Preparation and Evaluation of a Cholesterol Derivatized β-Cyclodextrin-bonded Phase for Achiral and Chiral HPLC

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A cholesterol mono-derivatized β-cyclodextrin was synthesized and bonded onto silica gel (SBA-15) to obtain a cholesterol mono-derivatized β-cyclodextrin-bonded stationary phase (CHCDP). The chemical structures of mono-derivatized β-cyclodextrin and CHCDP were characterized by infrared spectroscopy, mass spectrometry, elemental analysis and thermogravimetric analysis, correspondingly. Furthermore, the separation ability of CHCDP in terms of achiral compounds was systematically evaluated by separating benzene homologs, polycyclic aromatic hydrocarbons (PAHs) and some positional isomers. As a result, CHCDP completely separated five benzene homologs and nine PAHs within 30 min under the reversed-phase. In addition, the chiral chromatographic property of CHCDP was also evaluated by separating some racemic compounds including flavanones, triazoles, β-blockers, etc. The results showed that the CHCDP exhibited high enantioselectivities towards most of selected analytes. The enantioresolutions were in the range from 1.43 to 2.51 on CHCDP. Especially the resolutions of 2′-hydroxyflavanone, hexaconazole, Dns-serine and atenolol were as high as 1.94, 1.91, 2.15 and 1.57, respectively. Obviously, the CHCDP was a versatile stationary phase with chiral and achiral separation capabilities in multi-mode chromatography, which was related to the introduction of cholesterol to the port of cyclodextrin, enhancing the hydrophobic interaction of cyclodextrin with achiral compounds, while maintaining the inclusion complexion of it with chiral compounds as well.

Keywords HPLC, cholesterol mono-derivatized β-cyclodextrin-bonded phase, preparation and evaluation, chiral and achiral drug analysis

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Introduction

Due to the chirality of proteins, biological enzymes and antigen-antibodies, these macromolecules tend to exhibit high enantioselectivity in terms of acting with chiral compounds.1,4 In most cases, only one of the enantiomers has the desired efficacy, while the remaining enantiomers are often inactive, or even toxic. A case in point is that the R-propranolol is 300-times less effective than the S-propranolol in the treatment of hypertension, and worse is that the former can cause male infertility.2 Similar phenomena can also be found in chiral pesticides. For example, only one enantiomer of metolachlor has herbicidal activity, whereas the other enantiomer is not only inactive, but will also transform into a carcinogenic intermediate.1,2 Therefore, the separation and determination of enantiomers is of increasing significance for the safety analysis of drugs and food. In various chiral separation methods, owning enantiomers is of increasing significance for the safety analysis of drugs and food. In various chiral separation methods, owning

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which greatly enhances the efficiency of qualitative and quantitative analysis.6 However, the biggest factor that closely affects the separation efficiency is the enantioselectivity of CSPs, which is considered to be of crucial importance. In the current CSPs, the cellulose and cyclodextrin families are two types that have been widely used. As for cellulose-based CSPs, the ordered polymeric structure endows it with a high enantioselectivity, but the orderliness is easy to destroy during the bonding procedure.7 Therefore, this type of CSPs is usually used in a coating manner, which makes them have poor solvent tolerance, especially in the reversed phase mode.3 Cyclodextrin-based CSPs (CD-CSPs) are free from this issue, and they can be used in three HPLC modes, which also has an advantage in terms of combining the electrospray ionization mass spectrometry (HPLC-MS).

In 1984, Armstrong research group10 firstly prepared a stable ether-bonded β-cyclodextrin CSP. Since then, modifications to the ports of cyclodextrin have become a popular way to improve the chiral separation ability of CD-CSPs through introducing multiple sites. At present, the portal derivatization to cyclodextrin mainly focuses on the introduction of the polar substituents, such as the typical benzoylation and phenylcarbamated11–14 by adding excessive functional agents with preparing fully derivatized cyclodextrins. Zhong et al.11 prepared and evaluated electron-deficient nitro-containing π-acidic β-CD-CSPs, while were separated, and a variety compounds including chiral acids, chiral amines and alcohols, They believed that the inclusion effects of CD-CSPs are a main...
force for a successful enantioseparation. Li et al. in our research group also prepared a N-benzyl-phenethylamino-β-CD-CSP and separated the enantiomers of 23 chiral compounds in the reversed-phase and polar organic modes. In recent years, “Click Chemistry” has also been successfully applied to the preparation of derivatized CD-CSP with 1,2,3-triazole rings. Zhao et al. also used the “Click Reaction” to prepare a phenylamino derivatized bilayer CD-CSP, which exhibited a better separation ability to enantiomers of chiral benzfluorothiazide, fenoterol and isoxazolines. Obviously, the introduced groups on the ports of cyclodextrin provided great contributions to chiral separations.

Until now, most substituents arranged on the ports of cyclodextrin have been polar groups in nature; the introductions of non-polar substituents are rarely reported. However, it is known that cyclodextrin contains a large number of hydroxyl groups which are also featured by the strong polarity. Therefore, the introduction of polar substituents may further enhance the polarity, thus weakening the reversed-phase chromatographic performance of derivatized CD-CSPs. Moreover, the content of chiral compounds in actual samples is usually very low. Most of the achiral compounds tend to occupy the cavities and the ports of cyclodextrin, which will inevitably affect the enantiomeric separation of chiral compounds. It is necessary to introduce non-polar substituents to improve the achiral separation ability of CD-CSPs so as to reduce the interference on enantioseparations for chiral compounds.

In this study, we introduced a fat-soluble cholesterol to the port of cyclodextrin to prepare a cholesterol mono-derivatized β-cyclodextrin-bonded phase (CHCDP). On the one hand, an enhancement of the hydrophobic capacity promoted the chromatographic performance in the reversed-phase, which not only strengthened the capability to separate achiral compounds, but also to reduce the interference from some achiral compounds on the enantiomeric separations of chiral compounds. On the other hand, the introduction of a cholesterol group still remained the chiral recognition effect from the cavity and hydroxyls of cyclodextrin. Therefore, the CHCDP was a new multifunctional separation material which had abilities to separate achiral and chiral compounds.

Experimental

Reagents and chemicals

Triblock polymer P123 (MW ~5800), tetraethyl orthosilicate (TEOS), isocyanate propyl triethoxysilane were purchased from Sigma-Aldrich. β-Cyclodextrin (β-CD), cholesterol formyl chloride, p-toluene sulfonic chloride (TsCl), 1,3,5-trimethylbenzene (TMB) were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Racemic standards, such as triazoles, flavanones, dansyl amino acids, β-blockers, etc. were also purchased from Sigma-Aldrich. Acetonitrile and methanol were of HPLC grade, supplied by Tedia Co. Inc. (Shanghai, China). The glacial acetic acid (HOAc), triethylamine (TEA), N,N-dimethylformamide (DMF) and other reagents were of analytical grade, purchased from Sinopharm Group Chemical Reagent Co. Ltd (Shanghai, China). DMF was dehydrated by CaH2 and redistilled under reduced pressure.

Apparatus

A ZQ4000/2695 high-performance liquid chromatography coupled to a mass spectrometer system, with equipping a 2996 diode array detector and a chromatographic work station of Masslynx 4.1 Ver. (Waters, USA), was used for chromatographic separations. A Nicolet 5700 Fourier transformed infrared spectrometer (Thermo, USA) and a Vario EL III elemental analyzer (Elementar, Germany) were employed to characterize the chemical structure of the CDCHP. An AW-60 packing machine (Haskel, USA) was used to pack columns. Other instruments included a Milli-Q ultrapure water system (Millipore, USA) and a KQ-100E ultrasonic cleaner (Jiangsu Kunshan Ultrasonic Instrument Co., Ltd, China).

Preparation of cholesterol-derivatized β-CD bonded phase (CHCDP)

Preparation of SBA-15: The ordered mesoporous silica SBA-15 material was prepared by referring to reports and our laboratory experience. The SBA-15 was spherical particles with a pore diameter of about 25 nm and a specific surface area of about 420 m2/g. The synthetic method was briefly described as follows: a triblock polymer P123 (8.0 g, 1.3 mmol), a 208-ml of water, an 80-ml of DMF, 40-ml of 35% concentrated HCl and an appropriate amount of KCl was added to a 500-ml round-bottom flask with
magnetic stirring, and then TMB (8.0 g, 66.7 mmol) and TEOS (17.2 g, 1.3 mmol) were added with fast stirring at 35 °C for 24 h. The mixture was statically reacted in a hydrothermal autoclave reactor at 130 °C for 6 d. The solid was filtered and calcined at 550 °C for 8 h to remove the template. SBA-15 silica gel was obtained and stored in a desiccator before use.

The synthetic scheme of CHCDP was shown in Fig. 1.

(i) Synthesis of 6-OTs-CD: To a solution of 40 g β-CD in 200 mL of water was added a 15-mL of 8.25 mol/L NaOH with stirring for 1 h. The transparent solution was then cooled in an ice bath, and followed by dropwise adding a solution of 6.7 g TsCl in 30 mL acetoneitrile within 2 h. Then, the mixture was continuously stirred at room temperature for 2 h, and the pH of the filtrate was adjusted to neutral with HCl. After left standing overnight at 4 °C, the precipitate was recrystallized three times, and dried under a vacuum at 60 °C to obtain 5.1 g 6-OTs-CD (B) with a yield of about 11%.

(ii) Synthesis of 6-NH₂-CD: According to the reported method, 4.0 g of 6-OTs-CD was dissolved into 100 mL of 25% (v/w) ammonia; the mixture was stirred at 50 °C for 15 h under a nitrogen atmosphere. The mixture was then evaporated under a reduced pressure to give a light yellow solid, which was purified by a flash chromatography on a Sephadex C-25 column; a white powder of 6-NH₂-CD (C) was obtained with a yield of 33%.

(iii) Synthesis of cholesterol mono-derivatized β-CD (CHCD): a 1.5-g of 6-NH₂-CD, a 0.59-g of cholesterol formyl chloride and a 0.184-mL of triethylamine (acid binding agent) were dissolved into 10.0 mL of anhydrous DMF. The mixture was stirred at room temperature for 24 h to obtain a white turbid solution. Then, the solvent was removed by filtering, and the resulting precipitate was washed three times with acetone, which was dried in a vacuum to give the CHCD (D) with a yield of 85%.

(iv) Preparation of cholesterol mono-derivatized β-CD-bonded phase (CHCDP): a 1.2-g of CHCD was dissolved into 20 mL of anhydrous DMF with stirring, and then slowly added to a 0.4-mL of 3-isocyanatepropyltriethoxysilane. The mixture was then reacted at 80 °C for 2 h, followed by adding 3.0 g of SBA-15, and a small amount of anhydrous DMF. Then, elevating the temperature of the mixture was elevated to 110 °C and reacted for 24 h. Subsequently, the solid was filtered and collected which was successively washed by DMF, acetone, methanol and water to obtain the CHCDP.

Column packing

The HPLC column was packed by the traditional slurry method: 2.5 g of CHCDP was dissolved into a moderate amount of acetone as a homogenate. The methanol was used as a displacement agent to pack the CDCHP into a stainless-steel column (150 × 4.6 mm i.d.), during which the packing pressure was maintained at 34.5 MPa for 40 min, after the column was washed repeatedly by methanol and water. Naphthalene was used as a solute, methanol-water (70/30, v/v) was used as a mobile phase, the flow rate was set at 0.5 mL/min and the detection wavelength was 254 nm. Under the above condition, the column efficiency was determined to be 20311 plates/meter.

Chromatographic method

All standards were dissolved into methanol to prepare stock solutions with about 50 - 200 μg/mL concentration, which were filtered through a 0.22-μm organic filter membrane and degassed with ultrasonication. The racemic standards included flavonones, triazoles, amino acids, and β-blocks, and the achiral standards that included PAs (benzene, toluene, xylene, naphthalene, biphenyl, anacrine, anacracene, phenanthrene and fluoranthene), homologs (benzene, toluene, ethylbenzene, propylbenzene and butylbenzene) and cholesterol analogs (cholesterol and statins). The reversed-phase chromatography mobile phase was comprised of methanol and water, and a small amount of triethylamine/ glacial acetic acid (TEAA) buffer and formic acid (FA) were occasionally needed. The mobile phase in the polar organic mode was solutions constituted by different ratios of acetonitrile, methanol, triethylamine and glacial acetic acid (ACN/MeOH/ TEA/HOAc). All mobile phases were filtered through a 0.22-μm filter membrane and degassed before use. The flow rates of the mobile phases were generally 0.5 mL/min. The UV detection wavelengths were set according to the spectroscopic properties of the analytes within the range of 200 - 350 nm. The column temperature was 25 °C, and the injection volume was 2 - 5 μL.

Three parameters were used to evaluate the chromatographic performance, including: $k = (t_r - t_i)/t_i$, $α = k_2/k_1$ and $R_s = 1.18(t_{k2} - t_{k1})/(W_1 + W_2)$, where $k$ is the retention factor of each analyte, $t_i$ is the retention time of each analyte, $t_r$ is the dead time, $α$ is the selectivity factor of CDCHP, $R_s$ is the resolution; $W_1$ and $W_2$ are the half widths of two adjacent peaks.

Results and Discussion

Characterization of CHCD and CHCDP

The cholesterol-mono-derivatized β-cyclodextrin (CHCD) was characterized by the mass spectrum (Fig. S1) and the infrared spectrum (Fig. S2) in Supporting Information. ESI-MS (m/z): [M+Na]+ was calculated for 2523.85; found, 1569.51. According to the infrared spectra of CHCD and CHCDP (Fig. S2), the broad peak at 3448.81 cm⁻¹ was attributed to the remaining hydroxyl groups on the cyclodextrin and the silica gel. The 2941.66 cm⁻¹ absorption peak was ascribed into the stretching vibration absorption of C-H, which indicated that a layer of organic matter was bonded to the surface of SBA-15. The peak at 1654.48 cm⁻¹ was the stretching vibration of carbonyl on cholesterol, and 1584.24 cm⁻¹ was the stretching vibration of C=C on cholesterol, which indicated that cholesterol was successfully grafted to cycloextrin. The peaks at 1111.45 and 804.42 cm⁻¹ were the stretching and bending vibration absorption of the Si-O-Si and C-O moieties on of CHCDP. From the above data, it can be concluded that a successful immobilization of CHCD onto the SBA-15 occured, which could be further confirmed by the good separation ability of CHCDP.

Elemental analysis

The elemental analysis results of CHCDP showed: C, 4.98%; N, 0.25%; H, 1.14%. According to a calculation of the carbon content (4.98%), the loading efficiency of CHCDP was 0.13 μmol/m².

Chromatographic performance of CHCDP for achiral compounds

Among the three HPLC modes, including reversed-phase, normal-phase and polar organic modes, reversed-phase chromatography has attracted large-scale applications. Moreover, it is known that the hydrophobicity is a dominant force in reversed-phase chromatography. Therefore, in the present work, a cholesterol group was grafted to the β-cyclodextrin to enhance the hydrophobicity. Furthermore, some achiral analytes, including benzene homologs, PAs, positional isomers and some statin hypolipidemic drugs, were selected to investigate the reversed-phase chromatographic property of CHCDP.
Separations of benzene homologs and PAHs on CHCDP

The benzene homologs is a popular candidate in terms of the evaluation of the reversed-phase chromatographic properties of the stationary phases (i.e., C18 (ODS), C8 and phenyl columns). Therefore, we also used benzene five homologs as analytes to investigate the reversed-phase performance of CHCDP. These homologs have different amounts of the methylene group \((n = 0, 1, 2, 3, 4)\) with increasing hydrophobicity in turn, including benzene, toluene, ethylbenzene, propylbenzene and butylbenzene. As shown in Fig. 2, by using simple methanol–water (35:65, v/v) as a mobile phase, CHCDP was qualified to separate the five benzene homologs. We found that the increase of the methanol content in the mobile phase will shorten the retention time of homologs on CHCDP, which indicated that the CHCDP has a typical reversed-phase property. In addition, we found a linear relationship \((\ln k' = 0.4355n - 0.0702, R^2 = 0.9351, \ln \alpha(CH_2) = 0.4355, \alpha = k'_n/k'_1)\) between \(\ln k'\) and the number of methylene groups \((n)\) of these homologs, which was similar to that of ODS \((\ln k' = 0.5312n + 0.2985, R^2 = 0.9950, \ln \alpha(CH_2) = 0.5312)\). Obviously, the results showed that the introduction of cholesterol groups may enhance the hydrophobicity of the cyclodextrin, thus improving the reversed-phase property of CHCDP.

In order to further confirm the increase in the hydrophobicity of CHCDP, nine PAHs were also used as analytes, including benzene, toluene, xylene, naphthalene, biphenyl, anthracene, phenanthrene, anthracene and fluoranthene. These PAHs have different numbers of benzene rings with different hydrophobicity in nature. As shown in Fig. 3, by using a gradient elution comprised by methanol and water, all selected PAHs were completely separated on CHCDP within 30 min, which was an encouraging separation compared with the result obtained on the native cyclodextrin-based CSP. For example, such as phenanthrene and anthracene were not easy to be separated on the latter.\(^{17}\) However, CHCDP exhibited a high resolution \((R_s = 2.17)\) towards them, which should be attributed to a comprehensive effect from the hydrophobicity of the cholesterol group, and the inclusion complexation of β-cyclodextrin.

![Fig. 2](image2.png) Chromatogram of five benzene homologs. Methanol/water (v/v, 35/65). Benzene, toluene, ethylbenzene, propylbenzene, butylbenzene (7.97 - 26.57 min).

![Fig. 3](image3.png) Chromatogram of nine PAHs. Methanol/water in a gradient elution, methanol volume fraction: 25% (0 – 5 min), 25 – 50% (5 – 15 min), 50% (15 – 30 min), 25% (30 – 35 min). Benzene, toluene, xylene, naphthalene, biphenyl, anthracene, phenanthrene, anthracene, fluoranthene (11.00 – 23.75 min).

![Fig. 4](image4.png) Chromatograms of positional isomers of nitroaniline (left) and hydroxybenzoic acid (right). Methanol/water (35:65, v/v) for nitroaniline; and methanol/1% formic acid (5:95, v/v), respectively.
The separation of positional isomers by cyclodextrin-based columns was mainly based on the inclusion of the hydrophobic cavities with analytes, while the ODS columns mainly relied on the hydrophobic interaction to achieve the separations of isomers. Two representative positional isomers, nitroaniline (basic) and hydroxybenzoic acid (acidic), were selected as probes. Figure 4 (left) is a chromatogram of the positional isomer of nitroaniline when using methanol-water (35:65, v/v) as a mobile phase. It can be seen that the o-, m-, p-isomers of nitroaniline were completely separated. The p-nitroaniline was eluted at the end because it had a linear structure when entering the cavity of cyclodextrin deeply. The m- and o-isomers were also different regarding the depth of entering cavity due to the different substitution positions, thereby the three isomers were easily separated. Similarly, we also found that CHCDP exhibited high resolutions to o-, m-, p-hydroxybenzoic acids in Fig. 4 (right). Interestingly, the p-isomer among them was eluted in the middle, while the o-isomer was eluted at the end. This may be due to the fact that intramolecular hydrogen bonds of the o-isomer enhanced its hydrophobicity, being eluted at the end in reversed-phase chromatography. Obviously, CHCDP had a reversed-phase chromatographic property that was similar to the ODS column.

**Separation of cholesterol analogues**

In order to further investigate the contribution of the introduced cholesteryl group to the chromatographic property of CHCDP in the reversed-phase, the analogues of cholesteryl (statins) were selected as probes. Statin drugs\(^\text{18}\) (Fig. 5) were 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which can inhibit cholesterol synthesis in the human body. As shown in Fig. 5, CHCDP had strong retention strength to pravastatin (32 min) and atorvastatin (45 min) in the methanol-water (30:70, v/v) mobile phases. Obviously, there was a strong hydrophobic force between the cholesterol substituent and statin drugs, which was related to the larger alkyl skeleton of the cholesterol group on CHCDP. Therefore, the introduction of cholesterol could improve the hydrophobicity of CHCDP, thus enhancing separation performance in the reversed-phase chromatography.

**Evaluation of chiral chromatographic performance**

The chiral recognition of cyclodextrin mainly depended on its internally hydrophobic cavity and externally hydrophilic rims. Owing to encapsulation of the cavity towards enantiomers through size-fit and site-match, the inclusion complexes with
different stability formed, which made enantiomeric separation possible.11 In reversed-phase chromatography, the enantiomeric separation ability of cyclodextrin mainly depended on the inclusion effect and hydrogen bonding.24 Therefore, maintaining the inclusion capability of the CD’s cavity was an important strategy to design high-selectivity CD-CSPs. Among various CD-CSPs, the mono-derivatized cyclodextrin had a well-defined molecular structure with chiral sites, which are convenient for an illustration of the separation mechanisms. Moreover, the mono-derivatized CD-CSPs had a good chromatographic reproducibility.

In our previous attempt, we modified a long chain of the octadecane group to one port of cyclodextrin. As a result, the hydrophobicity was enhanced, but the reversed-phase chromatographic performance was not improved, which would not be conducive to an inclusion effect of cyclodextrin.18 As an alternative, for the purpose of alleviating blockage of the cavity, we selected fat-soluble cholesterol to modify the cyclodextrin.

**Reversed-phase chromatographic mode**

As we expected, the introduction of hydrophobic cholesterol not only enhanced the hydrophobicity of CHCDP, but also retained the inclusion ability from the cavity of cyclodextrin, as a result of enhancing the chiral recognition effect of CHCDP. In this study, some racemic drugs were selected as probes (Fig. 6) to evaluate the chiral chromatographic performance of CHCDP. The separation results are summarized in Table 1, and the separation chromatograms are shown in Figs. 7 and 8.

**Table 1: Separation results of flavanones and triazoles in the reversed-phase mode**

| No. | Enantiomers       | $k_1'$ | $k_2'$ | $\alpha$ | Rs    | Mobile phases (v/v)     |
|-----|-------------------|--------|--------|----------|-------|-------------------------|
| 1   | 2′-Hydroxyflavanone | 2.25   | 2.62   | 1.16     | 1.94  | MeOH/H2O/FA (30/70/0.1) |
| 2   | 4′-Hydroxyflavanone | 3.2    | 3.34   | 1.04     | 0.76  | MeOH/H2O/FA (30/70/0.1) |
| 3   | 6-Hydroxylflavanone | 2.16   | 2.25   | 1.04     | 0.35  | MeOH/H2O/FA (30/70/0.1) |
| 4   | Hexaconazole      | 2.85   | 3.49   | 1.23     | 1.91  | MeOH/H2O (35/65)        |
| 5   | Triticonazole     | 3.49   | 3.81   | 1.05     | 1.56  | MeOH/H2O (40/60)        |
| 6   | Triadimenol       | 3.09   | 3.29   | 1.07     | 0.98  | MeOH/H2O (20/80)        |
| 7   | Flutriatol        | 1.26   | 1.49   | 1.18     | 1.28  | MeOH/H2O (35/65)        |
| 8   | Diniconazonol     | 6.51   | 7.03   | 1.08     | 0.55  | MeOH/H2O (25/75)        |
| 9   | Uniconazol        | 6.03   | 6.44   | 1.07     | 0.51  | MeOH/H2O (25/75)        |

(1) Chiral separation of flavanones. The flavanones20 are effective components of Chinese herbal medicine, which has a variety of biological activity, such as inhibiting tumors, as well as exhibiting antioxidant and antibacterial efficacy. Moreover, the spatial structures of flavanones are closely related to their activities. Therefore, in this study, we evaluated the chromatographic performance of CHCDP by separating enantiomers of 2′-hydroxy, 4′-hydroxy and 6-hydroxy flavanones. The chemical structures, representative chromatograms and the separation results are shown in Fig. 6, Fig. 7, and Table 1, respectively.

Because the flavanones contained acidic phenolic hydroxyls, the formic acid solution was used to inhibit their ionization, which would promote the enantioseparations. The results showed that the three flavanones had long and similar retention times on CHCDP under the same mobile phase, but the resolutions were quite different; namely, the resolutions of 2′-hydroxyflavanone, 4′-hydroxyflavanone and 6-hydroxyflavanone were 1.94, 0.76 and 0.35, respectively. The possible separation mechanism was described as follows: during chiral recognition, the CHCDP was inclined to encapsulate the...
The polar organic mode for the separations of polar racemic \( \beta \)-blocker drugs are widely used chiral drugs for the treatment of cardiovascular and cerebrovascular diseases. However, the efficacy and toxicity of enantiomers are quite different.\(^{24-26}\) Due to the rapid development of these synthetic chiral drugs, there is still a lack of a systematic enantiomeric analysis method. Therefore, we used the newly prepared CHCDP to separate \( \beta \)-blockers to evaluate the chromatographic performance of it in the polar organic mode. The results of the resolutions of the seven \( \beta \)-blocker drugs (Fig. 9) are listed in Table 3, and representative chromatograms of racemic drugs are shown in Fig. 10.

The enantioseparation of triazole pesticides in reversed-phase chromatography

The triazole pesticides\(^{21}\) are a kind of important fungicides containing 1,2,4-triazolyl in their structures. Their enantiomers have different bactericidal activity and toxicity to human bodies, so it is necessary to assay enantiomeric residues of these pesticides. The following six triazole pesticides with similar structures were selected as chiral probes to evaluate the enantioseparation ability of CHCDP. The separation results and the representative chromatograms are shown in Table 1 and Fig. 7.

We found that the acetonitrile had a strong elution ability that made the interaction time between the analytes and CHCDP be too short, which was not conducive to the chiral separations. Therefore, the following separations were mainly conducted in the mobile phase consisting of the methanol-water. As shown in Table 1, CHCDP could effectively resolve all six triazole pesticides with similar structures were selected as chiral probes to evaluate the enantioseparation ability of CHCDP. The separation results and the representative chromatograms are shown in Table 1 and Fig. 7.

Interestingly, CHCDP exhibited an abnormal separation ability, which achieved relatively more efficient enantioseparations for the dansylated serine and threonine, while the enantioseparations of polar amino acids, such as dansylated serine and threonine, were very difficult. Interestingly, CHCDP exhibited an abnormal separation ability, which achieved relatively more efficient enantioseparations for the dansylated serine and threonine, while the enantioseparations of polar amino acids, such as dansylated serine and threonine, were very difficult.

The enantioseparation of amino acids in reversed-phase chromatography

Amino acids are amphoteric compounds that was usually require precolumn dansylation.\(^{15}\) We found that the separation efficiency by using only methanol-water as a mobile phase was unsatisfied. An appropriate concentration of TEAA buffer solution with certain pH values (pH 4.0 - 5.0) was required. The chiral separation results of five dansylated amino acids (Fig. 6) on CHCDP were listed in Table 2, and the representative chromatograms are shown in Fig. 8.

Combined with the separation data and chromatograms, we noticed that the resolutions (\( R_s = 0.87 - 2.15 \)) of the five dansyl-amino acids on CHCDP were significantly better than those of native cycloexdextrin CSP,\(^{13-15}\) indicating that the introduction of cholesterol to the cyclodextrin port facilitated the chiral resolution of the analytes. According to the literature,\(^{13-15}\) in general, hydrophobic amino acids, such as dansylated leucine and phenylalanine, could be separated on most derivatized CD-CSPs, while the enantioseparations of polar amino acids, such as dansylated serine and threonine, were very difficult. Interestingly, CHCDP exhibited an abnormal separation ability, which achieved relatively more efficient enantioseparations for the dansylated serine (\( R_s = 2.15 \)) and threonine (\( R_s = 2.09 \)) within 15 min. Obviously, in addition to inclusion, the other interactions, such as hydrogen bonding and steric hindrance, also played key roles in enantioseparations of the above polar analytes, in which the small volume of serine and threonine groups with relatively small hindrance could readily approach the ligand of CHCDP, thus being separated easily.

The enantioseparation of \( \beta \)-blocker drugs in polar organic mode

Clinically, the \( \beta \)-blockers are widely used chiral drugs for the treatment of cardiovascular and cerebrovascular diseases. However, the efficacy and toxicity of enantiomers are quite different.\(^{24-26}\) Due to the rapid development of these synthetic chiral drugs, there is still a lack of a systematic enantiomeric analysis method. Therefore, we used the newly prepared CHCDP to separate \( \beta \)-blockers to evaluate the chromatographic performance of it in the polar organic mode. The results of the resolutions of the seven \( \beta \)-blocker drugs (Fig. 9) are listed in Table 3, and representative chromatograms of racemic drugs are shown in Fig. 10.

The polar organic mode for the separations of polar racemic compounds was first proposed by Armstrong \textit{et al.}\(^{27}\) with using different volume ratios MeOH, ACN, TEA and HOAc as the mobile phase. Since then, this separation mode has been widely used in chiral separation. According to the separation data, some separation mechanisms were discussed as follows.

\( (1) \) Inclusion complexation. As shown in Table 3, CHCDP could completely separate atenolol (\( R_s = 1.57 \)), metoprolol

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### Table 2 Enantioseparation of dansyl-amino acids in reversed-phase chromatography

| No. | Enantiomer       | k\(_i\) | k\(_s\) | \( \alpha \) | \( R_s \) | Mobile phases (v/v)       |
|-----|------------------|--------|--------|-------------|--------|---------------------------|
| 10  | Dns-tt-leucine   | 6.26   | 6.59   | 1.05        | 1.17   | MeOH/H\(_2\)O/TEAA, 25/75/1 |
| 11  | Dns-tt-phenylalanine | 4.35   | 5.14   | 1.19        | 0.87   | MeOH/H\(_2\)O/TEAA, 25/75/1 |
| 12  | Dns-tt-tyrosine  | 4.52   | 4.91   | 1.10        | 1.37   | MeOH/H\(_2\)O/TEAA, 15/85/1 |
| 13  | Dns-tt-serine   | 1.42   | 1.85   | 1.30        | 2.15   | MeOH/H\(_2\)O/TEAA, 25/75/1 |
| 14  | Dns-tt-threonine | 2.28   | 3.17   | 1.38        | 2.09   | MeOH/H\(_2\)O/TEAA, 35/65/1 |
All four drugs had linear structures with long retention times in similar mobile phases. This indicated that the linear molecules were easily encapsulated by the cyclodextrin. Due to the difference in the stereo-structure of the enantiomers, the depth of entry into the cavity was different, and the interaction force with the stationary phase was different, thereby achieving effective separations; obviously, the introduction of cholesterol to the CD’s port did not affect inclusion of the cavity. Similarly, the resolutions of propranolol ($Rs = 1.08$), carvedilol ($Rs = 0.61$), and carteolol ($Rs = 0.73$) were also related to their molecular sizes and shapes during inclusion complexing.

(2) Hydrogen bonding. We found that these polar amino-propanol structures of β-blockers could not be resolved under reversed-phase chromatography. The reason was that water as an important hydrogen bond donor could greatly weaken the hydrogen bond interaction between analytes and CHCDP. On the contrary, these drugs could be completely separated when a non-aqueous polar organic solvent was used as the mobile phase. Obviously, the hydrogen bond between the hydroxyl at the chiral carbon of these analytes and CHCDP had an important contribution to the above chiral separations.

(3) Other interactions. Chiral recognition required the synergistic participation of various sites. For example, in addition to the above inclusion and hydrogen bonding, the steric hindrance of cholesterol might also be involved in chiral recognition. Carvedilol contained a large aromatic ring, which had a steric hindrance with cholesterol, and was not conducive to the inclusion of a solute in the cavity, so the enantioseparation on CHCDP was poor ($Rs = 0.61$).

Effect of TEA/HOAc in mobile phase on chiral separation

It was found that in the polar organic mode, if only with acetonitrile as the mobile phase, the chromatographic peaks of analytes were severely tailed, and the retention time was relatively long, and could even not be eluted. We found that a small amount triethylamine and glacial acetic acid (TEA and

| No. | Enantiomer | $k_1'$ | $k_2'$ | $\alpha$ | $Rs$ | MeOH/ACN/TEA/HOAc (v/v) |
|-----|------------|--------|--------|---------|------|------------------------|
| 15  | Atenolol   | 6.82   | 7.59   | 1.11    | 1.57 | 4/96/1/1               |
| 16  | Metoprolol | 2.05   | 2.31   | 1.13    | 1.48 | 5/95/1/1               |
| 17  | Esmolol    | 2.87   | 3.1    | 1.08    | 1.43 | 5/95/0.5/0.5           |
| 18  | Arotinolol | 4.15   | 4.46   | 1.07    | 1.54 | 10/90/0.5/0.5          |
| 19  | Propranolol| 2.87   | 3.02   | 1.05    | 1.08 | 1/99/1/1               |
| 20  | Carvedilol | 1.78   | 1.85   | 1.04    | 0.61 | 5/95/1/1               |
| 21  | Carteolol  | 2.46   | 2.56   | 1.04    | 0.73 | 5/95/1/1               |

(Rs = 1.48), esmolol ($Rs = 1.43$) and arotiolol ($Rs = 1.54$). All four drugs had linear structures with long retention times in similar mobile phases. This indicated that the linear molecules were easily encapsulated by the cyclodextrin. Due to the difference in the stereo-structure of the enantiomers, the depth of entry into the cavity was different, and the interaction force with the stationary phase was different, thereby achieving effective separations; obviously, the introduction of cholesterol to the CD’s port did not affect inclusion of the cavity. Similarly, the resolutions of propranolol ($Rs = 1.08$), carvedilol ($Rs = 0.61$), and carteolol ($Rs = 0.73$) were also related to their molecular sizes and shapes during inclusion complexing.
HOAc) could greatly improve the peak shape and the resolution of enantiomers (Fig. S4, Supporting Information). On the one hand, the addition of TEA to the mobile phase could effectively overcome the effect of residual acidic silanol groups of CHCDP on the basic β-blocker drugs. On the other hand, the addition of HOAc could inhibit the ionization of silanol groups and improve the peak shape as well. We optimized the ratios of MeOH/ACN and TEA/HOAc in the mobile phases (Table S1, Supporting Information). The resolutions of atenolol and metoprolol were 1.57 and 1.48 with the optimal volume ratio of TEA/HOAc (1%/1%), respectively; the resolution of esmolol was 1.43 with that of TEA/HOAc (0.5%/0.5%). Therefore, it was especially important to control the proportion and content of TEA/HOAc.

**Effect of temperature on the separation of arotinolol enantiomers**

Temperature can affect both the thermodynamic and kinetic processes of chromatography,28 so the effects of temperature on the chiral separation should be investigated and optimized to select the appropriate column temperature. Taking arotinolol as an example, the separations of arotinolol enantiomers at different temperatures (10 – 30°C) were carried out. The related chromatograms are shown in Fig. S3 (Supporting Information). It can be seen that with increases of the column temperature, the retention time of the arotinolol enantiomers decreased from 40.03 to 21.98 min, accompanied by a decrease of selective factors (α) from 1.28 to 1.03 and a decrease of the resolutions from 1.43 to 0.15, respectively. Obviously, with the increase of temperature, the molecular thermal motion was accelerated, and the inclusion complexes of two enantiomers with cyclodextrin became unstable, while their difference became small, making them difficult to be separated. From the separation results, the lower column temperature was conducive to the enantioseparations, but if the temperature was too low, the analysis time was prolonged, and the peak was also broadened. After a comprehensive consideration, the column temperature was set at 20°C.

**Conclusions**

In this study, a lipid-soluble cholesterol was firstly modified to the β-cyclodextrin port to obtain a new supramolecule with the hydrophobicity of cholesterol, the hydrophilicity of the portal hydroxyl group and the inclusion function of the cavity. The supramolecule was bonded onto the surface of ordered silica gel to prepare a novel 6-mono-cholesteryl-β-CD-bonded phase (CHCDP). After structural characterization, the achiral and chiral chromatographic performances of CHCDP were evaluated by using related probes. The results showed that CHCDP possessed separation abilities to both chiral and achiral compounds in multiple chromatographic modes. The introduction of a cholesterol group into the port of cyclodextrin not only improved the hydrophobicity, but also retained the cavity inclusion effect, which was a versatile chromatographic separation materials for achiral and chiral analysis.

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