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Chiral separation of salbutamol and bupivacaine by capillary electrophoresis using dual neutral cyclodextrins as selectors and its application to pharmaceutical preparations and rat blood samples assay

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Abstract

An attempt for the simultaneous separation of salbutamol (Sal) and bupivacaine (Bup) enantiomers was performed by capillary elecytrophoresis with a dual mixture of neutral cyclodextrins as chiral selector. The influence on the separation of several parameters such as buffer composition, pH, the concentration ratio of 2-hydroxypropyl-beta-cyclodextrin (HP-β-CD) to dimethyl-beta-cyclodextrin (DM-β-CD) was investigated. A better separation was obtained for Sal and Bup with the CD mixtures compared to the use of HP-β-CD or DM-β-CD alone. The best simultaneous separation of Sal and Bup enantiomers was achieved with a 20 mM HP-β-CD and 20 mM DM-β-CD at pH 2.5 in a triethanolamine (TEA)/phosphate buffer. This method-utilized chlorphenamine (Chl) as an internal standard was found to be linear in the range 0.5–100 μg/mL and 0.5–150 μg/mL for Sal and Bup enantiomers, respectively. The limits of detection for both enantiomers of Sal and Bup were 0.18 and 0.24 μg/mL, respectively. The proposed method was applied to monitor Sal and Bup enantiomers concentration change in rat blood samples obtained from a male rat after celiac doses administration 0–30 min of Sal and Bup racemate. The method could also be used to determine Sal enantiomers in a pharmaceutical aerosol.

Keywords: Chiral separation; Capillary electrophoresis; Mixtures of neutral cyclodextrin; Basic pharmaceutical; Acrosol

1. Introduction

It had been estimated that approximately 50% of the commercially available drugs obtained by chemical synthesis have a chiral center [1]. These isomers often exhibit different behavior in terms of pharmacological processes, therapeutic efficacy and biological processes. Thus, enantioselective analytical methods are necessary to meet the increasing demand for evaluation of the pharmacokinetic attributes of each enantiomer and to control the enantiomeric purity of pharmaceutical preparations.

Salbutamol (Fig. 1a) is a selective β_2-adrenoreceptor agonist widely used in the treatment of asthma and exercise induced asthma [2,3]. It is usually administered as a racemate. Studies have shown that the pharmacological action of salbutamol resides in the *R*-(−)-enantiomer, which moreover was found to undergo a faster metabolism in men than the *S*-(+)-enantiomer [4]. Bupivacaine (Fig. 1b) is a local anaesthetic and also used as the racemate. Bupivacaine enantiomers differ pharmacologically; *R*-(+)-bupivacaine is more toxic to the central nervous and the cardiovascular system than *S*-(−)-bupivacaine [5–7]. Sometimes the two drugs are administrated together in order to reduce the absorption of anesthetic by the tissues prolonging the anesthetic effect and alleviate pain of patients. Therefore, selective and robust methods for the separation and monitoring of salbutamol and bupivacaine enantiomers in biological fluids are of great importance.

A number of methods have been developed for chiral separations of salbutamol or bupivacaine included high-performance liquid chromatography (HPLC) [8,9], gas chromatography (GC) [7,10] and, more recently capillary electrophoresis (CE) [11–13]. In CE, the most widely used chiral selectors are cyclodextrins (CD). Among the different kinds of CDs, heptakis(2,6-di-*o*-methyl-β-CD) (DM-β-CD) and hydroxypropyl-β-CD) (HP-β-CD) were found to be the most

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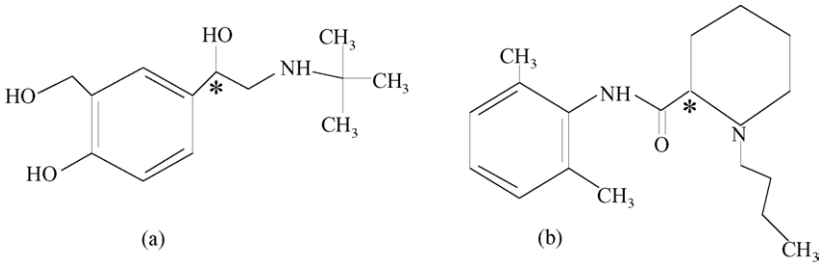

Fig. 1. Structures of the enantiomers (a) Sal and (b) Bup.

appropriate selectors for the separation of salbutamol or bupivacain enantiomers. For example, racemic salbutamol was baseline resolved using 20 mM DM-β-CD at pH 2.5 in a Tris/phosphate buffer [13]. Roig and Bergés [14] reported the use of 112 mM DM-β-CD in a citric acid/phosphate buffer (pH 2.5) for the separation of a complex mixture of salbutamol-related solutes, but the peak of salbutamol enantiomers tailed due to use of a too high CD concentration. Karen et al. [15] compared the performance of unmodified and dynamically coated capillaries using 30 mM DM-β-CD as chiral selector for the separation of salbutamol and bupivacaine enantiomers. Although migration times were shorter and peak symmetry was better on the dynamically coated capillaries, but peak tailing was not entirely eliminated and resolution was slightly decreased for salbutamol and bupivacaine. Kim et al. [16,17] attempted to separate salbutamol enantiomers with HP-β-CD, but they found that salbutamol enantiomers could not be adequately resolved at any HP-β-CD concentration. Amini et al. have systematically investigated the chiral separation of bupivacaine by β-CD, methyl-β-CD or HP-β-CD as a selector [18–20]. However, acceptable resolution was only achieved at a high CD concentration and with a polyacrylamide coated capillary, and the too high CD concentrations in the running buffer enhanced the viscosity of the background electrolyte (BGE), the background UV absorbance and noise. Therefore, the addition of HP-β-CD or DM-β-CD did not adequately resolve salbutamol and bupivacaine enantiomers, respectively. A more generally accessible method could be applied to develop routine drug analysis and metabolism studies, a further improvement of the chiral resolution is required.

Different studies presented the combination of selectors, showing the possibility of a changed and improved selectivity towards the enantiomers. Recently, several papers have reported exploring the possibility of combining two neutral CDs for the enantioseparation of chiral compounds which could not be adequately or hardly resolved using a single selector [21–24]. Although such dual CD system proved to be successful in enhancing the selectivity and improving the resolution, no work has been devoted to the simultaneous separation of salbutamol and bupivacaine enantiomers.

In this work, we attempted the simultaneous separation of salbutamol and bupivacaine enantiomers by using dual CD system. The combination of HP-β-CD and DM-β-CD has been tried and found effective for enhancing the selectivity and improving the resolution. The effects of a number of key experimental parameters, such as concentration ratio of HP-β-CD to DM-β-CD, buffer composition and pH were investigated and optimized. The method developed has been applied to the analysis of salbutamol in pharmaceutical aerosol and rat blood samples taken from a male rat after celiac doses administration 0–30 min of salbutamol and bupivacaine racemate.

2. Experimental

2.1. Chemicals

(±)-Chlorphenamine used as internal standard was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). (±)-Salbutamol hydrochloride and (±)-bupivacaine hydrochloride were purchased from Sigma Aldrich (St Louis, MO, USA). *R*-Salbutamol and *R*-bupivacaine were gifted by Dr. Xiaofeng Xu (Guangzhou University of Traditional Chinese Medicine). Salbutamol hydrochloride aerosol was obtained from Penglai Nuokang Pharmaceutical Co., Inc. products (Shandong, China). Dimethyl-β-cyclodextrin (DM-β-CD) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Hydroxypropyl-β-cyclodextrin (HP-β-CD, >97%) was purchased from Acros Organics. Phosphoric acid 85%, Tris(hydroxymethyl) aminomethane (Tris) and triethanolamine (TEA) were purchased from Beijing Chemical Factory (Beijing, China). All other reagents used were of analytical grade purity. Water for preparation of sample and buffer solution was deionized by a Milli-Q purification system with a 0.2-μm fiber filter (Barnstead, CA, USA).

2.2. Apparatus and analytical procedure

All experiments were performed on an Agilent HP^{3D} CE instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a UV–vis diode array detector. The analytes were detected at a wavelength of 195 nm for all experiments. The electropherograms and related data were collected with Agilent software (3D-CE Chemstation). An uncoated fused-silica capillary (Yongnian Optic Fiber Factory, Heibei, China) of 75 μm I.D. and 57 cm (effective length 48.5 cm) was used for separation. The capillary temperature was set at 25 °C. The new capillary was flushed successively for 30 min with 1 M NaOH, 30 min with water and then equilibrated with the operating buffer for 5 min. Washing between two runs consisted of successive rinsing of the capillary with water (1 min), 0.1 M NaOH (2 min), water (2 min), and finally with the running buffer (2 min). At the beginning of each

day and whenever the buffer solution was changed, the capillary was rinsed with 0.1 M NaOH for 2 min, water for 2 min, and the running buffer for 2 min. Samples were injected with pressure mode for 10 s at 50 mbar. A positive 18 kV separation voltage was applied and detection was performed at the cathode.

A stock solution containing 1 mg/mL (*R*,*S*)-Sal and 1.5 mg/mL (*R*,*S*)-Bup was prepared with water. Before analysis, this solution was only diluted with water to the required concentration. The internal standard solution Chl was prepared in water at a concentrations of 1 mg/mL as a stock solution. The solutions were stored at 4 °C and were stable for at least 3 months. The BGE was prepared by mixing a 50 mM phosphoric acid, proper weights of HP-β-CD and DM-β-CD in water and adjusting the pH to desired value with pH meter (IQ Scientific Instruments, Shanghai, China) by adding TEA or 0.5 mol/L Tris solution.

All running buffers and sample solutions were filtered through a 0.45 μm pore-size membrane filter before experiments.

2.3. Sample preparation

The determination of the Sal in the aerosol required a dilution of the aerosol with water. Different sample solutions were prepared by diluting different amounts of the aerosol in water in order to obtain the desired concentration. The resulting solution (1.0 mL) was spiked with 20 μL internal standard Chl and directly injected into the electrophoretic system.

Three male rats (weighing 230–250 g) were supplied water and food freely for 15 h before drug administration. A 2.0 mL mixture solution of 100 μg/mL rac-sal and 150 μg/mL rac-bup was injected to celiac of rat. The samples of blood (ca. 4 mL) were collected into Heparinised polystyrene tubes at time 0, 5, 10, 20 and 30 min after the application of the injection solution and centrifuged within 1 h of collection. The supernatant of centrifuged blood (ca. 2.5 mL) was immediately stored at −20 °C in polypropylene tubes until being defrosted for analysis.

Aliquots of rat blood samples (2.0 mL) were pipetted into glass tubes containing 20 μL internal standard Chl. The mixture was shaken for 1 min, deproteinized with 2.5 mL acetonitrile and vortex mixing for 2 min followed by centrifugation at 3000 rpm for 5 min. The organic layer was recovered and evaporated to dryness under a gentle nitrogen stream at ambient temperature. The residue was reconstituted with 0.40 mL acetonitrile, vortex mixed 10 s, sonicated for 3 min, centrifuged 1 min, and the supernatant was injected onto electrophoresis system. In this way, sample was concentrated five-fold.

2.4. Data analysis

The corrected peak areas were calculated using 3D-CE Chemstation. The enantiomeric resolution (R_s) was calculated according to the equation:

$$R_s = \frac{2(t_2 - t_1)}{w_2 - w_1} \quad (1)$$

where t_1 and t_2 are the migration times of the first- and second-migrating enantiomers and w_1, w_2 are their corresponding peak widths at the peaks base.

3. Results and discussion

3.1. Cooperative effect of DM-β-CD and HP-β-CD

Previous studies have shown chiral separation of Sal and Bup using DM-β-CD or HP-β-CD alone was not satisfactory [16,18,20]. In order to develop a more generally accessible method for biomedical analysis, further investigations on the use of the two CDs in the enantioseparation of Sal and Bup in single CD systems were performed. Addition of 15 mM HP-β-CD alone to a pH 2.6 phosphoric acid–triethanolamine buffer failed to resolve the (±)-Sal and (±)-Bup. A 15 mM of DM-β-CD alone poorly resolved (±)-Sal and (±)-Bup ($R_s < 0.50$). When 15 mM DM-β-CD and 15 mM HP-β-CD were simultaneously present in the same buffer, the baseline separation for Bup was obtained and the resolution for Sal was also reached to 1.0; in addition, the peak shapes and efficiencies using in combination were more favorable than with either DM-β-CD alone or HP-β-CD alone. This shows clearly that the combination of DM-β-CD and HP-β-CD can obviously improved separation and sensitivity.

3.2. Effect of the concentration ratio of DM-β-CD to HP-β-CD

The effect of the concentration ratio of DM-β-CD to HP-β-CD was studied by the addition of different amounts of DM-β-CD to a running buffer containing 15 mM HP-β-CD. As it can be seen in Fig. 2 that no separation was achieved for Sal and Bup in absence of DM-β-CD. The optimum concentration ratio of DM-β-CD to HP-β-CD is equal to 1:1 for simultaneous enantiomeric separation Sal and Bup. In order to further increase the resolution, higher concentrations of selectors were

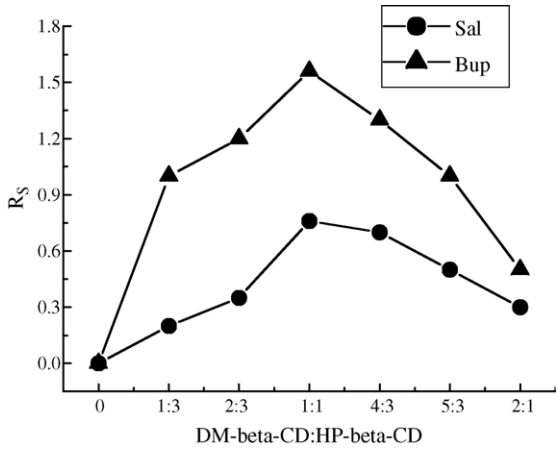

Fig. 2. Effect of the concentration ratio of DM-β-CD to HP-β-CD on chiral resolution (R_s). CE conditions: 50 mM phosphoric acid–triethanolamine buffer with 15 mM HP-β-CD and DM-β-CD at pH 2.6; capillary, 57 cm × 75 μm (I.D.); separation voltage, 18 kV; capillary temperature, 25 °C; UV detection, 195 nm.

investigated. As expected, chiral separation of the tested drugs increased rapidly with the total concentration of DM-β-CD and HP-β-CD up to 20 mM. The increase then got slowed down and the peak broaden, the efficiency decreased due to the high concentration in the running buffer enhances the viscosity of the BGE and causes more noise. Thus, the best CD concentrations in dual systems were defined as 20 mM DM-β-CD and 20 mM HP-β-CD.

3.3. Effect of the composition of running buffer and pH

In order to obtain the one best suit for separation of Sal and Bup from rat blood samples in uncoated capillary, various buffer composition and pH were studied. The better enantiomeric separations were obtained at pH 2.5 phosphoric acid/TEA buffer for simultaneous enantioseparation of the studied drugs. To accomplish efficient separation, the effect of buffer concentrations on chiral separation was also studied. Baseline separations were achieved for Bup and Sal at a 25 and 50 mM concentration buffer, respectively. Therefore, a 50 mM phosphoric acid/TEA buffer (pH 2.5) was chosen as the optimum.

3.4. Effect of applied voltage and temperature on enantioseparation

The influence of applied voltage was investigated from 10 to 25 kV. The current was increased with increasing applied voltage. No excessive Joule heating was generated until 20 kV. Above 18 kV baseline separation was not achieved for Sal. Thus, applied voltage was set at 18 kV. Numerous parameters, such as buffer viscosity, pK_a and complexation constants of the solute are directly affected by temperature. So, the influence of temperature on the chiral separation was performed at 15, 20, 25, 30, 35 and 40 °C. The enantiomeric resolution and the migration time were increased with decreasing of the temperature, which implies the increase in the stability of inclusion complexes. Over 25 °C baseline separation was not obtained for Sal. Therefore, 25 °C was chosen as optimization.

3.5. Sensitivity

Injection time affects injection sample volume, thereby influencing detection sensitivity. In order to increase detection sensitivity, injection time from 5 to 15 s was tested using hydrodynamic mode in 50 mbar. It was found that the optimum injection time was 10 s. In addition, rat blood sample after deproteinization was concentrated five-fold and the use of 195 nm as a wavelength for detection of Sal and Bup enantiomers, both permitting higher sensitivity, which is to be achieved. This higher sensitivity was needed to monitor drug concentration changes in rat blood for the study of drug metobolism.

3.6. Assay validation

Under the optimum conditions chosen, typical electropherogram for chiral separation Sal and Bup containing Chl in a single run are shown in Fig. 3. Chl was selected as internal standard because of its electrophoresis behaviors, migrating as a single peak and closing to those of salbutamol and bupivacaine enantiomers.

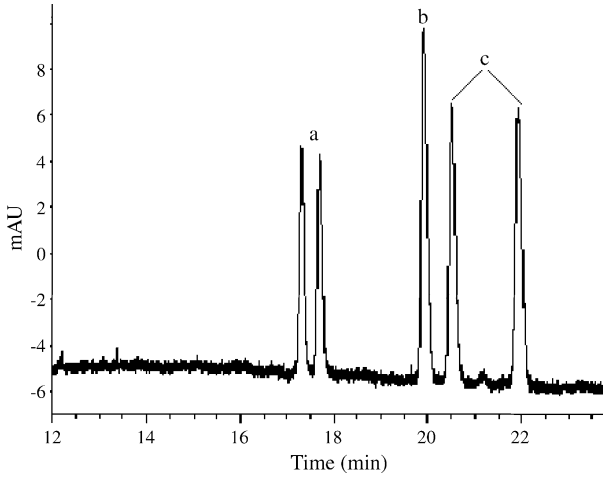

Fig. 3. Enantioseparation electropherogram from of a 20 μg/mL Sal and 30 μg/mL Bup mixture standard solution containing 30 μg/mL internal standard Chl. CE conditions: 50 mM phosphoric acid–triethanolamine buffer with 20 mM HP-β-CD and 20 mM DM-β-CD at pH 2.5; other conditions are the same as in Fig. 2. (a) Sal, (b) Chl and (c) Bup.

The validation of this method was performed to monitor Sal and Bup concentration changes in rat blood for the study of drug metabolism. Several blank rat blood samples from different male rat were tested for the presence of any interference. Typical electropherogram for blank blood and blood spiked with different concentration of Sal, Bup and Chl are shown in Fig. 4. The sample matrix can effect migration time of the Sal, Bup enantiomers and Chl peak in plasma sample, peaks identification were made with the method of standard solution addition. (*R*)-Form migrates first. No interfering endogenous peaks were found at the migration times of Sal, Bup and Chl peak. Therefore, drug-free rat blood samples were spiked with serial concentrations of Sal and Bup and also with a fixed concentration (20 μg/mL) of Chl. Peak-area ratios of analytes to internal standard were directly proportional to the concentrations of analyte in the range 0.5–100 μg/mL and 0.5–150 μg/mL for Sal and Bup enantiomer, respectively. The regression equations obtained for (*R*)-Sal, (*S*)-Sal, (*R*)-Bup and (*S*)-Bup were $y = 0.0632 + 0.0887x$, $y = 0.0625 + 0.0920x$, $y = 0.0550 + 0.1008x$ $y = 0.0567 + 0.1009x$, respectively. The calibration curves in all case showed good regression coefficients ($r^2 > 0.996$). For spiked rat blood samples, the limits of detection (LOD) defined as a signal-to-noise ratio of 3:1, were 0.18 and 0.24 μg/mL (9.3×10^{-7} mol/L and 8.7×10^{-7} mol/L) for both enantiomers of Sal and Bup, respectively.

The accuracy and precision of the method were determined by six-replicated analysis of blank rat blood samples spiked different concentrations of Sal and Bup within the range 0.5–150 μg/mL. The intra- and inter-day precision and accuracy data are summarized in Table 1. The intra-day precision and accuracy ranged from 2.5 to 4.4% and −1.5 to 4.3%, respectively, for all samples analyzed. Higher R.S.D. and RE values

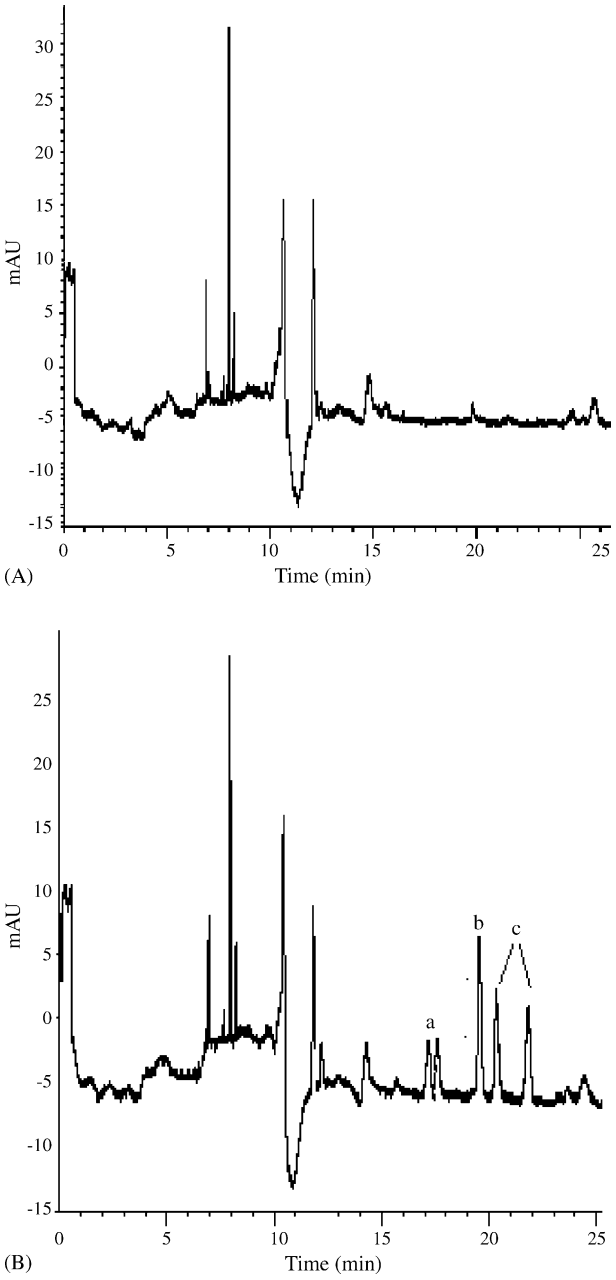

Fig. 4. Typical electrophergrams of (A) blank blood, (B) blood spiked with 10 μg/mL rac-Sal, 15 μg/mL rac-Bup and 20 μg/mL Chl. CE conditions are the same as in Fig. 3. (a) Sal, (b) Chl and (c) Bup.

were obtained in inter-day experiments, which ranged from 3.6 to 5.8% and 1.2 to −6.5%, respectively.

3.7. Analytical application

The developed method was devised to monitor Sal and Bup concentration change in rat blood for the study of drug metabolism. The electropherograms for rat blood samples obtained 30 min after celiac injection administration of rac-Sal and rac-Bup which is shown in Fig. 5. A blood concentration versus time profile for each enantiomer is summarized in Fig. 6. Results indicated that the rate of metabolism for Sal and Bup

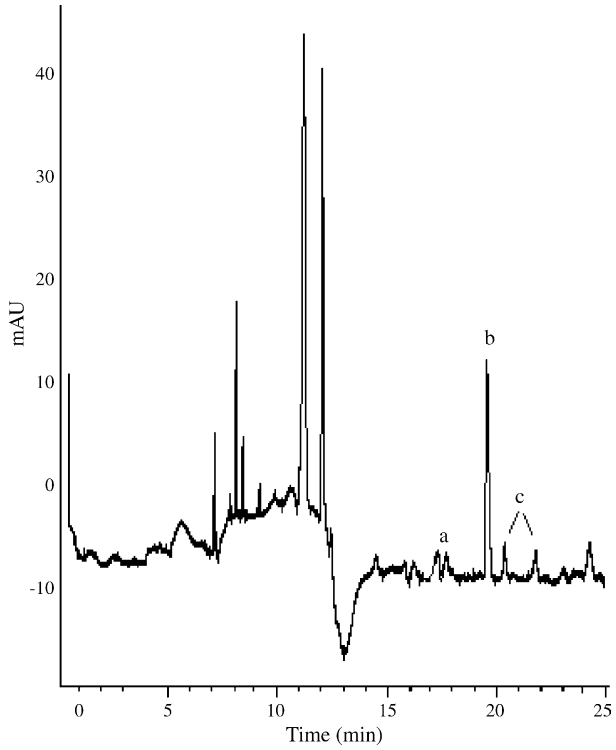

Fig. 5. Electropherograms from rat blood samples obtained 30 min after celiac injection administration of rac-Sal and rac-Bup. CE conditions are the same as in Fig. 3. (a) Sal, (b) Chl and (c) Bup.

enantiomers was different. The concentration of (*R*)-Bup and (*S*)-Bup was reduced to 1.17 and 1.22 μg/mL within 20 min, respectively. The rate of metabolism for Bup enantiomers was faster than for Sal enantiomers. Stereoselective effects for rac-Sal and rac-Bup in 30 min administration metabolism were not observed. These results were in agreement with the literatures [8,9], which inhaled after administration of rac-Sal [8] and after a 3 min infusion rac-Bup into the left coronary artery of a sheep [9].

The method was also applied to determine Sal in the pharmaceutical product (a aerosol solution with 0.140 mg/press of

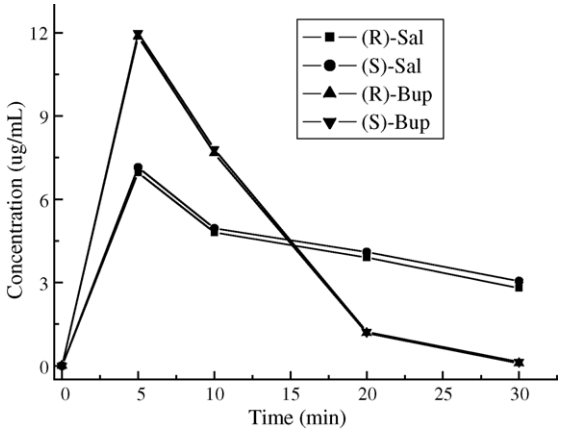

Fig. 6. Rat blood concentration–time profiles of Sal and Bup after celiac injection administration.

Table 1
Analysis of precision and accuracy of the method for quantification of Sal and Bup enantiomers in rat blood

	Intra-day				Inter-day			
	Concentration (μg/mL)	*n*	R.S.D. (%)	RE (%)	Concentration (μg/mL)	*n*	R.S.D. (%)	RE (%)
Blood, 10 μg/mL								
(R)-Sal	10.40	6	3.5	4.3	9.38	5	5.8	−6.2
(S)-Sal	10.28	6	3.0	3.5	9.35	5	5.5	−6.5
Blood, 20 μg/mL								
(R)-Sal	20.37	6	3.1	2.9	19.23	5	3.9	−3.8
(S)-Sal	20.50	6	2.5	3.0	19.52	5	4.2	−2.4
Blood, 100 μg/mL								
(R)-Sal	103.44	6	4.4	4.0	102.54	5	4.1	2.5
(S)-Sal	103.50	6	3.8	3.8	102.20	5	3.6	2.2
Blood, 15 μg/mL								
(R)-Bup	14.82	6	2.6	−2.2	14.43	5	5.7	−3.8
(S)-Bup	14.86	6	2.9	−1.5	14.56	5	5.3	−2.9
Blood, 30 μg/mL								
(R)-Bup	30.92	6	2.8	3.4	30.47	5	4.3	1.6
(S)-Bup	30.95	6	2.5	3.6	30.35	5	4.8	1.2
Blood, 150 μg/mL								
(R)-Bup	154.61	6	3.0	3.0	153.23	5	5.1	2.2
(S)-Bup	154.39	6	3.7	3.2	153.44	5	4.7	2.3

n, number of determinations; R.S.D., relative standard deviation; RE, the mean relative errors.

Sal). Fig. 7 shows a typical electrophergram of a 1.0 mL diluted aerosol solution (two-press aerosol was dissolved in 6.0 mL water) spiked with 20 μL internal standard (Chl, 1 mg/mL). Both enantiomers and internal standard were clearly separated without interference from excipients. The peaks were identified by standard addition under the exactly same conditions. The first migration peak was identified as *R*-forms and the second migration peak was identified as *S*-forms. The results obtained are shown in Table 2. The value obtained by the calibration, as well as the standard addition method was in excellent agreement with the labeled value. Therefore, this method is appropriate for the quality control of pharmaceutical solutions containing Sal enantiomers.

Table 2
Determination of Sal in a pharmaceutical aerosol

Calibration method[a] (μg/mL)	139.2 ± 0.1
Standard addition method[a] (μg/mL)	141.0 ± 0.2
Labeled value (μg/mL)	140

[a] Average of three determination.

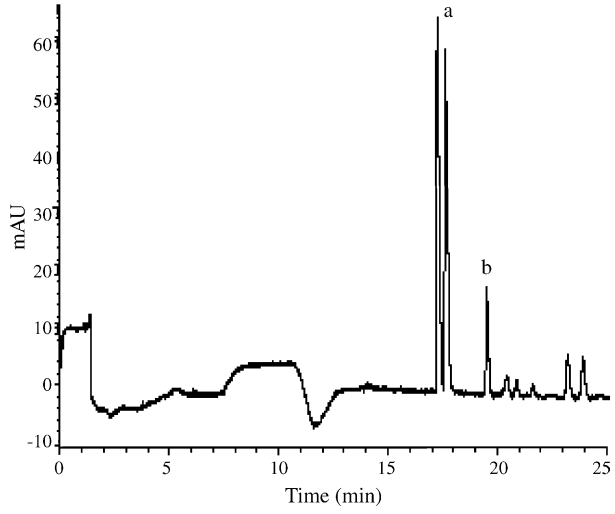

Fig. 7. Typical electrophergram of a 1.0 mL diluted aerosol solution (two-press aerosol was dissolved in 6.0 mL water) spiked with 20 μL internal standard (Chl, 1 mg/mL). Conditions were as same as in Fig. 3. Peaks: (a) (1) R-Sal; (2) S-Sal and (b) Chl.

4. Conclusion

This paper describes simultaneous separation of Sal and Bup enantiomers by capillary electrophoresis using dual neutral CD as chiral selector. It was found that dual neutral DM-β-CD and HP-β-CD have cooperative effect in improvement chiral separation of Sal and Bup. Under the selected experimental conditions, the validation of this method has been successfully used for the study of Sal and Bup concentration change in rat blood and determination of Sal in a pharmaceutical aerosol with satisfactory results. The results of drug metabolism indicated that the rate of metabolism for Bup enantiomers was faster than for Sal, but no enantioselective for rac-Sal and rac-Bup metabolism were observed. This CE method shows that dual CDs systems have a good precision, acceptable accuracy and sensitive and provide a convenient, simple sample pretreatment and without endogenous compound interferences' electropherograms that could further be used in the quality control during drug development and pharmacokinetic study.

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