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Journal of Chromatography B, 804 (2004) 13–18
JOURNAL OF CHROMATOGRAPHY B
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Short columns with molecularly imprinted monolithic stationary phases for rapid separation of diastereomers and enantiomers

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Abstract

Three molecularly imprinted monolithic columns with different length but almost identical column volume had been prepared. It was observed that the separation factors of diastereomers and enantiomers were almost unaffected by column length. However, the short column with dimension of 38 mm × 8 mm i.d. showed much lower resistance to flow rate so that it could be operated at much higher flow rates. By combining stepwise gradient elution with elevated flow rate, the diastereomers of cinchonine and cinchonidine and the enantiomers of Cbz-DL-Trp and Fmoc-DL-Trp were successfully separated within 3 min on the short column with dimension of 38 mm × 8 mm i.d.. Based on the above results, a cinchonine imprinted monolithic disk with dimension of 10 mm × 16 mm i.d. was further developed. The SEM image and the pore size distribution profile showed that large flow-through pores are present on the prepared monolith, which allowed mobile phase to flow through the disk with very low resistance. Chromatographic performances on the monolithic disk were almost unchanged compared with the long columns. A rapid separation of cinchonine and cinchonidine was achieved in 2.5 min at the flow rate of 9.0 ml/min. Furthermore, it was observed that there was almost no effect of the flow rate on the dynamic binding capacity at high flow rates. In addition, the effect of the loading concentration of analytes on the dynamic binding capacity, namely adsorption isotherm, was also investigated. A non-linear adsorption isotherm of cinchonine was observed on the molecularly imprinted monolith with cinchonine as template, which might be a main reason to result in the peak tailing of template molecule.

Keywords: Enantiomer separation; Stationary phases, LC; Molecularly imprinted monoliths; Cinchonine; Cinchonidine

1. Introduction

Molecularly imprinted polymers have becoming attractive as effective materials for functional separations due to their high selectivity for the objective compounds, namely, the imprinted molecules. Cases in point are chiral stationary phases in chromatography, affinity materials in solid phase extraction and artificial antibodies in immunoassays [1–3]. However, they also have major drawbacks as packing material for chromatography, such as the extremely tailed peaks and low efficiency. These poor properties originate from heterogeneous energy distribution of the adsorption sites on the surface of the stationary phases and slow mass transfer kinetics inside their particles [4,5]. In addition, they also result in the relatively long time spent in the separation, in particular for the enantioseparation.

Actually, the speed of separation can be increased by use of a higher flow-rate. However, a dominant limitation of increased speed is the peak broadening and the backpressure increasing due to resistance to mass transfer when conventional stationary phases with the shape of particle are used in liquid chromatography (LC) [6].

In recent years, monolithic supports as stationary phases in high performance liquid chromatography (HPLC) and capillary electrochromatography (CEC) have gained significant interest due to their ease of preparation, high reproducibility, versatile surface chemistries and fast mass transport. Large through-pores present in this type of stationary phase allow mobile phases to flow through the sorbent with low flow resistance at high flow rates and convection becomes a dominant mass transport mechanism, which is much more rapid than diffusion in conventional stationary phases [7–9]. A variety of applications of rapid separation on this type of stationary phase have been reported [10–12]. In 1993, Matsui et al. [13] employed the in situ polymerization technique to prepare molecularly imprinted monolithic polymer rods. This type of MIPs exhibited recognition ability

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doi:10.1016/j.jchromb.2004.01.015

for some imprint molecules such as theophylline, nicotine, diaminonaphthalene, cinchona alkaloid and enantiomers of phenylalanine anilide [13–17]. Subsequently, Schweitz et al. [18–21,23] and Lin et al. [22] used the same approach for the preparation of molecularly imprinted stationary phases for the separation of racemic mixtures in CEC. Using this technique, MIPs can be synthesized directly inside stainless steel columns or capillary columns without the tedious procedures of grinding, sieving and column packing. Furthermore, the preparation of this type of MIPs is more cost-efficient, as the required amount of template molecules is much lower. However, the prepared MIPs often suffer from high back-pressures and low efficiencies [16,24], which result in their poor application in practical separation. Several methods were used such as increasing the amount of cyclohexanol and adding the latex beads in polymerization mixture to increase the permeability of rod, but the results were unsatisfied [16]. Recently, we prepared the molecularly imprinted monolithic stationary phases with both good mass transfer properties and high stereoselectivity for liquid chromatographic separations of enantiomers of amino acid derivatives and diastereomers of cinchona alkaloids. In addition, an accelerating chiral separation process has also been achieved with 6 min at elevated flow rates on the monolithic stationary phases.

In this paper, we reported the short and disk columns with molecularly imprinted monolithic stationary phases for rapid separations of enantiomers and diastereomers at high flow rates. Additionally, the chromatographic behaviors on a molecularly imprinted monolithic disk were investigated.

2. Experimental

2.1. Materials

N-(Carbobenzyloxy)-L-tryptophan (Cbz-L-Trp) and *N*-(carbobenzyloxy)-DL-tryptophan (Cbz-DL-Trp) were obtained from Sigma (St. Louis, MO, USA). Fmoc-L-tryptophan (Fmoc-L-Trp) and Fmoc-D-tryptophan (Fmoc-D-Trp) were obtained from Fluka (Buchs, Switzerland). Cinchonine (CN), cinchonidine (CD) were purchased from Acros (Geel, Belgium). 4-Vinylpyridine (4-VP) and methacrylic acid (MAA) from Acros were distilled under vacuum. Ethylene glycol dimethacrylate (EDMA) from Sigma was extracted with 10% aqueous sodium hydroxide and water, and dried over anhydrous magnesium sulfate. 2,2′-azo-bis(isobutyronitrile) (AIBN) and toluene were dried prior to use. All other chemicals and solvents were of analytical or HPLC grade.

2.2. Preparation of chiral monolithic stationary phases

The stationary phase was directly prepared by in situ polymerization within the confines of four stainless steel chromatographic column tubes of 150 mm × 4 mm i.d., 100 mm × 5 mm i.d., 38 mm × 8 mm i.d. and 10 mm × 16 mm i.d.. The template molecule, free-radical initiator (AIBN), monomer and cross-linker (EDMA) were dissolved in porogenic solvents (toluene and dodecanol). The solution was sonicated for 5 min and deoxygenated with a stream of nitrogen gas for 5 min. The stainless steel tube sealed at the bottom was filled with the above polymerization mixture and then sealed at the top end of a column. The polymerization was allowed to proceed at 45 °C for 12 h. The seals were removed, the column was provided with fittings, and connected to an HPLC pump and washed exhaustively with methanol–acetic acid (4:1 v/v) to remove the porogenic solvents and the template molecules.

In this context, cinchonine, Cbz-L-Trp and Fmoc-L-Trp were used as the template molecules and the compositions of the polymerization mixtures were the same as those indicated in the previous paper [25].

2.3. Characterization of pore properties

The pore properties were determined by mercury intrusion porosimetry and its specific surface area was calculated from nitrogen adsorption/desorption isotherms using a combined BET sorptometer and mercury porosimeter (9310 Mercury Porosimeter, USA). Microscopic analysis of the monolith was performed in JSM-5600LV Scanning Electron Microscope (JEOL, Japan) at 20 keV.

2.4. High performance liquid chromatography

A Shimadzu LC-10A HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-10ATvp HPLC pumps with a limit of flow rate of 10 ml/min and a SPD-10Avp UV-Vis detector was used for all the chromatographic experiments. The data was acquired and processed with WDL-95 chromatographic workstation (National Chromatographic R&A Center, Dalian, China). An AT-130 temperature controller (Autoscience, Tianjin, China) was used to control the column temperature. The column was washed with mobile phase until a stable baseline was obtained before injection. Acetone was injected as a void marker under corresponding mobile phase. All separations were carried out at ambient temperature, except for the studies of the temperature effect on the separation. UV detection was performed at 280 nm.

The retention times were determined by injection of 10 μg of racemates or diastereomer mixtures (dissolved in 4 μl of the eluent). The triple injections were carried out and the average acted as the final data. Capacity factors, k', were calculated by using the equation $k' = (t_R - t_0)/t_0$, where t_R is the retention time of an analyte and t_0 is the elution time of the void marker. Separation factor (α) was defined as the ratio of the capacity factors of enantiomers or diastereomers.

2.5. Frontal analysis

Frontal breakthrough curves were measured as reported in the previous papers [26,27]. The dynamic binding capacity, q, was calculated as the equation, $q = (t_{50\%} - t_0)FC/V_c$, where $t_{50\%}$ is the time of 50% breakthrough, F the volumetric flow-rate, C the adsorbate concentration in the feed, V_c the volume of the column and t_0 is the elution time of the void marker.

3. Results and discussion

3.1. Effect of column length on separation and backpressure

According to theory for conventional chromatography, the column length largely affects the separation of analytes, in particular for small molecules. Thus, the separation of small molecules is usually carried out on a relatively long column. However, Podgornik and co-workers [28,29] recently reported the separation of small molecules such as organic acids and hydroxybenzoates on a thin monolithic column (3 mm thickness) due to the absence of pore diffusion in the monolithic column. Based on their results, we investigated the effect of column length on separation of enantiomers and diastereomers on the self-made molecularly imprinted monolithic columns with different lengths but almost identical column volume. As shown in Table 1, the separation factors on the three columns were almost unaffected by column length. It was indicated that the separation could be operated on a shorter column. Furthermore, an advantage by using a short column was that it exhibited a lower resistance to flow rate than the long column. As can be seen from Fig. 1, backpressure on a short column with length of 38 mm was only 3.63 MPa at the flow rate of 7.0 ml/min, whereas it had reached to 6.66 MPa on a long column with length of 150 mm at the flow rate of 2.5 ml/min. It implied a higher flow rate could be adopted on the short molecularly imprinted monolithic columns.

Table 1
Effect of column length on separation factor

	Separation factor		
	150 mm × 4 mm i.d. (1.88 ml)	100 mm × 5 mm i.d. (1.96 ml)	38 mm × 8 mm i.d. (1.91 ml)
Cinchonine/cinchonidine[a]	5.88	5.27	5.22
Cbz-DL-Trp[b]	1.85	2.08	1.87
Fmoc-DL-Trp[c]	2.21	2.28	2.21

Conditions: Isocratic elution was performed at the flow rate of 1.0 ml/min; detection wavelength: 280 nm.

[a] Mobile phase: acetonitrile–acetic acid (97:3, v/v); stationary phase: cinchonine imprinted monolith.

[b] Mobile phase: acetonitrile–acetic acid (99.7:0.3, v/v); stationary phase: Cbz-L-Trp imprinted monolith.

[c] Mobile phase: acetonitrile–acetic acid (99.8:0.2, v/v); stationary phase: Fmoc-L-Trp imprinted monolith.

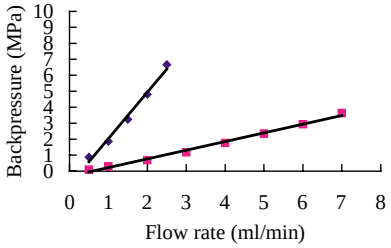

Fig. 1. Relationship of backpressure vs. flow rate on the cinchonine imprinted monolithic column with different column size. Conditions—mobile phase, acetonitrile–acetic acid (97:3, v/v); columns dimension: (■) 38 mm × 8 mm i.d. and (◆) 150 mm × 4 mm i.d.

In our previous paper [25], the combination of stepwise gradient elution with elevated flow rate had been used to accelerate the separation of enantiomers and diastereomers on the molecularly imprinted monolithic columns and almost all separations can be finished within 6 min. In this work, based on the above results obtained, much higher flow rates had adopted on a short monolithic column with dimension of 38 mm × 8 mm i.d. in order to achieve more rapid chiral separation process. As shown in Fig. 2a, the diastereomers of cinchonine and cinchonidine could be separated within 3 min at the flow rate of 7.0 ml/min on a cinchonine imprinted monolithic column. In comparison with its isocratic separation at the flow rate of 0.5 ml/min (Fig. 2b), the speed was increased by about 30-fold. In this manner, two recemates of amino acid derivates can also be resolved on the molecularly imprinted monolithic columns within 3 min as shown in Fig. 2c and 2d, respectively. Although their resolutions were lower than those obtained at low flow rates due to the slow adsorption/desorption process in molecularly imprinted polymers [4], it is impossible that such a rapid chiral separation process could be performed on the conventional column packed with molecularly imprinted polymers at high flow rates.

3.2. Molecularly imprinted monolithic disk

Based on the above observation that the column length has very less effect on separation factors of enantiomers and diastereomers on the molecularly imprinted monolithic columns, a molecularly imprinted monolithic disk with dimension of 10 mm × 16 mm i.d. was further developed and its chromatographic performances were investigated.

Fig. 3 showed the SEM image (a) and the pore size distribution profile (b) for the prepared cinchonine imprinted monolith. It can be clearly seen that the large flow-through pores with average diameter of 952 nm are present in this type of stationary phase. These pores allowed mobile phase to flow through the monolith with very low flow resistance. Moreover, a large specific surface area of 120 m^2/g was achieved due to the optimal preparation [25].

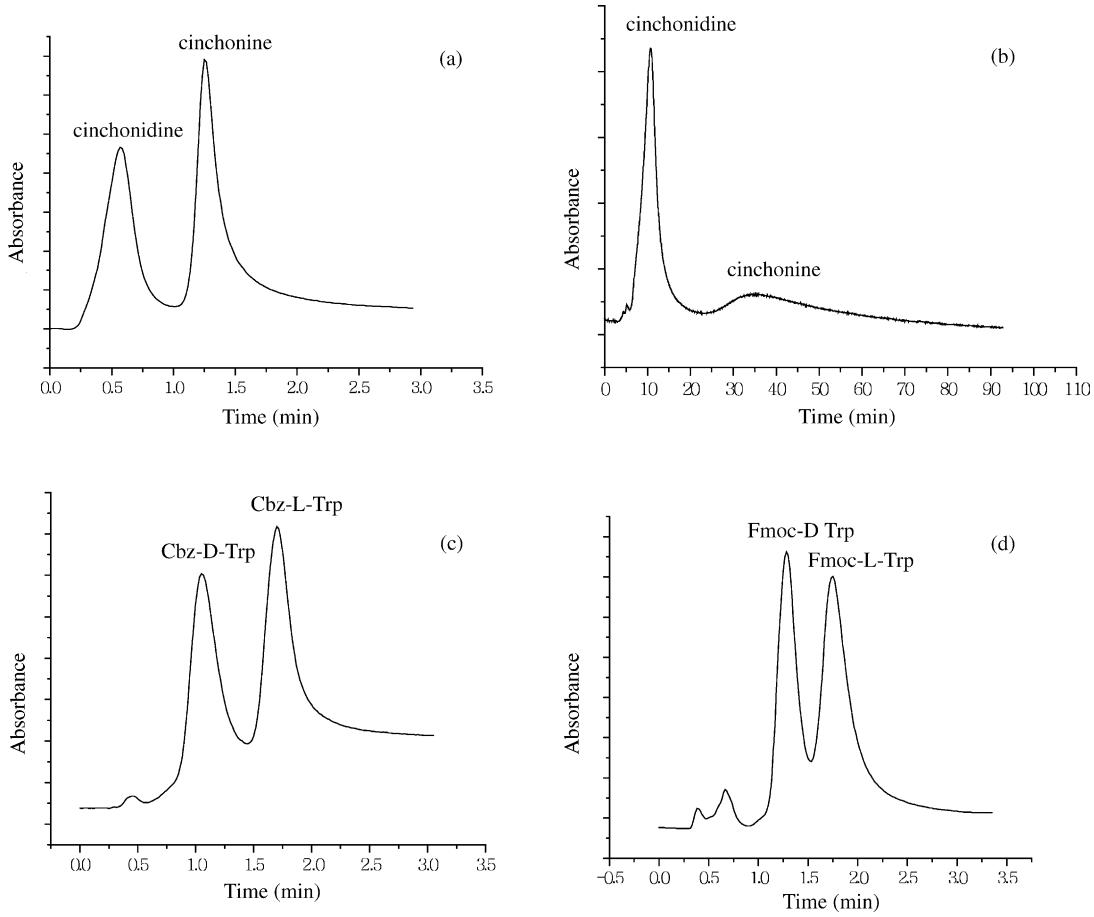

Fig. 2. Chromatographic resolution of diastereomers of cinchonine and cinchonidine and enantiomers of amino acid derivatives. (a) Stepwise gradient elution was performed on the short cinchonine imprinted monolithic column (38 mm × 8 mm i.d.) at the flow rate of 7.0 ml/min: 0–0.6 min, acetonitrile–acetic acid (97:3, v/v); 0.6–5 min, acetonitrile–acetic acid (90:10, v/v). (b) Isocratic elution was performed on the long cinchonine imprinted monolithic column (150 mm × 4 mm i.d.) at the flow rate of 0.5 ml/min with acetonitrile–acetic acid (97:3, v/v) mobile phase. (c) Stepwise gradient elution was performed on the Cbz-L-Trp imprinted monolithic column (38 mm × 8 mm i.d.) at the flow rate of 5.0 ml/min: 0–0.7 min, acetonitrile–acetic acid (99.7:0.3, v/v); 0.7–5 min, acetonitrile–acetic acid (99:1, v/v). (d) Stepwise gradient elution was performed on the Fmoc-L-Trp imprinted monolithic column (38 mm × 8 mm i.d.) at the flow rate of 5.0 ml/min: 0–0.6 min, acetonitrile–acetic acid (99.8:0.2, v/v); 0.6–5 min, acetonitrile–acetic acid (99:1, v/v).

As shown in Fig. 4a, the separation of diastereomers on the prepared disk was similar with that obtained on the long columns and the separation factor of diastereomers on the disk was about 5.75. It turned out that a multiple adsorption/desorption process also occurs in the disk [12,30]. Subsequently, effect of temperature on separation factor on the disk was investigated in the range of 30–70 °C. With increasing temperature, the separation factor increased, which was in agreement with the previous report obtained on the long column [25]. Likewise, the separation factor was also decreased with the increase of acetic acid concentration in mobile phase as observed on the long column. From these results, it was indicated that the performance of the monolithic disk was almost no difference with the long column. However, the disk showed a much lower resistance to flow rate and almost no pressure drop was shown on the disk even at the highest flow rate available for the analytical HPLC pump. Therefore, a rapid separation can be performed at the flow rate of 9.0 ml/min and the diastereomers of cinchonine and cinchonidine were fully separated within 2.5 min (Fig. 4b). This character allowed the separation of analytes on the molecularly imprinted monolithic disk in a low or moderate pressure system so that the requirement for equipment was much reduced.

3.3. Dynamic binding capacity and adsorption isotherm

For preparative or semipreparative scale separations of enantiomer the enantioselectivity and column binding capacity are the critical factors determining the throughput of pure enantiomers. Breakthrough curve on the monolithic disk was measured by frontal analysis method to determine the dynamic binding capacity. It was observed that the dynamic binding capacities of cinchonine and cinchonidine on the disk were 50.2 and 42.0 mg/g, respectively, by keeping the flow rate of 1.0 ml/min and the loading concentration at 0.2 mg/ml.

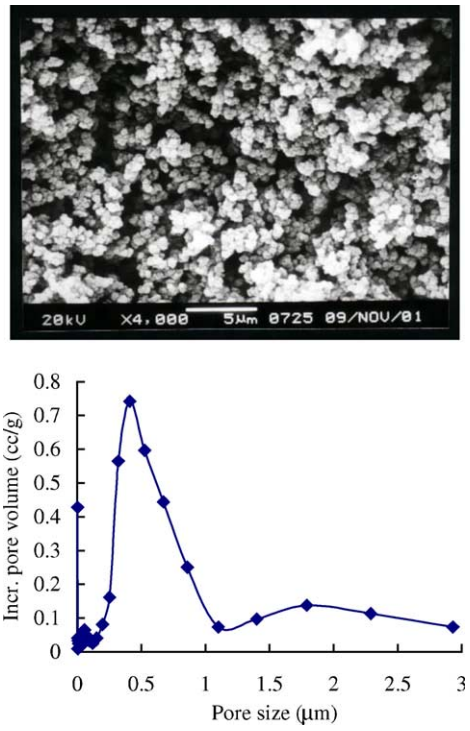

Fig. 3. SEM image and pore size distribution profile of the molecularly imprinted monolith inside the disk. (a) SEM image of magnified 4000-fold and (b) pore size distribution profile.

With respect to conventional stationary phases, flow rate has very large influence on the dynamic binding capacity because diffusion limits the adsorption of analytes on the binding sites with the increase of flow rate, as a result the dynamic capacity is compromised at elevated flow rate [31]. However, in the case of monolithic supports, convection becomes a dominant transport mechanism [9], as a consequence the dynamic binding capacity is largely independent of the flow rate, particularly at high flow rates [32]. The effect of flow rate on the dynamic binding capacity of cinchonine was investigated, and the obtained results were shown in Fig. 5. As can be seen, although the dynamic binding capacity was relatively lower at the high flow rates than that at the low flow rates, the dynamic binding capacity was also almost unaffected at the high flow rates. Therefore, a potential application of the molecularly imprinted monolithic disk in the high throughput separation of enantiomers could be expected.

The concentration of solute also affects the dynamic binding capacity through adsorption isotherm. In this study, the dynamic binding capacity of cinchonine and cinchonidine on the monolithic disk was measured by changing their concentration from 0.1 to 0.25 mg/ml, and the obtained results were shown in Fig. 6. It can be seen that the dynamic binding capacity of cinchonine almost linearly increased when its concentration was less than 0.2 mg/ml, but decreased when its concentration was higher than 0.2 mg/ml. This result suggests that a non-linear adsorption isotherm occurred

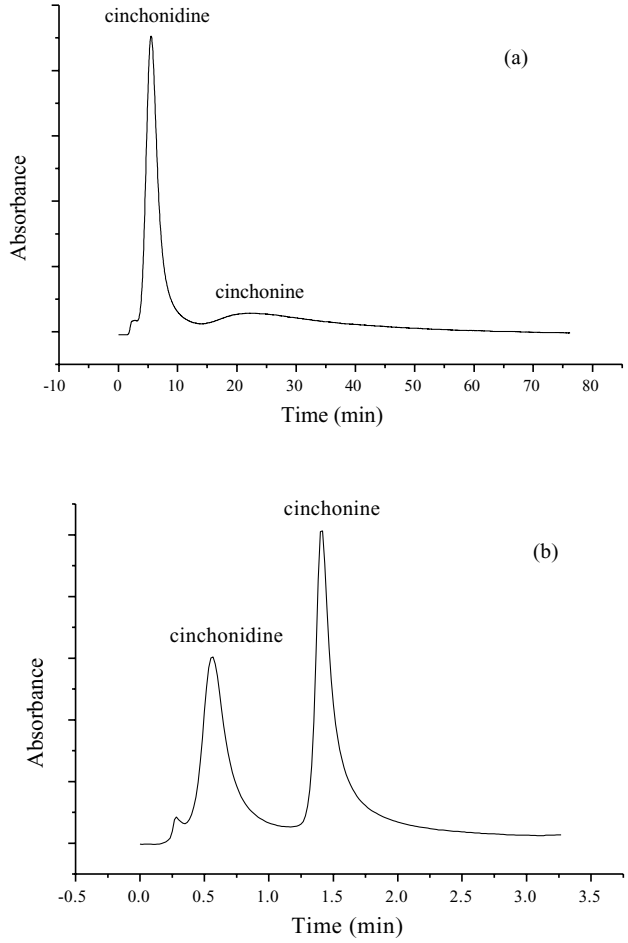

Fig. 4. Chromatographic resolution of diastereomers of cinchonine and cinchonidine on the cinchonine imprinted monolithic disk. (a) Isocratic elution was performed at the flow rate of 1.0 ml/min with acetonitrile–acetic acid (97:3, v/v) mobile phase. (b) Stepwise gradient elution was performed at the flow rate of 9.0 ml/min: 0–0.8 min, acetonitrile–acetic acid (97:3, v/v); 0.8–5 min, acetonitrile–acetic acid (90:10, v/v).

for adsorption of cinchonine on the cinchonine imprinted monolith, which may be caused from the heterogeneous distribution of the adsorption sites in imprinted monolith. Also a non-linear adsorption isotherm might be a main reason to result in peak tailing of template molecules on molecularly imprinted monolithic columns [33]. By contrast, the

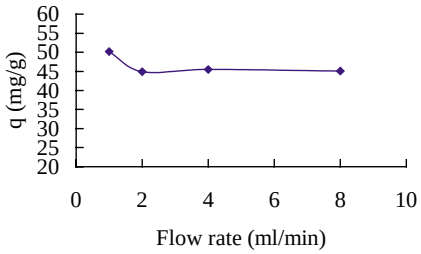

Fig. 5. Relationship of dynamic binding capacity of cinchonine vs. flow rate on the cinchonine imprinted monolithic disk. Conditions—loading phase, acetonitrile with cinchonine concentration of 0.2 mg/ml; UV detection, 280 nm; temperature, 25 ± 2 °C.

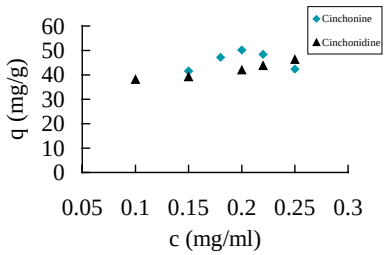

Fig. 6. Relationship of the dynamic binding capacity vs. the loading concentration of cinchonine or cinchonidine on the cinchonine imprinted monolithic disk. Conditions—loading phases: acetonitrile with different concentration of cinchonine or cinchonidine; flow rate, 1.0 ml/min; UV detection, 280 nm; temperature, 25 ± 2 °C.

adsorption isotherm of the cinchonidine was relatively straight over the whole range of concentration. Therefore, for keeping the relatively high dynamic binding capacity, a high loading concentration of cinchonine was not recommended in practice.

4. Conclusions

It was observed that the column length has very less effect on the separation factors of enantiomers and diastereomers on the molecularly imprinted monolithic columns. The short columns and disk with molecularly imprinted monolithic had been prepared for rapid chiral separation due to their low flow resistance to flow rate. By the combination of stepwise gradient elution and elevated flow rate, the diastereomers of cinchonine and cinchonidine and enantiomers of Cbz-DL-Trp and Fmoc-DL-Trp were successfully separated within 3 min. It was found that the chromatographic performances for separation of the diastereomers of cinchonine and cinchonidine on a cinchonine imprinted monolithic disk were almost unchanged comparing with the long column. However, a much lower resistance to flow rate on the disk even leads a possibility to perform the separation with the low or moderate pressure system. Furthermore, it was observed that the dynamic binding capacity on the disk was also almost unaffected at high flow rates, which provides us a potential application of the molecularly imprinted monolithic disk in the high throughput separation of enantiomers in future.

Acknowledgements

Financial supports from the National Natural Sciences Foundation of China (No. 20075032), the China State Key Basic Research Program Grant (001CB510202), the China State High-Tech Program Grant (2001AA233031-4) and the Knowledge Innovation program of DICP to Dr. Hanfa Zou are gratefully acknowledged.

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