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REGULAR ARTICLE

Novel chiral stationary phases based on 3,5-dimethyl phenylcarbamoylated β-cyclodextrin combining cinchona alkaloid moiety

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Abstract

Novel chiral selectors based on 3,5-dimethyl phenylcarbamoylated β-cyclodextrin connecting quinine (QN) or quinidine (QD) moiety were synthesized and immobilized on silica gel. Their chromatographic performances were investigated by comparing to the 3,5-dimethyl phenylcarbamoylated β-cyclodextrin (β-CD) chiral stationary phase (CSP) and 9-*O*-(*tert*-butylcarbamoyl)-QN-based CSP (QN-AX). Fmoc-protected amino acids, chiral drug cloprostenol (which has been successfully employed in veterinary medicine), and neutral chiral analytes were evaluated on CSPs, and the results showed that the novel CSPs characterized as both enantioseparation capabilities of CD-based CSP and QN/QD-based CSPs have broader application range than β-CD-based CSP or QN/QD-based CSPs. It was found that QN/QD moieties play a dominant role in the overall enantioseparation process of Fmoc-amino acids accompanied by the synergistic effect of β-CD moiety, which lead to the different enantioseparation of β-CD-QN-based CSP and β-CD-QD-based CSP. Furthermore, new CSPs retain extraordinary enantioseparation of cyclodextrin-based CSP for some neutral analytes on normal phase and even exhibit better enantioseparation than the corresponding β-CD-based CSP for certain samples.

KEYWORDS

chiral stationary phases, cinchona alkaloid, enantiomer separation, HPLC, β-cyclodextrin

1 | INTRODUCTION

Enantioseparation techniques have become a very important field in analytical chemistry, owing to the fact that chiral compounds may differ in pharmacodynamics activity, pharmacokinetic properties, and intrinsic activity at receptor sites.[1] Over the years, gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have been developed to support the enantioseparation of chiral drugs.[2] Moreover, HPLC is an effective analytical tool for the resolution of chiral compounds on both analytical and preparative scales.

Over recent years, with the ability to form inclusion complexes with analytes (in aqueous solvents),[3] β-cyclodextrin (β-CD)-based chiral stationary phases (CSPs) are able to provide unique enantioselectivities, which represent the popular chiral selector. It is important to note that β-CD functionalized with a broad variety of moieties, such as methyl group,[4–6] naphthylethyl

group,[7,8] and phenylcarbamate group,[9,10] possess the desirable chiral recognition capabilities. The β-CD-based CSPs exhibit enantioselectivity toward a broad spectrum of compounds, such as flavonoids,[11,12] β-blockers,[13–16] aromatic alcohols,[17,18] and binaphthol derivatives.[19,20]

Cinchona alkaloids are used as valuable drugs,[21,22] as chiral catalysis,[23,24] or as chiral resolution reagents.[25,26] Besides, cinchona alkaloids, especially quinine (QN) and quinidine (QD), as unique chiral anion exchangers, were proved to be powerful chiral selectors for the enantioseparations of chiral acids.[27–29] The early CSPs based on QN derivatives showed enantioselectivities to amino acid derivatives and carboxylic acids.[30] In particular, CSPs based on cinchona alkaloid derivatives are commercially available under the name Chiralpak QN-AX and Chiralpak QD-AX, which exhibit good enantioselectivity for various amino acids derivatives.[29,31,32] Subsequently, the enantioseparations of cinchona alkaloid-based CSPs were greatly extended by reacting hydroxyl groups of cinchona alkaloid with various chiral or achiral moieties. For example, the zwitterionic ion-exchange-type CSPs combining cinchona alkaloid-based chiral weak anion exchangers with strong cation exchangers, that is, the aminosulfonic acid derivatives, exhibit good enantioselectivity for various zwitterionic analytes such as α- and β-amino acids and peptides.[33] Furthermore, a series of cinchona alkaloid-based zwitterionic CSPs were employed in enantioseparation of amino acids, even underivatized amino acids.[34–36] Moreover, new types of CSPs based on cinchona alkaloid combining chiral peptoids[37,38] and crown ethers[39,40] showed specific enantioselectivities that were quite different from those on the original cinchona alkaloid CSPs. These observations proved that the enantioselectivity of the CSP can be modified by the combination of the carefully chosen chiral moiety. The extended application spectrum and new enantioselectivities can be expected owing to the synergistic effect of two combined chiral blocks.

Both the β-CD-based CSPs and cinchona alkaloid-based CSPs have their own distinct characteristics in enantioseparations. We herein report the evaluation of two novel β-CD-based CSPs combining cinchona alkaloid moiety. The purpose of the work is to extend the enantioselectivity to the enantiomers that can be resolved on β-CD-based CSP but also to the acidic enantiomers that can be separated on cinchona alkaloid-based CSP. As shown in Figure 1, QN or QD is used as a moiety, respectively, to bond 3,5-dimethyl phenylcarbamoylated β-CD on the surface of silica particles through thio-ene "click" reaction (CSP 2 and CSP 3). 3,5-Dimethyl phenylcarbamoylated β-CD CSP (CSP 1) and QN-AX were synthesized for comparison. Their enantioselectivities were evaluated by two sample sets totally containing 42 enantiomers.

2 | MATERIALS AND METHODS

2.1 | General information and material

The general synthesis of CSPs, including the synthetic details and the characterization of the CSPs, is described in the Supporting Information. The loadings of QN-AX, CSP 1, CSP 2, and CSP 3 were 0.157, 0.105, 0.023, and 0.027 mmol/g, respectively. It should be noted that CSP 1 was prepared by reacting underivatized cyclodextrin with modified silica followed by derivatization reaction, while CSP 2 or CSP 3 was synthesized via immobilization 3,5-dimethyl phenylcarbamoylated β-CD with much larger molecular weight on modified silica. CSP 1 has a higher loading owing to its less steric hindrance for surface bonding than CSP 2 and CSP 3. The chiral selector loading of CSP 1 was calculated by assuming that the β-CD moiety quantitatively derivatized because a large

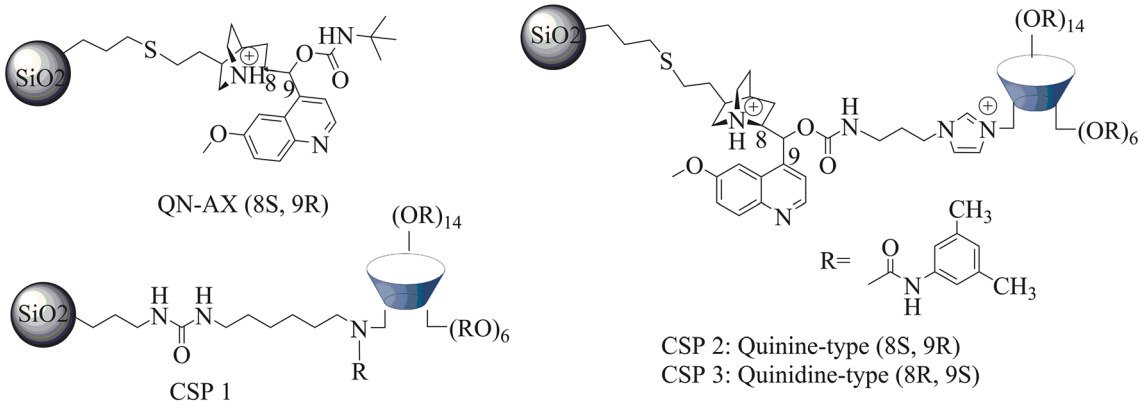


FIGURE 1 The schematic structure of QN-AX, CSP 1, CSP 2, and CSP 3. CSP, chiral stationary phase

excess of 3,5-dimethylphenyl isocyanate was used in the reaction. CSP 2 and CSP 3 have similar loadings, which is favorable for comparison.

Except for the chiral drug (±)-cloprostenol, which was purchased from Bide Pharmatech Ltd (Shanghai, China), the analytes for evaluation were from Daicel Chiral Technologies (China) Co., Ltd. (Shanghai, China). HPLC solvents methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), and *n*-hexane were of HPLC-grade quality from J&K Chemicals (China). HPLC-grade spherical silica gel (5-μm particle size; 10-nm pore size; 300 m^2/g surface area) was provided by Acchrom Technologies (Zhejiang, China). Formic acid (FA) and triethylamine (TEA) were purchased from Aladdin (Shanghai, China).

For column packing, each CSP (2.50 g) was slurried in MeOH (19 ml) and packed into an HPLC column (150 × 4.6 mm, id) with MeOH as propulsion solvent under a pressure of 500 MPa.

2.2 | Chromatographic evaluation

All the chromatographic separations were performed on Waters HPLC system consisting of a 515 HPLC pump, 7725i manual injector, model 1500 column heater, and 2489 UV/Vis detector (Waters, USA). The racemic analytes used for chiral separation evaluation were dissolved in HPLC-grade ethanol to form about 1 mg/ml of concentration. For chromatographic evaluations, the column temperature was held constantly at 25°C, and the flow rate was 1.0 ml/min. The injection volume was 1 μl.

The columns packed with CSP 1, CSP 2, and CSP 3 (150 × 4.6 mm, id, 5 μm) afforded efficiencies of 4.68×10^4, 4.18×10^4, and 4.41×10^4 plates/m, using benzene as a test probe under the normal phase (NP) mode (*n*-hexane/EtOH/MeOH = 95/5/1, v/v/v).

3 | RESULTS AND DISCUSSION

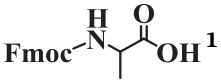
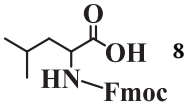
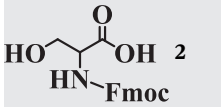
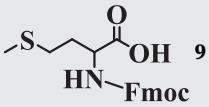
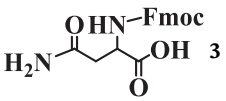
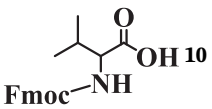
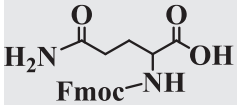
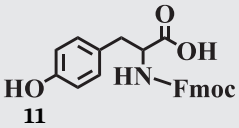
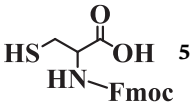
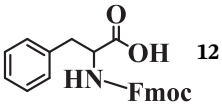
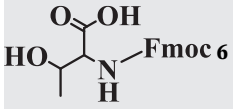
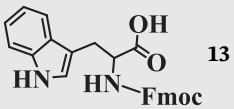
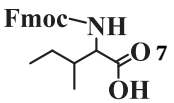
As shown in Figure 1, CSP 2 and CSP 3 contain 3,5-dimethyphenylcarbamate-β-CD combining QN and QD moiety, respectively. β-CD derivatives have been widely used as chiral selectors and showed wide enantioselectivity spectrums for the enantiomers with various structures. It has been known that the enantioselectivity of β-CD-type CSP was affected by the derivative groups.[41] The cinchona alkaloid-type CSPs, such as QN-based CSP and QD-based CSP, have a bulky quinuclidine moiety with a tertiary amino group protonated easily under acidic condition, which can provide an anion-exchange site together with other additional interactions such as hydrogen bonding and π–π interactions to achieve enantiomeric separation for acidic enantiomers. QN-type CSP and its pseudoenantiomeric QD-type CSP allow a switch in the elution order.[28] The separation behavior would change when the β-CD and cinchona alkaloid were combined in the CSPs. The application spectrum may be extended owing to the combination of two different functional groups. However, the absolute configurations of the chiral structures may influence the enantioselectivity. Diastereoisomers would be generated when β-CD, a cyclic oligosaccharide containing seven D-glucopyranose units, was connected to QN or QD. The synergistic effect between cyclodextrin and cinchona base would affect the enantioselectivity of the stationary phase, which could be illustrated via comparing CSP 2 and CSP 3. Herein, a sample set comprising 13 Fmoc-protected amino acids, (±)-cloprostenol, and 28 neutral analytes was evaluated on CSP 2 and CSP 3, as compared with QN-AX or CD-based CSP 1.

3.1 | Enantioseparation of Fmoc-amino acids

Polar organic (PO) mobile phases, which proved to be more advantageous than reversed-phase (RP) conditions because of the reduction of nonspecific interactions and high efficiency of mass transfer, are preferential mobile phases when employing QN-based or QD-based CSPs. In this section, the enantioseparations of 13 enantiomers of Fmoc-protected amino acids were evaluated by QN-AX, CSP 2, and CSP 3 with a polar organic mobile phase containing MeOH/ACN (10/90, v/v) as bulk solvent together with and 5 mM of FA and 2.5 mM of TEA as additives. The separation results are listed in Table 1.

It needs to be noted that all of these enantiomers were not resolved by CSP 1 that contains only 3,5-dimethyphenylcarbamate-β-CD chiral unit with the polar organic mobile phase (data are not shown). QN- and QD-based CSPs generally behave like pseudoenantiomers on which the enantiomeric elution order is reversed.[42] The elution orders of the Fmoc-protected amino acid enantiomers that can be resolved on CSP 2 and CSP 3 are all reversed, which indicates that the enantioseparations are dominated by the QN or QD moiety. Although the QN/QD moiety is deeply inside the large chiral selector molecule, there is sufficient space for the acidic analytes to diffuse to the QN/QD site because the size of derivated β-CD is much larger than that of QN/QD moiety. However, the enantioselectivity is changed by β-CD moiety. QN-AX and CSP 2 show different separation factors (α values) for the sample set. Especially for **4**, **5**, **12**, and **13**, higher α values were obtained on CSP 2, which suggests that the cyclodextrin derivative

TABLE 1 Enantioseparation of Fmoc-amino acids on QN-AX, CSP 2, and CSP 3

Enantiomer	CSP	k_1	α	Rs	EO	Enantiomer	CSP	k_1	α	Rs	EO
 1	QN-AX	4.92	1.16	2.30	D/L	 8	QN-AX	3.80	1.29	3.68	D/L
	CSP 2	2.45	0.00	0.00	-		CSP 2	1.73	0.00	0.00	-
	CSP 3	3.96	1.18	2.38	L/D		CSP 3	2.76	1.05	~0	L/D
 2	QN-AX	7.11	1.24	3.43	D/L	 9	QN-AX	6.52	1.23	3.16	D/L
	CSP 2	5.12	1.13	1.40	D/L		CSP 2	4.39	1.09	0.93	D/L
	CSP 3	5.18	1.31	3.82	L/D		CSP 3	4.17	1.13	1.77	L/D
 3	QN-AX	4.52	1.19	2.44	D/L	 10	QN-AX	4.48	1.34	4.33	D/L
	CSP 2	5.39	1.13	1.29	D/L		CSP 2	2.28	1.03	~0	-
	CSP 3	5.13	0.00	0.00	-		CSP 3	3.02	1.06	~0	-
 4	QN-AX	8.14	1.09	1.28	D/L	 11	QN-AX	9.28	1.12	1.74	D/L
	CSP 2	3.64	1.12	1.13	D/L		CSP 2	3.54	1.15	1.39	D/L
	CSP 3	6.06	1.14	1.81	L/D		CSP 3	6.03	1.14	1.89	L/D
 5	QN-AX	13.41	1.05	~0	-	 12	QN-AX	3.74	1.09	2.14	D/L
	CSP 2	8.07	1.11	~0	D/L		CSP 2	5.13	1.15	1.72	D/L
	CSP 3	5.75	1.19	2.37	L/D		CSP 3	5.30	1.14	1.95	L/D
 6	QN-AX	9.23	1.40	5.39	D/L	 13	QN-AX	5.03	1.24	1.71	D/L
	CSP 2	4.11	1.04	~0	D/L		CSP 2	6.27	1.59	4.96	D/L
	CSP 3	4.13	1.24	2.88	L/D		CSP 3	6.51	1.14	2.11	L/D
 7	QN-AX	4.43	1.30	3.66	D/L						
	CSP 2	2.06	0.00	0.00	-						
	CSP 3	2.90	1.10	1.13	L/D						

Note: Condition: flow rate, 1 ml/min; concentration of analyte, 1.0 mg/ml; mobile phase (Mp), MeOH/ACN (10/90, v/v) containing 5 mM of FA and 2.5 mM of TEA; detection, 260 nm.

Abbreviations: CSP, chiral stationary phase; EO, elution order; FA, formic acid; TEA, triethylamine.

unit is also to some extent involved in the enantiorecognition process of derivatized amino acids. Generally, the values achieved on CSP 3 are better than those on CSP 2. For instance, nine out of 13 Fmoc-protected amino acids were baseline separated (Rs ≥ 1.5) on CSP 3, whereas only two enantiomers (**12** and **13**) were baseline resolved on CSP 2. For **1**, **7**, and **8**, no separations were observed on CSP 2, whereas they were resolved by CSP 3. CSP 3 exhibits much better enantioselectivity for **2**, **5**, **6**, and **9** than CSP 2. The combinations of cinchona bases (QN and QD) and cyclodextrin derivatives produce two different diastereomeric isomers. When the Fmoc-protected amino acid molecule interacts with anion-exchange site on QN or QD, the recognition process is also affected by the structure of cyclodextrin derivative in some manners such as steric hindrance, hydrogen bond, and π–π interaction. Hence, the enantiorecognition ability of the CSPs can be ascribed to the synergistic effect of the two chiral moieties. In this case, the configuration of QD-β-CD is more favorable for the enantiorecognition of most of Fmoc-protected amino acid enantiomers.

3 and **13** are the two exceptions that were better separated on CSP 2 than on CSP 3. The structure of **3** contains a carboxyl group that can interact with anion-exchange site in cinchona base and an amide group that can form hydrogen bond with the carbamate group on β-CD. As shown in Figure 2A, **3** was separated on CSP 2, whereas it could not be resolved by CSP 3, which can be ascribed to their different absolute configurations. The configuration of cinchona-β-CD structure in CSP 2, compared with CSP 3, is more favorable for one of the enantiomers to form a more stable complex through the interaction of anion exchange and the hydrogen bond interaction with the carbamate group of β-CD moiety. It is interesting to note that **3** and **4**, which have a close structural similarity and differ only in the length of alkyl chain that bridging carboxyl group and amide group, achieved different enantioseparations on the CSPs. **4** was separated on CSP 2 and CSP 3 with higher α values than that on QN-AX

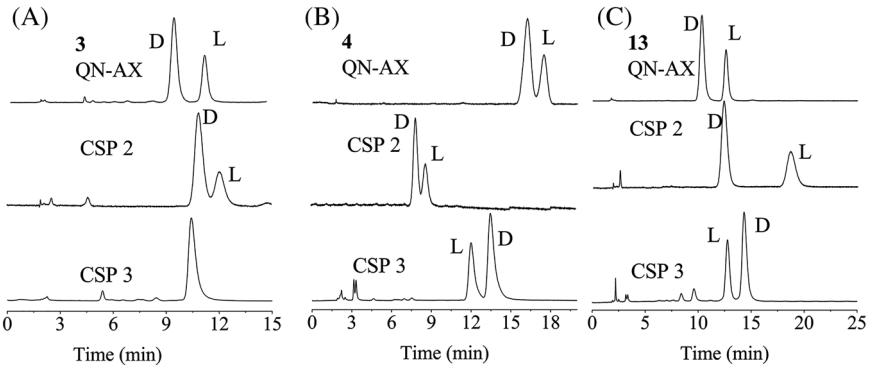


FIGURE 2 Enantiomeric separation of **3** (A), **4** (B), and **13** (C) on QN-AX, CSP 2, and CSP 3. Flow rate, 1 ml/min; mobile phase, MeOH/ACN (10/90, v/v) containing 5 mM of FA and 2.5 mM TEA; detection, 260 nm. CSP, chiral stationary phase; FA, formic acid; TEA, triethylamine

(see Figure 2B), which indicates that the longer and more flexible alkyl chain of **4** allowed one of its enantiomers to form a stable complex with the chiral selector whether the structure of the chiral selector was quinine-β-CD or quinidine-β-CD. For the separation of **13** (see Figure 2C), the separation factor on CSP 2 (1.59) is much higher than that on QN-AX (1.24) and CSP 3 (1.14). The stronger synergistic effects of anion exchange and π–π interaction between **13** and the chiral selector in CSP 2 than in CSP 3 may play a dominant role in enantiorecognition mechanism.

The influence of counterion concentration on the retentions and α values of **1**, **2**, **4**, and **13** was investigated on CSP 3. Counterion concentrations were varied in the range of 2.5 to 20 mM. The selectivity does not change significantly with the change of the concentration of the counterion (Figure 3B). The results obtained were depicted as plots of the logarithm of the retention factors ($\log k_1$) versus the logarithm of counterion concentration ($\log c$) of the first eluted enantiomer (Figure 3A). Based on the stoichiometric displacement model,[43] the slope value of $\log k_1$ versus $\log c$ should be 0.5, owing to the two positively charged sites in CSP. The values of the slopes in Figure 3A are slightly higher than this value (from 0.540 to 0.606). By comparing the retention factors of QN-AX with CSP 2, which differ in their QN residue loading of 0.157 to 0.023 mmol/g, a difference of a factor of ~3.4 is expected, assuming a linear correlation. According to Table 1, however, this is not the case; on the contrary, the factor varies between 2.6 and 0.73, which means that the permanently charged imidazolidine site is on the one hand dominating the electrostatic interactions, but on the other hand, there are also additional relatively strong synergistic selector–selectand interactions in force. Especially for analytes **12** and **13**, the retention as well as the overall stereoselectivity is affected.

3.2 | Enantioseparation of neutral analytes and cloprostenol

The separations of 28 neutral enantiomers and (±)-cloprostenol were carried out to further evaluate the chiral separation behaviors of the CSPs. Both NP and reverse phase were applied in the evaluation. It was observed that the low percentage (1%) of MeOH incorporated into mobile phase could improve separation performance of the columns under NP, especially for the improvement of the column efficiency. For instance, MeOH in the low percentage (1%) could enhance the resolution degree with higher α and Rs values of **18** on CSP 1 (α = 1.19, Rs = 1.62, while α = 1.12, Rs = 1.05 without MeOH,).

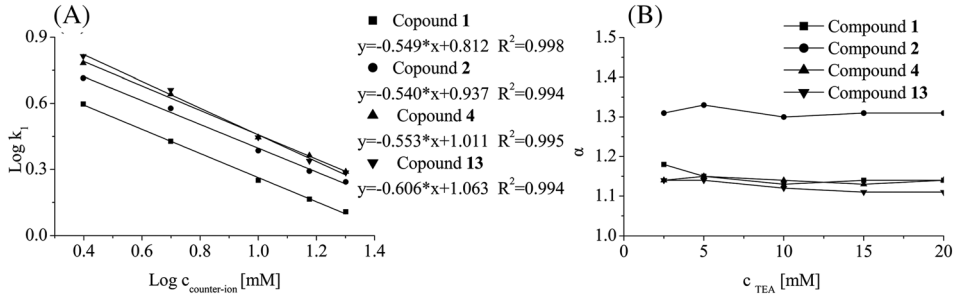


FIGURE 3 Effect of the counterion concentration (various concentrations of FA and TEA at a constant acid-to-base ratio of ranging from 5:2.5 to 40:20) in the mobile phase (MeOH/ACN) (10/90, v/v) on the retention (A) and selectivity (B) of analytes **1**, **2**, **4**, and **13** on CSP 3. Flow rate, 1 ml/min; detection, 260 nm. CSP, chiral stationary phase; FA, formic acid; TEA, triethylamine

TABLE 2 Enantioseparation of neutral analytes on CSP 1, CSP 2, and CSP 3

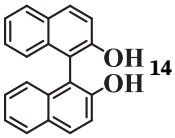
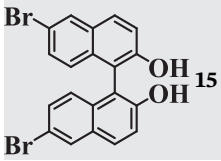
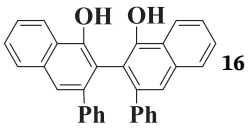
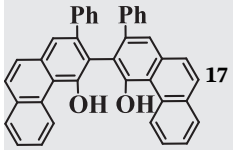
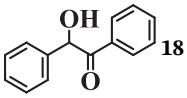
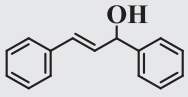
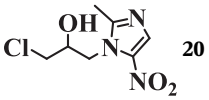
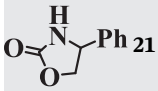
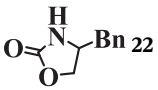
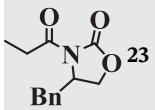
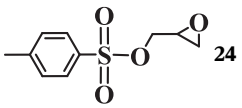
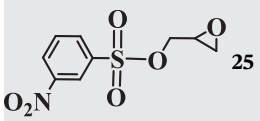
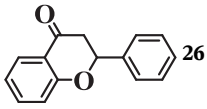
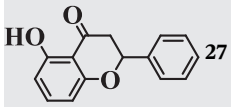
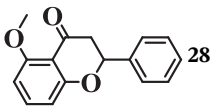
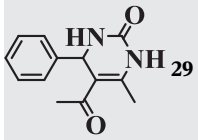
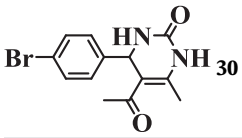
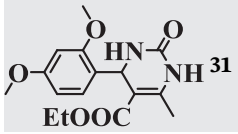
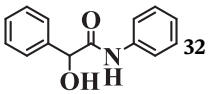
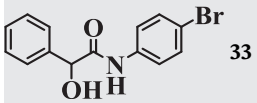
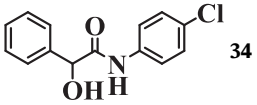
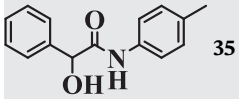
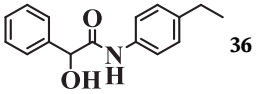
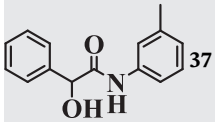
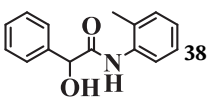
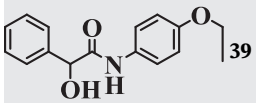
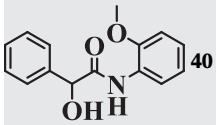
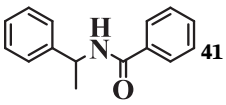
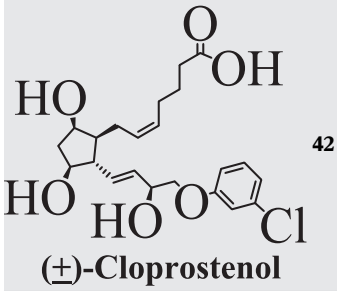
Enantiomer	NP						RP				
	CSP	k_1	α	Rs	EO	Mp	k_1	α	Rs	EO	Mp
 14	CSP 1	6.15	1.15	0.92	R/S	N-1	1.39	0.00	0.00	-	R-1
	CSP 2	7.32	1.11	1.03	R/S	N-1	1.41	0.00	0.00	-	R-1
	CSP 3	11.52	1.03	~0	-	N-1	2.18	0.00	0.00	-	R-1
 15	CSP 1	10.00	1.28	1.66	R/S	N-1	5.19	0.00	0.00	-	R-1
	CSP 2	15.25	1.24	2.61	R/S	N-1	3.40	1.08	~0	-	R-1
	CSP 3	15.89	1.06	1.09	R/S	N-1	5.02	1.02	~0	-	R-1
 16	CSP 1	3.04	1.46	2.05	R/S	N-1	6.24	1.20	~0	-	R-1
	CSP 2	5.37	1.27	1.80	R/S	N-1	5.24	1.06	~0	-	R-1
	CSP 3	4.84	1.14	1.86	R/S	N-1	7.11	1.08	~0	-	R-1
 17	CSP 1	3.47	1.27	1.02	R/S	N-1	11.28	0.00	0.00	-	R-1
	CSP 2	2.50	0.00	0.00	R/S	N-1	10.55	0.00	0.00	-	R-1
	CSP 3	6.10	1.09	~0	R/S	N-1	11.65	0.00	0.00	-	R-1
 18	CSP 1	4.33	1.19	1.62	-	N-1	9.59	1.02	~0	-	R-3
	CSP 2	4.29	1.03	~0	-	N-1	4.87	0.00	0.00	-	R-3
	CSP 3	4.14	1.02	~0	-	N-1	6.55	0.00	0.00	-	R-3
 19	CSP 1	3.24	2.26	6.17	-	N-1	3.70	1.92	3.76	-	R-2
	CSP 2	3.19	1.29	3.85	-	N-1	1.92	1.27	2.66	-	R-2
	CSP 3	2.96	1.30	4.49	-	N-1	1.75	1.31	2.26	-	R-2
 20	CSP 1	10.79	0.00	0.00	-	N-3	3.75	1.30	1.12	-	R-3
	CSP 2	4.47	1.87	8.90	-	N-3	2.10	1.25	2.16	-	R-3
	CSP 3	5.25	1.84	9.34	-	N-3	2.16	1.25	2.09	-	R-3
 21	CSP 1	12.29	0.00	0.00	-	N-3	5.10	0.00	0.00	-	R-3
	CSP 2	4.48	1.25	3.42	R/S	N-3	1.92	1.06	0.95	R/S	R-3
	CSP 3	5.69	0.00	0.00	R/S	N-3	1.92	0.00	0.00	-	R-3
 22	CSP 1	7.07	1.36	1.77	R/S	N-3	5.65	1.29	1.34	R/S	R-3
	CSP 2	4.13	1.13	1.94	R/S	N-3	2.42	1.09	0.95	R/S	R-3
	CSP 3	4.47	1.16	2.32	R/S	N-3	2.36	1.12	1.32	R/S	R-3
 23	CSP 1	1.71	1.59	3.39	R/S	N-3	5.84	1.32	1.58	R/S	R-3
	CSP 2	1..63	0.00	0.00	-	N-3	7.78	1.04	~0	-	R-3
	CSP 3	1.60	1.05	~0	-	N-3	7.34	1.06	0.79	R/S	R-3
 24	CSP 1	1.72	1.30	1.85	R/S	N-4	12.95	0.00	0.00	R/S	R-3
	CSP 2	3.20	1.71	6.40	R/S	N-4	6.34	1.09	1.16	R/S	R-3
	CSP 3	1.40	1.15	1.87	R/S	N-4	5.83	1.08	1.04	R/S	R-3
 25	CSP 1	3.20	1.34	2.54	R/S	N-4	8.45	1.15	~0	-	R-3
	CSP 2	2.91	1.84	5.09	R/S	N-4	4.35	1.18	1.98	R/S	R-3
	CSP 3	3.36	1.52	6.48	R/S	N-4	3.95	1.15	1.67	R/S	R-3

TABLE 2 (Continued)

Enantiomer	NP						RP				
	CSP	k_1	α	Rs	EO	Mp	k_1	α	Rs	EO	Mp
 26	CSP 1	3.66	0.00	0.00	-	N-2	8.00	1.41	2.78	-	R-2
	CSP 2	1.53	1.13	1.81	-	N-2	3.51	1.14	1.39	-	R-2
	CSP 3	4.32	0.00	0.00	-	N-2	4.35	1.12	1.56	-	R-2
 27	CSP 1	5.65	1.11	~0	-	N-2	5.46	1.26	1.40	-	R-2
	CSP 2	2.96	1.04	~0	-	N-2	2.17	0.00	0.00	-	R-2
	CSP 3	4.31	1.04	~0	-	N-2	2.17	1.06	~0	-	R-2
 28	CSP 1	2.37	1.86	4.92	-	N-2	13.64	1.92	4.15	-	R-2
	CSP 2	1.86	1.29	4.17	-	N-2	4.84	1.24	3.14	-	R-2
	CSP 3	2.03	1.44	4.13	-	N-2	4.65	1.29	3.40	-	R-2
 29	CSP 1	11.74	1.27	1.31	-	N-2	3.33	1.05	~0	-	R-3
	CSP 2	5.58	1.10	1.53	-	N-2	1.75	0.00	0.00	-	R-3
	CSP 3	9.05	1.09	1.47	-	N-2	1.97	0.00	0.00	-	R-3
 30	CSP 1	1.15	0.00	0.00	-	N-2	17.77	0.00	0.00	-	R-3
	CSP 2	6.05	1.30	4.17	-	N-2	4.42	1.10	1.21	-	R-3
	CSP 3	9.33	1.27	3.97	-	N-2	4.46	1.11	1.07	-	R-3
 31	CSP 1	7.61	1.15	~0	-	N-2	10.87	1.10	~0	-	R-3
	CSP 2	5.35	1.07	1.05	-	N-2	5.69	1.04	~0	-	R-3
	CSP 3	8.13	1.07	1.14	-	N-2	7.80	1.05	~0	-	R-3
 32	CSP 1	6.42	1.44	1.63	-	N-2	11.40	0.00	0.00	-	R-3
	CSP 2	5.49	0.00	0.00	-	N-2	8.52	0.00	0.00	-	R-3
	CSP 3	4.05	1.04	~0	-	N-2	6.12	0.00	0.00	-	R-3
 33	CSP 1	7.90	1.40	1.73	-	N-2	48.41	0.00	0.00	-	R-3
	CSP 2	6.67	1.07	1.23	-	N-2	15.87	1.11	1.44	-	R-3
	CSP 3	5.05	0.00	0.00	-	N-2	19.99	1.11	1.22	-	R-3
 34	CSP 1	7.34	1.40	1.92	-	N-2	30.01	0.00	0.00	-	R-3
	CSP 2	6.14	1.04	~0	-	N-2	11.77	1.11	1.42	-	R-3
	CSP 3	4.54	1.04	~0	-	N-2	14.60	1.12	1.43	-	R-3
 35	CSP 1	4.41	1.26	1.07	-	N-2	17.96	1.14	~0	-	R-3
	CSP 2	5.00	1.05	0.89	-	N-2	7.74	0.00	0.00	-	R-3
	CSP 3	3.06	0.00	0.00	-	N-2	9.41	0.00	0.00	-	R-3
 36	CSP 1	3.26	1.18	0.91	-	N-2	31.99	0.00	0.00	-	R-3
	CSP 2	4.37	1.06	0.91	-	N-2	12.59	0.00	0.00	-	R-3
	CSP 3	3.15	0.00	0.00	-	N-2	16.40	0.00	0.00	-	R-3
 37	CSP 1	3.26	1.18	0.91	-	N-2	15.88	1.03	~0	-	R-3
	CSP 2	4.49	1.09	1.54	-	N-2	7.76	0.00	0.00	-	R-3
	CSP 3	3.45	0.00	0.00	-	N-2	9.33	0.00	0.00	-	R-3
 38	CSP 1	2.98	1.24	1.01	-	N-2	8.62	1.11	~0	-	R-3
	CSP 2	3.11	0.00	0.00	-	N-2	5.04	0.00	0.00	-	R-3
	CSP 3	3.39	0.00	0.00	-	N-2	5.85	1.03	~0	-	R-3
	CSP 1	6.61	1.26	1.00	-	N-2	23.62	1.20	1.01	-	R-3

(Continues)

TABLE 2 (Continued)

Enantiomer	NP						RP				
	CSP	k_1	α	Rs	EO	Mp	k_1	α	Rs	EO	Mp
 **39**	CSP 2	5.48	1.03	~0	-	N-2	8.45	1.02	~0	-	
	CSP 3	5.30	0.00	0.00	-	N-2	11.24	1.05	~0	-	R-3
 **40**	CSP 1	3.81	0.00	0.00	-	N-2	17.44	0.00	0.00	-	R-3
	CSP 2	5.47	1.05	0.91	-	N-2	9.41	0.00	0.00	-	R-3
	CSP 3	4.33	0.00	0.00	-	N-2	11.54	0.00	0.00	-	R-3
 **41**	CSP 1	2.63	1.22	1.06	-	N-2	14.07	0.00	0.00	-	R-3
	CSP 2	5.16	0.00	0.00	-	N-2	2.30	0.00	0.00	-	R-3
	CSP 3	2.96	0.00	0.00	-	N-2	2.58	0.00	0.00	-	R-3
 **42** (±)-Cloprostenol	CSP 1	7.04	1.16	1.25	(-)	N-2					
	CSP 2	8.36	1.19	2.61	(-)	N-2					
	CSP 3	11.83	1.13	2.34	(-)	N-2					

Note: Condition: flow rate, 1 ml/min; concentration of analyte, 1.0 mg/ml; detection, 254 nm. Mobile phase (Mp): N-1, *n*-hexane/EtOH/MeOH = 95/5/1, v/v/v; N-2, *n*-hexane/EtOH/MeOH = 90/10/1, v/v/v; N-3, *n*-hexane/EtOH/MeOH = 80/20/1, v/v/v; N-4, *n*-hexane/EtOH/MeOH = 50/50/1, v/v/v; R-1, H_2O/MeOH/FA/TEA = 20/80/0.2/0.1, v/v/v/v; R-2, H_2O/MeOH/FA/TEA = 30/70/0.2/0.1, v/v/v/v; R-3, H_2O/MeOH/FA/TEA = 50/50/0.2/0.1, v/v/v/v.

Abbreviations: CSP, chiral stationary phase; EO, elution order; FA, formic acid; TEA, triethylamine.

In order to compare the chromatographic performances, each sample was evaluated on the CSP 1, CSP 2, and CSP 3 with the same mobile phase. The results are shown in Table 2. The summary of enantioseparation results including 28 neutral enantiomers and (±)-cloprostenol is shown in Figure 4. As shown in Figure 4, CSP 1 can only separate a few more samples than CSP 3 and performed roughly equal to CSP 2 under NP. It can be seen that CSP 2 and CSP 3 retain most of the chiral selectivity from β-CD structure. However, we also found the changes of enantioselectivities by connecting cinchona moiety with β-CD. For example, **20**, which could not be separated by CSP 1, was well separated by CSP 2 and CSP 3 with resolution factors (Rs value) up to 8.90 and 9.34, respectively. Similar results were observed on the enantioseparations of **22**, **25**, and **30**. CSP 2 and CSP 3 also exhibited different chiral selectivities for some enantiomers owing to the different configurations of cinchona bases. For example, **21**, **26**, and **37** were only baseline separated by CSP 2. Although cinchona moiety has a direct impact on the enantiorecognition, the elution orders of some analytes including compound **14~17**, **21~25**, and (±)-cloprostenol (**42**) still remain unchanged.

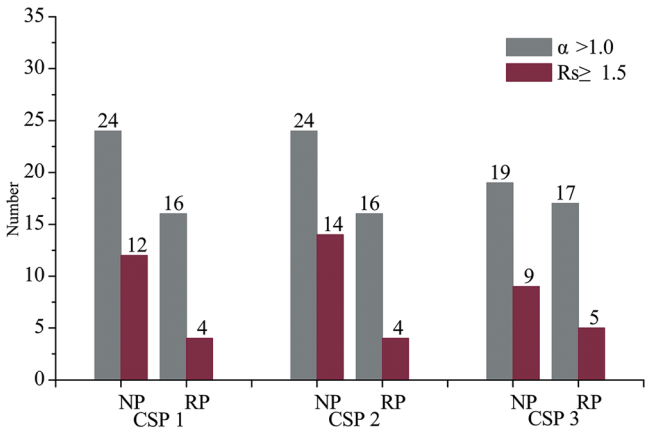


FIGURE 4 The number of chiral analytes which were separated on CSP 1, CSP 2, and CSP 3 under normal phase and reverse phase. CSP, chiral stationary phase

The number of samples separated under reversed-phase condition is less than that under NP condition. Furthermore, the number of enantiomers that can be resolved by the three columns is almost the same.

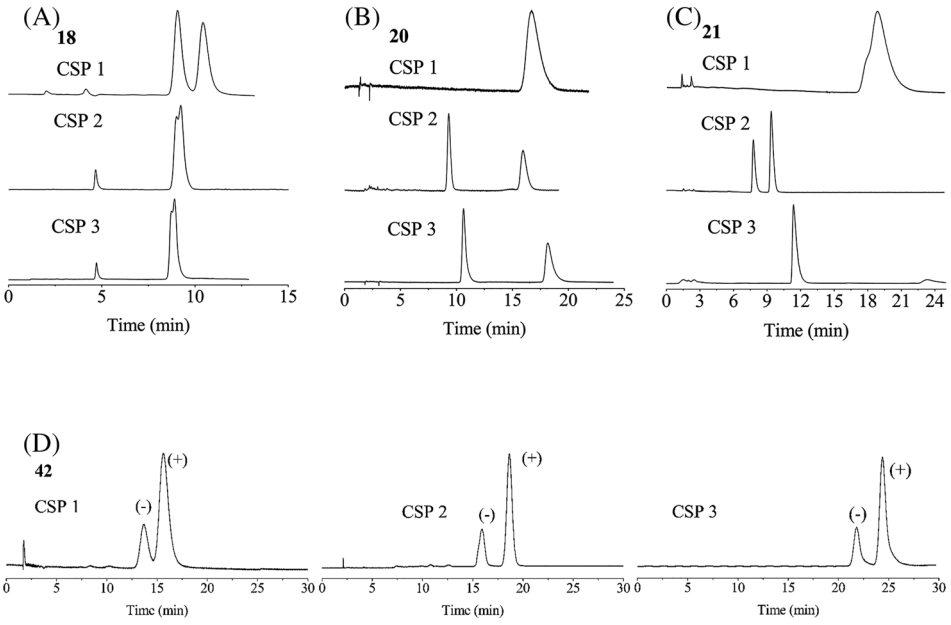


FIGURE 5 Enantiomeric separation of **18** (A), **20** (B), **21** (C), and **42** (D) on CSP 1, CSP 2, and CSP 3. Flow rate, 1 ml/min; mobile phase, *n*-hexane/EtOH/MeOH = 95/5/1, v/v/v for **18**; *n*-hexane/EtOH/MeOH = 80/20/1, v/v/v for **20**, **21**; *n*-hexane/EtOH/MeOH (90/10/1, v/v/v) containing 0.2% FA and 0.1% TEA for **42**; detection, 254 nm. CSP, chiral stationary phase; FA, formic acid; TEA, triethylamine

However, some of the enantiomers were only or much better resolved by CSP 1 under NP (**16**, **18**, **19**, **23**, **28**, **32**, **33**, and **34**), whereas some of them were only resolved by CSP 2 or (and) CSP 3 (**20**, **21**, **26**, **30**, and **37**). Figure 5 shows the enantioseparation chromatograms of some typical samples (**18**, **20**, and **21**). The difference of enantioselectivity between CSP 2 and CSP 3 under reversed-phase conditions was not as significant as that under NP conditions, which indicates that the retention mechanism in the reversed-phase mode was dominated by hydrophobic interaction provided by the structure of β-CD derivative.

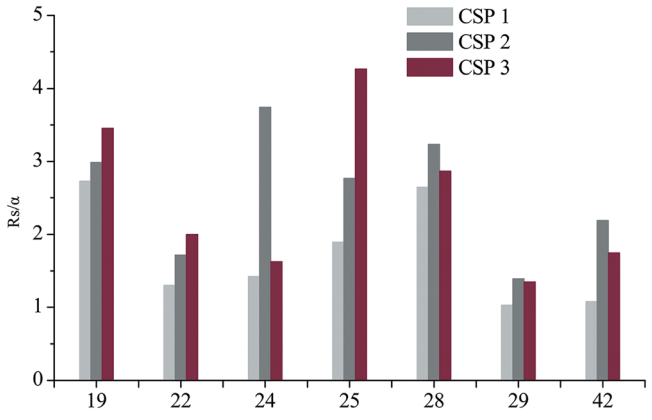


FIGURE 6 The value of Rs/α of **19**, **22**, **24**, **25**, **28**, **29**, and **42** on CSP 1, CSP 2, and CSP 3 under normal phase. Flow rate, 1 ml/min; mobile phase, *n*-hexane/EtOH/MeOH = 95/5/1, v/v/v for **19**; *n*-hexane/EtOH/MeOH = 90/10/1, v/v/v for **28**, **29**; *n*-hexane/EtOH/MeOH = 80/20/1, v/v/v for **22**; *n*-hexane/EtOH/MeOH = 50/50/1, v/v/v for **24**, **25**; *n*-hexane/EtOH/MeOH (90/10/1, v/v/v) containing 0.2% FA and 0.1% TEA for **42**; detection, 254 nm. CSP, chiral stationary phase; FA, formic acid; TEA, triethylamine

(±)-Cloprostenol (**42**) is an analogue of prostaglandin F2α that has been successfully employed in veterinary medicine. in vitro investigations confirmed stereoselective binding of (+)-cloprostenol to be 150 times more potent than (±)-cloprostenol on corpus luteum cells and 10 times more potent on myometrium cells.[44] For this purpose, the separation of (±)-cloprostenol was considered in the work. **42** was partially separated on CSP 1 and was baseline separated on CSP 2 and CSP 3 under NP (Figure 5). It is interesting to find that the column efficiencies of CSP 2 and CSP 3 are higher than those of CSP 1 in the separation of **42** as the same as for most of the neutral enantiomers in the sample set with normal mobile phase, especially for the enantiomers with high polarity. Figure 6 shows the values of Rs/α of the enantiomers that can be separated by three columns (**19**, **22**, **24**, **25**, **28**, **29**, and **42**). As mentioned in section 2.2, three columns have similar column efficiencies around 4.0×10^4 plates/m using benzene as probe. Therefore, the high column efficiency for some samples should be related to the structure of the chiral selector. The possible reason is that the steric hindrance of the cinchona moiety makes it difficult for the solute molecules to interact with the highly polar groups, such as the residual Si–OH groups on the surface of the stationary phase. Apparently, the high column efficiency always leads to high Rs value, which is beneficial to the enantioseparation of chiral drugs, because most of drug molecules have considerable polarity.

4 | CONCLUSION

Novel CSPs (CSP 2 and CSP 3) based on 3,5-dimethyl phenylcarbamoylated β-cyclodextrin combining with cinchona alkaloid moiety were prepared. 13 Fmoc-protected amino acids, chiral drug cloprostenol, and 28 neutral analytes were evaluated on CSP 2 and CSP 3 by HPLC as compared with QN-AX and CSP 1, which only comprises the 3,5-dimethyl phenylcarbamoylated cyclodextrin. Cinchona alkaloid unit and the imidazolium unit play dominant roles in the chiral recognition of Fmoc-amino acids, accompanied by the interactions of hydrogen bond, π–π interaction, and steric hindrance of cyclodextrin unit. CSP 2 and CSP 3 retain extraordinary enantioseparation of cyclodextrin-based CSP for some neutral analytes on NP, whereas hydrophobic interaction of β-CD is the more important chiral recognition mechanism in the reversed-phase mode. Moreover, better resolution factors were achieved on CSP 2 and CSP 3 for the reason that solute molecules are less accessible to highly polar residual groups on the silica surface because of the steric hindrance of cinchona alkaloid units. Overall, the novel CSPs conserved the enantioselectivities of both cyclodextrin and cinchona alkaloid moieties. It is a feasible way to combine two different chiral moieties with specific enantioselectivities to extend the application spectrum. It is important to note that the absolute configurations of chiral structure should be considered because of the synergistic effect of the two chiral units.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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