique that consumes an extremely small amount of sample and is capable of rapid, high-resolution separation, with reproducible results. In order to evaluate the feasibility of the CZE resolution of clenbuterol using β-cyclodextrins (β-CDs) as chiral additives, we studied the following analysis conditions: β-CD type and concentration, buffer type, concentration and pH, separation voltage and capillary temperature. Binding constants and thermodynamic parameters were evaluated for clenbuterol enantiomers using hydroxypropyl-β-cyclodextrin (HP-β-CD) as a chiral separator.

EXPERIMENTAL

Materials and Methods

All experiments were performed using a P/ACE MDQ capillary electrophoresis system equipped with diode array detection (Beckman Coulter, Fullerton, CA, USA). The temperature of the cartridge holding the capillary column was maintained at 20 ± 0.1 ºC by the liquid cooling system of the

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P/ACE instrument. The separations were monitored at 214 nm, with the analytical voltage maintained at 24 kV. The system was computer-controlled using the integrated P/ACE Station software (32 Karat^{TM} version 7.0). Uncoated fused silica capillaries (Hebei Yongnian Optical Fiber Factory, China), 60.2 cm × 50 μm i.d., were used with the detection window 10 cm from the capillary outlet. Before use, each capillary was rinsed with 1 mol l^{-1} NaOH (5 min), 0.1 mol l^{-1} NaOH (5 min), water (10 min) and running buffer (5 min). Conditioning between measurements included rinsing with 0.1 mol l^{-1} NaOH (3 min), water (3 min) and running buffer (2 min). We used a hydrodynamic injection mode employing 20 mbar of pressure for 5 s.

Chemicals

β-CD was purchased from Beijing Abxing Biological Technology Co., Ltd. in China, dimethyl-β-cyclodextrin (DM-β-CD, nominal degree of substitution, ~4.4) was purchased from Shandong Xinda Fine Chemical Co., Ltd. in China, and HP-β-CD (nominal degree of substitution, ~4.5) was obtained from Jiangsu Yiming Fine Chemical Co., Ltd. in China. Racemic clenbuterol hydrochloric acid was obtained from Shanghai Dazhong Pharmaceuticals, China. Other chemicals were of analytical grade. Water was deionized and doubly distilled.

Sample and Buffer Preparation

Racemic clenbuterol was dissolved in a 30 mmol l^{-1} HCl solution at a concentration of 250 μg ml^{-1}. Working solutions were made by further appropriate dilutions. All solutions were stored at 4 ºC. The running buffer was prepared by dissolving an exact amount of the chiral selector in a pH range of 2.0-4.5. The electrolyte solutions were filtered through a 0.45-μm membrane filter and sonicated prior to use. For pH measurements a model PHS 25 pH meter (Orion, model 818, Shanghai, China) equipped with a combined glass electrode was used.

RESULTS AND DISCUSSION

The most important step in developing a chiral separation method is choosing the type and concentration of the chiral selector. Optimization of a number of other experimental conditions, such as the buffer type, concentration and pH, and separation temperature and voltage, contribute to the highest degree of separation.

Effects of Type and Concentration of Chiral Selector

The use of native β-CD, HP-β-CD and DM-β-CD as chiral selectors for the resolution of clenbuterol, in a 50 mmol l^{-1} $H_3PO_4/H_2PO_4^-$ buffer (pH 2.5), containing 10 mmol l^{-1} β-CD, resulted in resolutions (*Rs*) of 2.33, 3.18 and 2.47, respectively. As noted in Fig. 1, better separation was achieved with HP-β-CD, perhaps because the mouth of the β-CD hydrophobic cavity is surrounded by secondary hydroxyl groups, considered to be important in chiral recognition. In a derivatized HP-β-CD, some hydroxyl groups are substituted with hydroxypropyl functional groups, allowing for a more stereospecific and stronger interaction between the hydroxyl groups and hydrogen-bonding moiety present in the clenbuterol structure [5].

According to Wren and Rowe's model [6,7], the enantiomeric pair can be best resolved at an optimal chiral selector concentration, which is dependent on the binding strength between the chiral selector and the solute, as solutes that interact weakly typically require higher chiral selector concentrations. The effect of several HP-β-CD concentrations (5-80 mmol l^{-1}) on the resolution of clenbuterol in

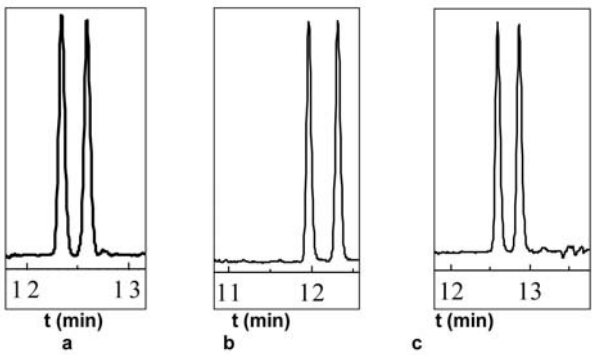

Fig. 1. Effect of chiral selector on the enantioseparation of clenbuterol. Conditions: 60/50 cm × 50 μm i.d. fused-silica capillary, 24 kV, 20 ºC, λ = 214 nm; 50 mmol l^{-1} H_3PO_4/HPO_4^- buffer (pH 2.5); injection: 20 psi during 5 s; (a) 10 mmol l^{-1} β-CD; (b) 10 mmol l^{-1} HP-β-CD; (c) 10 mmol l^{-1} DM-β-CD.

$H_3PO_4/H_2PO_4^-$ buffer (50 mmol l^{-1}, pH 2.5) is shown in Fig. 2. An increase in the concentration of HP-β-CD results in faster migration velocities of the HP-β-CD complex and better peak shape of the enantiomers. It is known that a difference in the mobility ($\Delta\mu$) between two enantiomers will reach a maximum at a particular chiral selector concentration, which depends on the affinity of the enantiomers for the chiral selector [6-8]. Considering the data shown in Fig. 2, it is clear that the optimum selector concentration range is quite narrow, at approximately 30 mmol l^{-1}. In other words, the enantiomers of clenbuterol show the greatest difference in mobility at 30-35 mmol l^{-1} of HP-β-CD.

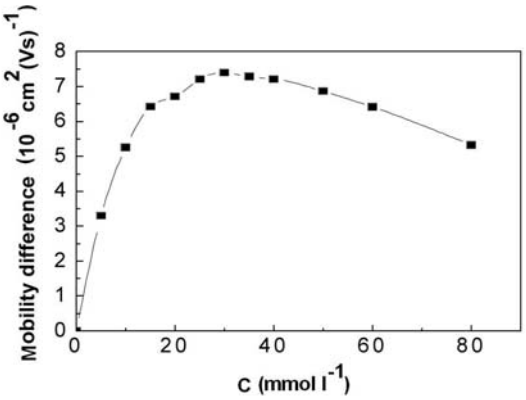

Fig. 2. Effect of HP-β-CD concentration on enantioseparation of clenbuterol. Conditions: 30 mmol l^{-1} HP-β-CD; other conditions as stated in Fig. 1.

Effect of Buffer

In this work, tris [hydroxymethyl] aminomethane (TRIS), and triethanolamine (TEOA), both of which are organic bases used to regulate pH, were also tested as counterions in phosphate, borate, acetate (Ac), and citrate (CIT) buffers at pH 2.5. When their effects on the separation selectivity were compared, a distinct difference was observed when using $H_3PO_4/H_2PO_4^-$ as the buffer (Table 1). A better chiral separation of clenbuterol was achieved when using 50 mmol l^{-1} $H_3PO_4/H_2PO_4^-$ buffer (pH 2.5) containing 30 mmol l^{-1} HP-β-CD with *Rs* of 6.78. It was found that the TEOA and TRIS cations compete with the analyte, to some extent, for the hydrophobic cavity of HP-β-CD, which reduces the enantiomeric resolution of clenbuterol [8]. For this reason, the $H_3PO_4/H_2PO_4^-$ buffer is generally more suitable for the chiral separation of clenbuterol.

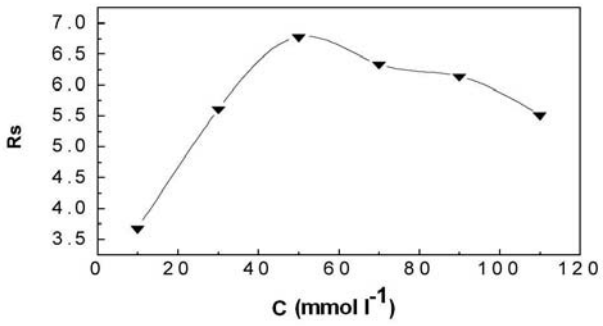

Fig. 3. Influence of phosphate buffer concentration (*C*) on resolution (*Rs*). Conditions: as given in Fig. 2.

The effect of the $H_3PO_4/H_2PO_4^-$ buffer concentration on the resolution of *R*/*S*-clenbuterol is demonstrated in Fig. 3. The influence of buffer concentration on the generation of electric current was tested. The current generated increases with increasing buffer concentrations. The better baseline separations were achieved with 40-60 mmol l^{-1} phosphate at pH 2.5 and 30 mmol l^{-1} of HP-β-CD. In order to prevent the generation of extensive Joule heat, a 50 mmol l^{-1} phosphate buffer concentration was chosen as optimal.

Table 1. Influence of Buffer on *Rs* and $\Delta\mu$ [10^{-6} cm^2 $(V\ s)^{-1}$]

	H_3PO_4/Na_2HPO_4	HCIT/NaCIT	HAc/NaAc	$H_3PO_4/Na_2B_4O_7$	H_3PO_4/TEOA	HCIT/TEOA	H_3PO_4/TRIS	HCIT/TRIS
Rs	6.78	3.74	2.67	5.95	6.74	3.37	5.70	2.38
$\Delta\mu$	7.39	1.57	2.65	6.90	7.18	6.32	6.69	6.23

Conditions: As given in Fig. 2.

Effect of pH

Clenbuterol is positively charged at acidic values and enantiomeric separation of cationic substances is best performed at low pH [8,9]. The baseline resolution was obtained in a pH range of 2.5-4.2; worse resolution and faster migration times were observed at higher pH (Fig. 4). This electrophoretic behavior of the analytes is probably related to the change in the electroosmotic flow (EOF), which at pH > 4.5 became considerable, resulting in short migration times. Accordingly, there was not enough time allowed for interactions between the used CD and the analyte. Also, the enantioseparation mechanism involves the formation of inclusion complexes that could be unstable at pH > 4.5. Furthermore, at a higher pH, the degree of the drug molecule protonation is smaller, which may allow the analyte molecule to fit into the chiral selector CD cavity better, producing a faster migration for the resultant negatively charged complex. Therefore, the appropriate pH was 2.5.

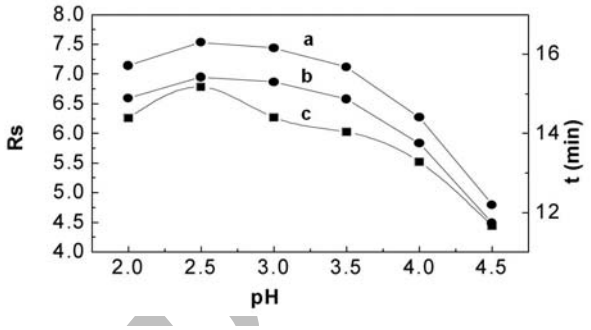

Fig. 4. Influence of pH on *Rs* and migration time: a) *Rs*; b) $t_{S\text{-clenbuterol}}$; c) $t_{R\text{-clenbuterol}}$. Conditions are as stated in Fig. 2.

Effect of Analytical Voltage and Capillary Temperature

The electrophoretic velocities are directly proportional to the electric field strength. The limiting factor here is Joule heating. The results show that the resolution of enantiomers increases with increasing voltage. The optimal voltage was set at 24 kV, which ensured short migration time, acceptable current generated and good resolution [8,10].

Changes in the capillary temperature can lead to changes in the mobility of the analyte (Fig. 5). A reduction in resolution at higher temperatures is due to a decrease in the formation constant of the analyte-chiral selector complex during the chiral separation and an increase in the solute diffusion. Apparently, the complex-formation interaction of clenbuterol with HP-β-CD is an exothermic reaction and the lower temperature supports the formation of the complex [10]. Therefore, the capillary temperature is maintained at 20 ± 0.1 ºC to optimize the analysis of the racemic drug.

The optimized conditions thus obtained for enantioseparation of clenbuterol are as follows: HP-β-CD concentration = 30 mmol l^{-1}, buffer solution = 50 mmol l^{-1} $H_3PO_4/H_2PO_4^-$, at pH 2.5, electric field strength = 24 kV, temperature = 20 ± 0.1 ºC, detection wavelength = 214 nm. The enantioseparation of clenbuterol obtained under the optimal conditions described in above mentioned sections is shown in Fig.6.

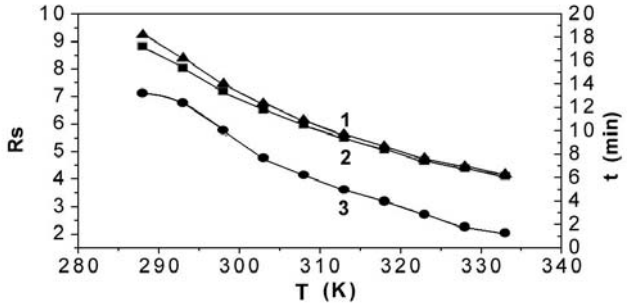

Fig. 5. Effect of temperature on *Rs* and migration time: 1) $t_{\boldsymbol{S}\text{-clenbuterol}}$; 2) $t_{\boldsymbol{R}\text{-clenbuterol}}$; 3) *Rs*. Conditions: as given in Fig. 2.

Calculation of Thermodynamic Parameters and Binding Constants

Temperature is an important factor in controlling chiral recognition processes. Conventionally, the relationships of capillary temperature with thermodynamic parameters are represented as [11,12]:

$$-\Delta\Delta G^0 = RT\ln\alpha \quad (1)$$

$$\Delta_{R,S}\Delta G^\circ = \Delta_{R,S}\Delta H^\circ - T\Delta_{R,S}\Delta S^\circ \quad (2)$$

$$\ln\alpha = \frac{\Delta_{R,S}\Delta H^0}{RT} + \frac{\Delta_{R,S}\Delta S^0}{R} \quad (3)$$

where $\Delta_{R,S}\Delta G°$, $\Delta_{R,S}\Delta H°$ and $\Delta_{R,S}\Delta S°$ represent the differences in free energy, enthalpy and entropy, respectively, for a given pair of enantiomers. The separation factor is α, R is the gas constant and T is the absolute temperature. Thermodynamic parameters were measured over a temperature range of 15-60 ºC using 30 mmol l^{-1} HP-β-CD in 50 mmol l^{-1} $H_3PO_4/H_2PO_4^-$ buffer (pH 2.5). Three determinations were made at each temperature. Figure 7 gives van't Hoff plots, which are highly linear ($r^2 > 0.9997$). The corresponding thermodynamic parameters can be obtained from the slope or intercept of the straight lines: $\Delta_{R,S}\Delta H$ = -0.626 kJ mol^{-1} and $\Delta_{R,S}\Delta S$ = -1.71 J mol^{-1} K^{-1}. These negative values and $|\Delta(\Delta H^0)| > |T\Delta(\Delta S^0)|$ indicate that the separation is enthalpy driven, the inclusion process is exothermic, and the entropy term is unfavorable to chiral recognition.

As electrophoretic mobility of the analyte increases with CD concentration, the adjusted electrophoretic mobility may therefore be determined by multiplying the experimentally-determined value by the ratio of the current at zero HP-β-CD concentration by that at the concentration of interest, which can be defined as:

$$\frac{1}{\mu_S - \mu_S^{eff}} = \frac{1}{(\mu_S - \mu_{S-CD})\cdot k_{S-CD}\cdot c} + \frac{1}{\mu_S - \mu_{S-CD}} \quad (4)$$

$$\frac{1}{\mu_R - \mu_R^{eff}} = \frac{1}{(\mu_R - \mu_{R-CD})\cdot k_{R-CD}\cdot c} + \frac{1}{\mu_R - \mu_{R-CD}} \quad (5)$$

where μ_S, μ_S^{eff}, $\mu_{S\text{-}CD}$, $K_{S\text{-}CD}$ and c represent the electrophoretic mobility, the apparent electrophoretic mobility of *S*-enantiomer, the electrophoretic mobility of inclusion, binding constant of the *S*-enantiomer to the CD and the CD concentration, respectively [12,13]. The *R*-enantiomer is described in the same way. According to Fig. 8, the plot of $1/(\mu_S\text{-}\mu_S^{eff})$ and $1/(\mu_R\text{-}\mu_R^{eff})$ *vs.* $1/C$ was highly linear (*S*-enantiomer: Y = 22.140x + 0.954, r^2 = 0.9988, $K_{S\text{-}CD}$ = 43.09 l mol^{-1}; *R*-enantiomer: Y = 36.719x + 0.826, r^2 = 0.9942, $K_{R\text{-}CD}$ = 22.50 l mol^{-1}). From the optimum concentration of the chiral selector calculation:

$$C = \frac{1}{\sqrt{K_{S-CD}K_{R-CD}}} \quad (6)$$

the optimum concentration is 32.5 mmol l^{-1}, which is approximately equal to the experimental value.

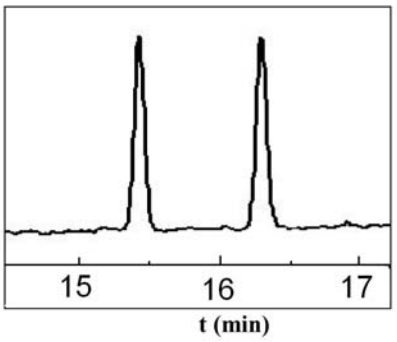

Fig. 6. Enantioseparation of clenbuterol under the optimal conditions given in Fig. 2.

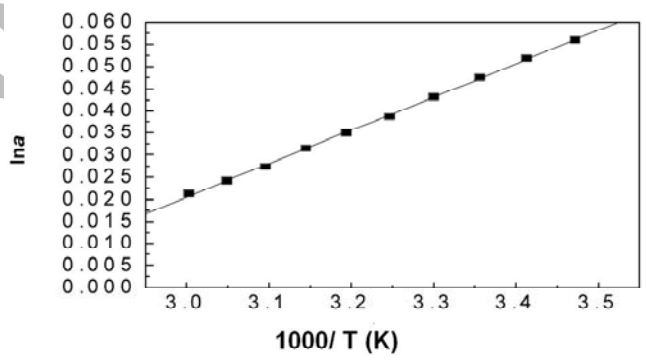

Fig. 7. lnα *vs.* $1/T$ for enantioseparation of clenbuterol. Conditions: as given in Fig. 2.

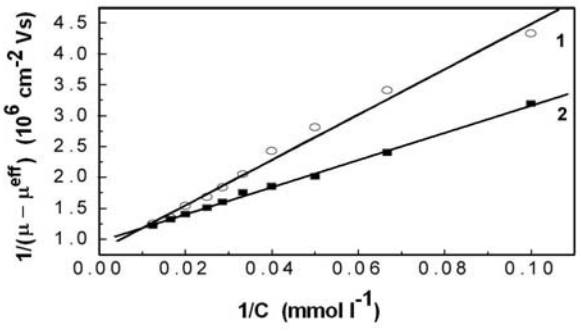

Fig. 8. $1/(\mu\text{-}\mu^{eff})$ *vs.* $1/C$ for the enantioseparation of clenbuterol: 1) *R*-clenbuterol; 2) *S*-clenbuterol. Conditions: As given in Fig. 1.

CONCLUSIONS

The optimized CZE method is suitable for the separation of clenbuterol if used with a low-pH $H_3PO_4/H_2PO_4^-$ buffer and a low EOF in an uncoated fused silica capillary. This method, tested using three types of β-CD chiral selectors, successfully achieved enantioseparation of *R,S*-clenbuterol, in which HP-β-CD is much more effective in the enantioseparation than the other forms of β-CD. Efficient chiral separation of clenbuterol was achieved within 17 min using the optimized conditions of 30 mmol l^{-1} HP-β-CD in $H_3PO_4/H_2PO_4^-$ buffer at pH 2.5, operated at 24 kV and 20 ± 0.1 ºC, using a detection wavelength of 214 nm (described in Fig. 6).

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of China (20776162) and by the Postdoctoral Science Foundation of Central South University.

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