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Preparation of molecularly imprinted polymer for sinomenine and study on its molecular recognition mechanism

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Abstract

A sinomenine (SIN) molecule-imprinted monolithic stationary phase (MIMSP) with specific recognition for SIN was prepared by in situ technique, utilizing methacrylic acid (MAA) as a function monomer, ethylene glycol dimethacrylate (EDMA) as a cross-linking agent, and low-polar solvents (toluene and dodecanol) as porogenic solvents. The selectivity of the polymers for SIN was evaluated by high performance liquid chromatography (HPLC). Some chromatographic conditions, such as the column temperature, the flow rate and the composition of the mobile phase, were changed in order to characterize the chromatographic procedure. SIN could be separated from some other structural analogues, including morphine, codeine, codethyline and magnoflorine, under optimized conditions. Scatchard analysis showed that two classes of binding sites existed in the SIN-imprinted polymers, with their dissociation constants estimated to be 7.257×10^{-5} and 3.828×10^{-3} mol l^{-1}, respectively. Compared with the SIN-imprinted polymers, the non-imprinted polymers prepared using the same method but in the absence of SIN did not exhibit the specific molecular selectivity, which suggests that the specific molecule-recognition ability of the SIN-imprinted polymers was largely ascribed to the imprinting effect.

Keywords: Molecularly imprinted polymer; Sinomenine; In situ technique

1. Introduction

Molecularly imprinted technique was introduced in 1972 by Wulff and Sarhan [1] and much advanced by the work of the Mosbach group in the 1980s [2]. This technique has been shown to be capable of producing materials with 'antibody-like' selectivity. Because molecularly imprinted polymers (MIPs) have predetermined selectivity, recognition and feasibility, they have been used in many fields. They are increasingly being used as selective supports in liquid chromatography, capillary electrophoresis, and solid-phase extraction, and as catalysts, bionic sensors and artificial antibodies [3–8].

MIPs can be prepared by both covalent and non-covalent methods. Non-covalent methods include bulk polymerization [9], in situ polymerization [10–12], suspension polymerization [13], and multistep-swelling polymerization [14]. Compared with other methods, in situ polymerization possesses several advantages, e.g. simple preparation procedure. Matsui and Huang [10–12] prepared MIMSP using cinchonine and amino acid derivatives as the template molecules, by which the rapid separation of their diastereomers and enantiomers was achieved. Recently, a monolithic MIPs with specific recognition ability for strychnine has been synthesized in our lab, and the molecular recognition mechanism was discussed [15].

Sinomenine (SIN) is one of the principal alkaloids isolated from *Sinomenium acutum Rehd. et Wils*. It has analgesic and anti-inflammatory effects, and is used clinically to cure rheumatoid arthritis and neuralgia [16]. So far SIN has been extracted from herbs mainly using aether or toluene as extraction solvent [17–19], with little attention to operation safety or environmental protection. Recently, the general determination methods of SIN have been thin-layer chromatographic scanning [20], HPLC [21], etc. whose selectivity and specificity are not good enough to accommodate the variety and complexity of biological samples. For these reasons, it is necessary to develop an effective method to extract SIN from herbs and biological fluids. In this paper, we prepared a SIN molecule-imprinted stationary phase (SIN-MIMSP) by in situ method, which shows specific recognition ability for the template molecule, i.e. SIN. Furthermore, we explored the

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possible recognition mechanism of the polymer by HPLC and Scatchard analysis.

2. Experimental

2.1. Materials

Methacrylic acid (MAA) was purchased from Tianjin Chemical Reagent Plant (Tianjin, China). 2-(Trifluoromethyl)-acrylic acid (TFMAA) and ethylene glycol dimethacrylate (EDMA) were obtained from Aldrich (Milwaukee, USA). 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Shanghai No. 4 Reagent Factory (Shanghai, China). SIN was purchased from Shaanxi Scidoor HI-tech Biology Co. Ltd with a labeled purity above 99.0% (Shaanxi, China). Morphine, codeine, codethyline and magnoflorinea were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of these compounds are illustrated in Fig. 1. Acetonitrile was of HPLC grade. All the other reagents were of analytical grade. MAA was distilled under vacuum to remove the inhibitor before polymerization. EDMA was purified according to the report [22]. Water was freshly distilled three times prior to use.

2.2. Preparation of polymers

Polymers were prepared by utilizing MAA as the functional monomer and EDMA as the cross-linking agent. The preparation procedure was as follows. Template (0.2 mmol), toluene, MAA, EDMA, dodecanol and AIBN were mixed and degassed by ultrasonication for 10 min. The mixture was purged with nitrogen for 5 min and then transferred into a stainless-steel column (100 mm×4.6 mm i.d.). The column was sealed and the mixture was kept at a certain temperature for 12 h. The resulting polymers were washed by using a mixture of methanol-acetic acid (4:1, v/v) to remove the template molecule, and then the residual acetic acid was removed using methanol. Non-imprinted polymers were prepared by the same procedure without the addition of template molecule.

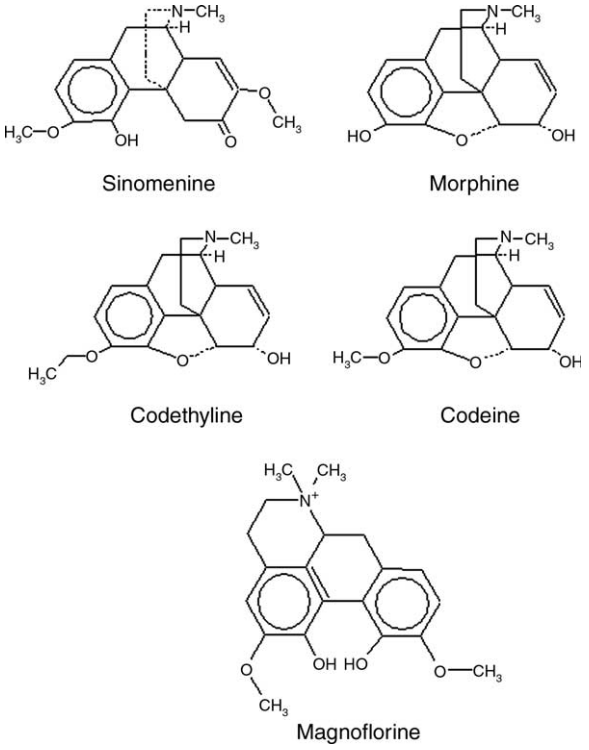

Fig. 1. Structures of sinomenine and its structural analogues.

2.3. Test of the morphologies of polymers

The morphologies of the MIP were analyzed by using a scanning electron microscope (HITACHI, S-570, Japan) at 20 keV, and the pore properties were determined by mercury intrusion porosimetry (9310 Mercury Porosimeter, USA).

2.4. Chromatography

The HPLC system was composed of a Spectra P200 pump, a Spectra 100 UV detector (Thermo Electron Co., Boston, USA) and an Anastar Chromatographic software. Detection was performed at 262 nm. The eluent used was specified in the legends of tables and figures. The retention factors were determined by the relation $k=(t_R-t_0)/t_0$, where t_R is the retention time of a given species and t_0 is the retention time of the void marker (acetone). The selectivity factors were calculated from the equation $S=k_{imprinted}/k_{non\text{-}imprinted}$, where $k_{imprinted}$ and $k_{non\text{-}imprinted}$ were the retention factors of SIN on the molecularly imprinted and non-imprinted polymers, respectively. The separation factors were calculated from the equation $\alpha=k_1/k_2$, where k_1 and k_2 were the retention factors of SIN and its analogues on the SIN-MIMSP, respectively.

2.5. Scatchard analysis

The polymers were pushed out of column. After that, 20 mg of the polymers were weighed into a 10 ml conical flask and mixed with 5.0 ml of SIN aqueous solution, the concentration of which varied from 0.1 to 4.5 mmol l^{-1}. The flasks were oscillated by an HZ-881S action shaker (Taicang City Scientific Instruments Factory, China) in a water bath for 16 h at 25 °C. Then the mixture was filtrated through a microporous membrane of 0.22 μm and the SIN concentration in the filtrate was measured by a SP-2102 UV (Shanghai Spectrum Instruments Co., Ltd, China) at 262 nm. The amount of SIN bound to the polymers was calculated by subtracting the concentration of free SIN from the initial SIN loading. The Scatchard equation $Q/[SIN]=(Q_{max}-Q)/K_d$ was used to estimate the binding parameters of the SIN-imprinted polymers, where Q was the amount of SIN bound to the polymer, Q_{max} was the apparent maximum number of binding sites, K_d was the equilibrium dissociation constant, and [SIN] represented the equilibrium concentration of SIN.

Table 1
Retention factors and selectivity factors for SIN on the polymers prepared by utilizing different functional monomers

Polymer	*k*		*S*
	Imprinted	Non-imprinted	
MAA-*co*-EDMA	2.53	0.12	21.77
TFMAA-*co*-EDMA	3.51	0.52	6.69

k, *S* refers to retention factor and selectivity factor, respectively. HPLC conditions: column size, 100 mm×4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–acetic acid (97:3, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 262 nm; loaded amount, 5 μg.

3. Results and discussions

3.1. Selection of a functional monomer

The MIPs for SIN were prepared using MAA or TFMAA as a functional monomer and EDMA as a cross-linker. The prepared MIPs were evaluated by chromatographic experiments to examine the effect of a functional monomer on the retentivity and selectivity for SIN. As shown in Table 1, the retention factors for SIN on MAA-*co*-EDMA polymers were smaller than those on TFMAA-*co*-EDMA polymers. However, the selectivity factor of the former was much higher than that of the latter because of a mass of non-specific adsorption in the SIN-imprinted TFMAA-*co*-EDMA polymers. Therefore, MAA was selected as a functional monomer.

3.2. Optimization of preparation conditions

We optimized the preparation method of the MIPs for SIN by changing some of the preparation factors, including the proportion of template to functional monomer (*T–M*), the degree of cross linking, the percentage of toluene in porogenic solvents, the initiator molar amount in the total moles of monomer and the preparation temperature. The results are shown in Table 2.

When the *T–M* was 1:2, the MIP-MIMSP exhibited no selectivity for SIN. When the *T–M* was 1:6, the MIP-MIMSP was too rigid to allow the mobile phase to flow through. Only when the *T–M* was 1:4 (MIP_2), did the SIN-MIMSP have both recognition ability and suitable column pressure.

With regard to the preparation temperature, the results showed that the polymers could not be synthesized at 45 °C, while at 60 °C the retention factors and separation factors decreased obviously. The retention factor at 50 °C (MIP_5) was slightly smaller than that at 55 °C, but the separation factor was bigger. At the same time, as the temperature increased from 50 to 60 °C, the peak shape gradually became worse. So we selected 50 °C as the optimum preparation temperature.

When the degree of cross linking was 85% (MIP_8), the MIP-MIMSP gave relatively higher retention and separation factors. The degree of cross linking affects the internal structure of the polymer, and in order to maintain good space structure, the degree of cross linking should usually be above 80% [23].

We used toluene and dodecanol as porogenic solvents and the toluene content directly affects the internal structure of the MIPs. It was observed that with a decrease in the content of toluene, the number of big particles polymerized increased, which caused low chromatographic efficiency; on the other hand, as the content of toluene increased, the number of small particles polymerized increased as well, which made the polymers rigid. Table 2 shows that when the toluene content was 20% (MIP_{12}), the retention and separation factors were the highest. At the same time, when the initiator was added in the amount of 5% moles of the total moles

Table 2
Retention factors and separation factors of SIN on the SIN-imprinted polymers prepared under different polymerization conditions

Polymer	Molar ratio[a]	Degree of cross linking (%)[b]	Toluene content in Porogen (v%)	Initiator molar amount of the total moles of monomer (%)	Preparation temperature (°C)	*k*	*α*
MIP_1	1:2	90	18	5	55	0.27	1.00
MIP_2	1:4	90	18	5	55	2.27	2.41
MIP_3	1:6	90	18	5	55	–[c]	–[c]
MIP_4	1:4	90	18	5	45	–[d]	–[d]
MIP_5	1:4	90	18	5	50	1.55	2.46
MIP_6	1:4	90	18	5	60	1.77	1.13
MIP_7	1:4	80	18	5	50	5.28	3.43
MIP_8	1:4	85	18	5	50	4.57	5.16
MIP_9	1:4	95	18	5	50	–[c]	–[c]
MIP_{10}	1:4	85	10	5	50	0.41	1.00
MIP_{11}	1:4	85	15	5	50	3.07	3.19
MIP_{12}	1:4	85	20	5	50	5.46	5.17
MIP_{13}	1:4	85	22	5	50	–[c]	–[c]
MIP_{15}	1:4	85	20	4	50	2.66	3.38
MIP_{16}	1:4	85	20	6	50	7.72	4.11

k, *α* refers to retention factor and separation factor, respectively. HPLC conditions: column size, 100 mm×4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–acetic acid (96:4, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 262 nm; loaded amount, 5 μg.

[a] The molar ratio refers to template/functional monomer.

[b] The degree of cross linking refers to the mole content of EDMA in the mixture of MAA and EDMA.

[c] The polymer was too rigid to allow the mobile phase to flow through.

[d] The temperature was too low to synthesize the polymer.

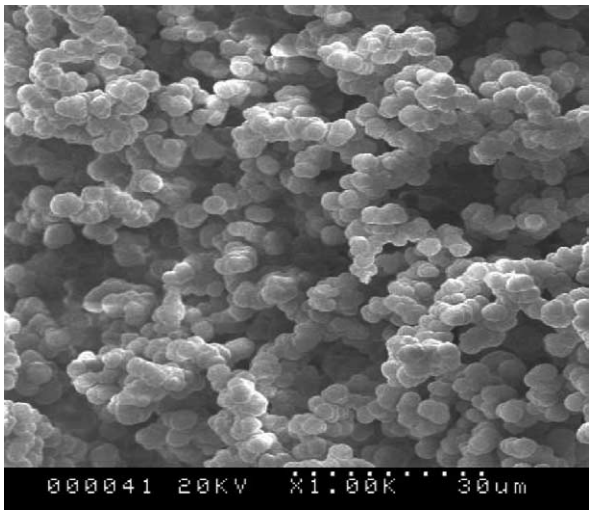

Fig. 2. The scanning electron microscopy (SEM) image of the SIN-MIMSP 1000×.

of monomer (MIP_{12}), the retention factor was relatively high and the separation factor was the highest.

Considering all of the above, the optimum preparation method was as follows: the *T–M* was 1:4; the degree of cross linking was 85%; the toluene content in porogenic solvents was 20%; the initiator molar amount of the total moles of functional monomer was 5%; the preparation temperature was 50 °C. As a result, we used MIP_{12} in the following experiments.

3.3. Morphology of polymers

Fig. 2 shows that the sizes and figures of all the particles were homogeneous. Fig. 3 shows that the range of pore size was about 2–7 μm in the SIN-MIMSP. Both results reveal that because macro-pores were present in this type of stationary phase, the mobile phases could be allowed to flow through with low resistance at high flow rate.

3.4. Retention properties of SIN on the SIN-MIMSP in organic mobile phases

The effects of flow rate, column temperature, and acetic acid content in the mobile phase on the retention properties of SIN

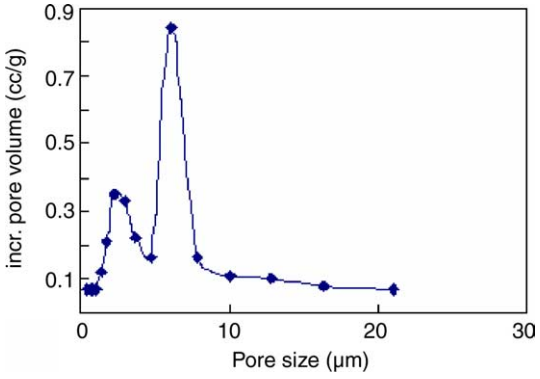

Fig. 3. Pore size distribution of SIN-MIMSP profile.

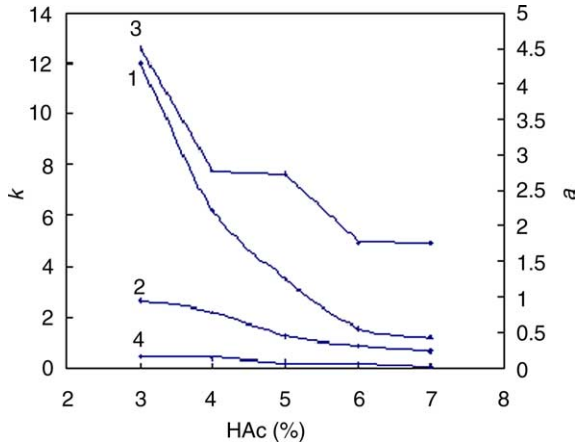

Fig. 4. Effect of the acetic acid content of mobile phase on the retention of SIN and morphine. 1. k_{SIN}; 2. $k_{morphine}$; 3. α; 4. $k_{SIN(non\text{-}imprinted)}$. HPLC conditions: column size, 100 mm×4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–acetic acid (v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 262 nm; loaded amount, 5 μg.

and morphine were investigated to clarify the retention and molecular recognition mechanism of SIN on the SIN-MIMSP in organic mobile phases, where the mobile phase used was acetonitrile-acetic acid. As shown in Fig. 4, on the non-imprinted polymers, the retention factor of SIN was less than 0.5 and was hardly affected by the acetic acid content, while the retention and separation factors of SIN and morphine on SIN-MIMSP decreased as acetic acid content increased. This result proved the existence of the hydrogen-bonding interactions between the target molecule and the MIPs [24].

Fig. 5 shows the effect of flow rate on the retention properties of SIN and morphine on SIN-MIMSP. With an increase of flow rate from 0.5 to 2.5 ml min^{-1}, the retention factors for both solutes decreased, but the degree of variation of morphine was not as large as that of SIN. So the separation factors decreased gradually as flow rate increased, with the highest separation factor obtained at the flow rate of 0.5 ml min^{-1}. That was mainly due to the slow mass transfer of SIN on the SIN-MIMSP [25]. Moreover, the column pressure remained very low during the whole experiment,

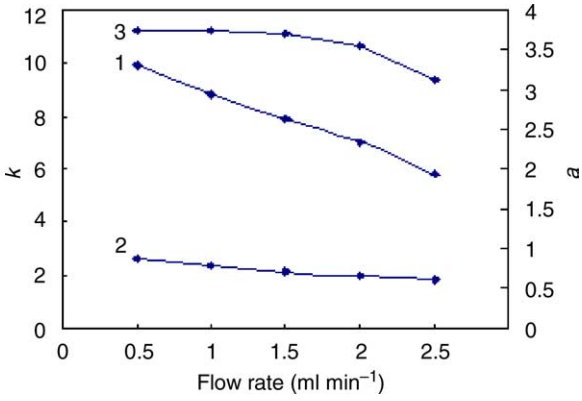

Fig. 5. Effect of flow rate on the retention of SIN and morphine. 1. k_{SIN}; 2. $k_{morphine}$; 3. α. HPLC conditions: column size, 100 mm×4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–acetic acid (96:4, v/v); detection wavelength, 262 nm; loaded amount, 5 μg.

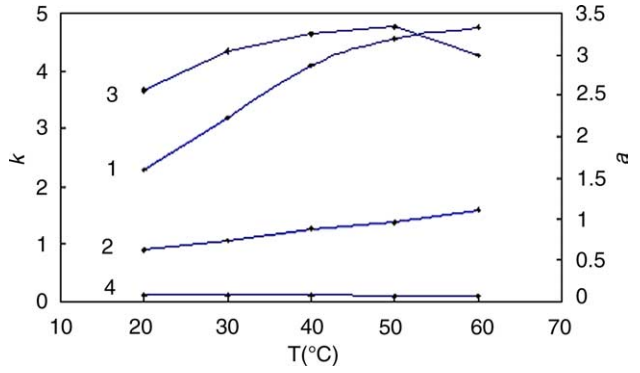

Fig. 6. Effect of temperature on the retention of SIN and morphine. 1. k_{SIN}; 2. $k_{morphine}$; 3. α; 4. $k_{SIN(non\text{-}imprinted)}$. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; mobile phase, acetonitrile–acetic acid (96:4, v/v); flow-rate, 0.5 ml min^{-1}, detection wavelength, 262 nm; loaded amount, 5 μg.

which can be explained by the existence of macro-pores in the polymers' backbone.

Fig. 6 shows the effect of column temperature on the retention properties of SIN and morphine on SIN-MIMSP, and of SIN on the non-imprinted polymers. On the non-imprinted polymers, retention of SIN was almost unchanged by increasing column temperature. But on the SIN-MIMSP, with an increase of column temperature from 20 to 60 °C, the retention and the separation factors of SIN and morphine increased, and the highest separation factor was obtained at 50 °C. These results proved that the two compounds have different values of thermodynamics on the MIP-MIMSP during the separation process [26].

3.5. Retention properties of SIN on SIN-MIMSP in aqueous mobile phases

The mobile phase pH and acetonitrile content were also tested in this experiment using phosphoric acid and potassium phosphate (PBS, 25 mM) and acetonitrile as the mobile phase. Fig. 7 shows the effect of pH on the retention properties of SIN and morphine on the SIN-MIMSP, and of SIN on the non-imprinted polymers. The results show that retention for SIN and morphine increased in the mobile phase pH range of 2–5 on both SIN-MIMSP and non-imprinted polymers. Note that retention on the non-imprinted polymers increased slightly. The results are explained by the fact that the degree of ionization of a carboxyl group becomes larger with the increase in mobile phase pH. When the mobile phase pH was 5, SIN could not be eluted. Because the p*K*a of MAA is 4.60 and that of SIN is 8.00. Thus, solutes were retained on the SIN-MIMSP by electrostatic interactions with a carboxyl group on the SIN-MIMSP, in addition to hydrophobic interactions with the polymers' backbone. At the same time, separation between SIN and morphine also increased with the increasing pH, and SIN showed much longer retention on the imprinted polymers than on the non-imprinted polymers, all of which can be explained by a molecular imprinting effect.

Fig. 8 shows the effects of acetonitrile content on the retention properties of SIN and morphine on the SIN-MIMSP, and of SIN on the non-imprinted polymers. As shown in Fig. 8, retention of SIN and morphine on the SIN-MIMSP gradually decreased as the acetonitrile content increased from 30 to 50%, while it increased in the range of 50–70% of acetonitrile content. When the acetonitrile content was 50%, the MIP-MIMSP gave the highest separation for SIN and morphine. These results can be explained by the fact that when the acetonitrile content was less than 50%, hydrophobic interactions were dominant, and when the acetonitrile content was more than 50%, electrostatic interactions became dominant. So in aqueous mobile phases, the retention of solutes in the SIN-MIMSP was mainly due to hydrophobic interactions, in addition to electrostatic interactions of the compounds with MAA. However, on the non-imprinted polymers, all of the retention factors of SIN were nearly zero, which could prove that the SIN-MIMSP recognition ability for SIN comes from the molecular imprinting process.

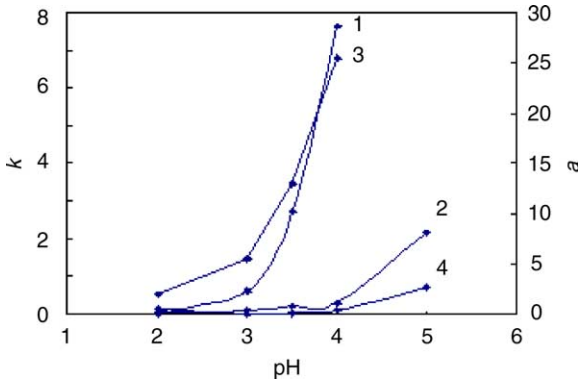

Fig. 7. Effect of the pH of mobile phase on the retention of SIN and morphine. 1. k_{SIN}; 2. $k_{morphine}$; 3. α; 4. $k_{SIN(non\text{-}imprinted)}$. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–PBS (50:50, v/v); flow-rate, 0.5 ml min^{-1}, detection wavelength, 262 nm; loaded amount, 5 μg.

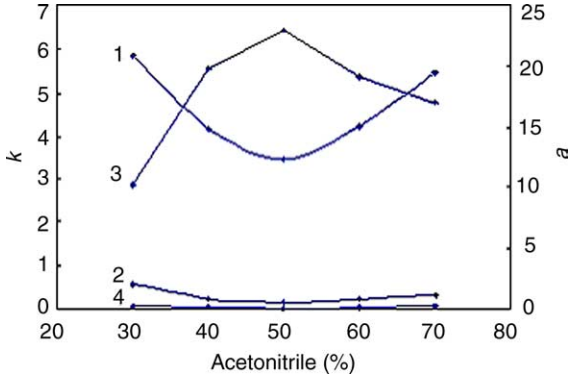

Fig. 8. Effect of the acetonitrile content of mobile phase on the retention of SIN and morphine. 1. k_{SIN}; 2. $k_{morphine}$; 3. α; 4. $k_{SIN(non\text{-}imprinted)}$. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–PBS (50:50, v/v); flow-rate, 0.5 ml min^{-1}, detection wavelength, 262 nm; loaded amount, 5 μg.

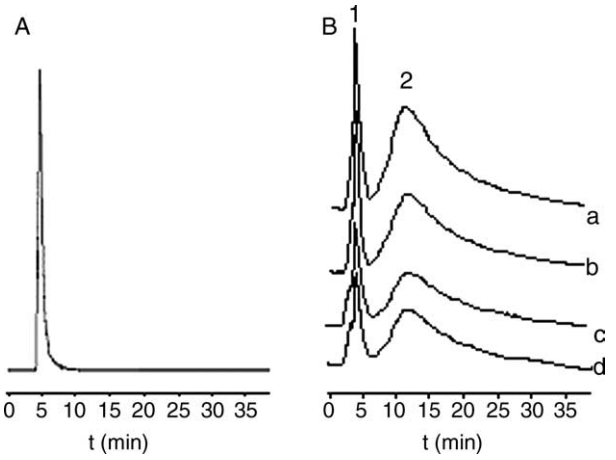

Fig. 9. Chromatograms of SIN and its analogues. A. Non-imprinted polymer; B. SIN-MIMSP. a. Codeine and SIN; b. Codethyline and SIN; c. Magnoflorine and SIN; d. Morphine and SIN. 1. The analogues; 2. SIN. HPLC conditions: column size, 100 mm×4.6 mm i.d.; column temperature, 25 °C; mobile phase, acetonitrile–PBS (50:50, v/v, pH 3.5); flow-rate, 0.5 ml min^{-1}, detection wavelength, 262 nm; loaded amount, 5 μg.

3.6. Separation of SIN and some of its analogues on the SIN-MIMSP

Fig. 9 shows the separation of SIN and some structural analogues, including morphine, codeine, codethyline and magnoflorine, on the SIN-MIMSP. On the non-imprinted polymers, no compounds could be separated, but on the SIN-MIMSP, SIN was completely separated from the other analogues. Their separation factors were all above 5.0. These results indicate that SIN-MIMSP could efficiently separate the target molecule from other similarly structural compounds.

3.7. Determination of binding parameters of the SIN-imprinted polymers

Fig. 10 shows the binding isotherms for SIN on the SIN-imprinted polymers and on the non-imprinted polymers. The binding amount increased gradually with the aqueous concentration of SIN in the initial solution, but the binding amount of SIN on the SIN-imprinted polymers was more than that on the non-imprinted polymers, which could be ascribed to the molecular-imprinting effect. The binding amount could reach a stable value because of some non-specific adsorption. This kind of binding isotherm was similar to that of biological receptors [27].

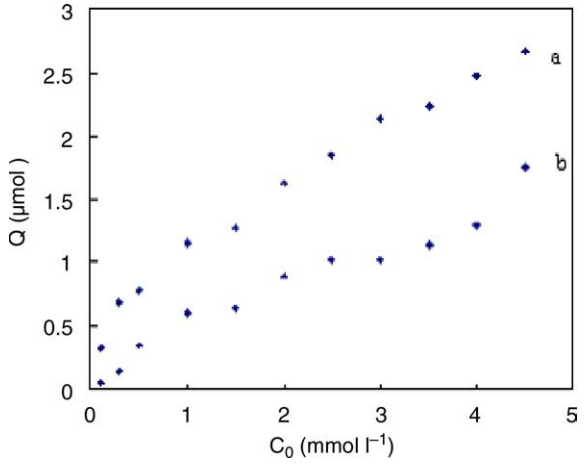

Fig. 10. Binding isotherm of polymers for SIN. a. SIN-MIMSP; b. non-imprinted polymers.

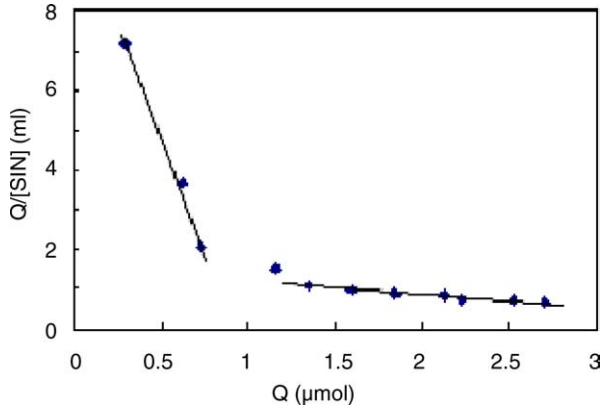

Fig. 11. Scatchard plots to estimate the binding nature of SIN-MIMSP.

The obtained data were plotted according to the Scatchard equation. As shown in Fig. 11, there were two distinct sections within the plot which could be regarded as straight lines. The results indicate that there are two classes of binding sites in the SIN-imprinted polymers. From the slope and intercept of the plot, the equilibrium dissociation constant K_{d1} and the apparent maximum number Q_{max1} of the higher affinity binding sites can be calculated to be 7.257×10^{-5} mol l^{-1} and 47.896 μmol g^{-1}, respectively. In the same way, K_{d2} and Q_{max2} of the lower affinity bonding sites were calculated to be 3.828×10^{-3} mol l^{-1} and 261.447 μmol g^{-1}, respectively.

4. Conclusions

A series of SIN-MIMSP were synthesized and their molecular recognition properties were studied. Among them, MIP_{12} showed the highest selectivity. It could recognize SIN from its analogues under suitable chromatographic conditions. The non-imprinted polymers had no selective ability, which proves that recognition ability can be ascribed to the imprinting process. The influence of chromatographic conditions on the retentivity and selectivity of the SIN-MIMSP demonstrated that hydrogen bonding between the binding sites and the substrates played an important role in molecular recognition in organic mobile phases, and that electrostatic and hydrophobic interactions both played important roles in aqueous mobile phases. According to Scatchard analysis, it was found that there were two classes of bonding sites in the SIN-imprinted polymers. All of these studies add evidence to the research about the use of alkaloid as a template in the field of molecularly imprinted technology, and support the application of SIN-MIP in the field of herbal extraction and biopharmaceutical analysis.

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