Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 93–100

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of a capillary electrophoresis method for the enantiomeric purity determination of RS86017 using experimental design

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ARTICLE INFO

Article history:
Received 20 November 2010
Received in revised form 10 January 2011
Accepted 18 January 2011
Available online 26 January 2011

Keywords:
Capillary electrophoresis
Enantiomeric purity
RS86017
Experimental design
Sulfobutyl ether-β-cyclodextrin

ABSTRACT

A selective capillary electrophoresis method for determination of enantiomeric purity of RS86017, a new antiarrhythmic agent with two chiral centers, was developed and validated using sulfobutyl ether-β-cyclodextrin as chiral selector. The concentration of the chiral selector and organic modifier, pH of background electrolyte (BGE), capillary temperature, and applied voltage were systematically optimized by using orthogonal design and concentration of chiral selector was further optimized. The optimal conditions included 25 mM phosphate buffer at pH 8.0, containing 28 mg/mL sulfobutyl ether-β-cyclodextrin and 20% acetonitrile as running buffer, an applied voltage of 22 kV, and a temperature of 20 °C. The detection wavelength was 206 nm. The obtained method was capable of separating RS86017 from its potential chiral impurities, the S,R-enantiomer, the R,R-diastereomer and the S,S-diastereomer with a short analysis time of 10 min. The separation was validated with respect to its selectivity, repeatability, linearity, precision, accuracy, limits of detection (LOD), limits of quantitation (LOQ) and robustness testing. The LODs and LOQs were 0.8 μg/mL and 2.5 μg/mL for all isomers of RS86017, respectively. Finally, the method was used to investigate the chiral purity of RS86017 in bulk samples.

1. Introduction

RS86017, (−)7R,13aS-N-p-chlorobenzyl-2,3-methylenedioxyl-9,10-dimethoxyl-5,8,13,13a-tetrahydro-6H-dibenzo[a,g] quinolizinium chloride, a new compound derived from berberine, having one chiral carbon and one chiral nitrogen, presents one enantiomer (SR86017) and two diastereomers (RR86017 and SS86017) which are shown in Fig. 1. Its racemic compound (CPU86017) possesses favourable antiarrhythmic effect in a variety of arrhythmia model and could block L-type calcium channel of myocardium and Ca^{2+}-related contractions of vascular smooth muscle [1,2]. However, CPU86017 is limited in clinical application because of its low solubility, poor absorption, low bioavailability and toxicity with intravenous injection. These problems can be improved if optical pure isomer is used and RS86017 plays a more important pharmacological activity compared to other stereoisomers. In addition to the antiarrhythmic effect, RS86017 can also reflect on response to anti-oxidative stress, blocking ET pathway so as to improve the cardiovascular activity and reduce side effect significantly [3,4]. According to the FDA's Draft Guidance for Industry on the development of stereoisomeric drugs, applications for an enantiomeric drug substance or applications for drug products containing an enantiomeric drug substance should include a stereochemically specific identity test and/or a stereochemically selective assay method. Therefore, to establish a reliable and effective enantioselective assay is essential for the development of pharmaceutical manufacturing process.

In recent years, CE has been widely used for enantiomer separation in terms of its high efficiency, short analysis time, flexibility, small sample volume requirement, low operation cost, less pollution, etc [5–8]. Applying cyclodextrins (CDs) and their derivatives as the chiral selectors is one of the most commonly used methods in CZE [9]. Native CDs have truncated cone shapes with hydroxyl groups around its narrower rim. The surface hydroxyl groups of the native CD can be modified chemically, leading to CDs with different properties. Modified CDs accommodate a wide range of analytes owing to their increased solubility and additional stereoselective bondings [10]. In our work, β-CD, hydroxypropyl-β-CD (HP-β-CD) and sulfobutyl ether-β-cyclodextrin (SBE-β-CD) as chiral selectors were chosen to separate the enantiomers. SBE-β-CD was chosen as the final chiral selector. SBE-β-CD is negatively charged within a very large pH range. Its own mobility, opposite to that of the electrosmotic flow (EOF), shows a strong resolving power, even if at very low concentration [11]. It is very effective for the discrim-

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doi:10.1016/j.jpba.2011.01.023

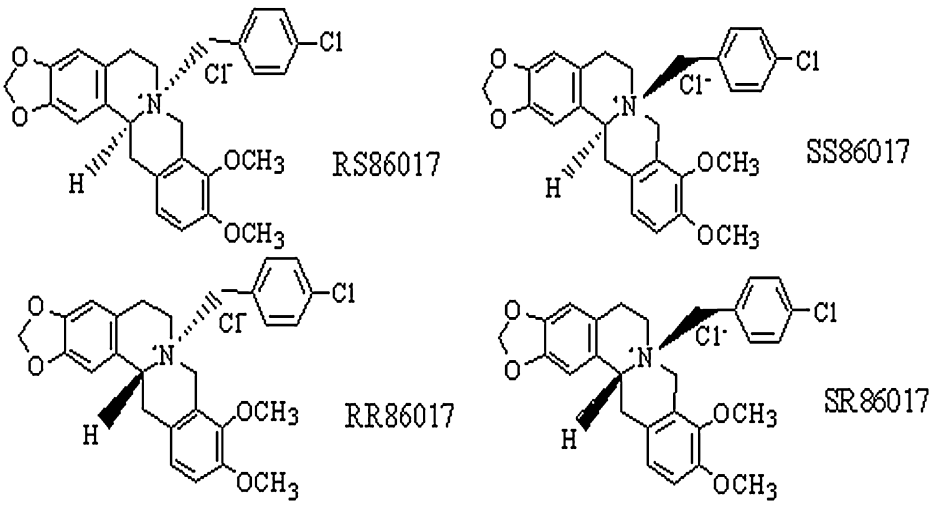

Fig. 1. Chemical structure of RS, SS, RR and SR86017.

ination of cationic and neutral isomers and has been successfully employed for the enantiomer separation of a wide number of drugs [12–14]. The SBE-β-CD we used represents a mixture of isomers with an average degree of substitution of about 6.1–7.1 for the sulfobutyl group. Applying SBE-β-CD as our chiral selector gave rise to a satisfactory separation of RS86017.

For the development of a chiral CE method, a satisfactory resolution of the stereoisomers of RS86017 should be achieved by optimizing the operating conditions. However, optimization of a capillary electrophoretic separation can be difficult due to the wide array of electrophoretic conditions. The technique of changing one variable one time, although widely used, involves a large number of independent runs. The interaction of different variables cannot be observed. Therefore, experimental designs are often employed for optimization in CE [15,16]. Orthogonal experimental design has been successfully applied in many scientific domains [17–19], which provided a simple, efficient, and systematic approach to optimize designs for performance, quality and cost. According to the statistic analysis, it allows a large number of factors to be screened and detects the main effects from the many less important ones. In this research, an orthogonal experimental design was used to find whether or not there was a significant change in the response for different levels of that factor.

Although Fan et al. separated one pair enantiomers of CPU86017 using CE with carboxymethyl-β-CD as chiral selectors [20], to the best of our knowledge, there is no literature about developing a stereoselective analytical method for simultaneously determining all stereo-isomers of RS86017. Thus the aim of this work was to develop and validate a chiral capillary electrophoresis method for simultaneous determination of the potential chiral impurities of RS86017.

2. Materials and methods

2.1. Chemicals and reagents

RS86017 and its isomeric impurities (S,R-enantiomer, R,R-diastereomer, and S,S-diastereomer) were by college of pharmacy of China pharmaceutical university. β-CD was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), HP-β-CD was from Wuhan Yuancheng Technology Development Co., Ltd. (Wuhan, China). SBE-β-CD was kindly provided by Dr. Wangwei.

Purified water (Robust, Guangdong, China) was used in the preparation of solutions. Sodium hydroxide, phosphoric acid, sodium dihydrogen phosphate and sodium phosphate were from Nanjing chemical reagent Co., Ltd. (Nanjing, China). Acetonitrile was purchased from Tedia Company, INC (USA).

2.2. Instrumentation

The experiments were carried out on HP^{3D} capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector and controlled by the HP^{3D}CE ChemStation software. The uncoated fused-silica capillaries of 50 μm i.d., with a total length of 48.5 cm (40 cm to the detector) purchased from Yong-nian Optical Fiber Factory (Yongnian, Hebai Province, China) was used for separation. The orthogonal experimental design was performed with Orthogonality Experiment Assistant version 3.1 (Sharetop Software Studio, China). Samples were injected hydrodynamically by applying 50 mbar pressure over 8 s. The detection wavelength was 206 nm. The capillary was at a temperature from15 to 35 °C and voltage was applied from 15 kV to 30 kV. The optimized value of the temperature and voltage was 20 °C and 22 kV, respectively. A new capillary was rinsed with 1 M sodium hydroxide (NaOH) for 30 min, 0.1 M NaOH for 30 min, followed by 30 min with water, and conditioned with background electrolyte (BGE) for 5 min. Between runs, the capillary was conditioned with 1 M HCl for 1 min, 1 M NaOH for 1 min, water for 1 min and BGE for 2 min.

2.3. Preparation of BGEs and samples

Sodium phosphate buffers (25 mM, 40 mM and 55 mM) at pH 2.5, 6.0, 7.0 and 8.0 were applied, pH was adjusted by phosphoric acid at the same concentration. BGEs were prepared in these phosphate buffers by dissolving organic modifier, SBE-β-CD, or other β-CDs at targeted levels. Stock solutions of standard RR, SS and SR86017 in the concentration of 500 μg/mL and RS86017 of 2.5 mg/mL were prepared in acetonitrile–water (1:9, v/v). The standard solutions (concentrations in the figures) were prepared by diluting the stock solutions with acetonitrile–water (1:9, v/v). All running buffers and analyte solutions were filtered through a 0.45 μm syringe filter before injection.

3. Results and discussion

3.1. Selection of initial conditions

For identification of a suitable chiral selector, mono-CD systems, namely β-CD, HP-β-CD, SBE-β-CD, were investigated for their abil-

ity to separate stereoisomers of RS86017. On the other hand, it has been shown many examples that dual CD systems based on combinations of charged and neutral CDs can result in efficient chiral CE separations, so a dual CD system which consisted of HP-β-CD and SBE-β-CD was conducted. Both systems were performed under the condition of 40 mM phosphate buffer as the BGE with capillary temperature at 20 °C. With the pH value of BGE set at 2.5, the concentrations of chiral selectors were varied. Applied voltage was set at 25 kV. Owing to the fact that apparent mobility (a combination of the mobility of free and complexed analyte and of the electroosmotic flow) was opposite to the electroosmotic flow for SBE-β-CD and the dual CD system, negative voltage was conducted. The results showed that β-CD did not provide any chiral separation of the two pairs enantiomers, while HP-β-CD had better chiral recognition abilities than β-CD, in which SR, RS86017 can get partly separated but RR, SS86017 cannot be satisfactorily resolved. SBE-β-CD exhibited potential separation for the two pairs of enantiomers of CPU86017, for which RR, SS86017 can get baseline separation while SR, RS86017 can be partly separated. The dual CD system (β-CD and SBE-β-CD) did not show any superiority compared with SBE-β-CD. From above results, SBE-β-CD was chosen as the final chiral selector. In the later screening design, the concentration range of SBE-β-CD was selected from 0 to 24 mg/mL.

Organic modifier plays an important role in CE enantioseparation. The addition of organic modifier can drastically influence both efficiency and resolution by changing viscosity of the BGE, electroosmotic flow, and stability of the analyte-CD complexation [21]. Methanol and acetonitrile, as two commonly used organic modifiers for fine-tuning the selectivity by enhancing the polarity of the background electrolyte, had been explored in this study. It was found that either methanol or acetonitrile could improve the resolution of SR86017 and RS86017, but a higher resolution was observed using acetonitrile as organic modifier. The resolution did not improve any more when the concentration of acetonitrile was tolerated up to 20% (v/v), therefore, the acetonitrile concentration range for the optimization of the resolution value was chosen from 0% to 20%.

The change of concentration of phosphate buffer provided the variation of ionic strength and would affect the chiral separation. As chiral selector we used is negatively charged, the high current will generate if the buffer concentration is high. On the basis of the preliminary experiment, we selected buffer concentrations from 25 to 55 mM for further investigation.

The pH of buffer had a marked effect on the separation, and it was shown that a high BGE pH offered a satisfactory separation, mainly due to the fact that the EOF became more significant and the difference between electrophoretic mobility of SBE-β-CD and electroosmotic flow would be more obvious. However, the pH cannot be over 8 in case of migration time being too short, which led to the poor resolution. Therefore, the BGE pH from 6 to 8 was studied subsequently.

Temperature and applied voltages had also been found to have influence on the resolution. With the change of temperature, buffer viscosity and complex stability would be different, and when voltage differ, the rate of eletroosmotic flow, migration time, Joule heating would change, too. As previously mentioned, positive voltage should be applied when the pH was over 5. Therefore, temperatures from 15 to 35 °C and applied voltages from 15 to 30 kV were used for the optimization of the resolution value, respectively.

3.2. Method optimization

3.2.1. Orthogonal experimental design

The development of an effective methodology to investigate and optimize significant factors is of great importance. At this stage, experimental design may be helpful for determining which variables mainly influence the separation and for optimizing the significant variables to give the best possible separation in a relatively low number of experiments. A good experimental design not only provides more information but also makes it possible to achieve the optimal experimental conditions. An orthogonal design was used to screen some significant factors from the investigated ones. There are six experimental factors chosen as variable parameters: SBE-β-CD concentration (0, 12 and 24 mg/mL), organic additive concentration [0, 10% and 20% (v/v)], buffer concentration (25, 40 and 55 mM), BGE pH (6, 7 and 8), run voltage (15, 22 and 30 kV) and temperature (15, 25 and 35 °C). Three levels were set for each variable parameter. A standard orthogonal table $L_{18}(6^3)$ was used for the design of the experiments, in which 18 meant the total number of experiments, 3 represented the number of levels and 6 stood for the factors' number. The interactions among factors examined were neglected in screening step. The ranges and intervals of the six variables were determined by preliminary experiments. The 18 individual experiments and the results of each run were summarized in Table 1.

Choosing a proper criterion to evaluate the separate condition turned to be another necessity. To find the best resolutions of the four stereoisomers, the sum of all resolutions should be considered at first, but due to the fact that the resolution of diastereoisomers (SS86017 and SR86017) was more greater than the two pairs of enantiomers, the sum of all resolutions could not reflect the separation of two pairs of enantiomers of CPU86017. The minimum resolution of the four stereoisomers was also tried to evaluate the separation information. But it could only reflect the resolution of one pair of enantiomers. Therefore, the sum of resolutions of the two pairs of enantiomers ($\sum$Rs), excluding the resolution of diastereoisomers (SS86017 and SR86017), was chosen as the criterion response, because the two pairs of enantiomers showed the same separate trend and could evaluate the enantioseparation quality.

To clarify the significance levels of different factors on the $\sum$Rs, the range analysis was conducted. Table 1 summarized the statistical level analysis of different factors on the $\sum$Rs. K1–K3 were the average resolution value under every level of an investigating variable. Range value (R) was the difference between the maximal and minimal value of the three levels. Based on the results of range analysis, the order of significance levels was SBE concentration, acetonitrile concentration, BGE pH, buffer concentration, voltage and temperature. The variance analysis (Table 2) revealed that only the concentration of the chiral selector had significant effect on the resolution value.

Data analysis of orthogonal experimental design showed that the optimal response was achieved when the temperature, running voltage, buffer concentration, organic additive concentration and BGE pH were 15 °C, 30 kV, 55 mM, 20% and 8, respectively. Finding the instrument would take a long time to get stable at 15 °C, and increasing temperature would not have significant effect on $\sum$Rs, we finally set the temperature at 20 °C, which is most commonly used and can be fast stabilized. If the applied voltage and the buffer concentration were set as 30 kV and 55 mM, poor reproducibility was observed which was probably as the result of increased current generation and Joule heating. Range and variance analysis showed that changing the level of voltage and buffer concentration from the optimal one to the second optimal one made little difference of resolution value. In order to get better reproducibility, running voltage and buffer concentration were maintained at 22 kV and 25 mM; organic additive concentration and BGE pH did not change, setting at 20% and 8, respectively.

Only the concentration of the chiral selector needed to be optimized due to the result of orthogonal experiment design. With the increase of SBE-β-CD concentration, it was found that the separation of four stereoisomers achieved better. To get more specified,

Table 1
Orthogonal experiments design chart, six variables, and three levels.

Experiments	(1)[a]	(2)[b]	(3)[c]	(4)[d]	(5)[e]	(6)[f]	Rs1[g]	Rs2[h]	∑Rs
1	15	15	0	0	25	6	0.00	0.00	0.00
2	15	22	12	10	40	7	4.40	2.40	6.80
3	15	30	24	20	55	8	5.90	5.67	11.57
4	25	15	0	10	40	8	0.65	0.49	1.14
5	25	22	12	20	55	6	3.72	2.50	6.22
6	25	30	24	0	25	7	3.31	0.29	3.60
7	35	15	12	0	55	7	2.81	1.08	3.89
8	35	22	24	10	25	8	3.87	1.67	5.54
9	35	30	0	20	40	6	0.80	0.78	1.58
10	15	15	24	20	40	7	1.77	0.00	1.77
11	15	22	0	0	55	8	0.00	0.00	0.00
12	15	30	12	10	25	6	3.34	1.75	5.09
13	25	15	12	20	25	8	2.72	2.64	5.36
14	25	22	24	0	40	6	4.01	0.69	4.70
15	25	30	0	10	55	7	0.64	0.40	1.04
16	35	15	24	10	55	6	4.04	1.63	5.67
17	35	22	0	20	25	7	0.34	0.00	0.34
18	35	30	12	0	40	8	2.14	1.04	3.18
K1	4.205	2.972	0.677	2.563	3.322	3.872			–
K2	3.678	3.935	5.090	4.213	3.190	2.907			–
K3	3.360	4.337	5.477	4.467	4.732	4.465			–
R	0.845	1.365	4.800	1.904	1.542	1.558			–

[a] Temperature (°C).
[b] Voltage (kV).
[c] SBE-β-CD concentration (mg/mL).
[d] Acetonitrile concentration (v/v).
[e] Buffer concentration (mM).
[f] The BGE pH.
[g] Resolution value of RR86017 and SS86017.
[h] Resolution value of SR86017 and RS86017.

a further study on the influence of SBE-β-CD concentration should be processed.

3.2.2. Effect of SBE-β-CD concentration

A continuative investigation, with a 25 mM phosphate buffer (pH 8.0) containing 20% acetonitrile and with a constant voltage of 22 kV at 20 °C, would enable to determine the effect of SBE-β-CD concentration on enantioseparation through data analysis. The resolution values of Rs1 (resolution value of RR86017 and SS86017) and Rs2 (resolution value of SR86017 and RS86017) were shown in Fig. 2. The results indicated that baseline separation of four stereoisomers could be achieved even the SBE-β-CD concentration was at 12 mg/mL. The ∑Rs varied with the concentration of SBE-β-CD, and it achieved the highest value when the SBE-β-CD concentration was at 28 mg/mL. The highest value of ∑Rs was 11.07 (Fig. 3) and the resolution value of RR, SS86017 and SR, RS86017 were 6.34 and 4.73, respectively, which was high enough to control chiral purity of RS86017. Thus, satisfactory separation with the optimal conditions for determining the enantiomeric impurities of RS86017 was achieved.

A reason for the comparatively good separation of RS86017 can be the fact that positively charged analytes achieve higher separation efficiency using negatively charged β-CDs [22,23]. It is known that the enantioselectivity of CDs is linked to hydrogen-bonding interaction, hydrophobicity, steric effects, and electrostatic force. Diastereoisomeric complexes formed by the analytes and SBE-β-CD were carried towards the detector by the electroosmotic flow. The apparent mobility for the complexes is a combination of the mobility of free and complexed analyte and of the electroosmotic flow [11]. With the SBE-β-CD concentration increased, the decrease in apparent mobility would result in a general increase in migration time, and ∑Rs gradually improved. As broad peaks were obtained, ∑Rs did not improve any more when the SBE-β-CD concentration was over 28 mg/mL.

The optimal method was thus selected at 20 °C, using 28 mg/mL SBE-β-CD in 25 mM phosphate buffer (pH 8.0) containing 20% acetonitrile, with an applied voltage of 22 kV. The detection wavelength was 206 nm. However, a relatively large RSD for migration time (over 15%) was observed under the optimal conditions, which was probably due to the interactions between the negatively charged CD-analytes and the inner wall of capillary [24]. To solve this problem, several flushing procedures were conducted. Compared with 0.1 M NaOH, 1 M NaOH was better reproduced, but the migration time still prolonged after several runs. Trying combined flushing steps, we found that repeated rinsing of the capillary with 1 M HCl, 1 M NaOH, water and BGE after each run, could ensure the reproducibility of migration time.

Additionally, it was found that the solvent of analyte solutions had substantial influence on the resolution value and sensitivity. Using pure acetonitrile to dilute the sample stock solution prevented the separation, instead, with the increasing percentage of water, the resolution value would improve evidently, but the analytes were difficult to dissolve if too much water was used. Using 20% acetonitrile as analyte solvent, we found the LOD was 1.5 μg/mL, which is two times of 10% acetonitrile. Therefore, 10% acetonitrile, which could dissolve analytes properly, was finally used.

Table 2
An ANOVA table for normalized experimental responses in the $L_{18}(3)^6$.

Factor	DOF[a]	SSD[b]	F[c]	Type of effect
Temperature	2	2.185	0.107	
Voltage	2	5.905	0.289	
SBE-β-CD concentration	2	85.334	4.182	Significant
Acetonitrile concentration	2	12.819	0.628	
Buffer concentration	2	8.764	0.430	
The BGE pH	2	7.423	0.364	

[a] Degrees of freedom.
[b] Sum of squares of deviations.
[c] Critical value is 3.890 ($P < 0.05$) and 2.810 ($P < 0.1$).

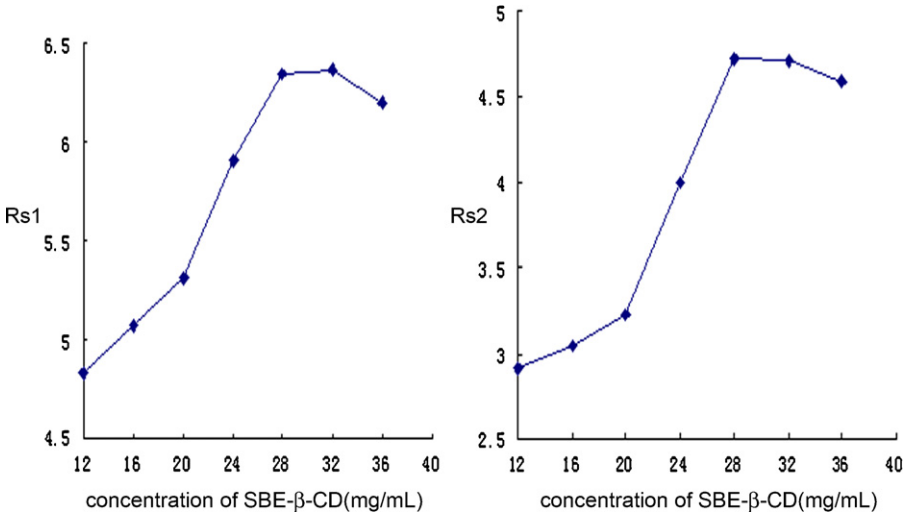

Fig. 2. The effect of SBE-β-CD concentration on Rs1 and Rs2. Separation solution: 28 mg/mL SBE-β-CD in 25 mM phosphate buffer (pH 8.0) containing 20% acetonitrile; capillary, 48.5 cm × 50 μm i.d. (effective length, 40 cm); applied voltage, 22 kV; detection wavelength, 206 nm; injection, 50 mbar over 8 s; analyte concentration, 25 μg/mL for RR, SS, SR86017 and 40 μg/mL for RS86017.

3.3. Method validation

The optimal method was subsequently validated according to the ICH guideline Q2 (R1) with respect to selectivity, repeatability, linearity range, precision, accuracy, LOD, LOQ and robustness.

3.3.1. Selectivity

Selectivity of the method was demonstrated by analyzing test solutions spiked of 0.1% (w/w) RR, SS, and SR86017 shown in Fig. 4. Acceptable resolution of RS86017 and its isomers was achieved. Compared with the resolution value obtained from the experimental design, Rs1 was maintained. Though Rs2 was less, it can still be sensitive enough to detect isomer impurity. This problem may occur as a result of high concentration of RS86017, increasing the peak width of main compound.

3.3.2. Repeatability

The repeatability of migration times and peak areas of RR, SS, SR and RS86017 were determined in six measurements. The concentration of four isomers was all at 25 μg/mL. The RSD of migration times were 1.95%, 2.01%, 1.65% and 1.53% for RR86017, SS86017, SR86017 and RS86017. The RSD of peak area were 2.02%, 2.82%, 2.16% and 1.64% for RR86017, SS86017, SR86017 and RS86017.

3.3.3. Linearity range

The linearity of RR, SS and SR86017 in standard solutions was investigated at six concentration levels. All calibration curves were linear over the concentration range from 2.5 μg/mL to 100 μg/mL. And the correlation coefficients (r^2) of the calibration line were all over 0.998. The linear equations for RR, SS and SR86017 were $Y_1 = 2.3241X_1 - 0.4369$, $Y_2 = 3.2532X_2 - 3.6848$, $Y_3 = 3.195X_3 + 0.2021$, respectively (X represents the concentration of enantiomer: μg/mL; Y represents peak area of enantiomer in electropherogram).

3.3.4. Accuracy and precision

The method accuracy was assessed as recovery obtained from three isomers when spiking the test solutions (2.5 mg/mL) with known concentrations of three isomers (2.5, 12.5 and 25 μg/mL of each isomer). The recovery values for RR, SS and SR86017 were as follows: 97.36 ± 4.72%, 97.42 ± 4.82% and 97.57 ± 4.73%. The values of RSD were all less than 5.0%.

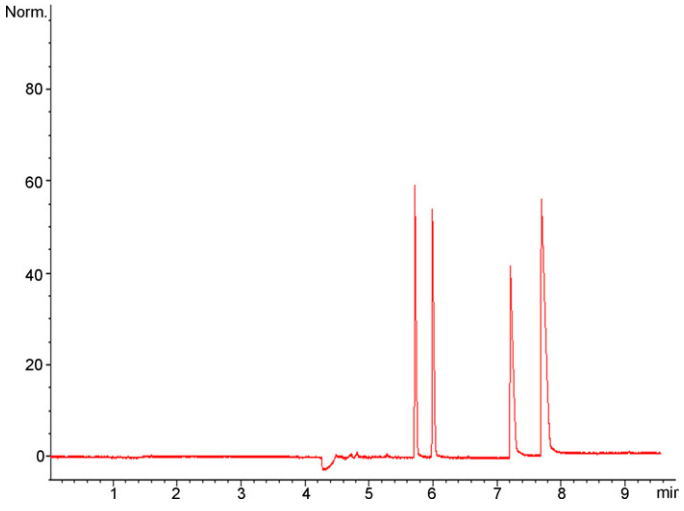

Fig. 3. Electrophoretic separation of CPU86017. The migration order was RR, SS, SR and RS86017. The separate conditions are as in Fig. 2.

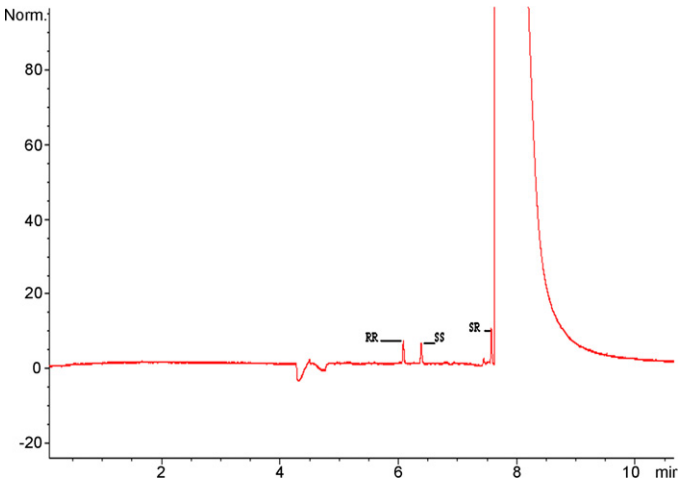

Fig. 4. RS86017 standard (2.5 mg/mL) containing 0.1% RR, SS and SR86017. RR, SS and SR represent RR, SS and SR86017. Other conditions are as in Fig. 2.

Table 3
The Plackett–Burman design.

Experiments	(1)[a]	(2)[b]	(3)[c]	(4)[d]	(5)[e]	(6)[f]	Dummy1	Dummy2
1	29	26	7.9	21	19	21	−1	1
2	27	24	7.9	21	21	23	−1	1
3	27	24	7.9	19	19	21	−1	−1
4	29	26	8.1	19	21	23	−1	1
5	29	24	8.1	19	19	21	1	1
6	27	24	8.1	21	21	21	1	1
7	29	24	7.9	19	21	23	1	−1
8	29	24	8.1	21	19	23	−1	−1
9	27	26	7.9	19	19	23	1	1
10	27	26	8.1	21	19	23	1	−1
11	29	26	7.9	21	21	21	1	−1
12	27	26	8.1	19	21	21	−1	−1

[a] SBE-β-CD concentration (mg/mL).
[b] Buffer concentration (mM).
[c] The BGE pH.
[d] Acetonitrile concentration (v/v).
[e] Temperature (°C).
[f] Voltage (kV).

The test solution containing 0.1% isomer impurities was used to evaluate precision by replicate testing for six times. The RSD values of peak areas, 3.58%, 3.35%, 3.56% and 4.13%, were obtained for RR, SS, SR and RS86017, respectively. The RSD values of migration times for RR, SS, SR and RS86017 were 2.45%, 2.59%, 3.18% and 3.22%.

3.3.5. LODs and LOQs

LODs and LOQs of each isomer of RS86017 were based on signal to noise ratio, 3:1 and 10:1, respectively. The LODs and LOQs for RR, SS, and SR86017 were 0.8 μg/mL and 2.5 μg/mL, corresponding to a relative concentration of 0.03% and 0.1% of the main compound based on a concentration of RS86017 of 2.5 mg/mL.

3.3.6. Robustness

During the robustness testing, the analysis method must prove to be able to remain unaffected by small, but deliberately introduced variations in method variables. If the variables have significant effect on the responses, one should change the method or control experiment procedure more strictly. As the purpose of the study was to establish a CE method to control the chiral impurities of RS86017, the standard solution of 2.5 mg/mL RS86017 containing 0.1% isomer impurities was used to the robustness testing. Statistical experimental designs such as Plackett–Burman and fractional factorial designs are often applied to minimize the number of experiments [25]. The robustness of the method was examined by a Plackett–Burman design. The factor interactions can be negligible due to the small variations in the factor levels [26,27].

In the test, six variables which potentially might affect the results were investigated on six responses. The method variables were investigated at the upper (+) and lower (−) values with regard to the nominal one which is the optimal value in the procedure. The six variables included the SBE-β-CD concentration, buffer concentration, pH of BGE, organic additive concentration, temperature and voltage. Two dummy factors were included in the design to estimate the experimental error. The dummy factor is an imaginary variable of which the change between the levels does not represent a physical change in the method [28]. The experimental designs are given in Table 3.

Table 4
The factor effects and *P* values on the different responses.

Factor	S1[a]	S2[b]	S3[c]	S4[d]	∑Rs[e]	At[f]
(a) Effects on						
SBE-β-CD concentration	0.2833	0.0950	0.3433	443.7	−0.3867	0.6137
Buffer concentration	−0.3167	−0.8250	−0.0700	−22.0	−0.6200	−0.1450
The BGE pH	0.2433	0.7917	0.3300	737.6	2.4367	0.0323
Acetonitrile concentration	0.0767	−0.3950	−0.1233	245.6	−0.2867	0.0363
Temperature	0.0700	0.5583	0.1200	240.7	−0.6700	−0.3117
Voltage	−0.5133	−0.2517	−0.1733	−643.2	−0.2067	−0.8933
Dummy1	−0.1333	0.4250	0.1000	256.5	0.3767	−0.0357
Dummy2	0.1333	−0.3783	−0.1200	−296.7	0.0467	0.0450
(b) *P* values						
SBE-β-CD concentration	0.482	0.859	0.133	0.168	0.373	0.021[g]
Buffer concentration	0.437	0.191	0.704	0.934	0.193	0.366
The BGE pH	0.541	0.205	0.144	0.057	0.007[g]	0.828
Acetonitrile concentration	0.842	0.480	0.515	0.390	0.495	0.807
Temperature	0.856	0.338	0.526	0.399	0.168	0.107
Voltage	0.243	0.644	0.377	0.079	0.616	0.007[g]
Dummy1	0.731	0.450	0.593	0.372	0.384	0.811
Dummy2	0.731	0.497	0.526	0.313	0.908	0.763

[a] The peak area of RR86017.
[b] The peak area of SS86017.
[c] The peak area of SR86017.
[d] The peak area of RS86017.
[e] The sum of resolutions of the two pairs of enantiomers.
[f] The analysis time.
[g] Significance at $\alpha = 0.05$ level.

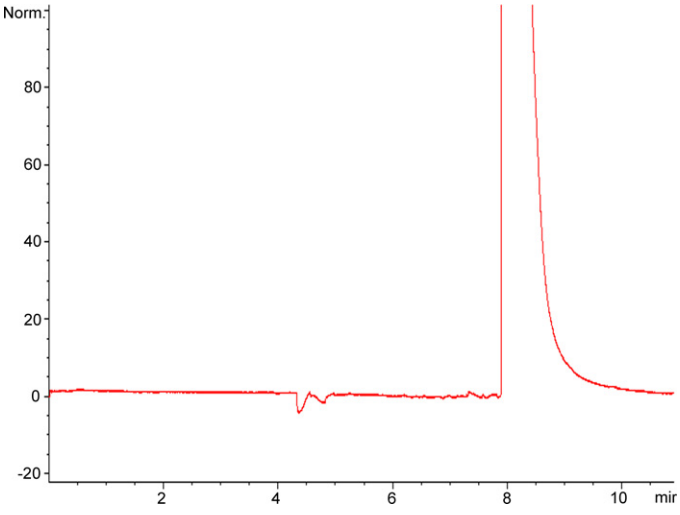

Fig. 5. Electropherogram of RS86017 in bulk samples (2.5 mg/mL). The separate conditions are as in Fig. 2.

The peak areas of RS86017 and its isomers, the analysis time (the last migrating peak) and ∑Rs, which were considered appropriate for describing the quality of the chiral separation, were used as the responses. The effects of the different factors (E_x) on the considered responses and the corresponding P values could be detected in Table 4. The E_x is calculated as follows:

$$E_x = \frac{2\left(\sum Y(+1) - \sum Y(-1)\right)}{N}$$

$\sum Y(+1)$ and $\sum Y(-1)$ are the sums of responses where factor x is at (+) or (−) value, respectively, and N is the experiment numbers.

It was found that none of the six studied variables had a significant effect on the peak areas of RS86017 and its isomers. The migration time was significantly influenced by the SBE-β-CD concentration and the voltage. ∑Rs on the other hand was obviously influenced by the BGE pH (t-test, $\alpha = 0.05$).

As the purpose of the study was to determine the isomer impurities of RS86017, the method is adequately robust for routine analysis for quality control of RS86017. To get repeatability of migration time and ∑Rs, the other experimental conditions like SBE-β-CD concentration, voltage and BGE pH should be controlled strictly.

3.4. Application in bulk samples

The method was applied to determine chiral impurities of RS86017 in bulk samples. The result was compared with the RS86017 standard which contained 0.1% RR, SS, SR-86017, showing that the concentration of the chiral impurities in RS86017 bulk samples are far below 0.1% (Figs. 4 and 5). Thus, a sensitive and effective method of determining chiral impurities at a concentration of 0.1% can be estimated so that the impurity control of RS86017 can be regarded as reliable.

4. Conclusions

A selective capillary electrophoresis method for the simultaneous separation of RS86017 from its potential chiral impurities, the S,R-enantiomer, the R,R-diastereomer and the S,S-diastereomer, was developed and validated. By using experimental designs, optimum separation was successfully achieved and four stereoisomers of RS86017 could be separated in 10 min with satisfactory resolution, linearity, and sensitivity. The method was also applied to estimate the enantiomer impurities of RS86017 bulk samples. It demonstrated that the method we developed could be applied for the control of enantiomeric purity of RS86017, and the whole procedure described might be a very useful tool for the quality control of the enantiomeric drug products.

Acknowledgement

The authors thank to Major scientific and technological specialized project for "significant new formulation of new drugs" of China (Grant No. 2009ZXJ09004-070), Program for New Century Excellent Talents in University (No. NCET-08-0847) and the Fundamental Research Funds for the Central Universities (CPU-JPK2009001) for the financial supports.

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