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Uniform-sized molecularly imprinted polymer for (*S*)-naproxen selectively modified with hydrophilic external layer

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Abstract

A uniform-sized molecularly imprinted polymer (MIP) for (*S*)-naproxen selectively modified with hydrophilic external layer has been prepared. First, the molecularly imprinted polymer for (*S*)-naproxen was prepared using 4-vinylpyridine and ethylene glycol dimethacrylate (EDMA) as a functional monomer and cross-linker, respectively, by a multi-step swelling and thermal polymerization method. Next, a 1:1 mixture of glycerol monomethacrylate (GMMA) and glycerol dimethacrylate (GDMA) was used for hydrophilic surface modification, and it was added directly to the molecularly imprinted polymer for (*S*)-naproxen 4 h after the start of molecular imprinting. The retention factors of all solutes tested were decreased with the surface modified molecularly imprinted polymer, compared with the unmodified molecularly imprinted polymer. However, chiral recognition of racemic naproxen was attained with the surface modified molecularly imprinted polymer as well as the unmodified molecularly imprinted polymer. Further, bovine serum albumin was completely recovered from the surface modified molecularly imprinted polymer. These results revealed that the chiral recognition sites of (*S*)-naproxen remained unchanged with hydrophilic surface modification, and that the molecularly imprinted polymer for (*S*)-naproxen was selectively modified with hydrophilic external layer. Preliminary results reveal that the surface modified molecularly imprinted polymer could be applicable to direct serum injection assays of (*S*)-naproxen.

Keywords: Molecular imprinting; Stationary phases, LC; Enantiomer separation; Naproxen

1. Introduction

The molecularly imprinted polymers (MIPs), which can afford specific recognition against an imprint molecule and moderate recognition against the structurally related compounds, are used for chromatographic separations, solid-phase extractions, membranes, antibody-mimics and sensors [1–4]. They are called artificial antibodies or receptors. Since they are stable, easy to prepare and inexpensive, they can be an attractive alternative or complement to natural antibodies and receptors in the above applications. Among those, molecularly imprinted solid-phase extractions are very promising, and have been used for selective enrichment and pretreatment of analytes in complex matrices such as biological fluids and environmental samples [3,4].

On the other hand, a lot of restricted access

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materials (RAMs) were prepared and used for enrichment and pretreatment of the analytes in complex matrices, especially in biological fluids, by HPLC [5–7]. With the RAMs, large molecules such as serum proteins are eluted in the void volume without destructive accumulation, but small molecules such as drugs can reach the hydrophobic or ion-exchange sites and be separated. They can be utilized as an analytical column or precolumn for enrichment and pretreatment of analytes in complex matrices. However, the RAMs so far developed could not be used for selective enrichment of an analyte, because hydrophobic or ionic interaction could play an important role in the recognition of the analyte with such materials.

We prepared uniform-sized MIPs for (*S*)-naproxen [8,9] and propranolol [10], where a typical multi-step swelling and polymerization method [11] with water as the suspension medium was used, and evaluated the obtained MIPs by using a mixture of phosphate buffer and acetonitrile as a mobile phase. However, the MIP for (*S*)-naproxen prepared by us gave similar enantioselectivity for naproxen than that prepared with non-aqueous bulk polymerization techniques by Kempe and Mosbach [12]. Uniform-sized polymer-based materials had an advantage that in situ surface modification could be made to introduce another functionality to the materials [13,14]. It is difficult to make such surface modification to the polymer block prepared by bulk polymerization method, which is a typical preparation method for MIPs.

The aim of this study is to make a uniform-sized MIP for (*S*)-naproxen selectively modified with hydrophilic external layer, through a combination of molecular imprinting and hydrophilic surface modification techniques. Further, we preliminarily applied the obtained MIP to direct serum injection assay of (*S*)-naproxen.

2. Experimental

2.1. Materials

Ethylene glycol dimethacrylate (EDMA) and 4-vinylpyridine were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Wako Pure Chemical Industry (Osaka, Japan), respectively. Both monomers were purified by general distillation techniques in vacuo to remove the polymerization inhibitor. 2,2′-Azobis(2,4-dimethylvaleronitrile)(V-65) and potassium peroxodisulfate were purchased from Nacalai Tesque (Kyoto, Japan), and used without further purification. Glycerol monomethacrylate (GMMA) and glycerol dimethacrylate (GDMA) were gifts from Fuso Chemical (Osaka, Japan). (*S*)-(+)-Naproxen and racemic naproxen were purchased from Tokyo Chemical Industry (Tokyo, Japan). (*S*)-(+)-Ibuprofen and racemic ibuprofen were purchased from Aldrich Chemical (Milwaukee, WI, USA). (*S*)-(+)-Flurbiprofen and racemic flurbiprofen were donated by Kaken Pharmaceutical (Tokyo, Japan). Racemic ketoprofen and pranoprofen were donated by Chugai Pharmaceutical (Tokyo, Japan) and Yoshitomi Pharmaceutical (Osaka, Japan), respectively. Other reagents and solvents of an analytical-reagent grade were used without further purification.

Water purified with a Nanopure II unit (Barnstead, Boston, MA, USA) was used for the preparation of the eluent and the sample solution.

2.2. Preparation of surface modified MIPs

Uniformly sized, polystyrene seed particles utilized as the shape template were prepared by an emulsifier-free emulsion polymerization and purified by a previously reported method [15]. The size of the seed particle was ca. 1 μm in diameter. Preparation of uniform-sized MIP materials as well as the surface modified MIP materials was carried out by a multi-step swelling and polymerization method as shown in Fig. 1. The MIPs for (*S*)-naproxen were prepared with the EDMA/toluene (porogen) ratio 5:5 and 7:3, which were termed MIPs 1 and 3, respectively. A 1:1 mixture of GMMA and GDMA was used for hydrophilic surface modification, and the surface modified materials of MIPs 1 and 3 were termed MIPs 2 and 4, respectively. A water dispersion of the uniformly sized, polystyrene seed particles (0.107 g/ml), 0.83 ml, was admixed with a micro-emulsion prepared from 0.48 ml of dibutyl phthalate as activating solvent [16], 0.02 g of sodium dodecyl sulfate and 5 ml of distilled water by sonication. This first-step swelling was carried out at room temperature for 15

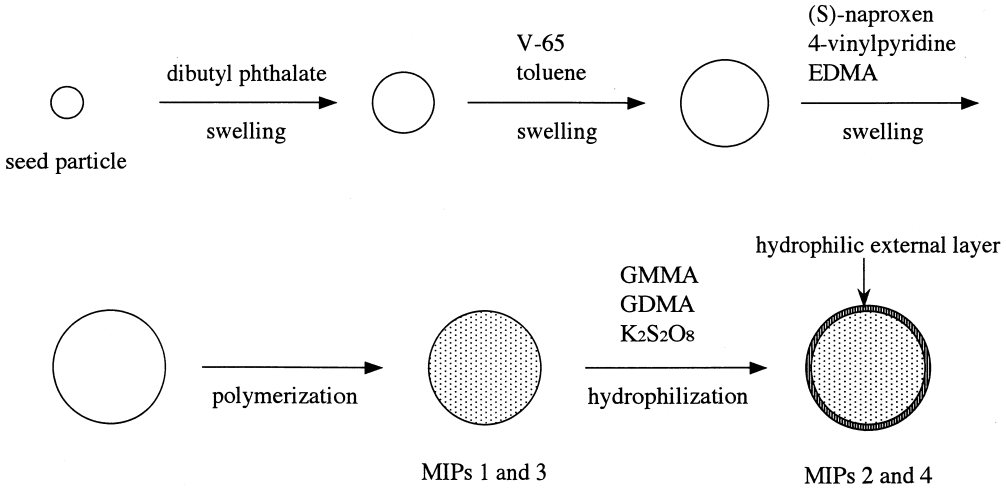

Fig. 1. Synthetic scheme of surface modified MIP for (*S*)-naproxen. Abbreviations: V-65, 2,2′-azobis(2,4-dimethylvaleronitrile); EDMA, ethylene glycol dimethacrylate; GMMA, glycerol monomethacrylate; GDMA, glycerol dimethacrylate.

h with stirring at 125 rpm until micro oil droplets were completely disappeared. To the swollen particles, a micro-emulsion prepared from 0.375 g of V-65, 5 ml of toluene for MIPs 1 and 2 (or 3 ml of toluene for MIPs 3 and 4), 12.5 ml of water and 10 ml of 4.8% polyvinylalcohol solution was added. This second-step swelling was carried out at room temperature for 2 h with stirring at 125 rpm. To the dispersion of swollen particles, a dispersion of 5 ml of EDMA for MIPs 1 and 2 (or 7 ml of EDMA for MIPs 3 and 4), 0.63 g of 4-vinylpyridine, 0.02 g of sodium dodecyl sulfate, 12.5 ml of water and 10 ml of 4.8% polyvinylalcohol solution was added. This third-step swelling was carried out at room temperature for 2 h with stirring at 125 rpm. When the template molecule was added, 0.46 g of (*S*)-naproxen was admixed with the monomers utilized to prepare the dispersion for the third-step swelling. After the third-step swelling was completed, the polymerization procedure was started at 50°C under argon atmosphere with slow stirring. After 4 h of polymerization, the hydrophilic monomers (0.5 ml of GMMA and 0.5 ml of GDMA), with 0.02 g of potassium peroxodisulfate, were added to the polymerizing materials. After a further 20 h of stirring at 70°C, the dispersion of polymerized particles was poured into 250 ml of a hot water–methanol mixture and the supernatant was discarded after sedimentation of the particles. The polymer particles were redispersed into methanol and the supernatant was again discarded after sedimentation. This procedure was repeated three times in methanol and twice in tetrahydrofuran (THF). The resulting 5- to 6-μm polymer particles were collected using a membrane filter, washed with THF and then with acetone and finally dried at room temperature.

The prepared materials were packed into a stainless-steel column (4.6 mm I.D.×100 mm) by a slurry technique using methanol as the slurry medium to evaluate their chromatographic characteristics.

2.3. Chromatography

2.3.1. Estimation of pore volumes of MIPs

The HPLC system used was composed of an LC-9A pump, an SPD-6A spectrophotometer, a Rheodyne 7125 injector with a 20-μl loop and a C-R6A integrator (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.2 ml/min. Detection was performed at 254 nm. The micropore volume of the MIP (Eva) was evaluated by elution volume of benzene minus that of hexylbenzene. The mesopore volume (Evb) was evaluated by elution volume of hexylbenzene minus that of polystyrene, whose molecular weight (MW) is 580. The macropore volume (Evc) was estimated by elution volume of polystyrene (MW=580) minus that of polystyrene

(MW=20,000,0000). The total pore volume (Evtot) was the sum of the Eva, Evb and Evc.

2.3.2. Evaluation of MIPs

The same HPLC system described above was used. The flow-rate was maintained at 1.0 ml/min. Detection was performed at 220 or 254 nm. Retention factors were calculated from the equation $k = (t_R - t_0)/t_0$, where t_R and t_0 are retention times of retained and unretained solutes, respectively. The retention time of unretained solute, t_0, was measured by injecting a solution whose organic modifier content was slightly different from that of the eluent used. The enantioseparation factor is calculated from the equation $\alpha = k_2/k_1$, where k_1 and k_2 are the retention factors of the first and second eluted enantiomers, respectively. The number of theoretical plates is calculated from the equation $N = 16(t_R/w)^2$, where w is the baseline peak width. The data cited are average values of two replicate measurements. All separations were carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The eluents are prepared by using phosphoric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate and acetonitrile. The eluent used was specified in the legends of tables and figures.

2.4. Recovery of BSA from surface modified MIPs

The recovery of BSA from unmodified or surface modified MIPs at the first injection was calculated based on the peak area of BSA sample (1 mg) by taking the area obtained without a column as 100%.

3. Results and discussion

3.1. Preparation of surface modified MIPs

In a previous paper [8,9], we reported characterization of uniform-sized MIP for (*S*)-naproxen prepared by a multi-step swelling and thermal or redox polymerization technique with water as a suspension medium, where 4-vinylpyridine and EDMA were used as a functional monomer and cross-linker, respectively. The MIP prepared by the thermal polymerization method gave higher column efficiency than that prepared by the redox polymerization method. On the other hand, selective, hydrophilic surface modification of uniform-sized polymer-based stationary phase prepared by a multi-step swelling and thermal polymerization technique was reported by Hosoya et al. [13]. When the hydrophilic monomers, GMMA and GDMA, in the presence of a water-soluble initiator were added directly to the polymerization medium after the establishment of the hydrophobic poly(styrene–co-divinylbenzene) materials, they partitioned between the water phase and the porogenic solvent according to its partition coefficient. The resulting polymers had hydrophobic inner surfaces and hydrophilic outer surfaces. In this study, we tried to make the uniform-sized MIP for (*S*)-naproxen by a multi-step swelling and thermal polymerization technique, to modify it with hydrophilic external layer, and to apply it to the direct serum injection assays.

Table 1 shows the pore volumes of MIPs 1–4, which are evaluated using a size-exclusion chromatographic method [17]. The MIP 1 gave larger macro-

Table 1
Pore volumes of unmodified and surface modified MIPs for (*S*)-naproxen estimated by size exclusion chromatographic method[a]

MIP	EDMA/toluene ratio (v/v)	GMMA/GDMA ratio (v/v)	Eva (ml)[b]	Evb (ml)[c]	Evc (ml)[d]	Evtot (ml)[e]
1	5:5	None	0.16	0.09	0.36	0.61
2	5:5	5:5	0.14	0.06	0.26	0.46
3	7:3	None	0.16	0.08	0.20	0.44
4	7:3	5:5	0.16	0.06	0.17	0.39

[a] HPLC conditions: column, 100×4.6 mm I.D.; eluent, tetrahydrofuran; flow-rate, 0.2 ml/min; detection, 254 nm.
[b] Eva: micropore volume.
[c] Evb: mesopore volume.
[d] Evc: macropore volume.
[e] Evtot: total pore volume (Eva+Evb+Evc).

pore and total pore volumes (Evc and Evtot, respectively) than the MIP 3, while the micropore and mesopore volumes (Eva and Evb, respectively) of both materials were almost the same. By increasing the EDMA/toluene ratio from 5:5 to 7:3, the total pore volumes were decreased. After hydrophilic surface modification, all pore volumes, especially macropore volumes, of the MIP 1 were decreased. On the other hand, the mesopore and macropore volumes of MIP 3 were decreased. These results reveal that the total pore volumes of MIPs 1 and 3 are decreased by hydrophilic surface modification, and that the decrement of the total volume of MIP 1 is larger than that of MIP 3.

3.2. Retentivity and enantioselectivity of surface modified MIPs

Fig. 2 shows the retentivity and enantioselectivity of naproxen on unmodified and surface modified MIPs for (*S*)-naproxen. The retention factors of (*S*)-naproxen were decreased by hydrophilic surface modification (compare MIPs 1 and 2, or MIPs 3 and 4), while similar or higher enantioselectivity was observed with the surface modified MIPs (MIPs 2 and 4), compared with the unmodified MIPs (MIPs 1 and 3). As reported previously [9], racemic ibuprofen and flurbiprofen were partially resolved with the MIP for (*S*)-naproxen. Similarly, the surface modified MIPs could resolve racemic ibuprofen and flurbiprofen, and gave similar or higher enantioselectivity, compared with unmodified MIPs (data is not shown). These results revealed that the chiral recognition sites of (*S*)-naproxen remained unchanged with hydrophilic surface modification. Table 2 shows batch reproducibility data of MIPs 1 and 2. With regard to the retentivity and enantioselectivity of naproxen, both MIPs gave similar reproducibility. These results reveal that unmodified and surface modified MIPs for (*S*)-naproxen could be reproducibly prepared. Fig. 3, parts A–C, shows the retention factors of 2-arylpropionic acid derivatives, other acidic compounds and neutral compounds, respectively, on the unmodified and surface modified MIPs. The retention factors of all solutes tested were decreased by hydrophilic surface modification of the MIPs. This is due to a decrease in hydrophobicity of the MIP materials by hydrophilic modification using GMMA and GDMA. Fig. 4, parts A and B, shows the separation of racemic ketoprofen and ibuprofen, and (*R*)- and (*S*)-naproxen on the MIPs 1 and 2, respectively. The numbers of theoretical plates of the

Table 2
Batch reproducibility of MIPs 1 and 2[a]

	MIP 1		MIP 2	
	k_2	α	k_2	α
Batch 1	16.6	1.63	16.2	1.65
Batch 2	15.2	1.57	14.1	1.58
Batch 3	14.9	1.56	14.0	1.58
Average±SD	15.6±0.9	1.59±0.04	14.8±1.2	1.60±0.04

[a] The retention factor of (*S*)-naproxen (k_2) and enantioseparation factor (α) of naproxen were measured under the HPLC conditions as in Fig. 1.

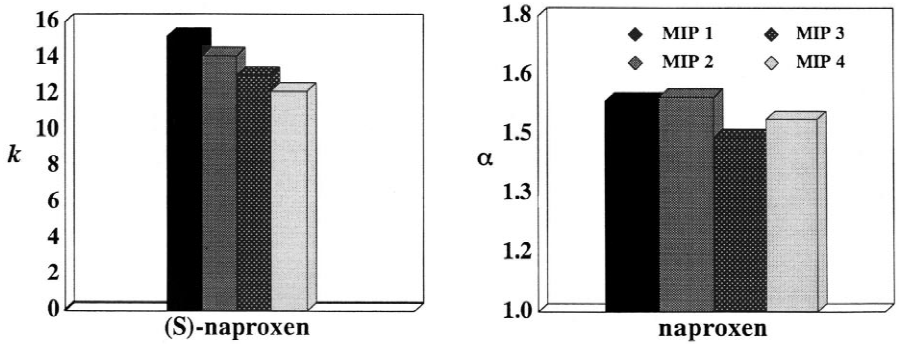

Fig. 2. Retention factor of (*S*)-naproxen (A) and enantioseparation factor of naproxen (B) on unmodified (MIPs 1 and 3) and surface modified (MIPs 2 and 4) MIPs for (*S*)-naproxen. HPLC conditions: mobile phase, 20 m*M* phosphate buffer (pH 3.2)/CH_3CN=50:50 (v/v); flow-rate, 1.0 ml/min; injection volume, 20 μl; loaded amount, 0.5 μg.

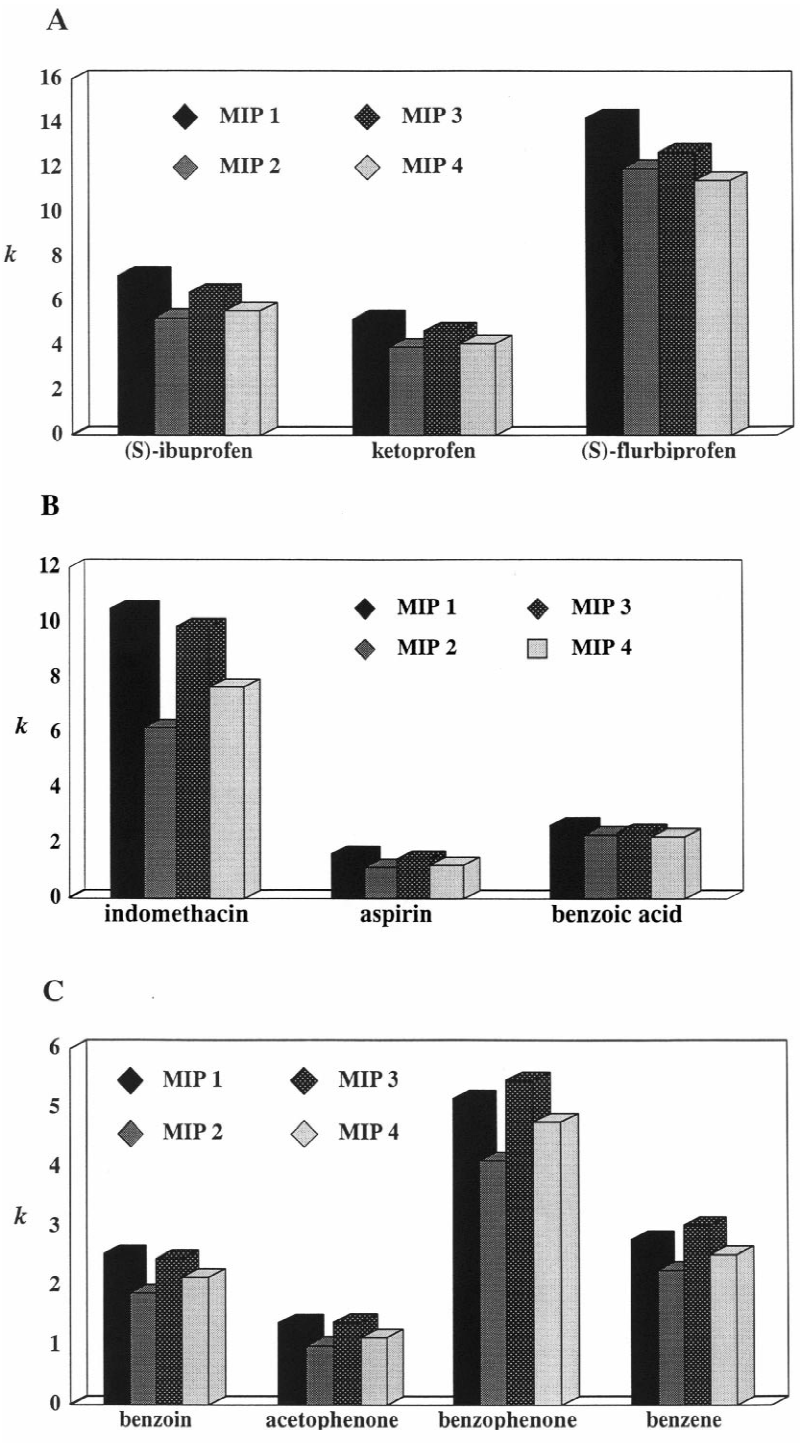

Fig. 3. Retention factors of 2-arylpropionic acid derivatives (A), other acidic compounds (B) and neutral compounds (C) on unmodified (MIPs 1 and 3) and surface modified (MIPs 2 and 4) MIPs for (*S*)-naproxen. HPLC conditions as in Fig. 2.

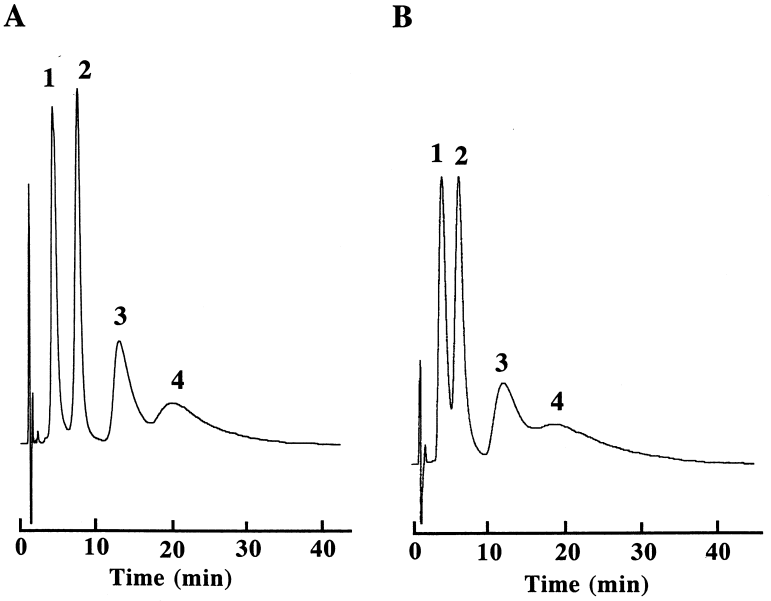

Fig. 4. Separation of racemic ketoprofen and ibuprofen, and (*R*)- and (*S*)-naproxen on unmodified (MIP 1) (A) and surface modified (MIP 2) (B) MIPs. Peak assignments: 1, racemic ketoprofen; 2, racemic ibuprofen; 3, (*R*)-naproxen; 4, (*S*)-naproxen. HPLC conditions as in Fig. 2.

MIP 1 were 121 and 37 for (*R*)- and (*S*)-naproxen, respectively, estimated from a chromatogram in Fig. 4, part A, and those of the MIP 2 were 43 and 9. The column efficiency of the MIP 2 was lower than that of the MIP 1. This might be due to mass transfer limitation by hydrophilic surface modification of the MIP and/or swelling of hydrophilic external layers in an aqueous-rich eluent.

The MIP 2 gave higher retentivity and enantioselectivity for naproxen than the MIP 4. In the following study, we used the MIP 2.

3.3. Recovery of BSA from unmodified and surface modified MIPs

Table 3 shows the recovery of BSA from MIPs 1–4 at the first injection of 1 mg of BSA using a mixture of phosphate buffer and acetonitrile as an eluent. The MIP 1 showed only about 10% recovery of BSA, while the MIP 3 showed about 30–40% recovery. BSA could access to the macropores and interact with hydrophobic polymer backbone, and the macropore volume of the MIP 1 is larger than that of the MIP 3. This could be the reason the MIP 3 gave higher BSA recovery than the MIP 1. After hydrophilic surface modification of the MIPs 1 and 3, BSA was almost completely recovered from the MIPs 2 and 4. The results described above reveal that the MIP for (*S*)-naproxen is selectively modified with hydrophilic external layer, and that direct serum injection assays of naproxen could be attained using the surface modified MIP.

Table 3
Recovery (%) of BSA from unmodified and surface modified MIPs[a]

MIP	Eluent	
	pH 7.1	pH 3.4
1	9.5	6.9
2	100.9	97.0
3	42.1	32.7
4	97.0	96.8

[a] HPLC conditions: column, 100×4.6 mm I.D.; eluent, 50 m*M* phosphate buffer/CH_3CN=90:10 (v/v); flow-rate, 1.0 ml/min; wavelength, 280 nm.

3.4. Preliminary application of surface modified MIP for direct serum injection assay of (S)-naproxen

Fig. 5, parts A and B, shows chromatograms of control serum and control serum spiked with (*S*)-naproxen, respectively, on the surface modified MIP, MIP 2. Fig. 6, parts A and B, shows chromatograms of control serum and control serum spiked with (*S*)-naproxen, respectively, on the surface modified, non-imprinted material. Serum proteins were eluted in the void volume on both the surface modified, MIP and non-imprinted materials. However, on the non-imprinted materials (*S*)-naproxen was overlapped with ordinary components of serum samples, while on the MIP materials (*S*)-naproxen was completely separated from serum components. These preliminary results reveal that the surface modified MIP should have selective internal layer and hydrophilic external layer, and that it could be applicable to direct serum injection assays of (*S*)-naproxen.

Further study is in progress in our laboratory.

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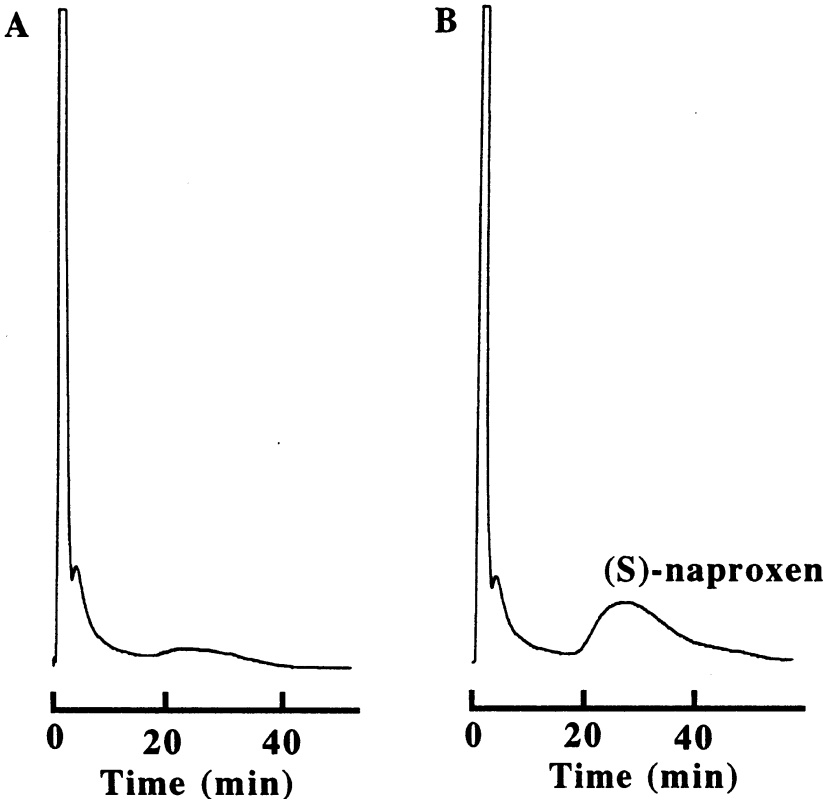

Fig. 5. Chromatograms of control serum (A) and control serum spiked with (*S*)-naproxen (B) on the surface modified MIP (MIP 2). HPLC conditions: column, 100×4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 6.04)/CH_3CN=80:20 (v/v); flow-rate, 1.0 ml/min; wavelength, 220 nm; injection volume, 20 μl; loaded amount, 2.5 μg (*S*)-naproxen.

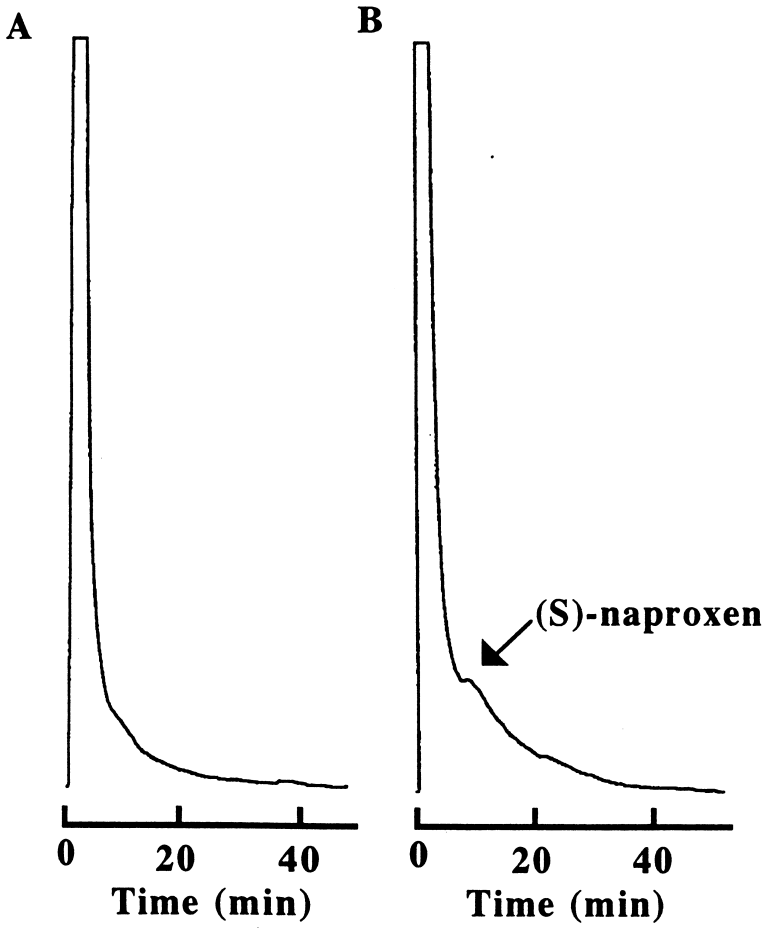

Fig. 6. Chromatograms of control serum (A) and control serum spiked with (*S*)-naproxen (B) on the surface modified, non-imprinted polymer. HPLC conditions as in Fig. 5.

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