

Preparation and evaluation of monolithic molecularly imprinted stationary phase for *S*-naproxen

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Abstract: An *S*-naproxen (*S*-NAP) molecularly imprinted monolithic stationary phase (MIMSP) with specific recognition for *S*-NAP and naproxen (NAP) was prepared by *in situ* technique, utilizing 4-vinylpyridine (4-VP) 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 *S*-NAP and NAP was evaluated by high performance liquid chromatography (HPLC). The binding characteristics were tested by Scatchard analysis. Racemic NAP could be specifically separated to some extent. At the same time, NAP could be separated from ibuprofen under optimized conditions. Scatchard analysis showed that two classes of binding sites existed in the *S*-NAP-imprinted polymers, with their dissociation constants estimated to be 1.045 and 5.496 μM , respectively. The results demonstrate that *S*-NAP and NAP can be recognized specifically on the obtained MIMSP.

Keywords: molecularly imprinted polymer; naproxen; *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 multi step-swelling polymerization [14]. Compared with other methods, *in situ* polymerization possesses 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 laboratory, and the molecular recognition mechanism was discussed [15].

Naproxen (NAP), 2-(6-methoxy-naphthyl) propanoic acid, is one of the principal non-steroidal anti-inflammatory drugs. It is widely used for the treatment of pain, inflammation, and fever in clinic [16,17]. As shown in Figure 1,

its chemical structure possesses an asymmetric α carbon atom and, therefore, occurs as *S*-naproxen and *R*-naproxen. To ensure its safety and therapeutic effect, only enantiomerically pure naproxen is in increasing demand in market. For this reason, the enantioseparation of naproxen is of considerable interest. So far, Lei [18] has used bulk polymerization to separate naproxen, which has some disadvantages, such as irregular particles and low column capacity. Haginaka [19] used multi step-swelling polymerization to achieve enantioseparation of naproxen. But preparation process was long and time-consuming. In this paper, we prepared an *S*-NAP molecule-imprinted stationary phase (MIMSP) by *in situ* method, which showed a specific recognition ability for the template molecule. Furthermore, we explored the possible recognition mechanism of the polymer by HPLC and Scatchard analysis.

2 Experimental

2.1 Materials

4-Vinylpyridine (4-VP) was purchased from Aldrich (Milwaukee, USA), and ethylene glycol dimethacrylate (EDMA) was obtained from Aldrich (Milwaukee, USA). 2, 2'-Azobisisobutyronitrile (AIBN) was purchased from Shanghai No.4 Reagent Factory (Shanghai, China). NAP was purchased from Zhejiang Chetou Pharmaceutical Co., Ltd. (Zhejiang, China). *S*-NAP was obtained from Fluka (USA). Ibuprofen was obtained from Wuhan Huazhong Pharmaceutical Co., Ltd. (Wuhan, China). Acetonitrile was of HPLC grade. All the other reagents were of analytical grade. 4-VP was distilled under vacuum to remove the inhibitor before polymerization. EDMA was purified. Water was freshly distilled three times prior to use.

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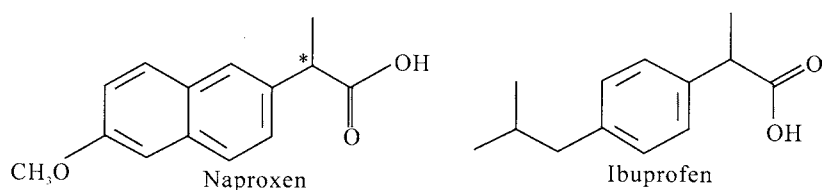


Figure 1 Structures of naproxen and ibuprofen

2.2 Preparation of MIMSP

MIMSP was prepared by utilizing 4-VP as the functional monomer and EDMA as the cross-linking agent. The preparation procedure was as follows. Template (0.2 mM), toluene, 4-VP, 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 50 °C for 12 h. The resulting polymers were washed using a mixture of methanol-acetic acid (4 : 1, v/v) to remove the template molecule, and then the residual acetic acid was removed by washing with methanol. Non-imprinted polymers were also prepared by the same procedure but without the addition of template molecule.

2.3 Test of the morphologies of polymers

The morphologies of the MIPs were analyzed using a scanning electron microscope (Hitachi, S-570, Japan) at 20 keV.

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 254 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 (determined by injecting acetone). The $k_{\text{imprinted}}$ and $k_{\text{non-imprinted}}$ were the retention factors of NAP 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 are the retention factors of NAP and ibuprofen on S-NAP-MIMSP, respectively.

2.5 Scatchard analysis

MIMSP was pushed out of the column. After that, 20 mg of the polymers were weighed into a 10 mL conical flask and mixed with 5.0 mL of S-NAP aqueous solution, the concentration of which varied from 0.1 to 4.0 mM. 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 filtered through a 0.22 μm membrane and the S-NAP concentration in the filtrate was measured by a SP-2102 UV (Shanghai Spectrum Instruments Co., Ltd., China) at 254 nm. The

amount of S-NAP bound to the polymers was calculated by subtracting the concentration of free S-NAP from the initial S-NAP loading. The Scatchard equation $Q/[S-NAP] = (Q_{\text{max}} - Q)/K_d$ was used to estimate the binding parameters of the NAP-imprinted polymers, where Q is the amount of S-NAP bound to the polymer, Q_{max} is the apparent maximum number of binding sites, K_d is the equilibrium dissociation constant, and $[S-NAP]$ represents the equilibrium concentration of S-NAP.

3 Results and discussion

3.1 Optimization of preparation conditions

We optimized the S-NAP-MIMSP preparation procedure by changing the preparation conditions, including the proportion of template to functional monomer (T-M), the degree of cross linking and the percentage of toluene in porogenic solvents. The results are shown in Table 1. When the T-M ratio was 1 : 2 or 1 : 3, the polymers could not be synthesized. When the T-M ratio was 1 : 6, S-NAP-MIMSP was too rigid to allow the mobile phase to flow through. Only when the ratio was 1 : 4 (MIP3), did NAP-MIMSP have both recognition ability and suitable column pressure.

When the degree of cross linking was 80% (MIP3), S-NAP-MIMSP showed relatively higher retention and separation factors. When the degree of cross linking was 85% or 90%, the pressure of the column was too high to allow the mobile phase to flow through. The degree of cross linking affects the internal structure of polymers, and in order to maintain good space structure, the degree of cross linking should usually be above 80% [20].

We used toluene and dodecanol as porogenic solvents. It was observed that with the decrease of the content of toluene, the number of big pores polymerized increased, which caused low chromatographic efficiency; on the other hand, as the content of toluene increased, the number of small pores polymerized increased as well, which made the polymers rigid. Table 1 shows that when the toluene content was 15% (MIP3), the retention and separation factors were the highest.

Considering all of the above, the optimum preparation conditions were as follows: the T-M ratio 1 : 4, the degree of cross linking 80%, the toluene content in porogenic solvents 15%, the initiator molar amount of the total moles of functional monomer 5%, and the preparation temperature 50 °C. As a result, we used MIP3 in the following experiments. Figure 2 and Figure 3 show the chromatograms of (S)-naproxen and racemic naproxen on MIP3.

Table 1 Retention factors and separation factors of *R*-NAP and *S*-NAP on the *S*-NAP-imprinted polymers prepared under different polymerization conditions

Polymer	Molar ratio ^a	Degree of cross-linking (%) ^b	Toluene content in porgene (%)	k_1	k_2	α
MIP ₁	1 : 2	80	15	/	/	/
MIP ₂	1 : 3	80	15	/	/	/
MIP ₃	1 : 4	80	15	6.00	4.13	1.45
MIP ₄	1 : 6	80	15	—	—	—
MIP ₅	1 : 4	75	15	5.64	5.45	1.03
MIP ₆	1 : 4	85	15	—	—	—
MIP ₇	1 : 4	90	15	—	—	—
MIP ₈	1 : 4	80	10	4.46	4.38	1.02
MIP ₉	1 : 4	80	18	—	—	—
MIP ₁₀	1 : 4	80	20	—	—	—

k_1 , k_2 refer to retention factor of *R*-NAP and *S*-NAP. α refers to separation factor. HPLC conditions: column size, 100 mm × 4.6 mm i. d.; column temperature, 25 °C; mobile phase, acetonitrile-phosphoric acid and potassium phosphate (50 : 50, v/v); flow-rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μ g. ^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 4-VP and EDMA. /, The polymer was too rigid to allow the mobile phase to flow through. —, The temperature was too low to synthesize the polymer.

3.2 Morphology of polymers

Figure 4 shows the morphologies of MIMSP prepared in this work using the scanning electron micrography. It can be seen from the images that the sizes and figures of the polymerized products were almost homogeneous and that micro-, mid-, and macro-pores existed. The result revealed that because macropores were present in MIMSP, the mobile phases could be allowed to flow through with low back pressure at a relatively high flow rate.

3.3 Retention properties of NAP on NAP-MIMSP in organic mobile phases

The effects of flow rate, column temperature, and acetic acid content in mobile phase on the retention properties of NAP and ibuprofen were investigated to clarify the retention and molecular recognition mechanism of NAP on *S*-NAP-MIMSP in organic mobile phases, where the mobile phase used was acetonitrile-acetic acid. As shown in Figure 5, the retention factors of NAP and ibuprofen on *S*-NAP-MIMSP decreased as acetic acid content increased. This result proved the existence of the hydrogen-bonding interactions between the target molecule and the MIPs [21].

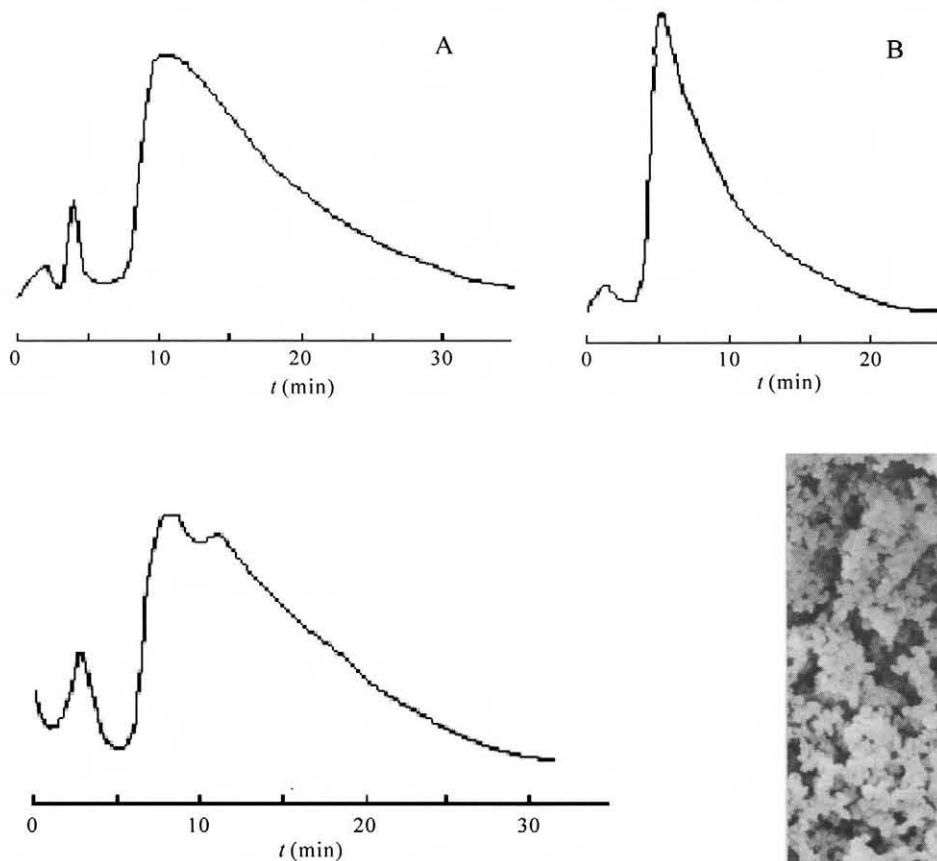


Figure 2 Chromatograms of (*S*)-naproxen on *S*-NAP-MIMSP(A) and non-imprinted polymer (B). HPLC conditions: column size, 100 mm × 4.6 mm i. d.; column temperature, 25 °C; mobile phase, acetonitrile-PBS (50 : 50, v/v, pH 3.0); flow rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μ g.

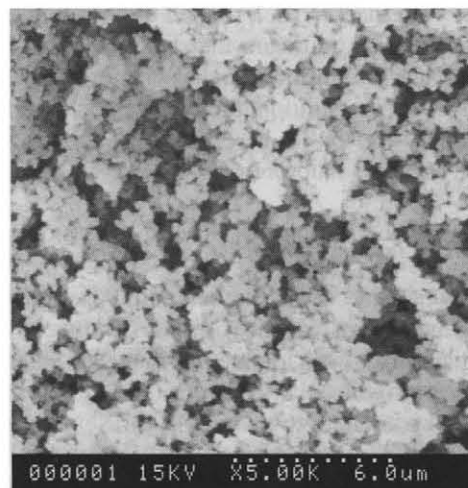


Figure 3 The enantioseparation chromatogram of naproxen. HPLC conditions: column size, 100 mm × 4.6 mm i. d.; column temperature, 25 °C; mobile phase, acetonitrile-PBS (50 : 50, v/v, pH 3.0); flow rate, 1.0 mL/min; detection wavelength, 254 nm.

Figure 4 The scanning electronmicroscopy (SEM) image of *S*-NAP-MIMSP (5 000 ×)

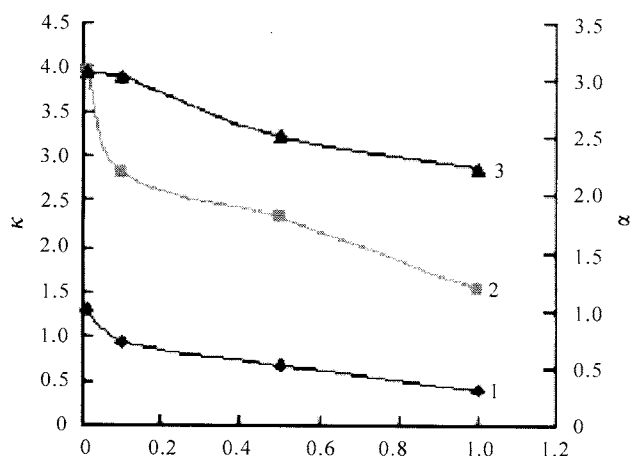


Figure 5 Effect of the acetic acid content of mobile phase on the retention of NAP and ibuprofen. 1, $k_{\text{ibuprofen}}$; 2, k_{NAP} ; 3, α . HPLC conditions: column size, 100 mm \times 4.6 mm i. d.; column temperature, 25 $^{\circ}\text{C}$; mobile phase, acetonitrile-acetic acid (99.99 : 0.01, v/v); flow-rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μg .

Figure 6 shows the effect of flow rate on the retention properties of NAP and ibuprofen on *S*-NAP-MIMSP. With an increase of flow rate from 0.5 to 2.0 mL/min, the retention factors for both solutes decreased, but the degree of variation of ibuprofen was not as large as that of NAP. It was mainly due to the slow mass transfer of NAP on *S*-NAP-MIMSP [22]. Moreover, the column pressure remained very low during the whole experiment, which can be explained by the existence of macro-pores in the polymers' backbone.

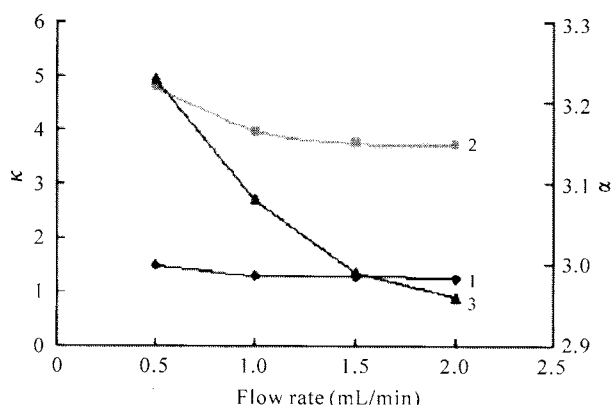


Figure 6 Effect of flow rate on the retention of NAP and ibuprofen. 1, $k_{\text{ibuprofen}}$; 2, k_{NAP} ; 3, α . HPLC conditions: column size, 100 mm \times 4.6 mm i. d.; column temperature, 25 $^{\circ}\text{C}$; mobile phase, acetonitrile-acetic acid (99.99 : 0.01, v/v); detection wavelength, 254 nm; loaded amount, 5 μg .

Figure 7 shows the effect of column temperature on the retention properties of NAP and ibuprofen on *S*-NAP-MIMSP. On *S*-NAP-MIMSP, with an increase of column temperature from 20 to 60 $^{\circ}\text{C}$, the retention factors of NAP and ibuprofen increased and then decreased, and the highest separation factor was obtained at 30 $^{\circ}\text{C}$. These results proved that the two compounds have different values of thermodynamics on *S*-NAP-MIMSP during the separation

process [23].

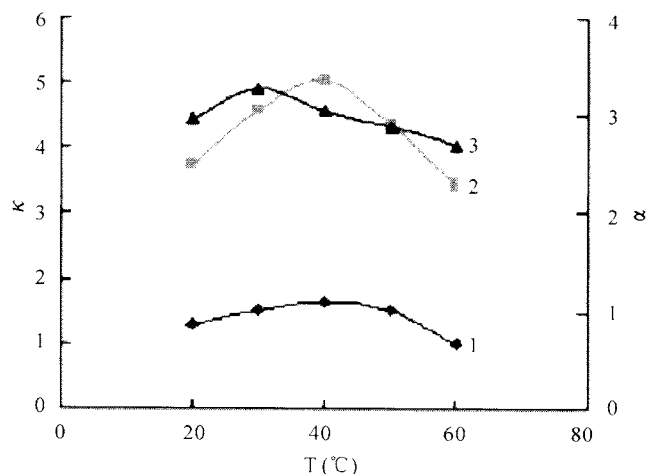


Figure 7 Effect of temperature on the retention of NAP and ibuprofen. 1, $k_{\text{ibuprofen}}$; 2, k_{NAP} ; 3, α . HPLC conditions: column size, 100 mm \times 4.6 mm i. d.; mobile phase, acetonitrile-acetic acid (99.99 : 0.01, v/v); flow rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μg .

3.4 Retention properties of NAP on *S*-NAP-MIMSP in aqueous mobile phases

The mobile phase pH and PBS content were also tested in this experiment when 25 mM phosphate buffer solution (PBS) and acetonitrile were used as mobile phase. Figure 8 shows the effect of pH on the retention properties of NAP on *S*-NAP-MIMSP and on the non-imprinted polymers. The results showed that retention for NAP increased and then decreased in the mobile phase pH range of 2–6 on *S*-NAP-MIMSP. When the mobile phase pH was 4, the retention for NAP reached the highest. This result could be explained by the protonation level of the 4-VP and the degree of deprotonation of NAP in the different mobile phase pH. Because the pK_a of NAP is 4.00, thus, NAP was retained on *S*-NAP-MIMSP by electrostatic interactions with the N-H groups in functional monomers on *S*-NAP-MIMSP, in addition to hydrophobic interactions with the polymers' backbone. At the same time, the retention of NAP also changed markedly with the increase of pH, and NAP 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.

Figure 9 shows the effects of PBS content on the retention properties of NAP on *S*-NAP-MIMSP and on the non-imprinted polymers. As shown in Figure 9, retention of NAP on *S*-NAP-MIMSP gradually decreased as the PBS content increased from 30% to 40%, while it increased in the range of 40%–70% of PBS content. When the PBS content was 40%, the MIMSP gave the lowest separation for NAP. These results can be explained by the fact that when the PBS content was less than 50%, hydrophobic interactions were dominant, and when PBS content was more than 50%, electrostatic interactions became dominant. So in aqueous mobile phases, the retention of solutes on *S*-NAP-MIMSP was mainly due to hydrophobic interactions,

in addition to electrostatic interactions of the compounds with 4-VP. However, on the non-imprinted polymers, the retention factors of NAP were nearly zero, which could prove that *S*-NAP-MIMSP recognition ability for NAP largely comes from the molecular imprinting process.

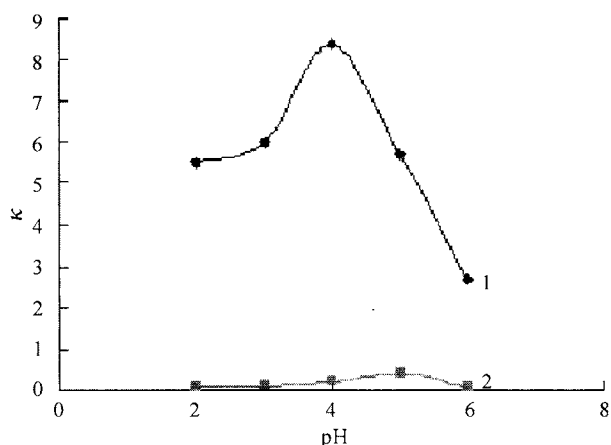
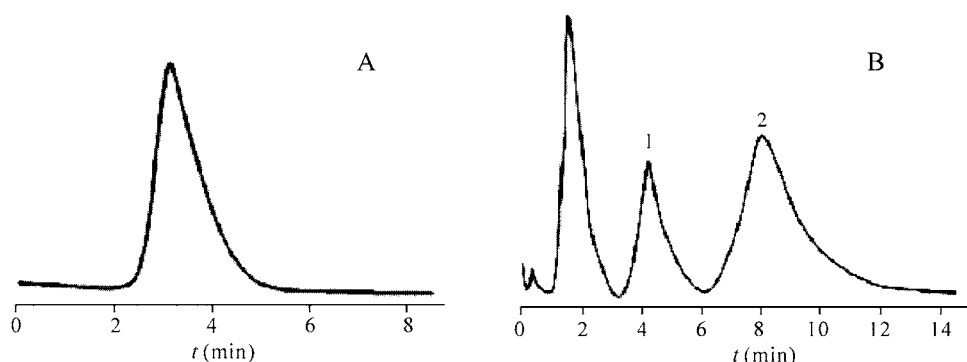


Figure 8 Effect of the pH of mobile phase on the retention of NAP. 1, k_{NAP} ; 2, $k_{\text{NAP(non-imprinted)}}$. HPLC conditions: column size, 100 mm \times 4.6 mm i. d.; column temperature, 25 $^{\circ}\text{C}$; mobile phase, acetonitrile-PBS (50 : 50, v/v); flow rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μg .

3.5 Separation of NAP and ibuprofen on *S*-NAP-MIMSP

Figure 10 shows the separation of NAP and ibuprofen on *S*-NAP-MIMSP and on the non-imprinted polymers. On the non-imprinted polymers, NAP and ibuprofen could not be separated, but on *S*-NAP-MIMSP, NAP was completely separated from ibuprofen. These results indicated that *S*-NAP-MIMSP could efficiently separate the target molecule from its structurally similar compound.

Figure 10 Chromatograms of NAP and ibuprofen. A, Non-imprinted polymer; B, *S*-NAP-MIMSP. 1, Ibuprofen; 2, NAP. HPLC conditions: column size, 100 mm \times 4.6 mm i. d.; column temperature, 25 $^{\circ}\text{C}$; mobile phase, acetonitrile-acetic acid (99.99 : 0.01, v/v); flow rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μg .



The obtained data were plotted according to the Scatchard equation. As shown in Figure 12, there were two distinct sections within the plot which could be regarded as two straight lines. The results indicated that there were two classes of binding sites existing in *S*-NAP-MIMSP. From the slope and intercept of the plots, the equilibrium dissociation constant K_{d1} and the apparent maximum number Q_{max1} of the higher affinity binding sites can be calculated to be 1.045 μM and 5.496 μM , respectively. In the same

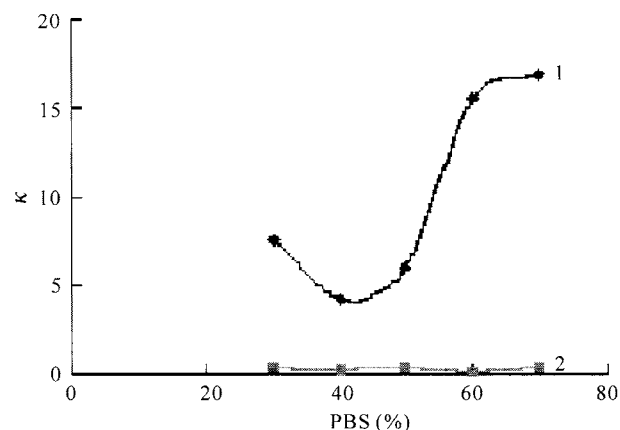


Figure 9 Effect of the PBS content of mobile phase on the retention of NAP. 1, k_{NAP} ; 2, $k_{\text{NAP(non-imprinted)}}$. HPLC conditions: column size, 100 mm \times 4.6 mm i. d.; column temperature, 25 $^{\circ}\text{C}$; mobile phase, acetonitrile-PBS (50 : 50, v/v); flow rate, 1.0 mL/min; detection wavelength, 254 nm; loaded amount, 5 μg .

3.6 Determination of binding parameters of *S*-NAP-imprinted polymers

Figure 11 shows the binding isotherms for *S*-NAP on *S*-NAP-MIMSP and on the non-imprinted polymers. The binding amount increased gradually with the aqueous concentration increase of *S*-NAP in the initial solution, but the binding amount of *S*-NAP on MIMSP 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 [24].

way, K_{d2} and Q_{max2} of the lower affinity bonding sites were calculated to be 3.429 μM and 1.000 μM , respectively.

4 Conclusions

An *S*-NAP-MIMSP was synthesized by *in situ* technique, utilizing 4-vinylpyridine (4-VP) as a function monomer, ethylene glycol dimethacrylate (EDMA) as a cross-linking agent, and toluene and dodecanol as porogenic solvents.

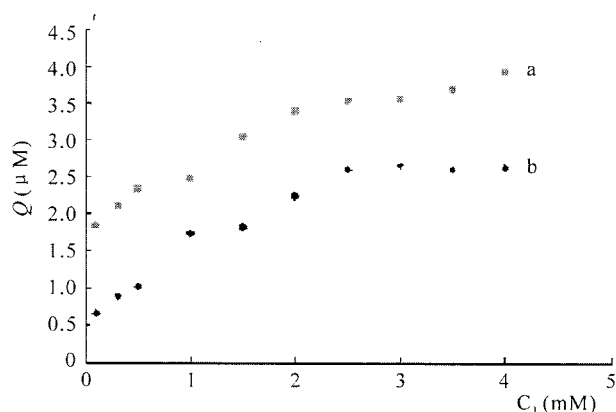


Figure 11 Binding isotherm of polymers for *S*-NAP. a, *S*-NAP-MIMSP; b, non-imprinted polymers.

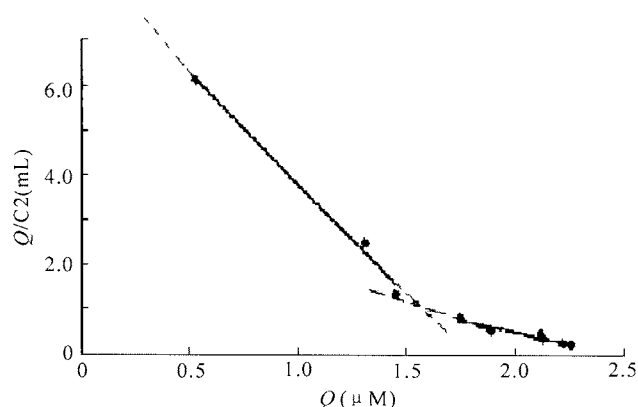


Figure 12 Scatchard plots to estimate the binding nature of *S*-NAP-MIMSP.

Racemic NAP could be specifically separated to some extent. At the same time, NAP could be separated from ibuprofen under the optimized conditions. The effects of chromatographic conditions on the retention and selectivity of *S*-NAP-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. Scatchard analysis showed that two classes of binding sites existed in *S*-NAP-MIMSP, with their dissociation constants estimated to be 1.045 and 5.496 μM , respectively. All of these results add evidence to the future extensive research about the use of *S*-NAP as a template in the field of molecularly imprinted technology, and support the application of *S*-NAP-MIMSP in the field of biopharmaceutical analysis.

Acknowledgments

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