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Enantiomeric separation of β_2-agonists on macrocyclic antibiotic chiral stationary phases in high performance liquid chromatography

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The enantiomeric resolution of six β_2-agonists including bambuterol, clenbuterol, clenproperol, fumoterol, mabuterol and terbutaline, was studied on three macrocyclic antibiotic chiral stationary phases (CSPs): Chirobiotic V, T and R. By varying the mobile phase compositions for different CSPs, all the six compounds were successfully separated on Chirobiotic V and T CSPs. The thermodynamic parameter variation was calculated. The chiral recognition mechanism was discussed, the ionic interaction was confirmed to be the most dramatic interaction responsible for the chiral recognition. No enantioseparation for all the β_2-agonists was found on Chirobiotic R CSP.

1. Introduction

The adrenergic β_2-agonists are drugs mainly for the treatment of pulmonary diseases, as well as hypertension, heart failure and coronary artery disease (Clarkson and Thompson 1997; Salpeter et al. 2003; Anzueto 2006). Chemically, they are hydroxylamine-containing compounds whose amino groups are always secondary; and their molecules contain at least one aromatic ring. The majority of β_2-agonists are chiral compounds, and their enantiomers may exhibit different pharmacological and toxicological effects (Vakily et al. 2002). For instance, L-mabuterol was reported to be 2.3 and 2.6 times more potent than the racemates in relaxing the isolated trachea and in inhibiting the increase in the bronchial resistance in guinea pigs (Murai et al. 1984). Consequently, the chiral analysis of β_2-agonists is of great importance. It is crucial to develop effective analytical methods for the separation and identification of β_2-agonist stereoisomers and for the determination of the enantiomeric excess (ee) values of these compounds.

The liquid chromatographic resolution of racemic compounds on chiral stationary phases (CSPs) has been known to be a very useful means of determining enantiomeric composition of chiral compounds. Many CSPs have been developed and used for the chiral resolution of a varity of racemates. The important CSPs include Pirkle types (Pirkle and Burke 1991), derivatized polysaccharides (Girod et al. 2000; Li et al. 2004), cyclodextrin and its derivatives (Lubda et al. 2003), protein phases (Allenmark et al. 1984), chiral crown ethers (Zhang et al. 2005; Hyun et al. 2006), macrocyclic antiobiotic (Ward and Farris 2001) and ligand exchange CSPs (Davankov 1989). Among theses CSPs, cyclodextrins and macrocyclic antiobiotic based CSPs are most diverse and widely applicable as they have achieved a great reputation in the field of chiral resolution. The macrocyclic antibiotic CSPs were developed recently (Chirobioc^{TM} Handbook 1999), they contain numerous functional groups and many stereogenic centers, these sites offer the possibility of different interactions with enantiomers of various chiral compounds.

Among the six β_2-agonists investigated in this study, the enantiomers of mabuterol and clenbuterol have generally been separated on macrocyclic antibiotic CPSs (Aboul-Enein and Serignese 1999; Lu et al. 2005), clenbuterol and terbutaline were also resolved in packed capillary electrochromatography using vancomycin silica stationary phase (Desiderio et al. 2001; Fanali et al. 2002). However, there are few reports about the enantioseparation of other three β_2-agonists: bambuterol, clenproperol and fumoterol.

As an effort to elucidate the usefulness of macrocyclic antibiotic CSPs in the resolution of β_2-agonists, in the present paper, the direct liquid chromatographic enantioseparation of six racemic β_2-agonists on three macrocyclic antibiotic CSPs, such as Chirobiotic V, T and R, is reported. The enantioseparation was performed in all three mobile phase modes: reversed-phase mode (RPM), normal-phase mode (NPM) or a newly developed polar-organic mode (POM). Factors affecting the enantiomeric resolution on all CSPs were investigated, the thermodynamic parameter variation was calculated and the chiral recognition mechanism was discussed.

2. Investigations and results

Besides cyclodextrin-bonded phases (Risley and Strege 2000), only the macrocyclic antibiotic phases are considered to be multimodal CSPs (Chirobioc^{TM} Handbook

1999). Multimodality means that the stationary phases can be used in different chromatographic modes, RPM or NPM. It has recently been established that separations are also possible with a new type of mobile phase, referred to as the polar organic mode (POM). The enantiomeric resolution of six racemic β_2-agonists, such as bambuterol, clenbuterol, clenproperol, fumoterol, mabuterol and terbutaline, was performed on three macrocyclic antibiotic CSPs in RPM, NPM and POM. All the analytes were chromatographed and detected without pre- or post-column derivatization. Retention and selectivity were controlled by altering the concentration and nature of the buffer and/or organic modifier and variation of the temperature sometimes had a beneficial effect on the resolution.

2.1. Enantioseparation of β_2-agonists

The POM would be the first choice of mobile phase since β_2-agonists contain more than one functional group (phenyl and hydroxyl) on the stereogenic center, which are capable of interacting with the stationary phase. In this chromatographic mode, the enantiomers of all the six β_2-agonists, bambuterol, clenbuterol, clenproperol, fumoterol (only one pair of peaks), mabuterol and terbutaline, could be successfully resolved. In the POM, enantiomeric separation is markedly influenced by the type of the polar organic solvents, both retention and selectivity are also controlled by the ratio and the concentration of organic acid, glacial acetic acid (AcOH) and base, anhydrous triethylamine (TEA) in the mobile phase.

In the case of macrocyclic antibiotic CSPs, methanol is the dominant solvent, therefore, methanol was chosen as the solvent of the mobile phase in this study. The concentration and the ratio of the buffer components (AcOH-TEA) were varied to achieve the best resolution. Without TEA in the mobile phase, only one peak with distorted shape was detected for all compounds tested. The addition of acetic acid only did not play a major role in chiral discrimination, however, both peak sharpness and symmetry were improved when acetic acid was added to mobile phases containing methanol and TEA. The best chiral resolution was observed at the ratio of 1 : 1 of TEA to AcOH. At this constant ratio, the effect of the content of buffer in the mobile phase on the retention and chiral selectivity was studied. Decrease of the buffer components content from 0.1% to 0.01% increased the retention factors, which resulted in higher enantioselectivity and better resolution. However, further decrease of the content to lower value induced longer retention time and broader peaks which led to poor resolution.

Then a mobile phase of MeOH−AcOH-TEA (100 : 0.01 : 0.01) was applied in most cases and enantiomeric separation was achieved for all six β_2-agonists in the POM on Chirobiotic V and T CSPs, the retention and separation data are summarized in Table 1. The representative chromatograms are depicted in Fig. 1 and Fig. 2.

The enantiomeric elution order was determined by running the chromatograms of the optically pure isomer of the reported β_2-agonists under identical HPLC conditions. It has been observed that the (−)-enantiomer was eluted first followed by the (+)-enantiomer of all the reported adrenergic β_2-agonists on both Chirobiotic V and T CSPs, and no inversion of the elution order was observed when varying the eluent composition.

Resolution of the investigated β_2-agonists could not be achieved on Chirobiotic R CSP in POM.

Table 1: Chromatographic separation data: retention factors of the first eluted peak (k_1'), separation factors (α) and resolution factors (Rs) of β_2-agonists on Chirobiotic V and T CSPs in the polar organic mode with methanol-acetic acid-triethylamine (100 : 0.01 : 0.01, v/v/v) as mobile phase

Analytes	Chirobiotic V			Chirobiotic T		
	k_1'	α	Rs	k_1'	α	Rs
Bambuterol	8.33	1.02	0.18	12.23	1.23	2.68
Clenbuterol	5.43	1.14	2.50	9.70	1.24	3.75
Clenproperol	6.62	1.08	1.60	11.58	1.16	2.13
Fumoterol	8.56	1.28	4.11	16.29	1.06	0.42
Mabuterol	4.34	1.16	2.55	7.87	1.25	3.73
Terbutaline	5.06	1.09	1.80	9.78	1.34	5.17

Flow rate: 1.0 mL/min. Detection: 254 nm. Temperature: 20 °C

2.2. Thermodynamic parameter variations

To study the effects of temperature on enantioselectivity and on variation of thermodynamic properties, the experiments were carried out at 10, 20, 30 and 40°. Increasing temperature induced both the retention time and the enantioselectivity to decrease slightly. Data are summarized in Table 2.

The difference in the interaction with the stationary phase between two enantiomers leading to chiral discrimination can be expressed as the difference in the free energy $-\Delta\Delta G^{\circ}_{R,S}$ calculated from the separation factor (α) according to the following Van't Hoff equations:

$$-\Delta\Delta G^{\circ}_{R,S} = RT \ln k_2'/k_1' = RT \ln \alpha \quad (1)$$

$$\ln \alpha = \frac{-\Delta\Delta G^{\circ}_{R,S}}{RT} = -\frac{\Delta\Delta H^{\circ}_{R,S}}{RT} + \frac{\Delta\Delta S^{\circ}_{R,S}}{R} \quad (2)$$

Eqs. (1) and (2) correspond to the first and second eluted enantiomer respectively, $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ are the differences between two enantiomers in enthalpy and entropy, respectively, R is the ideal gas constant, T is absolute temperature.

The results given in Table 3 show that binding of two enantiomers to a given chiral site may involve different amounts of energy simply because one of the enantiomers, for steric reasons, might be forced to adopt an energetically less favorable conformation.

If the interaction mechanism is based on the same type(s) of interaction(s) in relation to temperature, then a plot of lnα versus 1/T would be linear with a slope of $(-\Delta\Delta H^{\circ})/R$ and an intercept of $(\Delta\Delta S^{\circ})/R$. In this study, the Van't Hoff plots were all linear for these β_2-agonists. By these plots, we can obtain the $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ values, which are also listed in Table 3. All these values are negative which indicate that the processes were controlled enthalpically. This is consistent with the results reported in the literature from various chromatographic data using many CSPs, such as imprinted CSPs (Sellergren and Shea 1995), modified cellulose CSPs (Fulde and Frahm 1999; Cirilli et al. 2004), chiral brush-type CSPs (Kontrec et al. 2000), crown ether-based CSPs (Hyun 2003; Hyun et al. 2005) and cyclodextrins CSPs (Zhang et al. 2001).

3. Discussion

Vancomycin, teicoplanin and ristocetin antibiotics are the chiral selectors in the Chirobiotic V, T and R columns, respectively. The structures of these antibiotics are very

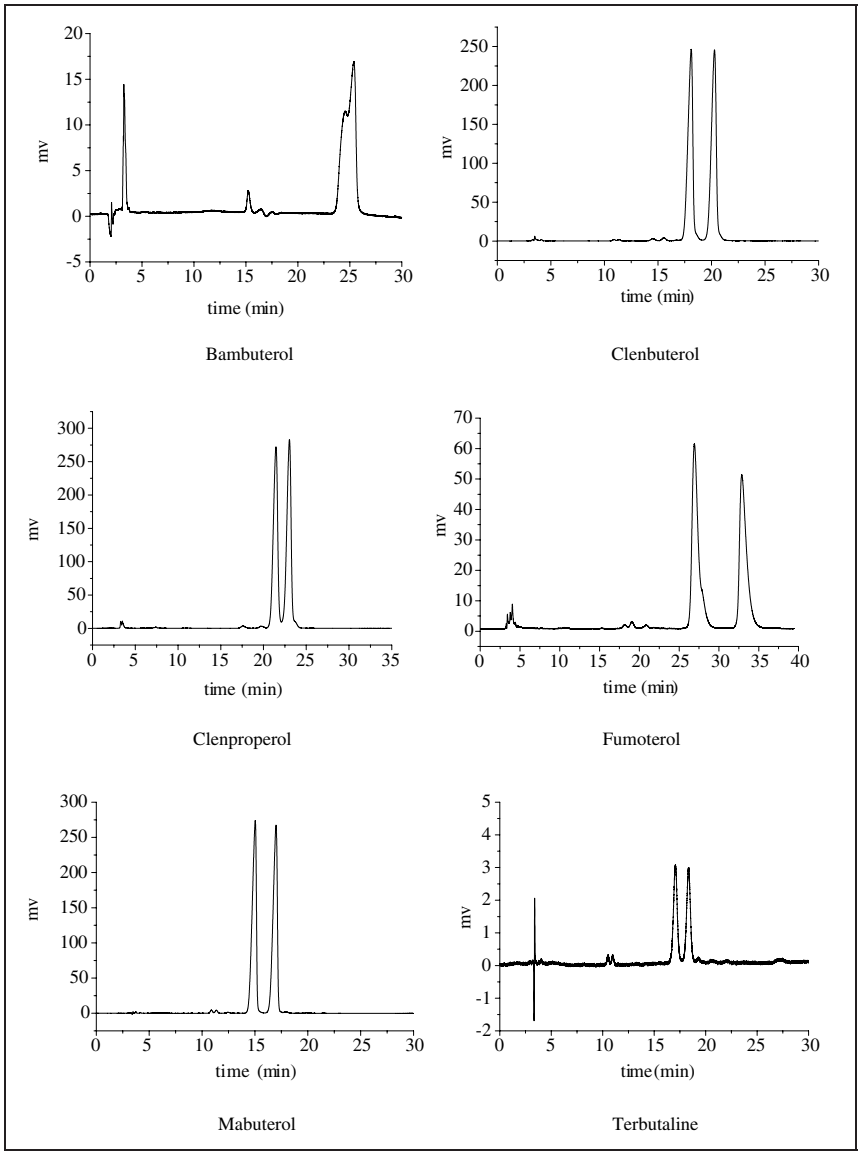

Fig. 1:
Representative chromatograms of β_2-agonists investigated on Chirobiotic V CSP with methanol-acetic acid-triethylamine (100 : 0.01 : 0.01, v/v/v) as mobile phase.
Flow rate: 1.0 mL/min. Detection; 254 nm. Temperature: 20 °C

complex, containing different chiral centers, inclusion cavities, and various functional groups. Because of the complex structures of the antibiotics, the possible interactions occurred between these CSPs and enantiomers include π-π complexation, hydrogen bonding, inclusion complexation, dipole interactions, steric interactions and ionic interactions (Chirobioc™ Handbook 1999; Ward and Farris 2001). The adrenergic β_2-agonists studied contain phenyl rings, hydroxyl, halogens, amino and amide groups, which can interact with the complimentary functional groups on the chiral selectors to induce enantiomeric resolution. The POM can enhance all the interactions (Gasper et al. 1996; Ward and Farris 2001), it is therefore not surprising that in this mode Chirobiotic V and T CSPs provided much better enantiomeric resolution for these β_2-agonists than in other two modes. Furthermore, the best enantioresolution was achieved with both TEA and AcOH in the mobile phase, which indicates the successful enantioseparation obtained in this mode is essentially due to ionic interactions.

In the reversed phase mode (RPM), chiral recognition was only found for mabuterol and terbutaline. With the optimum mobile phase, 0.1% TEAA (pH 6.0) −30% ethanol at a flow-rate of 0.6 mL/min, baseline resolution for terbutaline was achieved and mabuterol was also partly resolved on Chirobiotic V CSP, with separation factors (α) of 1.11 and 1.04, resolution factors (Rs) of 1.65 and 0.22, respectively. On Chirobiotic T CSP, only terbutaline enantiomers can be separated partly with the resolution factor of 0.50 with the optimum mobile phase of 0.1% TEAA (pH 4.1) −20% methanol at a flow-rate of 1.0 ml/min. To find the optimum mobile phases, we investigated different factors influencing the enantiomeric separation. Because both the analytes and the CSPs are ionizable, the pH of the mobile phase would affect the ionization state of the analytes as well as the ionization state and conformation of chiral selectors. At the optimum pH, the ionization state of the analytes and the CSPs is most suitable for the ionic interactions which induced the best enantioresolution in RPM. In this study, it was found that the retention time of mabuterol and terbutaline increased with the increase of alcohol content on both Chirobiotic V and T CSPs, which is not a reversed phase chromatographic behavior (that should be the opposite). This indicates that enantioseparation of the analytes can be successful in POM which is consistent with the results in this study. The point also shows the critical role of ionization, ionic interaction is likely the dramatic interaction responsible for the chiral recognition.

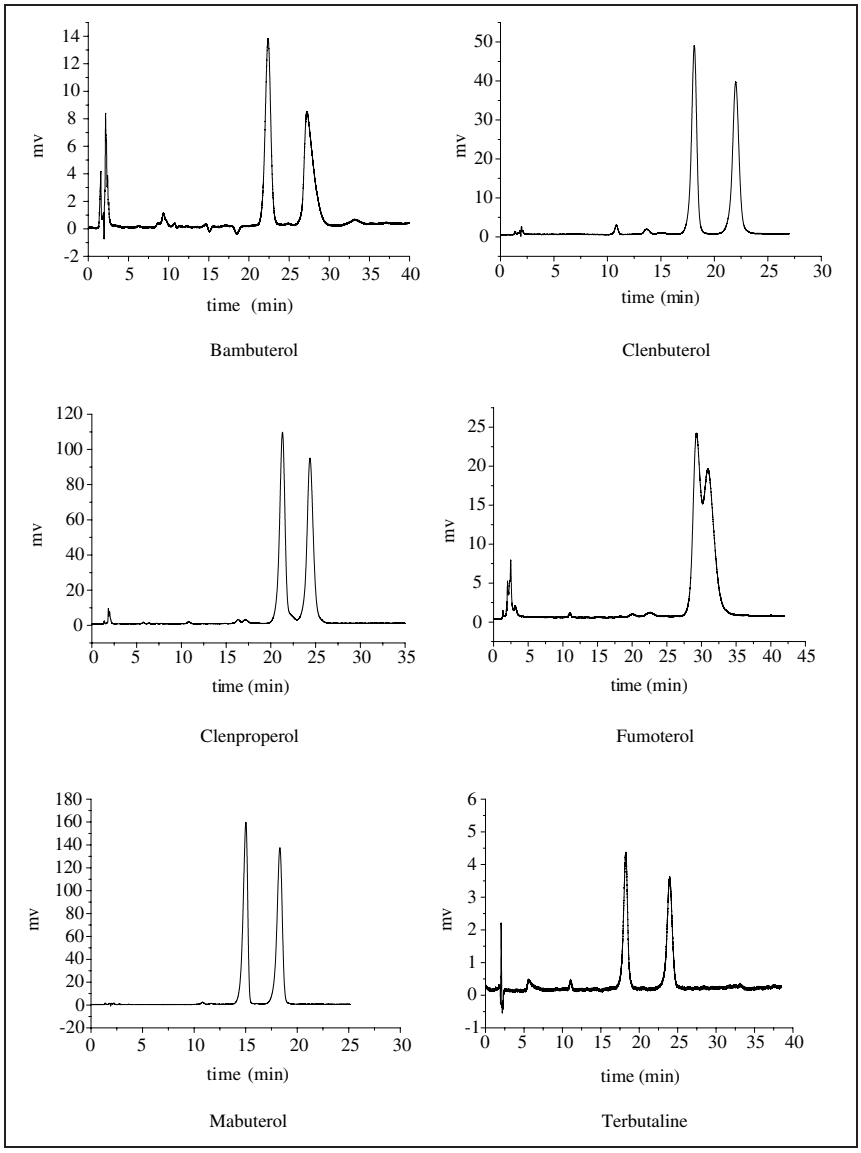

Fig. 2:
Representative chromatograms of β_2-agonists investigated on Chirobiotic T CSP with methanol-acetic acid-triethylamine (100 : 0.01 : 0.01, v/v/v) as mobile phase.
Flow rate: 1.0 mL/min. Detection; 254 nm. Temperature: 20 °C

Table 2: Effects of temperature on retention factors of the first eluted peak (k'_1), separation factors (α) and resolution factors (Rs) of β_2-agonists on Chirobiotic V and T CSPs with methanol-acetic acid-triethylamine (100 : 0.01 : 0.01, v/v/v) as mobile phase

Analytes	T	Bambuterol			Clenbuterol			Clenproperol			Fumoterol			Mabuterol			Terbutaline		
	(L)	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs
Chirobiotic	10	8.46	1.04	0.93	6.27	1.16	3.00	7.18	1.10	2.00	9.06	1.29	4.67	5.06	1.18	3.09	1.39	1.10	2.40
V CSP	20	8.19	1.04	0.80	5.93	1.14	2.67	7.03	1.09	2.00	8.66	1.25	4.11	4.82	1.16	3.08	1.28	1.10	2.33
	30	8.10	1.03	0.38	5.43	1.14	2.57	6.62	1.08	1.71	8.56	1.21	3.54	4.34	1.15	2.80	1.21	1.09	1.80
	40	7.44	1.02	–	4.69	1.13	2.50	5.89	1.08	1.60	8.46	1.18	2.59	3.72	1.14	2.55	1.15	1.09	1.45
Chirobiotic	10	13.29	1.27	2.91	10.62	1.25	3.75	12.39	1.16	2.17	17.61	1.06	0.62	8.52	1.26	3.73	11.07	1.34	5.17
T CSP	20	12.81	1.23	2.68	10.14	1.24	3.47	11.91	1.16	2.13	17.38	1.06	0.55	8.38	1.25	3.47	10.12	1.34	4.56
	30	12.45	1.21	2.60	9.73	1.21	3.00	11.58	1.14	1.81	17.09	1.05	0.42	7.91	1.21	3.21	9.78	1.29	4.00
	40	12.23	1.19	2.53	9.70	1.19	2.74	11.50	1.13	1.20	16.29	1.04	0.19	7.87	1.18	2.89	9.39	1.26	3.75

Flow rate: 1.0 mL/min. Detection: 254 nm.

In the normal phase mode (NPM), no enantioresolution was found for all the β_2-agonists with the variation of the mobile phase composition on all investigated CSPs. This confirms the essential role of ionic interactions for the chiral recognition which is difficult or impossible in normal phase mode.

In this paper, the enantiomeric resolution of the investigated β_2-agonists could not be achieved on Chirobiotic R CSP. In the structure of ristocetin antibiotics, the chiral selector of Chirobiotic R column, the dramatic difference is the lack of carboxyl and more sugar moieties compared to teicoplanin, the chiral selector of Chirobiotic T column,

Table 3: Thermodynamic parameters $\Delta\Delta H^\circ$ (cal/mol), $\Delta\Delta S^\circ$ (cal/mol · K) and $\Delta\Delta G^\circ_{R,S}$ (cal/mol) of β_2-agonists on Chirobiotic V and T CSPs

Analytes	Chirobiotic V			Chirobiotic T		
	$\Delta\Delta H^\circ$	$\Delta\Delta S^\circ$	$-\Delta\Delta G^\circ_{R,S}$ (20 °C)	$\Delta\Delta H^\circ$	$\Delta\Delta S^\circ$	$-\Delta\Delta G^\circ_{R,S}$ (20 °C)
Bambuterol	−118.43	−0.33	20.44	−374.39	−0.85	123.84
Clenbuterol	−139.46	−0.20	79.75	−301.92	−0.62	121.56
Clenproperol	−114.23	−0.22	50.61	−167.97	−0.29	82.68
Fumoterol	−528.84	−1.36	129.62	−116.17	−0.29	31.58
Mabuterol	−198.42	−0.38	88.20	−401.72	−0.95	124.19
Terbutaline	−111.91	−0.20	53.25	−389.30	−0.78	161.98

however, much difference exists in the results of enantioresolution. These points indicate that the carboxyl is likely one important chiral recognition site, which may produce ionic interaction with the analytes, and confirm the essential role of ionic interactions for enantioresolution. The inclusion cavities and the sugar moieties of the CSPs can provide the chiral groove baskets for the enantiomers to fit in different fashion stereogenically which may also contribute to the chiral discrimination (Chirobioc™ Handbook 1999). Therefore, the non-occurrence of chiral resolution of the reported adenergic β_2-agonists on Chirobiotic R column may be explained on the basis that the chiral grooves and the different types of interactions, especially the ionic interactions, of Chirobiotic R column are not suitable for the enantiomeric resolution of β_2-agonists.

4. Experimental

4.1. Apparatus

The chromatography was performed on a Jasco (Japan) modular liquid chromatography system equipped with UV-975 detector and UV-980 pump. Chiral columns, Chirobiotic V (250 × 4.6 mm I.D., 5 μm), Chirobiotic R (250 × 4.6 mm I.D., 5 μm) and Chirobiotic T (150 × 4.6 mm I.D., 5 μm) were purchased from Advanced Separation Technologies, Inc. (Whippany, NJ, USA). The detection was set at 254 nm. The temperature was regulated and controlled by a type IC-2000 thermostat.

4.2. Chemicals and reagents

All β_2-agonist racemates and enantiomers used in this study were provided by the Laboratory of Medicinal Chemistry, Shenyang Pharmaceutical University of China. The methanol and ethanol were of HPLC grade from Concord Technology Co. LTD (Tianjin, China). Acetic acid and triethylamine of super purity were purchased from Yuwang Reagent Company (Shandong, China). All solvents were filtered through a 0.45 μm filter and degassed before use.

4.3. Sample preparation

The solutions of all β_2-agonists were prepared in methanol to a concentration of 1.0 mg · mL^{-1}. From each solution, 2 μL were injected into the HPLC system.

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