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# Direct separation and quantitative determination of clenbuterol enantiomers by high performance liquid chromatography using an amide type chiral stationary phase

Yaru Song [a,*], Defa Wang [a], Yuping Hu [a], Xiaoxia Chen [a], Yuguo Jiao [b], Dongyan Hou [c]

[a] *Department of Chemistry, Beijing Normal University, Beijing 100875, PR China*
[b] *Department of Biochemistry, Central University for Nationalities, Beijing 100081, PR China*
[c] *Department of Chemistry, Anshan Normal University, Anshan 114005, PR China*

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**Abstract**

Enantiomers of clenbuterol were directly separated by a new high performance chromatographic method on Chirex 3005 column. Several parameters such as mobile phase composition, column temperature and flow rate were studied. Baseline enantioseparation was achieved, using the optimized mobile phase of *n*-hexane–1,2-dicholoethane–methanol (54:38:8, v/v/v) at 17 °C and 1.0 ml/min, with the separation factor ($\alpha$) 1.43 and the resolution factor ($R_S$) 1.81. The mechanism of separation was also discussed. Standard linear calibration cures were established for the *R*- and *S*-enantiomers, over the range of 26.1–1045.8 and 5.7–229.6 nmol/ml, with the correlation coefficient of 0.9999 for both. The limits of detection were 0.47 and 1.04 nmol/ml for *R*- and *S*-enantiomers, respectively. Recovery and precision of the method were also evaluated, which had been successfully used to monitor and identify quantitatively the profile of the clenbuterol enantiomers in human serum.


*Keywords:* Clenbuterol; Enantiomer separation; Chiral stationary phase; HPLC

## 1. Introduction

Clenbuterol, belonging to the family of β-adronists, is a sympathomimetic drug with potent $\beta_2$-adrenoceptor stimulating properties and used for the treatment of pulmonary diseases. Its bronchodilatory effect on smooth muscle is the main pharmacological action responsible for alleviation of asthmatic attacks brought about by infection or allergies, as well as treating other respiratory disorders [1–3]. Besides the smooth muscle relaxation effect, clenbuterol is used to increase the muscle growth [4] and reduce the fat of livestock in agriculture [5]. It is also one of the drugs abused by athletes as a performance-enhancing agent [6]. It was reported that clenbuterol

* Corresponding author. Tel.: +86-10-6220-7843; fax: +86-10-6265-8917.
*E-mail address:* onlywdf_1977@163.com (Y. Song).

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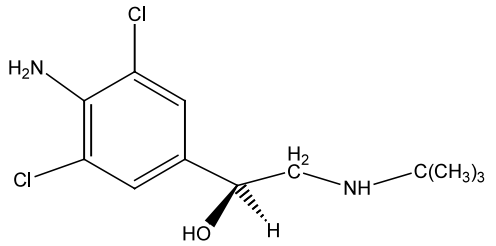
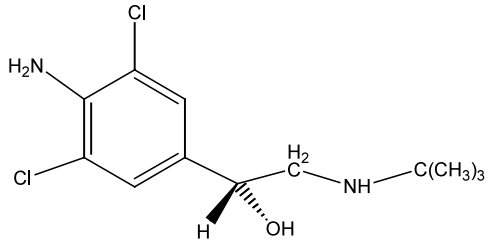



(-)-R-Clenbuterol




(+)-S-Clenbuterol

Fig. 1. The absolute configurations of (−)-*R*- and (+)-*S*-clenbuterol.

might be used to treat of cardiomyopathy, a clinical condition caused by several heart diseases [7]. Though it is prohibited from being used in cattle feed, there is also evidence of human poisoning from the consumption of meat products containing clenbuterol residue [8].

Clenbuterol is chemically known as 4-amino-3,5-dichloro-α-[(1,1-dimethylethyl)amino]-methylbenzenemethanol, or 1-(4-amino-3,5-dichlorophenyl)-2-*tert*-butylaminoethanol. There is one chiral center in its molecule and the (−)-*R* and (+)-*S* configurations are shown in Fig. 1.

Chirality is a prominent feature of most biological processes and the enantiomers of bioactive molecules often possess different biological effect [9]. It was reported that the $\beta_2$-agonistic as well as the $\beta_1$-antagonistic effect of clenbuterol resides in the (−)-isomer and the (+)-isomer does not seem to contribute to the pharmacological effects [10]. There was also a report that the antidepressant-type effect of (±)-clenbuterol is caused by the (−)-enantiomer, and the (+)-form has been shown to act as a pure $\beta_1$-blocker in the heart, where it antagonized the effects of isoproterenol [11]. Therefore, enantioseparation of clenbuterol is very important for preparative and analytical purposes.

High performance liquid chromatography (HPLC) and capillary electrophoresis [3,5,9,12–19] were commonly used to resolve the enantiomers of clenbuterol. Comparing to capillary electrophoresis, HPLC possess low noise, smooth baseline and possibility for preparing the single enantiomer. Macrocyclic antibiolic CSP and Pirkle type CSP such as Chirex 3022, 3020 and 3018 have been used to resolute clenbuterol on HPLC. There are two main types of Pirkle type CSP, π-acceptor and π-donor phase. One kind of popular π-acceptor phases containing *N*-(3,5-dinitrobenzoyl), such as Chirex 3005, are capable of separating a large range of compounds including a π-donor aromatic group. But in Cleveland's report [19], the enantioseparation of Clenbuterol, a typical π-donor analyte, was unsuccessful on column Chirex 3005.

Aim to evaluate the feasibility of enantioseparation of clenbuterol on Chirex 3005, different chromatography conditions were studied in this paper. Results show that Chirex 3005 is applicable to assay clenbuterol enantiomers, not only qualitatively but also quantitatively. In addition, this method has simple mobile phase system and time saving sample preparation, compare to previously published methods.

## 2. Exeprimental

### 2.1. Chemicals

Methanol was provided by Beijing Chemical Plant (Beijing, China). 1,2-Dichloroethane and *n*-hexane were purchased from Tianjin Bodi Chemical Company (Tianjin, China). All reagents were dried and redistillation to further purification before using. The mixture of (±)-clenbuterol enantiomers got from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with the ratio of (−)/(+) = 82/18. Human serum was a gift from Beijing Normal University Hospital.

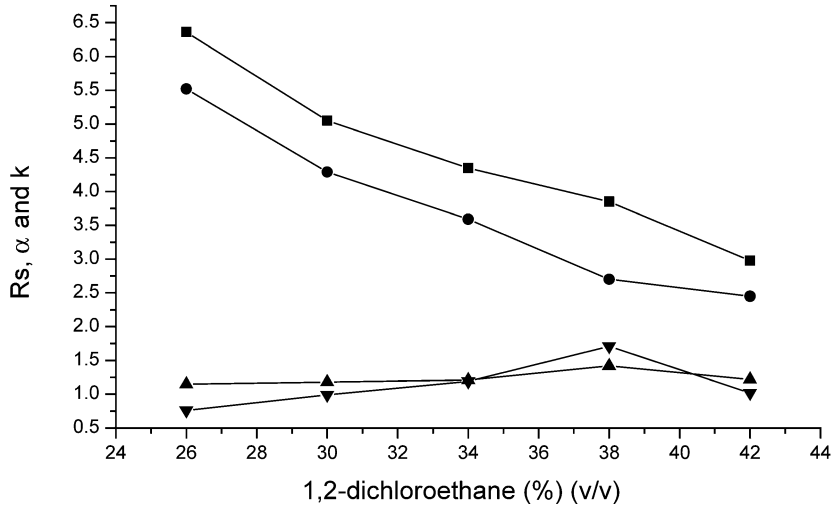



Fig. 2. Effects of concentration of 1,2-dichloroethane in mobile phase on the enantioseparation. Column, Chirex 3005 (250 × 4.6 mm i.d.); mobile phase composed of *n*-hexane, 1,2-dichloroethane and methanol; the volume percentage of methanol, 8%; flow rate, 1.0 ml/min; column temperature, 25 °C; UV detector, 247 nm; sample concentration, 318.8 nmol/ml; injection volume, 20 μl; ▼, $R_S$; ▲, $\alpha$; ●, $k_1$; ■, $k_2$.

### 2.2. Equipments

Agilent 1100 series HPLC was used, including G1311A pump, G1316 column temperature controller, and G1365B multiple wavelength detector (Agilent, USA). The amide type CSP used in this study was made of (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid with dimensions of 250 × 4.6 $mm^2$, known as the Chirex 3005 column, purchased from Phenomenex (USA). The polarimetry was performed by a 241-MC polarimeter from Perkin–Elmer (USA).

### 2.3. Assay conditions

The chromatograms were monitored by UV detection and the wavelength of 247 nm was selected. Samples were dissolved in methanol. The injection volume was fixed at 20 μl. The mobile phase was *n*-hexane–1,2-dichloroethane–methanol (54:38:8 v/v/v). The optimized column temperature and flow rate were 17 °C and 1.0 ml/min, respectively. Chromatographic data were acquired and analyzed with HP ChemStation Rev.A.08.03 (Agilent, USA).

### 2.4. Sample preparation and extraction

One milliliter of blank serum spiked with clenbuterol in a 13 × 100 mm glass tube was vortex mixed for 10 s and left for 10 min. 1,2-Dichloroethane (0.5 ml) was added and the mixture was vortex for 10 min and centrifuged for another 10 min. The organic layer was transferred to a clean glass tube. Another 0.5 ml of 1,2-dichloroethane was added and the process was repeated. The organic phase was combined and evaporated to dryness under $N_2$ and the residue was redissolved in 1 ml of methanol and filtered. A 20 μl aliquot was injected onto the HPLC system. Blank serum sample (none spiked) was processed in the same way to detect interfering peaks.

### 2.5. Chromatographic parameters

The Chemstation provided the chromatographic parameters of elution time $t_R$, stereochemical resolution factor $R_S$ and peak area $A$. The capacity factors $k$ were calculated using equation $k = (t_R - t_0)/t_0$ where $t_0$ is the void time. The separation factor $\alpha$ was calculated using the equation $\alpha = k_2/k_1$ where $k_2$ and $k_1$ are the capacity

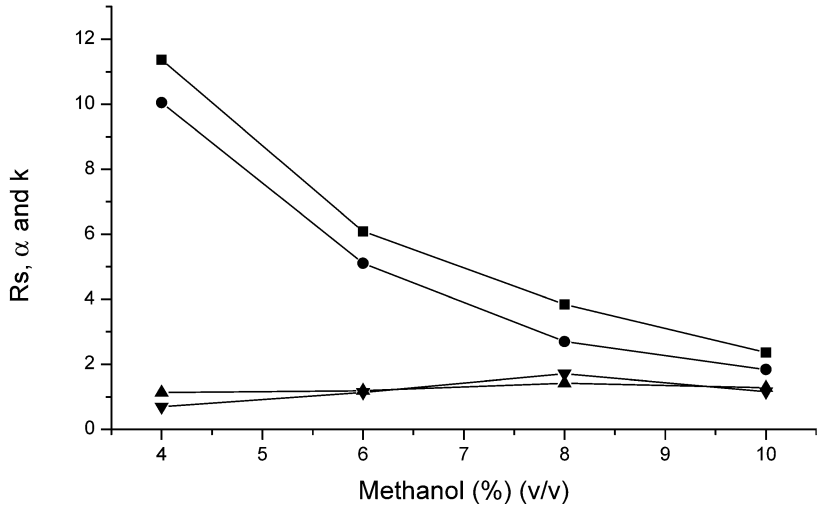



Fig. 3. Effects of concentration of methanol in mobile phase on the enantioseparation. Column, chirex 3005 (250 × 4.6 mm i.d.); mobile phase composed of *n*-hexane, 1,2-dichloroethane and methanol; the volume percentage of 1,2-dichloroethane, 38%; flow rate, 1.0 ml/min; column temperature, 25 °C; UV detector, 247 nm; sample concentration, 318.8 nmol/ml; injection volume, 20 μl; ▼, $R_S$; ▲, $\alpha$; ●, $k_1$; ■, $k_2$.

factors for the second and first eluted peaks, respectively.

## 3. Results and discussion

### 3.1. Effects of assay conditions on the separation

#### 3.1.1. Content of 1,2-dichloroethane in the mobile phase

Being as one of components of the mobile phase, the percentage of 1,2-dichloroethane had strong effects on the separation. As described in Fig. 2, increasing the content of 1,2-chloroethane, the capacity factor ($k$) decreased, correspondingly. This means that the elution times ($t_R$) were shorten by adding 1,2-dichloroethane into the mobile phase. But $\alpha$ and $R_S$ appeared maximum values of 1.42 and 1.71, when 38% (v/v) of 1,2-dichloroethane was added, and the capacity factors $k$ were 3.85 and 2.70 for each enantiomer.

#### 3.1.2. Content of methanol in the mobile phase

Similar to 1,2-dichloroethane, the percentage of methanol had very strong effects on the chiral separation (Fig. 3). The capacity factor ($k$) reduced by adding methanol in the mobile phase. $R_S$ and $\alpha$ both rose when the percent varying from 4 to 8% and decreased when it got 10%. Fig. 3 presents that 8% of methanol led to highest $\alpha$ and $R_S$ and low capacity factors.

Comparing Figs. 2 and 3, it could be concluded that, both being as polar modifier, methanol had much more ability of elution than 1,2-dichloroethane. The decreasing of capacity factors was faster when adding methanol than 1,2-dichloroethane. This was because that methanol was not only a polar modifier but also a competitor to the clenbuterol, whose adsorption on the CSP was weakened by the interaction between methanol and the stationary phase.

#### 3.1.3. Column temperature

So far, a few papers had reported the influence of temperature on the resolution of enantiomers by HPLC. The different chiral stationary phases used include protein phase [20–22], Pirkle phase [23,24], cyclodextrin phase [25], and some others [26,27].

It was often found that a decrease in temperature caused an increase in enantioselectivity. In this paper, six value of temperatures, 13, 17, 21, 25, 29 and 33 °C were selected to evaluate the effects on separation. One result was that rising of

Table 1
Linear regression of $\ln k = a/T + b$ ($n = 6$; $286 < T < 306$ K)

| | $a$ | $b$ | $r$ |
|---|---|---|---|
| $k_2$ | 0.22067 ± 0.02372 | 0.61342 ± 0.08025 | 0.98 |
| $K_1$ | 0.20728 ± 0.01723 | 0.30138 ± 0.05828 | 0.99 |

temperature shorten elution time and there was a linear relationship between $\ln k$ and $1/T$. According to the Van't Hoff equation, $k$ dependency of temperature was expected to be:

$$\ln k = a/T + b.$$

The parameters $a$ and $b$ were related to the molar change of enthalpy and entropy for the adsorption and solution process, respectively. If a small temperature range was considered, both parameters could be regarded as constants. So the linear regressions were obtained, presented in Table 1.

Being as the other result, there was an optimized temperature condition with the highest $R_S$. Decreasing the column temperature from 33 to 17 °C, $R_S$ rose from 1.66 to 1.81, because of the value of $\alpha$ increasing [25]. While the temperature dropped down to 13 °C, the value of $R_S$ descent, too. This was because that $R_S$ was directly proportional to the $N^{1/2}$ (the theoretical plate number), which would decrease when the column temperature was too low [24].

### 3.1.4. Flow rate of mobile phase

Experiments shown that flow rate of mobile phase had influences on the separation. This was because, in theory of random walk model, flow rate had strong effect on the theoretical plate height ($H$), which was inversely proportional to $N$. As the Fig. 4 described, raising flow rate not only shorten the elution times and capacity factors, but also varied $R_S$ and $\alpha$, greatly. When it was 1.0 ml/min, the highest values of $R_S$ 1.81 and $\alpha$ 1.43 were got with the elution times of 14.91 and 11.30 min for $S$- and $R$-enantiomers.

### 3.1.5. Chromatograms and elution order

As Section 3, the optimized of assay conditions were $n$-hexane–1,2-dichloroethane–methanol (54:38:8, v/v/v), the column temperature 17 °C and the flow rate 1.0 ml/min, which resulted in the highest $R_S$ (1.81) and $\alpha$ (1.43). The capacity factors were 3.97 and 2.77 for each enantiomer, which benefited from the short elution times of 14.01 and 11.30 min, respectively. The typical chromatogram for the enantioseparation of standard clenbuterol enantiomers is shown in Fig. 5(A). The ratio of

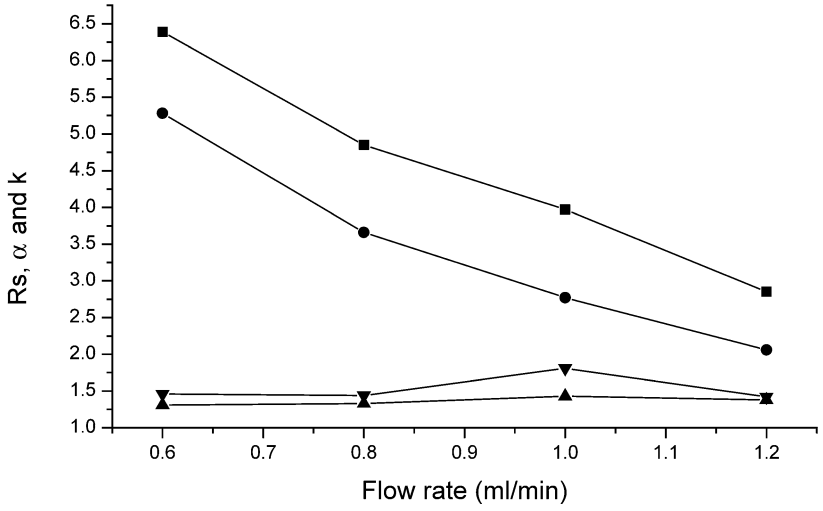



Fig. 4. Effects of flow rate on the enantioseparation. Column, chirex 3005 (250 × 4.6 mm i.d.); mobile phase composed of $n$-hexane, 1,2-dichloroethane and methanol (54:38:8 by volume); column temperature, 17 °C; UV detector, 247 nm; sample concentration, 318.8 nmol/ml; injection volume, 20 μl; ▼, $R_S$; ▲, $\alpha$; ●, $k_1$; ■, $k_2$.

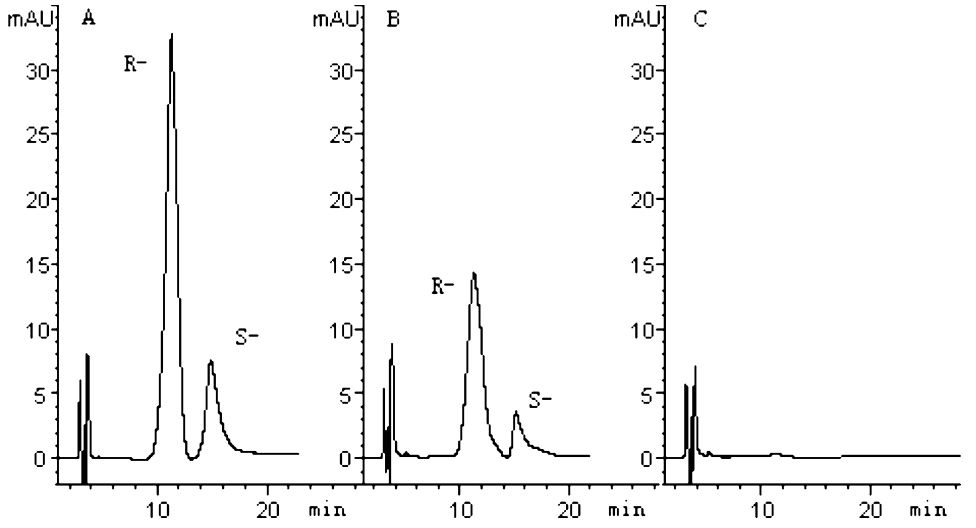



Fig. 5. Chromatograms of standard sample (A), serum sample (B) and plasma blank (C). Column, chirex 3005 (250 × 4.6 mm i.d.); mobile phase composed of *n*-hexane–1,2-dichloroethane–methanol (54:38:8 v/v/v); flow rate, 1.0 ml/min; column temperature, 17 °C; UV detector, 247 nm; sample concentration, injection volume, 20 μl; standard sample, 318.8 nmol/ml; spiked serum, 191.3 nmol/ml.

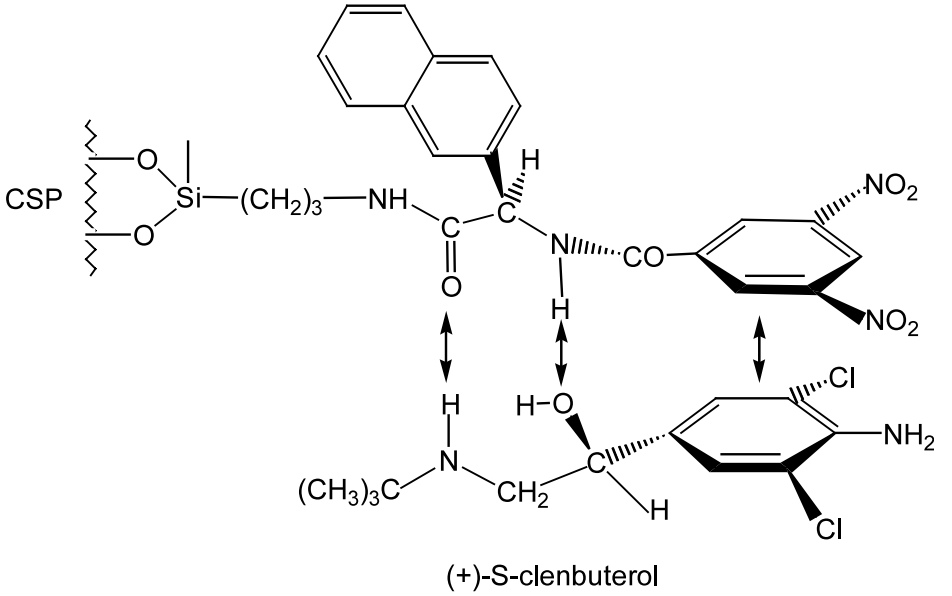



Fig. 6. Proposed stereochemical interaction between (+)-*S*-clenbuterol and the Pirkle chiral stationary phase.

areas of the two peaks was almost 82–18, equal to that of the amount of *R*- to *S*-enantiomers in their mixture, which was got by detecting the rotation of the mixture using the 241-MC digital polarimeter. So the elution order could be told that the (−)-*R*-clenbuterol eluted first and the (+)-*S*-enantiomer eluted later.

The processed chromatogram was obtained and compared with the blank human serum and spiked serum sample. No obvious interfering peaks were

Table 2
Repeatability of the method

| Standard solution (nmol/ml) | | | | RSD (%) | | | |
|---|---|---|---|---|---|---|---|
| *R* | *S* | $k_R$ | $k_S$ | $Area_S$ | $Area_R$ | $R_S$ | α |
| Inter-assay variability ($n = 5$) | | | | | | | |
| 26.1 | 5.7 | 2.05 | 0.73 | 1.58 | 3.84 | 3.81 | 0.54 |
| 65.4 | 14.3 | 1.31 | 0.90 | 1.70 | 3.58 | 5.69 | 1.34 |
| 261.4 | 57.4 | 1.65 | 0.38 | 3.19 | 4.39 | 5.02 | 1.54 |
| 522.8 | 114.8 | 0.35 | 0.38 | 1.63 | 4.01 | 2.08 | 0.36 |
| 1045.8 | 229.6 | 0.41 | 0.31 | 2.65 | 4.18 | 4.93 | 0.48 |
| Intra-assay variability ($n = 3$) | | | | | | | |
| 26.1 | 5.7 | 1.57 | 1.64 | 2.70 | 4.91 | 5.76 | 1.37 |
| 65.4 | 14.3 | 1.77 | 1.27 | 5.13 | 4.16 | 6.15 | 1.71 |
| 261.4 | 57.4 | 1.43 | 0.45 | 6.74 | 5.48 | 6.32 | 1.07 |
| 522.8 | 114.8 | 0.50 | 0.69 | 2.49 | 6.90 | 6.28 | 1.01 |
| 1045.8 | 229.6 | 0.93 | 0.61 | 6.66 | 5.05 | 5.71 | 1.73 |

found at the retention time of clenbuterol. Representative chromatograms of spiked serum and blank serum are shown in Fig. 5(B and C).

### 3.2. Mechanism of the enantioseparation

Resolution of enantiomers on Pirkle type chiral stationary phase had been reported [28–30]. The mechanism of this resolution had been proposed to be due to the transient formation of diastereromeric complexes between the enantiomers and the chiral stationary phase [31,32]. The relative stability of these complexes results in differing rate of elution of the enantiomers. The complexes formed as a result of π-bonding, electrostatic bonding, hydrogen bonding and a steric interaction [33]. As shown in Fig. 6, a more stable complex was formed with stationary phase and (+)-*S*-clenbuterol, which possessed three-point interaction with the stationary phase. One is due to π-bonding between the π-basic 4-amino-3,5-dichloro-phenyl group of the clenbuterol and the π-acidic 3,5-dinitrobenzoyl group of the chiral stationary phase. Others come from hydrogen bondings of the hydroxy group and amino group around the chiral center of clenbuterol to the amide groups of the stationary phase. But due to mirror of the (+)-*S*-isomer in the absolute configuration, the (−)-*R*-isomer was weakly fixed to the CSP, with at most two-point bonding. These interactions lead to a more rapid elution of the (−)-enantiomer.

### 3.3. Analytical characteristics

#### 3.3.1. Calibration curve

Under the optimized working conditions, standard calibration curves were constructed over the range of 26.1–1045.8 nmol/ml for (−)-*R* and 5.7–229.6 nmol/ml for (+)-*S*, with the straight-line equations: $A_R = 20.94 + 10.13C_R$ (correlation coefficient $R = 0.9999$) for *R*-enantiomer and $A_S = -11.76 + 10.14C_S$ (correlation coefficient $R = 0.9999$) for *S*-enantiomer. The limits of detection concentration were calculated to be 0.47 and 1.04 nmol/ml for *R*- and *S*-enantiomers, respectively, when a signal-to-noise ratio of 3 was used as the criteria.

#### 3.3.2. Precision

Under the optimized condition, five concentrations of standard solution were used to evaluate the precision of this method. As shown in Table 2, RSDs (relative standard deviation) of retention time and selectivity were always less than 4% in both inter- and intra-assays, and those of peak area and resolution were less than 7%. These mean this method has enough precision for qualitative and quantitative analysis.

Table 3
Recovery of the method

| | Sample added (nmol/ml) | Average recovery (%) ($n = 3$) | RSD (%) |
|---|---|---|---|
| (−)-*R* | 156.9 | 90.37 | 1.00 |
| | 287.6 | 89.09 | 0.98 |
| (+)-*S* | 34.4 | 89.51 | 2.08 |
| | 63.1 | 89.65 | 1.22 |

### 3.3.3. Recovery

Recoveries of clenbuterol enantiomers were established by analysing two concentrations of serum samples spiked standard clenbuterol, under the optimized separation conditions. As shown in Table 3, the recovery of almost 90% for both enantiomers was obtained.

## 4. Conclusion

Chirex 3005 is applicable to assay clenbuterol enantiomers, on HPLC, not only qualitatively but also quantitatively. The operating conditions had strong effect on the enantioseparation. The optimized separation conditions included the mobile phase *n*-hexane–1,2-dichloroethane–methanol (54:38:8, v/v/v), 17 °C of column temperature and 1.0 ml/min of flow rate. This method is simple, fast and reliable.

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