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Article

Capillary Electrophoresis of Clenbuterol Enantiomers and NMR Investigation of the Clenbuterol/Carboxymethyl-β-cyclodextrin Complex

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A capillary electrophoretic method has been established for the separation of the enantiomers of clenbuterol. The effects of pH value, composition of the background electrolyte, concentration of carboxymethyl-β-cyclodextrin (CM-β-CD), capillary temperature and running voltage have been investigated. The two enantiomers were separated in an uncoated capillary with phosphate buffer (50 mmol/L, pH 3.5) containing 10 mmol/L CM-β-CD. The capillary temperature was at 15°C and applied voltage was at 20 kV. The inclusion complex of CM-β-CD and clenbuterol was synthesized and characterized by two-dimensional rotating frame spectroscopy (2D ROESY). Based on the 2D ROESY analysis, an inclusion structure of the clenbuterol/CM-β-CD complex was proposed, in which clenbuterol penetrated CM-β-CD in a tilted manner due to the interaction of intermolecular hydrogen bonds between clenbuterol and CM-β-CD.

Introduction

Cyclodextrins (CDs), cyclic oligosaccharides whose molecules have hydrophilic outer surfaces and a hydrophobic cavity at the center, have been extensively investigated because they act as host molecules or containers in the formation of inclusion compounds with many guest molecules in aqueous solution (1, 2). Clenbuterol is a beta 2 adrenergic agonist. These drugs are usually administered as racemates, whereas the pharmacological activity usually resides in the *R*-enantiomer (3). For instance, the *R*-enantiomer of clenbuterol is responsible for the mimetic effect on β_2-receptors, while the *S*-enantiomer reveals a blocking effect on the β_1 -receptors (4). Clenbuterol is a moderate accumulation drug that primarily exists in the liver, fur and retina of animals; it can result in human food poisoning, ultimately including muscle tremor, tachycardia, palpitation and dizziness (5).

Huang (6) reported a comparative study to improve chiral resolutions in simultaneous enantioseparation of β-agonists by capillary electrophoresis (CE) via a CD inclusion complexation modified with ionic liquids (ILs). Clenbuterol enantiomers were baseline-resolved by CE using hydroxypropyl-β-cyclodextrin (HP-β-CD), sulfated β-cyclodextrin (SCD) and dimethyl-b-cyclodextrin (DM-β-CD) (7–9). Na (10) reported the chiral separation of clenbuterol by CE using modified nanoparticles as chiral selectors. Two-dimensional nuclear magnetic resonance (2D NMR) of the inclusion complexes of β-CD, HP-β-CD and DM-β-CD have been reported (11–17). Jug (18) established a novel method to exploit experimental (NMR) and theoretically calculated data obtained by a molecular modelling technique to obtain deeper insight into inclusion geometry. Currently, there is no relevant literature on how clenbuterol and cyclodextrins interact.

The primary objective of this study was the development of a rapid enantiomeric separation of clenbuterol. The inclusion complex of clenbuterol and CM-β-CD was synthesized and characterized. Based on the results from two-dimensional rotating frame spectroscopy (2D ROESY), an inclusion structure of the clenbuterol/CM-β-CD complex was proposed. Through this clenbuterol/CM-β-CD inclusion model, the split theoretical investigation was studied.

Experimental

Reagents and samples

All reagents employed for the preparation of the background electrolyte (BGE) were of analytical grade. Acetic acid, sodium hydroxide, sodium acetate, phosphoric acid, boric acid and monopotassium phosphate were purchased from Beijing Chemical Plant (Beijing, China). β-CD, HP-β-CD, CM-β-CD and trimethyl-b-cyclodextrin (TM-β-CD) were purchased from Fluke Corp. (Seattle, WA); DM-β-CD was obtained from Acros Organics (Geel, Belglum); γ-CD was bought from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); clenbuterol was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Buffers and standard solutions were prepared with Milli-Q water and filtered through a 0.45 μm pore size membrane filter (Millipore, Milford, MA).

Apparatus

All CE experiments were performed on a Beckman P/ACE MDQ CE system (Beckman Coulter, Fullerton, CA) equipped with a photodiode array detector. The detector was operated at 254 nm. Samples were injected into the capillary by voltage (10 kV for 5 s). The CE separation was conducted in 75 μm i.d. bare fused silica capillaries (48.5 cm total length, effective length 40 cm; Hebei Yongnian Optical Fiber Factory, Hebei, China). Electropherograms were acquired and processed by P/ACE MDQ Station software 32 Karat (Beckman Coulter).

The NMR data were obtained by a Bruker Advance DRX 400 MHz superconducting NMR spectrometer. NMR spectra of the inclusion complex were obtained by a Bruker Avance II spectrometer NMR using D2O as solvent; the relaxation delay was 2.0 s and mixing time equaled 2.00 ms. The NMR spectra

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of pure clenbuterol and CM-β-CD were also achieved by the same procedure for comparison. The complex was prepared by mixing (at 1:1M ratio) clenbuterol and CM-β-CD; the concentration of the complex was 15 mmol/L in deuterium oxide for the NMR measurement.

Buffer and sample preparations

Phosphate, acetate and borate buffers were prepared in Milli-Q water and the pH was adjusted with phosphoric acid, acetic acid or sodium hydroxide. β-CD, γ-CD, CM-β-CD, DM-β-CD, TM-β-CD and HP-β-CD were weighed directly into the BGE. Stock standard solutions were prepared by dissolving the appropriate amount of clenbuterol in Milli-Q water up to a final concentration of 200 μg/mL.

Preparation of clenbuterol and CM-β-CD complex

The complex was prepared by mixing (at 1:1M ratio) clenbuterol and CM-β-CD according to the freeze-drying method. Briefly, a mixture of CM-β-CD (0.015 mmol) and clenbuterol (0.015 mmol) was diluted in 1 mL of phosphate buffer (50 mmol/L, pH 3.5). The mixture was sonicated for 30 min. The resulting solution was frozen at −80°C and then subjected to lyophilization in a freeze-drier (Labconco, Kansas City, MO) for 24 h to obtain a powder.

Results and Discussion

Optimization

Effect of CD type

CDs and their derivatives are the most widely used types of chiral additives for CE. β-CD, γ-CD, CM-β-CD, DM-β-CD, TM-β-CD and HP-β-CD were investigated to evaluate their effect on the enantiomeric separation under the following conditions: 10 mM chiral selector and 50 mM phosphate buffer at the desired pH of 3.5.

Figure 1 shows that the enantiomers of clenbuterol were baseline separated by the use of β-CD, DM-β-CD and CM-β-CD.

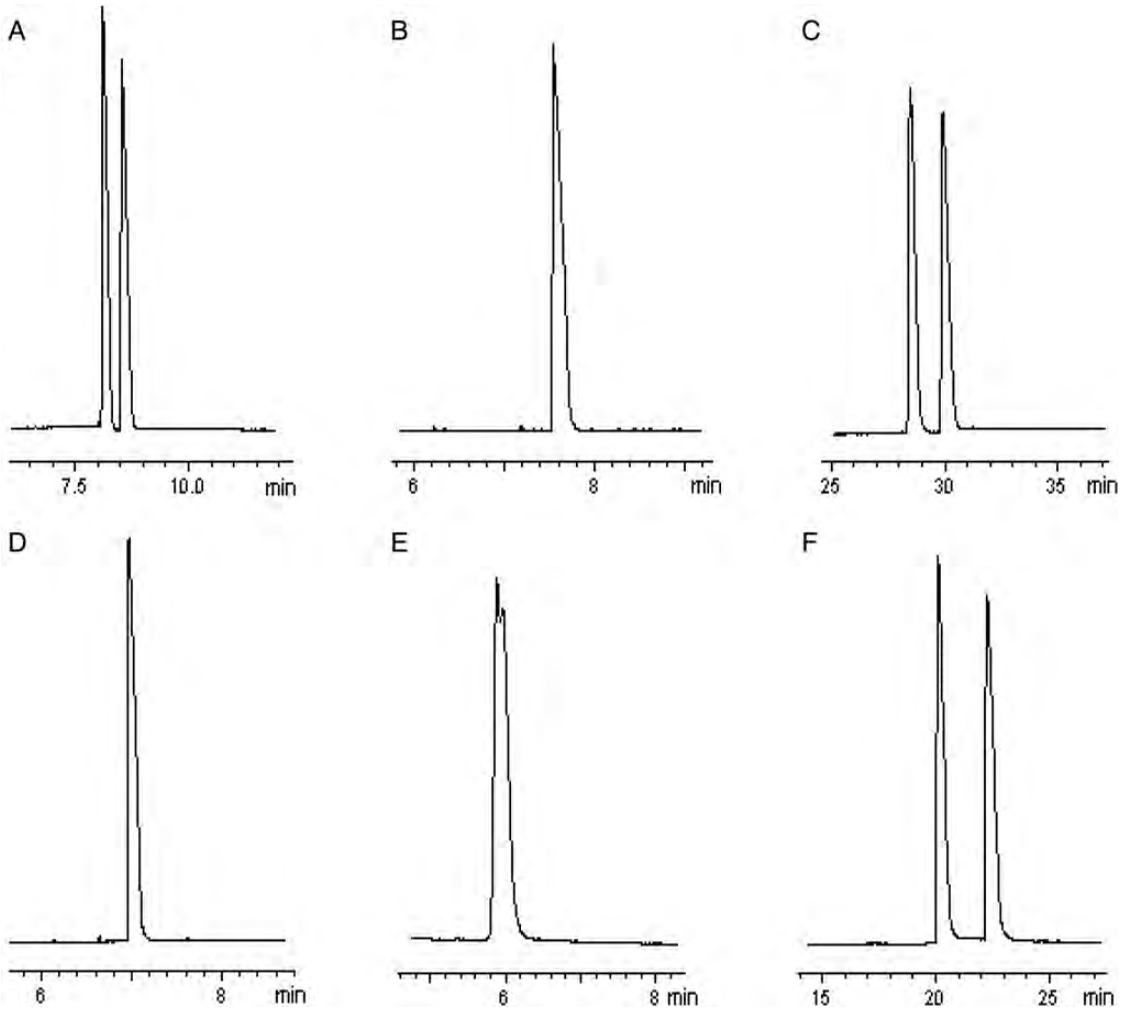

Figure 1. Chiral separation of racemic clenbuterol (200 μg·mL^{-1}) using as chiral selector different CDs. A: β-CD B: γ-CD C: DM-β-CD D: TM-β-CD E: HP-β-CD F: CM-β-CD CE conditions: phosphate buffer (50 mmol•L-1, pH 3.5) containing CM-β-CD (10 mmol•L-1); sample injection method (10 kV × 5 s); detection wavelength: 200 nm; running voltage: 20 kV; column temperature: 15°C; capillary total length: 48.5 cm (effective length: 40 cm).

An excellent baseline enantiomeric separation of clenbuterol was achieved with CM-β-CD at pH 3.5. Therefore, CM-β-CD was chosen as the chiral additive throughout the present experiment.

Effect of chiral *selector concentrations*

The concentration of the chiral selector in the running buffer is one of the most important factors to be considered in chiral CE analysis. In this study, CM-β-CD was added to the buffer system improve the separation and to investigate the CE behavior of clenbuterol. A series of electrolyte solutions was tested containing CM-β-CD at concentrations ranging from 1 to 15 mmol/L. As shown in Figure 2A, when the concentration was increased from 5 to 10 mmol/L, the resolution improved considerably, whereas further increase of the selector concentration was accompanied by only a moderate improvement. However, the migration times increase with the concentration of CM-β-CD. Taking into consideration both migration time and resolution, the optimal baseline separation was obtained at 10 mmol/Lof CM-β-CD concentration.

Effect of background electrolyte composition, concentration and pH

The appropriate choice of buffer composition is an important aspect of achieving resolution in CE. The effects of three different buffer systems (50 mM acetate, pH 5.0; 50 mM phosphate, pH 3.5; and 50 mM borate, pH 9.0) were applied on the enantiomer separation using 10 mmol/L CM-β-CD. The enantiomer separations of clenbuterol using phosphate buffer as BGE were better than those using acetate and borate buffers.

The effect of buffer concentrations upon migration time and resolution was investigated in the range of 30 ~ 70 mmol/L. Although the buffer concentration increased from 30 to 50 mmol/L, the enantiomer separation was greatly improved. However, little influence on the separation could be observed when the concentration changed in the range from 50 to 70 mmol/L. To prevent excessive generation of Joule heat and to obtain good baseline separation, 50 mmol/L phosphate buffer was chosen in this experiment. The two enantiomers could be separated to baseline.

Using 50 mmol/Lphosphate buffer (containing 10 mmol·L-1 CM-β-CD) the effects of buffer pH 2.5 ~ 6.5 on the separation were studied. As shown in Figure 2B, the optimized resolution was obtained at pH 3.5 for clenbuterol.

Effect of applied voltage and capillary temperature

The effect of applied voltage on resolution was investigated in a range from 15 to 25 kV, and the optimum potential was found to be 20 kV, because this resulted in a shorter migration time and a better resolution. An optimum potential of 20 kV was indicated.

The effect of temperature (15 ~ 25°C) on the separation was studied at an applied voltage of 20 kV. An increased resolution was found in the enantiomeric separation when the temperature was decreased using CM-β-CD as the chiral selector. An optimum capillary temperature of 15°C was selected.

2D NMR analyses

2D ROESY is a powerful tool for investigating inter-molecular and intra-molecular interaction. The presence of cross-peaks, which are generated by nuclear Overhauser effects (NOE), between the protons of guest molecules and CDs provides useful information about the dynamics and averaged relative inter-/intra-molecular proton distances of these species within 0.4 nm in solution (19, 20). Based on the results obtained from the 2D ROESY spectra, the spatial conformations of inclusion complexes may be determined. Thus, to gain more conformational information, 2D ROESY was used to study the inclusion complexes.

Figure 3A shows a partial contour plot of 2D ROESY spectra of the inclusion complex of clenbuterol and CM-β-CD. The inspection of this ROESY map allows a spatial proximity to be established between the guest protons and the inner protons of CM-β-CD. The ROESY spectrum of the clenbuterol/CM-β-CD complex showed appreciable correlation of the H-4 and H-6 protons of clenbuterol with the H-3, H-5 and H-6 protons of CM-β-CD. These results indicated that the benzene ring (head) of clenbuterol was close to the side of the narrower rim of CM-β-CD and the carbon chain (tail) of clenbuterol was far from both the narrower and wider rim sides of CM-β-CD. Therefore, four possible models were proposed, in which clenbuterol was partially inserted into the cavity of CM-β-CD (Models 1 and 2) and clenbuterol penetrated into the cavity (Models 3 and 4), as shown in Figure 3B. Because Ha/H6, Ha/H5, Hb/H6 and Hb/H5 interactions occurred, Models 1 and 2, in which Ha and Hb were far from H5 and H6 and could not produce the NOE signal, were excluded. In

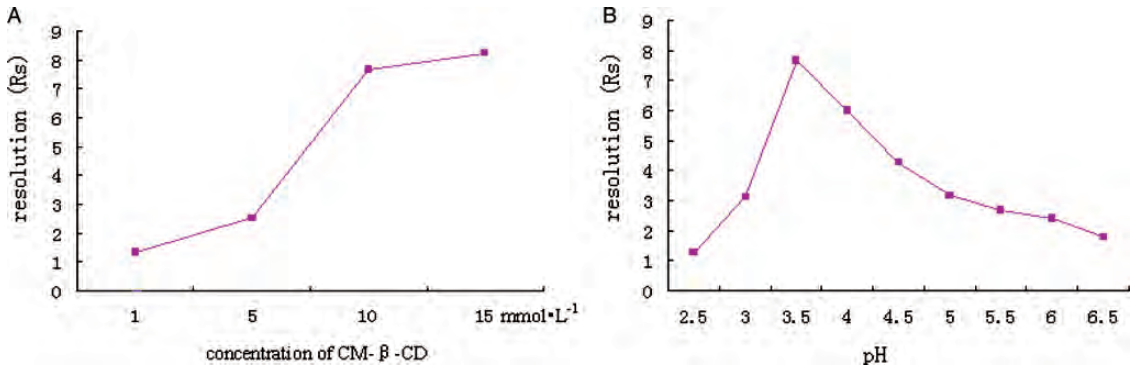

Figure 2. A: Effect of the concentration of CM-β-CD on chiral separation Clenbuterol; B: Effect of pH on chiral separation Clenbuterol.

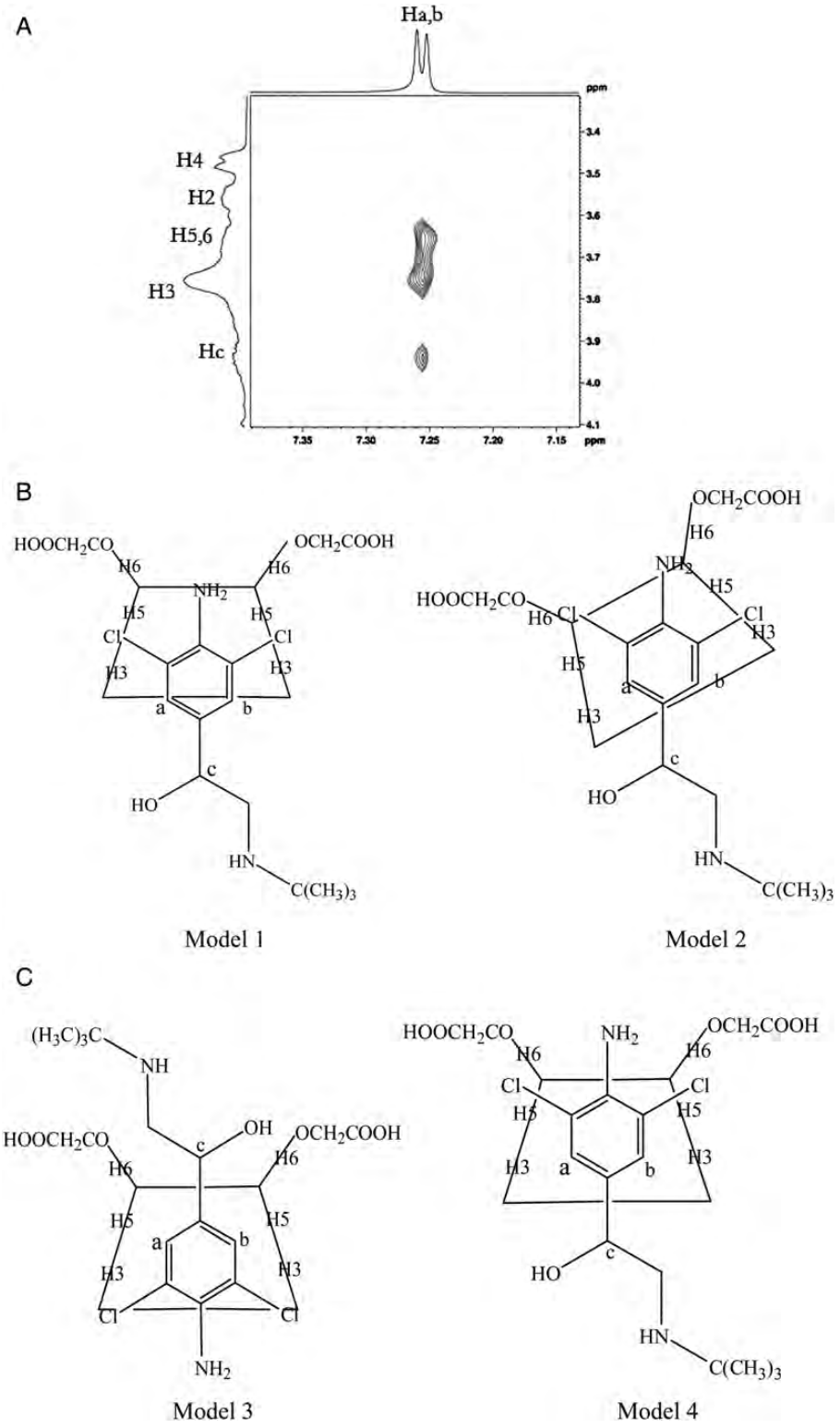

Figure 3. (A) Parts of 2D ROESY spectrum of clenbuterol/CM-β-CD complex in D2O, (B) possible models of entry of clenbuterol into CM-β-CD cavity.

Model 3, Hc was close to H6. Hc and H6 should have interactions, but this is not consistent with the 2D ROESY experimental results. Therefore, Model 3 was also excluded. When clenbuterol penetrated CM-β-CD in a tilted manner (Model 4), the experimental observations made in the ROESY study were described consistently. Therefore, Model 4 was adopted as the model of the formation of the complex between clenbuterol and CM-β-CD.

Conclusions

A method enabling the rapid enantiomeric separation of clenbuterol was developed in this study. The method has been demonstrated to be successful for the direct enantioseparation of clenbuterol. The inclusion complex of clenbuterol and CM-β-CD was synthesized and characterized. Based on the results from 2D ROESY, an inclusion structure of the clenbuterol/CM-β-CD complex was proposed, in which clenbuterol penetrated CM-β-CD in a tilted fashion under the influence of the intermolecular hydrogen bond between clenbuterol and CM-β-CD.

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