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Chiral separation of bupivacaine hydrochloride by capillary electrophoresis with high frequency conductivity detection and its application to rabbit serum and pharmaceutical injection

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Conductivity detection was employed to detect the enantiomers of bupivacaine hydrochloride (Bup), which were separated by high performance capillary electrophoresis. A computer-aided technique was used to calculate the binding energies, and the interaction between Bup enantiomers and cyclodextrins (CDs) is preliminarily discussed. Factors affecting the separation efficiency such as the types and concentration of chiral selectors, running buffer, pH value, separation voltage and capillary inside diameter and length were studied. Under optimized conditions, a baseline separation of Bup enantiomers was achieved in less than 15 min in 4 mM NH_4Ac-NaAc-HAc (pH 4.00) -0.48 mM sulfobutyl ether-β-cyclodextrin running buffer at a separation voltage of 12 kV. The lowest detectable concentration was 0.052 μg/mL. The proposed method was applied to chiral separation of Bup enantiomers in rabbit serum and pharmaceutical injections.

1. Introduction

It had been estimated that approximately half of the drugs which are used at present have a chiral center. These drugs are used clinically mostly in the form of the racemate. Most of these isomers exhibit different pharmacological and pharmacokinetic behavior. In practice, one of them is more active while the other may produce side-effects or even toxicity in some cases. Therefore, analytical methods of chiral separations have become more important in recent times and there is a continuing trend to the use of single-enantiomer formulations. High performance liquid chromatography (HPLC) is regarded as a prime separation method and has good reproducibility, but requires expensive chirality columns and the pretreatment or derivatization of the sample. High performance capillary electrophoresis (HPCE) is an attractive alternative method. HPCE is a powerful technique for chiral separations due to its high separation efficiency, speed of analysis, low reagent consumption and small sample requirement. Therefore, chiral separation has become an important field of application of high performance capillary electrophoresis.

Bupivacaine hydrochloride (Bup) is a local anesthetic agent in frequent clinical use with characteristics such as long duration of action, strong action and low toxicity. The chemical structure of Bup has a chiral carbon atom (Fig. 1) and has two enantiomers, *R*-(+)- Bup and *S*-(-) - Bup (Tahraoui et al.1996). Clinical tests show cardiac toxicity to be mainly caused by *R*-(+)- Bup, which has clinical significance for enantiomeric separation.

Only limited work has been reported on the chiral separation of Bup, mainly by HPLC (Dong et al. 2006; Da Silva et al. 2005; Gu et al. 1998) and HPCE-UV (Fan et al. 2003; Wei et al. 2006; Phinney and Sander 2005; Amini et al. 1998; Amini et al. 1999; Jäverfalk et al. 1998). Chiral separation of bupivacaine hydrochloride was studied by Dong et al. (20066) and Da Silva et al. (2005) using a chiral column in HPLC. Gu et al. (1998) separated and determined bupivacaine hydrochloride enantiomers in blood by HPLC. In HPCE, the most common chiral selectors are cyclodextrins (CDs). Carboxymethyl-poly-β-CD (CM-p-β-CD) (Fan et al. 2003), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) (Wei et al. 2006; Phinney and Sander 2005) have been found to be selective for the separation of the enantiomers of bupivacaine hydrochloride among many CDs. Fan et al. (2003) used CM-p-β-CD as the chiral selector and achieved a useful resolution. Wei et al. (2006) achieved simultaneous separation of salbutamol and bupivacaine enantiomers with 20 mmol HP-β-CD and 20 mmol DM-β-CD at pH 2.5 in a triethanolamine (TEA)-phosphate buffer. Unmodified and dynamically coated capillaries using 30 mmol DM-β-CD as chiral selector were tested for the separation of salbutamol and bupivacaine enantiomers (Phinney and Sander 2005).

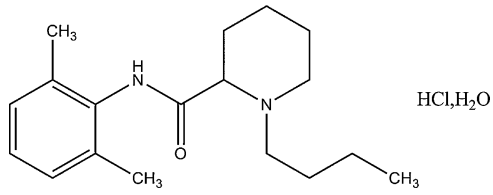

Fig. 1: Chemical structure of bupivacaine

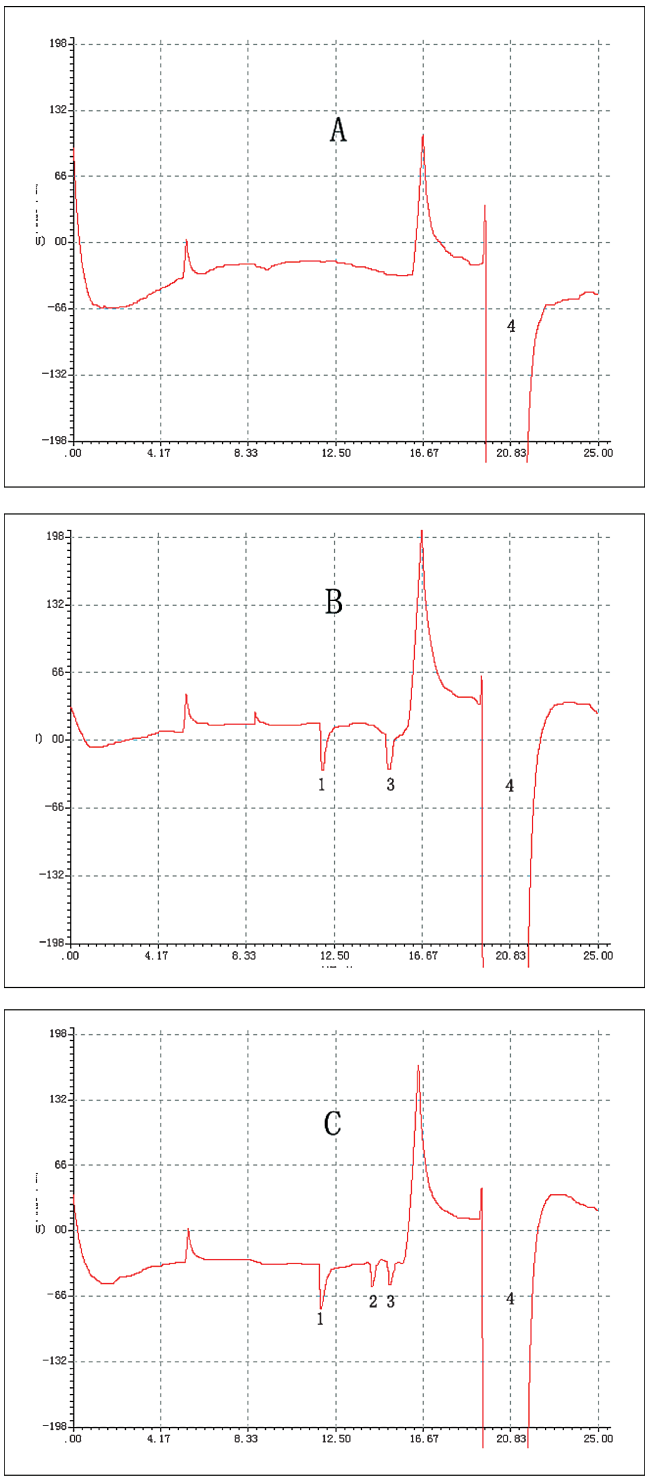

Fig. 2: Typical electrophergrams of (A) blank serum, (B) serum spiked with standard of S-(-)- Bup and internal standard, (C) serum spiked with Bup enantiomers and internal standard. HPCE conditions: 4 mM NH_4Ac-NaAc-HAc buffer with 0.48 mM SEB-β-CD at pH 4.00, separation voltage, 12 kV. (1) Internal standard, (2) R-(+)- Bup, (3) S-(-)- Bup, (4) system peak

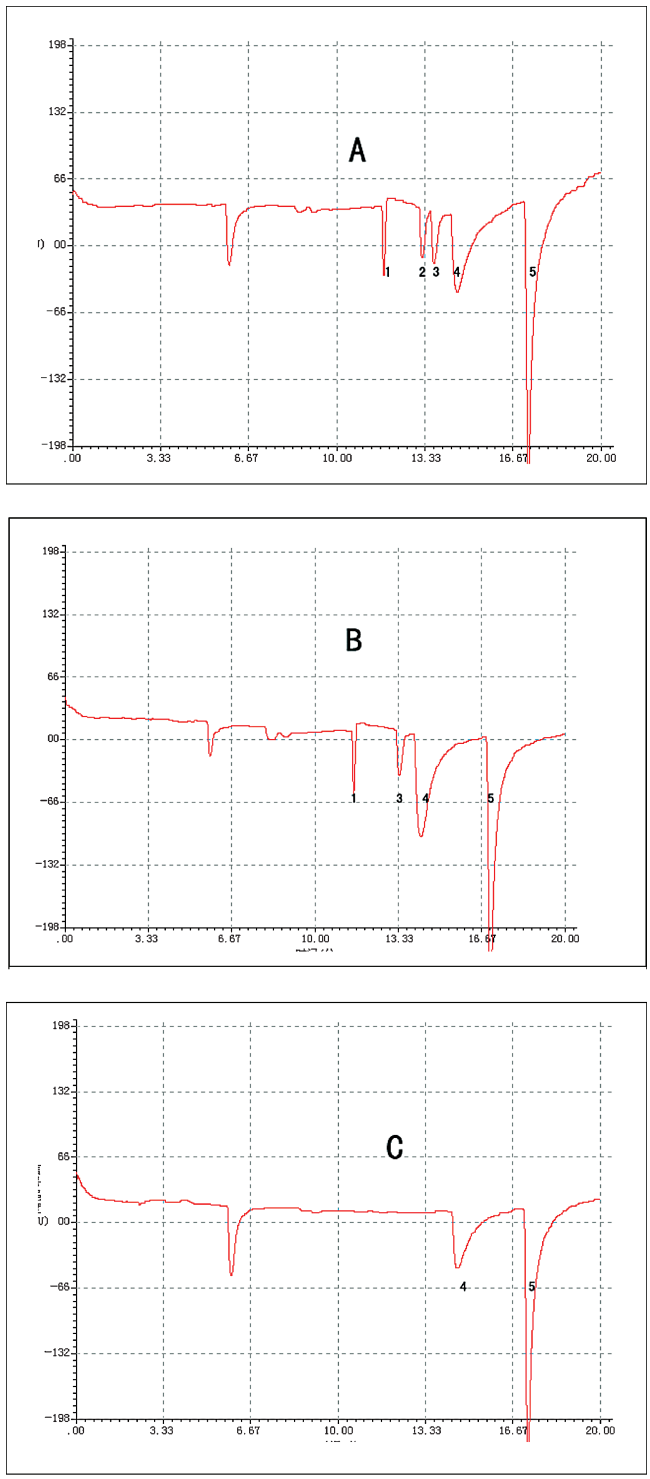

Fig. 3: Typical electropherogram of (A) Bup injection,(B) S-(-)- Bup standard, (C) Physiological saline solution. HPCE conditions as in Fig. 2. (1) Internal standard, (2) R-(+)- Bup, (3) S-(-)- Bup, (4) and (5) system peaks

All the HPCE studies mentioned above used UV detection. One of the major drawbacks of extensive use of HPCE-UV is its low sensitivity, limiting its application in trace analysis. As an alternative, many applications to both inorganic and organic cations and anions have been reported using HPCE with conductivity detection, enabling non-UV-absorbing compounds to be analyzed directly with good sensitivity without chemical derivatization. The main advantages of conductivity detection are its universality for all ionic species and its low cost. The main requirement for a sensitive and stable output is the use of buffers of low conductivity. In this study, high performance capillary electrophoresis with conductivity detection was employed for the first time in the chiral separation and detection of the optical isomers of Bup. The key experimental parameters, such as type and concentration of CDs, and buffer composition and pH were investigated and optimized. Also, binding energies were calculated by a computer-aided technique to enable a preliminary discussion of the stereoselectivity between Bup enantiomers and CDs. The method developed has been applied to the chiral sep-

aration and determination of Bup enantiomers in rabbit serum and pharmaceutical injections.

2. Investigations and results

2.1. Sample preparation

Weigh accurately 5.2 mg of S-(-)- Bup CRS, into a 50 ml volumetric flask, then dissolve in water to volume and mix well giving a standard stock solution at a concentration of 104 μg/mL. Codeine phosphate in water at a concentration of 52 μg/mL and ranitidine hydrochloride in water at a concentration of 42 μg/mL were used as internal standard solutions in rabbit serum and pharmaceutical injection, respectively.

The test solution of Bup injection consisted of a racemic mixture of bupivacaine, sodium hydroxide as pH regulator and sodium chloride as isotonic agent. This solution was simply diluted with water to the desired concentration (ca. 2.25 μg/mL).

Rabbit serum samples (0.5 mL) were pipetted into plastic centrifuge tubes containing 20 μL of codeine phosphate internal standard. The mixture was shaken for 10 min with 0.3 mL of 2 M sodium hydroxide and 3 mL chloroform-ether (10:1) followed by centrifugation at 2500 rpm for 5 min. The supernatant was recovered and evaporated to dryness on a water bath at 60 °C. The residue was then dissolved in 100 μL of the methanol (1:1) running buffer.

2.2. Electrophoretic procedure

The electrochemical detection cell including the platinum micro-disk working electrode was cleaned ultrasonically for 10 min before the experiment. At the beginning of each day and whenever the buffer solution was changed, the capillary was rinsed with 0.1 M sodium hydroxide solution for 5 min followed by water for 5 min, and the capillary was then equilibrated with the running buffer for 5 min. This process was repeated every five injections. The running buffer was obtained by dissolving a suitable amount of SBE-β-CD in a solution prepared by mixing acetic acid, ammonium acetate and sodium acetate in an appropriate ratio to give a suitable pH value. Samples were injected into the capillary with a positive voltage of 10 kV for 3 s, the separation voltage being 12 kV. All running buffers, standard and sample solutions were filtered through a 0.45 μm pore-size membrane before the experiment.

2.3. Sample analysis

Electrophoresis chromatograms under the conditions chosen for blank serum and serum spiked with Bup and codeine are shown in Fig. 2. Bup enantiomers were clearly separated and there was no interference by endogenous components from the rabbit serum or the internal standard.

After this, drug-free rabbit serum samples were spiked with a series of concentrations of Bup and also with a fixed concentration (10.4 μg/mL) of the internal standard. The ratio of the chromatographic peak area of S-(-)- Bup to that of the internal standard correlated linearly with the concentration over the range from 0.52 to 10.4 μg/mL. The regression equation obtained for S-(-)- Bup was $Y = 0.2362\rho + 0.077 (r = 0.9931)$. The limit of detection (LOD) was 0.26 μg/mL (S/N = 3).

The accuracy and precision of the method were determined by five-replicated analyses of blank serum samples spiked with different concentrations of Bup within the range 0.52 to 10.4 μg/mL. The relative recovery of the concentrations 0.52, 2.60, 10.40 μg/mL were 110.9% (RSD = 2.04%), 97.2% (RSD = 5.45%), and 100.1% (RSD = 3.05%), respectively. The intra-day precision ranged from 2.48 to 5.62% and the inter-day precision ranged from 4.29 to 6.42% (Table 1).

The average extraction recoveries of S-(-)- Bup were 74.68% (RSD = 6.91), 73.80% (RSD = 9.62), 76.59 (RSD = 4.99), respectively.

Prepared drug-free rabbit serum samples were spiked with Bup and internal standard and kept at −20 °C, 4 °C and 25 °C, respectively, for the 24 h stability test. At −20 °C, RSDs of S-(-)-Bup and R-(+)- Bup were 1.78% and 1.12%. At 4 °C, RSDs of S-(-)-Bup and R-(+)- Bup were 2.36% and 1.80%. At 25 °C, RSDs of S-(-)-Bup and R-(+)- Bup were 1.86% and 1.29%. The results showed the samples were stable and the analysis of samples is possible in these conditions.

Five mg/kg 0.5% Bup injections were given to rabbits in a calm state, blood was taken from the heart 30 min later, and 0.5 mL of serum was then isolated. Preparation of serum was conducted as described in Section 2.2. Assay results for Bup in rabbit serum under optimized conditions are shown in Table 2.

The method was also applied to determine Bup in a pharmaceutical product. Preparation of S-(-)- Bup and Bup injection solution was conducted as described in Section 2.2, The electrophoresis chromatogram is shown in Fig. 3. The Bup content of Bup injection was determined using the regression equation. The total Bup contents were 34.7 mg/5 mL (batch number: 070906), 36.2 mg/5 mL (batch number: 080201), and 35.2 mg/5 mL (batch number: 080301), all meeting the requirement of the labeled content of 37.5 mg/5 mL. The limit of detection (LOD) was 0.052 μg/mL (S/N = 3). Therefore, this method is appropriate for the quality control of pharmaceutical solutions containing Bup enantiomers.

3. Discussion

3.1. Effect of cyclodextrin type and concentration

Interaction with the chiral selector (CDs) and substrate is an important factor in enantioseparation, and thus the choice of a suitable chiral selector is of crucial importance. The effect of three different chiral selectors (neutral β-CD, HP-β-CD and the negatively charged SBE-β-CD) at the same concentration on enantioseparation was studied. Bup was completely separated using SBE-β-CD as chiral selector when the other conditions were identical, while no separation was achieved for Bup with the other two CDs. The results show that under the same experimental conditions, SBE-β-CD has a better capability for identification of Bup enantiomers than β-CD and HP-β-CD.

The structure of β-CD, HP-β-CD, SBE-β-CD and Bup were simulated by ChembioOffice3D software for further study of the interaction of different CDs with Bup enantiomers. The results show that the maximum width of Bup is 0.7195 nm (Fig. 4), and the mean diameter of the larger port of β-CD is 0.6308 nm (the average distance between corresponding hydrogen atoms with a radius of 0.053 nm), the mean diameter of the larger port of HP-β-CD is 0.6118 nm and the mean diameter of the larger port of SBE-β-CD is 0.7651 nm. This means that β-CD and HP-β-CD cannot provide enough space for Bup and could not form inclusion complexes with Bup enantiomers.Therefore, they can hardly play a role in chiral recognition. In contrast, SBE-β-CD can provide enough space for Bup, and thus the hydrophobic part of Bup can fit into the hydrophobic cavity of SBE-β-CD to form an inclusion complex, displaying activity in chiral recognition. Thus, SBE-β-CD was chosen as the chiral selector. Next, the effect of SBE-β-CD concentration from 0.32∼0.72 mM on enantioseparation was studied. Acceptable resolution was achieved only at a high CD concentration in the running buffer, which enhanced the viscosity of the background electrolyte (BGE). Figure 5 shows that Bup enantiomers were

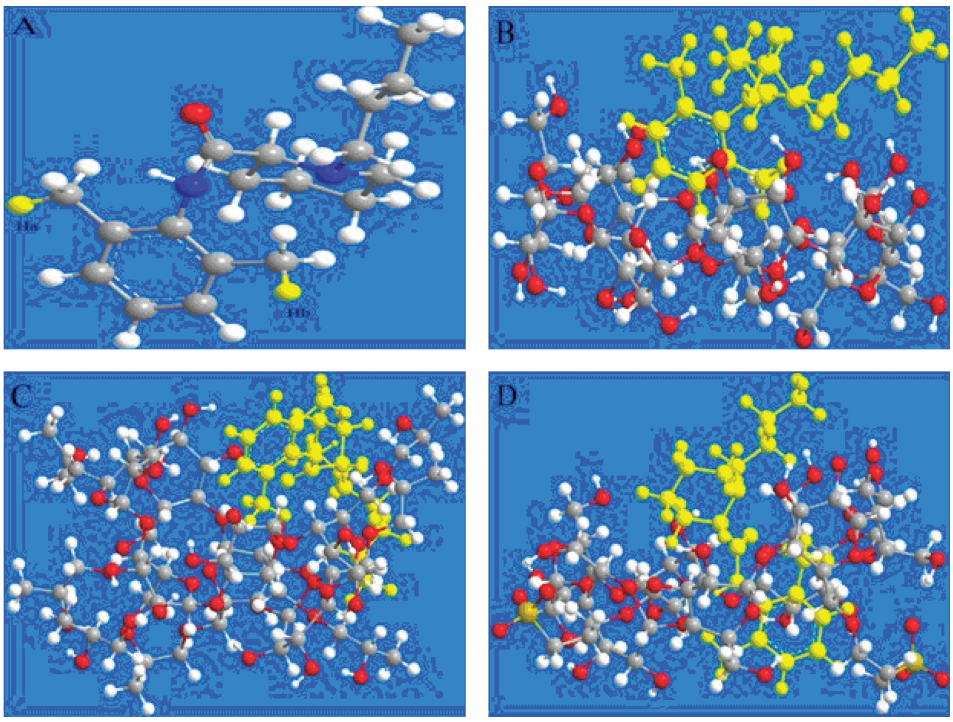

Fig. 4: Structure simulation of bupivacaine (A) complexes with β-CD(B), HP-β-CD(C) and SBE-β-CD(D)

Table 1: Analysis of relative recovery and precision of the method for quantification of Bup enantiomers in rabbit serum (n = 5)

Concentration (μg/mL)	Actual measurement (μg/mL)	Relative recovery (%)	RSD (%)	Intra-day (RSD %)	Inter-day (RSD %)
0.52	0.58	110.9	2.04	3.04	4.29
2.60	2.53	97.2	5.45	2.48	6.42
10.40	10.41	100.1	3.05	5.62	6.33

only partly separated at concentrations of SBE-β-CD below 0.48 mM, while the Bup peak was near to electroosmotic flow (EOF) at SBE-β-CD concentrations above 0.48 mM. Therefore, a SBE-β-CD concentration of 0.48 mM was selected.

3.2. Computer simulation of principle of separation

Molecular simulation and theoretical calculations (Zhou et al. 2006) used a computer-aided technique including ChembioOffice 3D, Pcmodel9 and Scigress Explorer. The chiral recognition process between enantiomers and cyclodextrin was simulated, and then the parameters of molecular mechanics during this process were calculated. The order of the peaks of *S*-(-)-Bup and *R*-(+)-Bup in capillary electrophoresis was determined based on the results.

First, optimised molecular models of the Bup enantiomers and SBE-β-CD were built with ChembioOffice 3D software, then Pcmodel 9 was used for further optimization of the molecular structure models, and finally Scigress Explorer software was used to dock the optimal molecular structure of the Bup enantiomers and SBE-β-CD and calculate the binding energy of the inclusion complexes between the Bup enantiomers and SBE-β-CD.

Armstrong (1986) considered that cyclodextrin and enantiomers may produce non-enantiomers which have different electrophoretic behavior. In this process, factors contributing to the conformational energy may be as follows: First, the match of the cavity size and organic molecules. Second, hydrogen bond formation between the solute and hydroxyl groups at the cavity

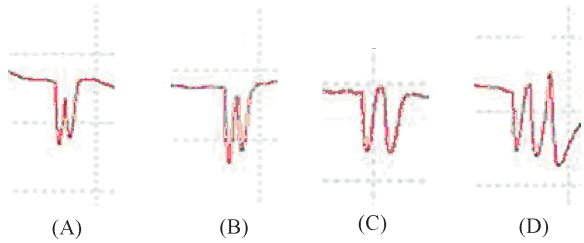

Fig. 5: Effect of SBE-β-CD concentration on enantioseparation (A) 0.32 mM, (B) 0.40 mM, (C) 0.48 mM, (D) 0.56 mM. HPCE conditions: 4 mM NH_4Ac-NaAc-HAc buffer with SEB-β-CD at pH 4.00; separation voltage, 12 kV

Table 2: Determination of Bup in rabbit serum (n = 3)

No.	Concentration of S-(-)-Bup (μg/mL)	Concentration of R-(+)-Bup (μg/mL)
1	1.1010	1.0246
2	1.0298	0.9051
3	1.0176	0.9904

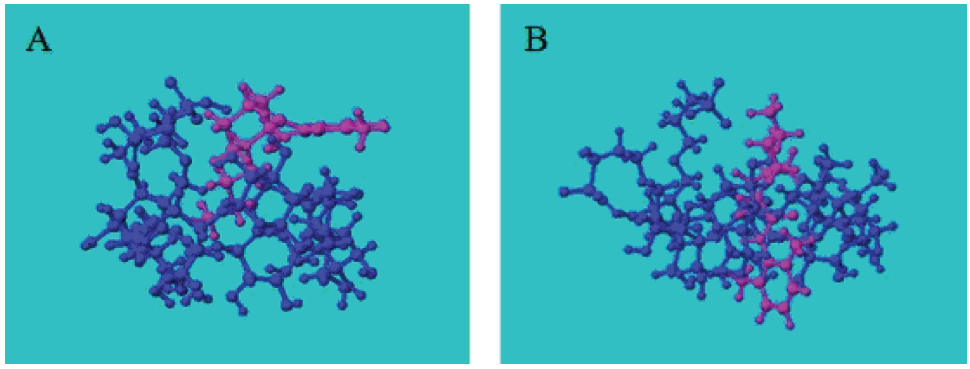

Fig. 6: Docking pattern of SBE-β-CD complexes with *R*-(+)-Bup (A) and *S*-(-)-Bup (B), respectively

edge. Third, interaction between the solute and the hydrophobic cavity. Fourth, van der Waal's forces of the edge atoms. Inclusion complexes were formed by interaction of the enantiomers with cyclodextrin. With a smaller binding energy, the inclusion complex is more stable, and the migration time in capillary electrophoresis is longer.

According to the results calculated, the conformational energy formed by R-(+)-Bup and SBE-β-CD is -3.61312 kcal/mol and by S-(-)-Bup and SBE-β-CD it is -14.3260 kcal/mol (Fig. 6). It can be seen that the conformational energy formed by S-(-)-Bup and SBE-β-CD is smaller, leading to a longer migration time. The results also indicated that in the acidic environment, inclusion complexes formed by R-(+)-Bup and SBE-β-CD were not complete, so an entity was formed with NH_4^+ and SBE-β-CD in the peripheral environment which carried more positive charge than that of the entity formed by S-(-)-Bup, NH_4^+ and SBE-β-CD. The theory is consistent with the results.

3.3. *Effect of running buffer composition and pH*

In order to select the most suitable conditions for separation of Bup enantiomers in an uncoated capillary, various buffer compositions and pH values were studied. Tris-Cit, Cit-CitNa, NaH_2PO_4-H_3PO_4, NH_4Ac-HAc, NaAc-HAc and NH_4Ac-NaAc-HAc were used as the running buffer at different concentrations. The results showed that only in NH_4Ac-NaAc-HAc running buffer were Bup enantiomers able to achieve enantioseparation and good reproducibility. This may be because Na^+ in the running buffer combined with silanols in capillary, reducing Zeta potential and EOF and prolonging migration time. Therefore, a NH_4Ac-NaAc-HAc buffer was selected.

In capillary electrophoresis, Joule heat will be generated if the concentration of the running buffer is too high, resulting in poor peak shape. The influence of NH_4Ac concentration was studied in the range from 2 to 10 mM. The results showed that the peaks of the enantiomers were disturbed if the concentration of NH_4Ac was too low; An increase in detection background and the running current occurred when the concentration of NH_4Ac became higher. At a concentration of 4 mM, Bup enantiomers could be well separated with high sensitivity. NaAc concentration was studied in the range from 2 to 5 mM using a background electrolyte containing 4 mM NH_4Ac. The results showed that when the concentration of NaAc was lower than 4 mM, good separation could not be achieved. When it was above 4 mM, the analysis time was longer and the signal-to-noise ratio (S/N) become poor because of the increase in background noise and running current. At a concentration of 4 mM, there was well-defined separation of Bup enantiomers. Therefore, a running buffer of 4 mM NaAc was selected.

The effect of pH on the separation of Bup enantiomers was studied. 4 mM NH_4Ac-NaAc was used as the background electrolyte and its pH was adjusted with glacial acetic acid. The effective mobilities of the Bup enantiomers decreased with pH in the range between 3.81 and 5.06 as can be observed in Fig. 7. This behavior could be because Bup is a drug with a p*K*a of 8.1 (Cherkaoui and Veuthey 2002), and the whole atom is positive within the pH range investigated, with direction of mobility the same as EOF and opposite to that of SBE-β-CD. A lower pH produces more opportunity for Bup to interact with SBE-β-CD. However, with a decrease of pH, background noise and running current increase. Considering both of these factors, a pH of 4.00 was selected for the following experiments.

3.4. *Effect of capillary diameter and length*

The effect of capillary diameter (75, 50, 25 μm) on separation was also investigated. Since negatively charged SBE-β-CD was added to the running buffer, the background conductance was greatly increased. Experimental results showed that under the same electrophoresis conditions the EOF of 75 and 50 μm capillary columns is much greater than that of the 25 μm column, resulting in an increase in background current and Joule heat and thus baseline instability. The effect of capillary length on separation was also investigated. Taking into account resolution and separation time, a capillary column with a length of 60 cm is better than 50 cm and 70 cm columns. Therefore, a 60 cm × 25 μm capillary column was selected.

3.5. *Selection of separation voltage and injection methods*

Increased separation voltage can shorten separation time, but the background current increases, leading to reduced column effi-

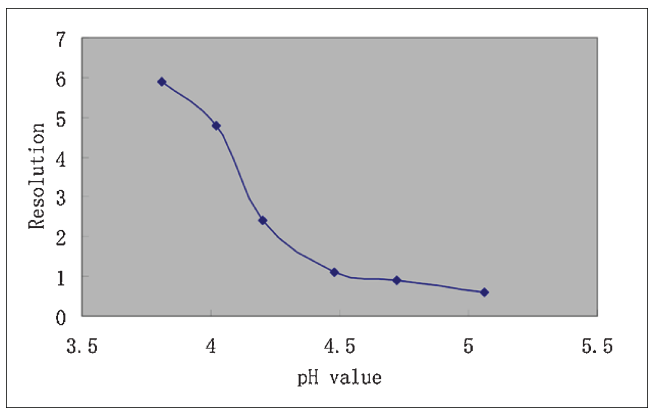

Fig. 7: Effect of pH on enantioseparation. CE conditions: 0.48 mM SEB-β-CD, other conditions as in Fig. 3

ciency of and resolution. Considering these factors, a separation voltage of 12 kV was chosen. In this study electrokinetic injection was used to accumulate the sample concentration and this greatly enhanced the sensitivity. Taking into account the peak height, separation efficiency and sample precision, an injection time of 3 s was chosen.

3.6. High sensitivity of conductivity detection

One of the major advantages of high frequency conductivity detection is its high sensitivity. Under optimized conditions, the limits of detection (LOD) were 0.052 μg/mL *in vitro* and 0.26 μg/mL (S/N = 3) *in vivo*. Compared with LODs of 0.24 μg/mL (Wei et al. 2006) and 20 μg/mL (Martinez-Pla et al. 2004) *in vitro* with UV detection, high frequency conductivity detection has a clear advantage in trace analysis.

3.7. Conclusions

Capillary electrophoresis with conductivity detection was employed for enantiomeric separation of bupivacaine hydrochloride in the convenient CZE mode for the first time. The method was characterized by its higher sensitivity and resolution and lower operating cost. A computer-aided technique was used preliminarily to calculate the binding energies and the interaction of the Bup enantiomers with CDs. The sequence of appearance of peaks in the experiment agrees with theory. Under the selected experimental conditions, the validated method was used successfully for chiral separation and determination of bupivacaine hydrochloride enantiomers in rabbit serum and in a pharmaceutical injection.

4. Experimental

4.1. Apparatus and reagents

The high performance capillary electrophoresis instrument includes: high voltage power supply, high frequency conductivity detector, and capillary electropherosis data workstation (School of Chemistry and Chemical Engineering, Sun Yat-sen University). Model Orion 828 pH meter (USA), DL-360 ultrasonic washing device (Ningbo Electronic Instrument Factory). The uncoated fused silica capillaries were 25 μm I.D.(Yongnian Ruipu Chromatogram Equipment Co., Hebei, China) and total and effective lengths were 60 and 55 cm.

All chemicals were of analytical reagent grade. Sulfobutyl ether-β-cyclodextrin (SBE-β-CD) was purchased from Xinda Fine Chemicals Co., Ltd (Shandong, China). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Boao Biological Technical Ltd. (Shanghai,China). Acetic acid and phosphoric acid were purchased from Guanghua Chemical Factory Co., Ltd (Guangdong, China). Ammonium acetate, citric acid and sodium citrate were purchased from Guangzhou Chemical Reagent Factory (Guangdong, China). Sodium acetate and disodium hydrogen phosphate were purchased from Fuchen Chemical Reagent Factory (Tianjin, China). *S*-(-)-Bup (Lot number: 27070801, purity: 99.4%) was kindly donated by the Department of Anesthesiolgy of Guangzhou First Municipal People's Hospital (Guangdong, China). Bupivacaine hydrochloride injection (Batch number: 070906, 080201, 080301) was purchased from Hefeng Pharmaceuticals Company (Shanghai, China). Ultrapure water (junction resistance 18.2 Ω/cm) was used to prepare solutions.

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