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Uniformly Sized Molecularly Imprinted Polymer for (*S*)-Nilvadipine. Comparison of Chiral Recognition Ability with HPLC Chiral Stationary Phases Based on a Protein

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Uniformly sized molecularly imprinted polymers (MIPs) for (*S*)-nilvadipine have been prepared by a multistep swelling and polymerization method using methacrylic acid, 2-(trifluoromethyl)acrylic acid, 2-vinylpyridine, or 4-vinylpyridine (4-VPY) as a functional monomer and ethylene glycol dimethacrylate (EDMA) as a cross-linker. The chiral recognition abilities of the MIPs for nilvadipine and other dihydropyridine calcium antagonists were evaluated using a mixture of sodium phosphate buffer (or water) and acetonitrile or only acetonitrile as the mobile phase. The (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers gave the highest resolution for nilvadipine among the MIPs prepared. In addition, the enantioseparation of nilvadipine was attained using the (*S*)-nilvadipine-imprinted EDMA polymers, without use of a functional monomer. ^{1}H NMR and molecular modeling studies suggested a one-to-one hydrogen-bonding-based complex formation of (*S*)-nilvadipine with 4-VPY in chloroform. These results reveal that the (*S*)-nilvadipine-imprinted EDMA polymers could recognize the template molecule by its molecular shape, and that in addition to this recognition, hydrophobic and hydrogen-bonding interactions seems to play important roles in the retention and chiral recognition of nilvadipine on the 4-VPY-*co*-EDMA polymers in hydroorganic mobile phases. By optimizing chromatographic conditions such as column temperature and flow rate, the baseline separation of nilvadipine enantiomers was attained with a short analysis time and with a column efficiency comparable to commercially available chiral stationary phases based on a protein, such as ovomucoid or α_1-acid glycoprotein.

Molecularly imprinted polymers (MIPs) have the ability to recognize target molecules specifically and have been used in a variety of molecular recognition-based applications, such as separation media,[1−3] artificial antibody mimics,[4−6] and sensing devices.[7,8] In some cases, the specificity for a target molecule is comparable to that observed with biological systems.[9] With molecular imprinting techniques, nonaqueous bulk polymerization techniques[10] are usually employed to obtain MIPs. However, to produce HPLC packing materials, the block polymers obtained need to be crushed, ground, and sieved. MIPs are also unsuitable for HPLC packing materials because of their random shape and size distribution. To overcome this problem, uniformly sized MIPs have recently been prepared using a multistep swelling and polymerization method.[11] This method is advantageous because it allows facile preparation of uniformly sized and monodispersed particles and in situ modification and also because it is suitable for preparing HPLC packing materials.[12] In addition, we prepared the uniformly sized MIP for (*S*)-naproxen, which showed molecular recognition ability equivalent to that prepared by nonaqueous bulk polymerization techniques and could be used as an HPLC stationary phase after simple washing.[13]

Dihydropyridine calcium antagonists (DHPs), a major class of calcium antagonists, are clinically used to treat angina pectoris, hypertension, and subarachnoid hemorrhage. Except for nifedipine, the DHPs have an asymmetric carbon at position 4 of the dihydropyridine ring and therefore are optically active. Most of these drugs are now clinically used as a racemic mixture, but the

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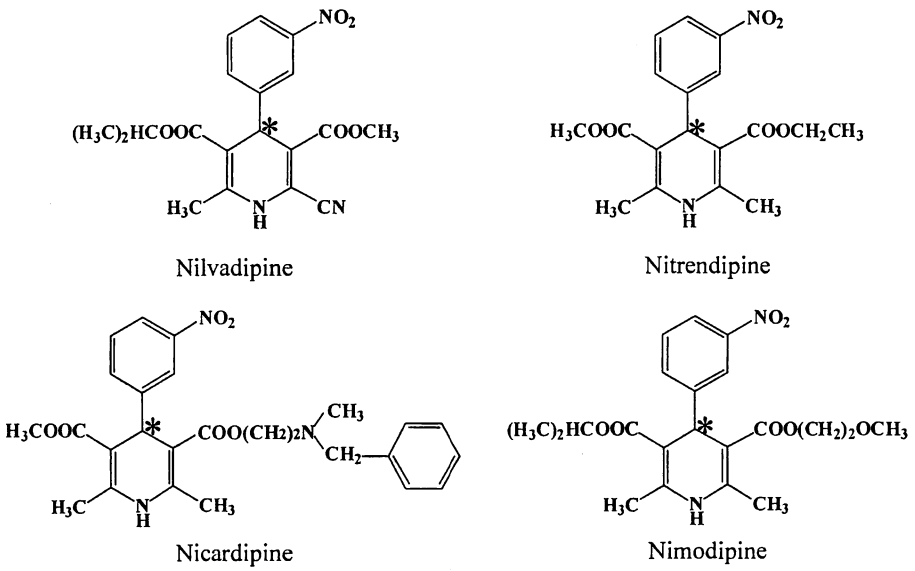

Figure 1. Structures of dihydropyridine calcium antagonists used in this study.

pharmacological effects between DHP enantiomers are likely to be quantitatively or qualitatively different.[14-16] For example, (*S*)-nilvadipine is ~100 times more potent than its antipode for relaxing potassium-induced contractions of isolated dog coronary arteries.[17] DHPs have been enantioseparated with HPLC chiral stationary phases based on (+)-poly(triphenylmethyl methacrylate),[15] α_1-acid glycoprotein (α_1-AGP),[18] ovomucoid (OVM),[18,19] derivatized polysaccharides,[20,21] and Pirkle-type[22] selectors. However, there has been no report of the MIPs being employed for the chiral recognition or separation of DHPs.

In this study, uniformly sized (*S*)-nilvadipine-imprinted polymers were prepared using methacrylic acid (MAA), 2-(trifluoromethyl)acrylic acid (TFMAA), 2-vinylpyridine (2-VPY), or 4-vinylpyridine (4-VPY) as a host functional monomer and ethylene glycol dimethacrylate (EDMA) as a cross-linker by a multistep swelling and polymerization method. The molecular recognition properties of the MIPs for nilvadipine and the other DHPs were evaluated using a mixture of phosphate buffer (or water) and acetonitrile or only acetonitrile as the mobile phase. ^{1}H NMR and molecular modeling studies of (*S*)-nilvadipine and 4-VPY were performed. Based on the results obtained, the retentive and enantioselective mechanisms of nilvadipine on the (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymer are discussed. Furthermore, the enantioseparation of nilvadipine, following optimization of the MIP preparation methods and HPLC separation conditions of nilvadipine on the MIP, is compared with that on commercially available chiral stationary phases based on a protein, i.e., OVM or α_1-AGP.

EXPERIMENTAL SECTION

Materials. EDMA and TFMAA were purchased from Tokyo Chemical Industry (Tokyo, Japan). MAA, 4-VPY, and 2-VPY were purchased from Wako Pure Chemical Industry (Osaka, Japan). The monomers were purified by a general distillation technique in vacuo to remove the polymerization inhibitor. 2,2′-Azobis(2,4-dimethylvaleronitrile) (V-65) was purchased from Wako Pure Chemical Industry (Osaka, Japan) and used without further purification. (*S*)- and (*R*)-nilvadipine were gifts from Fujisawa Pharmaceutical Co. (Osaka, Japan). A racemic nilvadipine sample was prepared by mixing the two enantiomers equally. Nitrendipine, nicardipine, and nimodipine racemates were purchased from China Drug and Bioproduct Inspection Institute (Beijing, China). The structures of DHPs used in this study are illustrated in Figure 1. Other reagents and solvents of analytical-reagent grade were used without further purification. Water purified with a Nanopure II unit (Barnstead, Boston, MA) was used for the preparation of the mobile phase and the sample solution.

Preparation of MIPs. Uniformly sized MIPs for (*S*)-nilvadipine as well as nonimprinted polymers (NIPs) were prepared according to the method reported previously.[13] Briefly, a water dispersion of the uniformly sized, polystyrene seed particles (0.407 g mL^{-1}), 0.17 mL, was admixed with a microemulsion prepared from 0.48 mL of dibutyl phthalate as an activating solvent, 0.02 g of sodium dodecyl sulfate, and 10 mL of distilled water by sonication. This first-step swelling was carried out at room temperature for 15 h with stirring at 125 rpm until the oil microdroplets completely disappeared. To the swollen particles, a microemulsion prepared from 0.375 g of 2,2′-azobis(2,4-dimethylvaleronitrile) as an initiator, 5 mL of toluene, chloroform, or cyclohexanol as a porogenic solvent, 12.5 mL of water, and 10 mL of 4.8% poly(vinyl alcohol) solution as a dispersion stabilizer 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 25 mmol of EDMA as a cross-linker, a functional monomer (whose type and amount used are shown in Table 1), 12.5 mL of water, and 10 mL of 4.8% poly(vinyl alcohol) solution was added. This third-step swelling was

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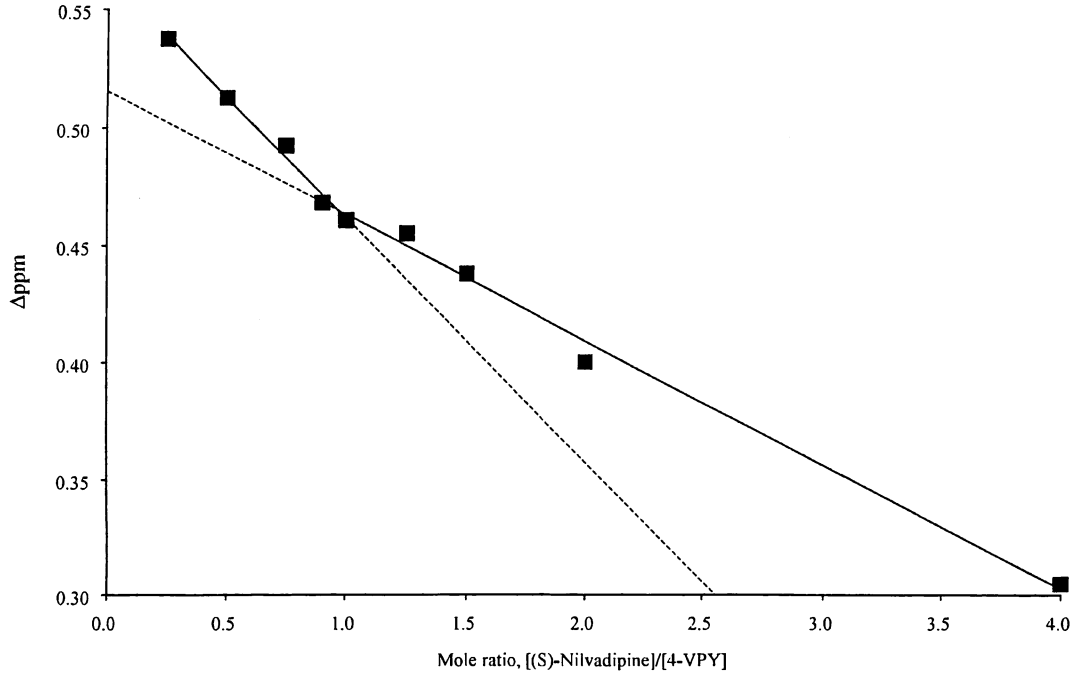

Figure 2. Mole ratio method for the complexation of (*S*)-nilvadipine and 4-VPY. Δppm is the difference between the chemical shift of NH proton of (*S*)-nilvadipine with and without addition of 4-VPY.

of 4-VPY, the chemical shift of the NH proton of (*S*)-nilvadipine resulted in drastic downfield shifts. This could be largely due to an increase in the basicity of the $CDCl_3$ solution with an increase in the molar fraction of 4-VPY. Thus, we used a mole ratio method, where a constant concentration of 4-VPY is used. The differences between the chemical shifts of the NH protons of (*S*)-nilvadipine with and without addition of 4-VPY were plotted against the mole ratio of (*S*)-nilvadipine to 4-VPY. As shown in Figure 2, with a decrease of the mole ratio of (*S*)-nilvadipine to 4-VPY, the downfield shift of the NH proton becomes larger. Furthermore, the plot reveals a one-to-one stoichiometry for the complex formation. These results show a one-to-one hydrogen-bonding-based complex formation of (*S*)-nilvadipine with 4-VPY in chloroform.

Figure 3A shows the energy map for the hydrogen-bonding complex model with the dihydropyridine and pyridine rings of (*S*)-nilvadipine and 4-VPY molecules, respectively, disposed coplanarly. The complex model indicates the minimum energy at the N—H···N atom distance of 2.5 Å. Figure 3B depicts the AM1-optimized structure of the minimum energy complex model. The stabilization due to H-bonding can be calculated using the following formula: ΔH stabilization = ΔH formation (the hydrogen-bonded complex) − ΔH formation ((*S*)-nilvadipine) − ΔH formation (4-VPY). Thus, the complex model is stabilized by 1.9 kcal mol^{-1} by the intermolecular hydrogen bonding between the $N-H_{(S)\text{-nilvadipine}}$ and $N_{4\text{-VPY}}$ residues. A similar result was obtained for the hydrogen-bonded model with the dihydropyridine and pyridine rings of (*S*)-nilvadipine and 4-VPY molecules, respectively, disposed perpendicularly to each other. The ^1H NMR and molecular modeling results imply that the hydrogen-bonding interaction between (*S*)-nilvadipine and 4-VPY is plausible in a nonpolar solvent.

Retention Properties of DHPs on the MIPs for (*S*)-Nilvadipine. As shown in Table 1, 10 MIPs for (*S*)-nilvadipine,

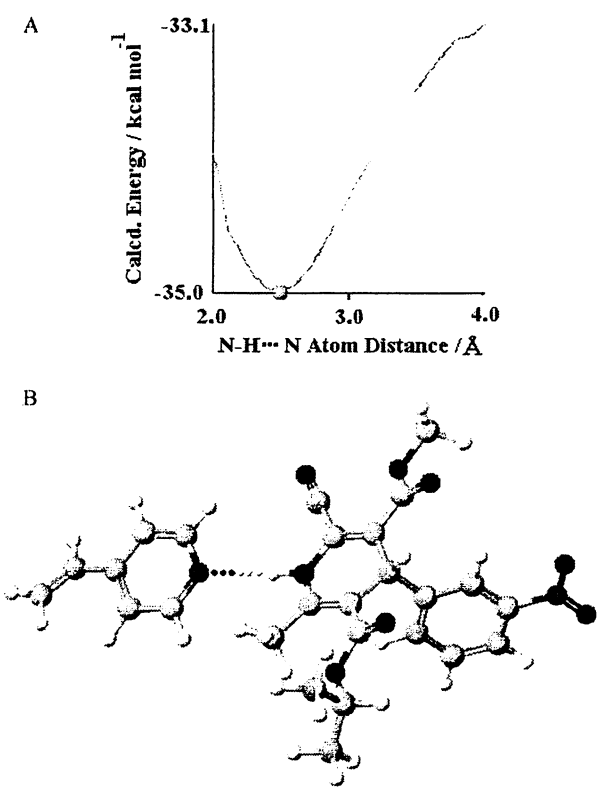

Figure 3. Hydrogen-bonding potential map for the (*S*)-nilvadipine and 4-VPY complex (A) and the AM1-optimized structure of the minimum energy complex (B).

MIPs 1−10, were prepared and their molecular recognition abilities were compared using a mixture of phosphate buffer and acetonitrile as the mobile phase. Figure 4A shows the effect of mobile-phase pH on the retention properties of (*S*)-nilvadipine on MIPs 1, 2 and 5, where the mobile phases used were a mixture

Table 1. Preparation of (*S*)-Nilvadipine-Imprinted Polymers

	amount (mmol)				
		functional monomer			
polymer	template	type	amount	cross-linker	porogen[a]
MIP 1	2	none	0	25	toluene
MIP 2	2	MAA	7	25	toluene
MIP 3	2	TFMAA	6	25	toluene
MIP 4	2	2-VPY	6	25	toluene
MIP 5	2	4-VPY	6	25	toluene
MIP 6	1	4-VPY	3	25	toluene
MIP 7	2	4-VPY	3	25	toluene
MIP 8	4	4-VPY	6	25	toluene
MIP 9	4	4-VPY	6	25	chloroform
MIP 10	4	4-VPY	6	25	cyclohexanol

[a] Five milliliters of porogen was used.

carried out at room temperature for 2 h with stirring at 125 rpm. When the template molecule was added, (*S*)-nilvadipine was admixed with EDMA or EDMA and a functional monomer by sonication 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 stirring at 125 rpm for 24 h. The dispersion of polymerized beads was poured into 200 mL of water to remove the suspension stabilizer, and the supernatant was discarded after sedimentation of the beads. The polymer beads were redispersed into methanol, and the supernatant was again discarded after sedimentation. This procedure was repeated three times in methanol and twice in tetrahydrofuran; then the polymer beads were filtered on a membrane filter and washed with tetrahydrofuran and acetone, followed by drying at room temperature.

The prepared beads were packed into a stainless steel column (100 mm × 4.6 mm i.d.) by a slurry packing technique using methanol–2-propanol as the slurry solvent and methanol as the packing solvent to evaluate their chromatographic characteristics.

NMR Studies. ^{1}H NMR spectra were recorded on a JEOL FT-NMR model ECP-500 (Tokyo, Japan) at 35 °C. The sample solution contained (*S*)-nilvadipine at various concentrations of 2.5–40 mM and 10 mM 4-VPY in $CDCl_3$. ^{1}H NMR measurement was also carried out for the same (*S*)-nilvadipine solution in the absence of 4-VPY. Chemical shifts are referenced to tetramethylsilane.

Molecular Modeling. Semiempirical MO calculations were carried out with CAChe MOPAC version 94 implemented in CAChe programs[23] run on a Windows 98-based desktop PC. Molecular geometries of (*S*)-nilvadipine and 4-VPY are optimized by the AM1 method. In all cases, the PRECISE option was used to provide higher accuracy within the calculation. The initial molecular geometry for (*S*)-nilvadipine was taken from the X-ray structure data[24] and that of 4-VPY was generated by the CAChe Editor. The hydrogen-bonded complex model was constructed by arranging the dihydropyridine and pyridine rings of (*S*)-nilvadipine and 4-VPY molecules, respectively, in the same plane. The structures and heat of formation values of the hydrogen-bonding model were calculated at the N–H···N atom distances of 2.0–4.0 Å between the dihydropyridine ring of (*S*)-nilvadipine and the pyridine ring of 4-VPY with the fixed N–H···N angle of 180°. The simulation was also performed on the hydrogen-bonding complex model with the dihydropyridine and pyridine rings of (*S*)-nilvadipine and 4-VPY molecules, respectively, disposed perpendicularly to each other.

Evaluation of MIPs. The HPLC system used was composed of a Jasco 880-PU pump, a 875-UV/VIS detector (both from Jasco, Tokyo, Japan), a Rheodyne 7125 injector with a 5-μL loop, and a C–R6A integrator (Shimadzu, Kyoto, Japan). The flow rate was maintained at 1.0 mL min^{-1} (unless otherwise stated). Detection was performed at 236 nm for DHPs. The retention factor (k) was 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 the unretained solute, t_0, was measured by injecting methanol. The enantioseparation factor (α) was 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. Resolution (Rs) was calculated from the equation $Rs = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, where t_{R2} and t_{R1} are the retention times of the first- and second-eluted enantiomers, respectively, and w_1 and w_2 are the baseline peak widths of the first- and second-eluted enantiomers, respectively. The selectivity factor (S) was calculated from the equation $S = k_{MIP}/k_{NIP}$, where k_{MIP} and k_{NIP} are the retention factors of a solute on the MIP and NIP, respectively. The number of theoretical plates (N) was calculated from the equation $N = 16(t_R/w)^2$. The column temperature was controlled by a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The mobile phases were prepared using phosphoric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, trisodium phosphate, or acetonitrile. The mobile phases used are specified in the legends of tables and figures.

Sample Preparation. As DHPs were insoluble in water and unstable to light, the samples were first dissolved in methanol at a concentration of ~1 mg mL^{-1}, kept away from light, and diluted to a concentration of 0.1 mg mL^{-1} with water before use. A 5-μL aliquot of the sample solution was loaded onto the column.

Comparison of (*S*)-Nilvadipine-Imprinted 4-VPY-*co*-EDMA Polymers with Chiral Stationary Phases Based on a Protein. The OVM (Ultron ES-OVM, 150 mm × 4.6 mm i.d.) and α_1-AGP (Chiral-AGP, 100 mm × 4.0 mm i.d.) columns were obtained from Shinwa Chemical Industries (Kyoto, Japan) and ChromTech AB (Norsborg, Sweden), respectively. The k, α, Rs, and N values of nilvadipine on the (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers (MIP 8) were compared with those on OVM or α_1-AGP columns.

Safety Considerations. Monomers (EDMA, TFMAA, MAA, 4-VPY, 2-VPY) are toxic by inhalation or in contact with skin. These compounds should be handled with special care.

RESULTS AND DISCUSSION

Interaction between (*S*)-Nilvadipine and 4-VPY. The interaction between (*S*)-nilvadipine and 4-VPY at the prepolymerization stage has been investigated by ^{1}H NMR spectroscopy. First, we used a continuous variance method, where a molar fraction of (*S*)-nilvadipine and 4-VPY was changed in order to evaluate their interactions. However, with an increase of the molar fraction

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Table 2. Retention Factor of DHPs on MIPs for (*S*)-Nilvadipine and NIPs[a]

	retention factor									
solute	MIP 1	NIP 1	MIP 2	NIP 2	MIP 3	NIP 3	MIP 4	NIP 4	MIP 5	NIP 5
(*S*)-nilvadipine	20.2	14.3	10.1	6.53	11.0	5.82	27.6	17.5	24.5	12.6
nitrendipine	12.7	9.91	7.05	5.00	7.36	4.42	17.2	11.9	11.3	8.04
nicardipine	19.2	17.5	48.0	36.2	205	55.8	22.3	16.0	14.7	10.7
nimodipine	12.9	10.1	7.01	5.01	7.21	4.26	17.2	11.9	10.6	7.59

[a] HPLC conditions as in Figure 4 except that the mobile phase used was 20 mM phosphate buffer (pH 6.0)–acetonitrile (60:40, v/v).

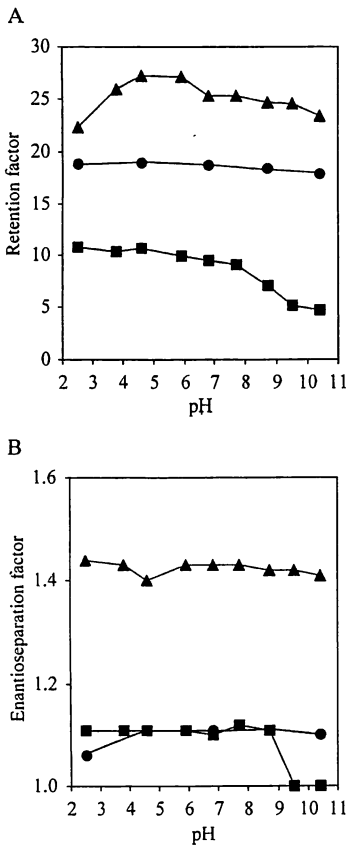

Figure 4. Effect of mobile-phase pH on the retention (A) and enantioseparation (B) factors of (*S*)-nilvadipine on MIPs 1, 2, and 5. Key: ●, MIP 1; ■, MIP 2; ▲, MIP 5. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; column temperature, 25 °C; mobile phase, 20 mM phosphoric acid or sodium phosphate, or both–acetonitrile (60:40, v/v); detection, 236 nm; flow rate, 1.0 mL min^{-1}. Loaded amount, 0.5 μg.

of 20 mM phosphoric acid or sodium phosphate, or both, and acetonitrile. Since the decomposition of nilvadipine was observed above mobile-phase pH 10.5, the pH range examined was from pH 2.5 to 10.5. On these polymers, the retention factor of (*S*)-nilvadipine increased in the order of MIPs 2, 1, and 5. On MIP 1, it remained unchanged from pH 2.5 to 10.5. This suggests that nilvadipine might have no acid–base properties in a mixture of phosphate buffer and acetonitrile. On MIP 2, it was gradually decreased with an increase of the mobile-phase pH, and the decrease was accelerated around pH 7.5. This result could be explained by the deprotonation of the carboxyl groups in the polymers, whose average pK_a value is reported to be ~9.[25] The retention tendency observed for (*S*)-nilvadipine on MIP 3 was similar to that on MIP 2 except that (*S*)-nilvadipine was retained more on MIP 3 and that a decrease in the retention factor was accelerated around pH 6. The latter could be ascribable to differences in the pK_a values of MAA and TFMAA, which are 4.5 and 3.0, respectively.[26] On MIP 5, the retention factor of (*S*)-nilvadipine remained unchanged from mobile-phase pH 4 to 10.5, while a slight decrease was observed below pH 4. This could be explained by the protonation of pyridyl groups in the polymer, whose average pK_a value is reported to be below 3.[27] The retention tendency observed for (*S*)-nilvadipine on MIP 4 was similar to that on MIP 5 except that (*S*)-nilvadipine was retained more on MIP 4.

Table 2 shows the retention factors of (*S*)-nilvadipine, nitrendipine, nicardipine, and nimodipine on the various MIPs for (*S*)-nilvadipine (MIPs 1–5) and the respective NIPs (NIPs 1–5), where a mixture of phosphate buffer (pH 6.0) and acetonitrile was used as the mobile phase. (*S*)-Nilvadipine was retained the most among the DHPs tested on MIPs 1, 4, and 5, while nicardipine was retained the most on MIPs 2 and 3. The former is due to a molecular imprinting effect, while the latter is due to ionic interactions of the tertiary amine group of nicardipine with a carboxyl group in the polymers. On the polymers prepared using basic functional monomers, i.e., 4-VPY and 2-VPY, (*S*)-nilvadipine was retained more than on those using acidic monomers, i.e., MAA and TFMAA.

As shown in Figure 4B, the enantioseparation factors of nilvadipine on MIPs 1, 2, and 5 were not affected by the mobile-phase pH over the range tested except for 9.5–10.5 in the case of MIP 2, where no enantioseparation was attained. Furthermore, the enantioseparation factor for nilvadipine on MIP 1 was almost the same with that on MIP 2 except in the mobile-phase pH range of 9.5–10.5 and that on MIP 5 was higher than those on MIPs 1 and 2. Table 3 shows the retention factor, enantioseparation factor, and resolution of nilvadipine on the various MIPs for (*S*)-nilvadipine (MIPs 1–5) and the respective NIPs (NIPs 1–5). The NIPs did not show enantioselectivity for nilvadipine, but all of the MIPs did. The enantioseparation factors of 1.11, 1.11, and 1.13, respectively, for nilvadipine were obtained with MIPs 1, 2, and 3, respectively. It is interesting that MIP 1, which was prepared without use of a functional monomer, could recognize the template molecule, (*S*)-nilvadipine, and that the recognition occurs based on the molecular shape.[28] On the other hand, MIP 5 gave higher retention and enantioseparation factors for nilvadipine than MIPs

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(26) The pK_a values of MAA and TFMAA were determined from the titration curves of MAA and TFMAA with a 0.1 M NaOH solution.
(27) Haginaka, J.; Sanbe, H.; Takehira, H. *J. Chromatogr., A* **1999**, *857*, 117–125.

Table 3. Retention Factor, Enantioseparation Factor, and Resolution of Nilvadipine Racemate on MIPs for (*S*)-Nilvadipine and NIPs[a]

	nilvadipine			
polymer	k_R	k_S	α	Rs
MIP 1	16.9	18.9	1.11	0.67
NIP 1	13.6	13.6	1.00	
MIP 2	9.65	10.7	1.11	0.45
NIP 2	7.85	7.85	1.00	
MIP 3	13.2	14.9	1.13	0.68
NIP 3	9.79	9.79	1.00	
MIP 4	27.0	30.0	1.12	0.82
NIP 4	18.2	18.2	1.00	
MIP 5	19.4	27.2	1.40	1.18
NIP 5	13.5	13.5	1.00	

[a] HPLC conditions as in Figure 4 except that the mobile phase used was 20 mM phosphate buffer (pH 4.0)–acetonitrile (60:40, v/v).

Table 4. Retention Factor, Enantioseparation Factor, and Resolution of Nilvadipine Racemate on MIP 8[a]

concn of acetonitrile (%)	nilvadipine			
	k_R	k_S	α	Rs
40	20.0	32.1	1.60	1.51
60	2.84	3.80	1.37	0.99
80	0.58	0.78	1.35	0.64
100	1.36	1.66	1.22	0.59

[a] HPLC conditions as in Figure 4 except that the mobile phase used was a mixture of H_2O and acetonitrile.

1, 2, and 3. The results suggest that, besides the molecular shape recognition, the hydrogen-bonding interactions of the NH proton of nilvadipine with a pyridyl group in the polymers as well as the hydrophobic interactions with the polymer backbones could play important roles in the retention and chiral recognition of nilvadipine in the hydroorganic mobile phase. Although MIP 4 showed longer retention for nilvadipine than MIP 5, the former gave lower enantioselectivity than the latter. Enantioseparations of the other DHPs (nitrendipine, nicardipine, nimodipine) could not be achieved on the MIPs prepared.

Table 4 shows the effect of acetonitrile content on the retention factor, enantioseparation factor, and resolution of nilvadipine on MIP 8. With an increase in the acetonitrile content, the retention and enantioseparation factors of nilvadipine decreased when the hydroorganic mobile phase was used. When 100% acetonitrile was used as the mobile phase, the retention factor of nilvadipine slightly increased. These results reveal that, in addition to the molecular shape recognition, hydrophobic and hydrogen-bonding interactions can play important roles in the hydroorganic mobile phase, and hydrogen-bonding interactions can work for the recognition when acetonitrile is used as the mobile phase.

Selectivity of (*S*)-Nilvadipine-Imprinted Polymers for DHPs and Acidic, Basic, and Neutral Compounds. Figure 5 shows the selectivity factor for DHPs and acidic, basic, and neutral solutes on the various MIPs (MIPs 1–5). Among the MIPs, MIP 5 gave the highest selectivity factor of 2.01 for (*S*)-nilvadipine. The selectivity factors for nitrendipine, nicardipine, and nimodipine

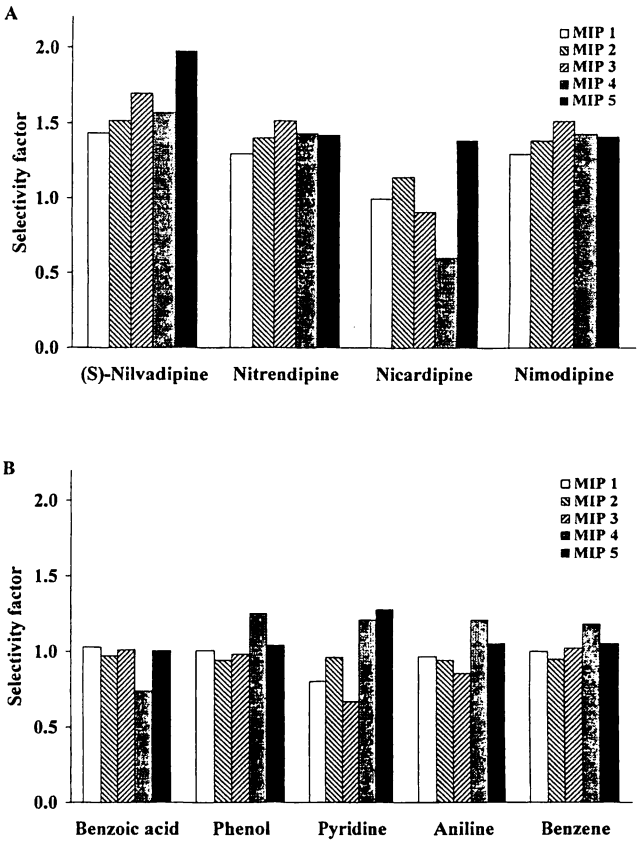

Figure 5. Selectivity factor of DHPs (A) and neutral, acidic, and basic solutes (B) on MIPs 1–5. HPLC conditions as in Figure 4 except that the mobile phase used was 20 mM phosphate buffer (pH 4.0)–acetonitrile (60:40, v/v).

Table 5. Retention Factor, Enantioseparation Factor, and Resolution of Nilvadipine Racemate on MIPs 5–8[a]

	nilvadipine			
polymer	k_R	k_S	α	Rs
MIP 5	19.4	27.2	1.40	1.18
MIP 6	16.3	20.2	1.24	0.88
MIP 7	18.0	24.1	1.34	1.07
MIP 8	19.5	30.5	1.57	1.49

[a] HPLC conditions as in Figure 4 except that the mobile phase used was 20 mM phosphate buffer (pH 4.0)–acetonitrile (60:40, v/v).

were 1.41, 1.40, and 1.42, respectively, on MIP 5, while those for benzoic acid, phenol, benzene, pyridine, and aniline were ~1. The results indicate that MIP 5 shows the highest selectivity for nilvadipine and moderate selectivity for other DHPs, but little selectivity for structurally unrelated acidic, basic, and neutral compounds.

Optimization of Preparation Methods for the (*S*)-Nilvadipine-Imprinted 4-VPY-*co*-EDMA Polymers. Next, we optimized preparation methods for the (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers. Table 5 shows the effect of a molar ratio of the template molecule to the functional monomer on the retention factor, enantioseparation factor, and resolution of nilvadipine on MIPs 5–8. With an increase of the molar amounts of the template molecule and functional monomer, the retention factor, enantioseparation factor, and resolution increased (see

(28) Hosoya, K.; Yoshizako, K.; Shirasu, Y.; Kimata, K.; Araki, T.; Tanaka, N.; Haginaka, J. *J. Chromatogr., A* **1996**, *728*, 139–147.

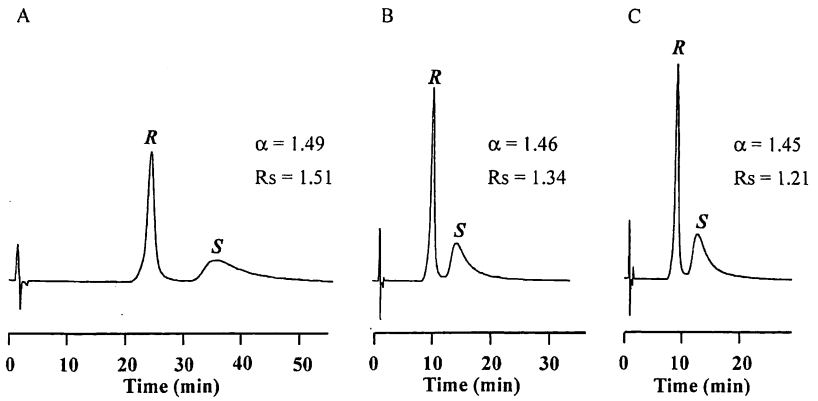

Figure 6. Effect of flow rate on the separation of nilvadipine enantiomers on MIP 8. Flow rate: (A) 0.5, (B) 1.0, and (C) 1.5 mL min^{-1}. HPLC conditions, column size, 100 mm × 4.6 mm i.d.; column temperature, 40 °C; mobile phase, 20 mM sodium phosphate buffer (pH 4.0)–acetonitrile (55:45, v/v); detection, 236 nm; loaded amount, 0.5 μg.

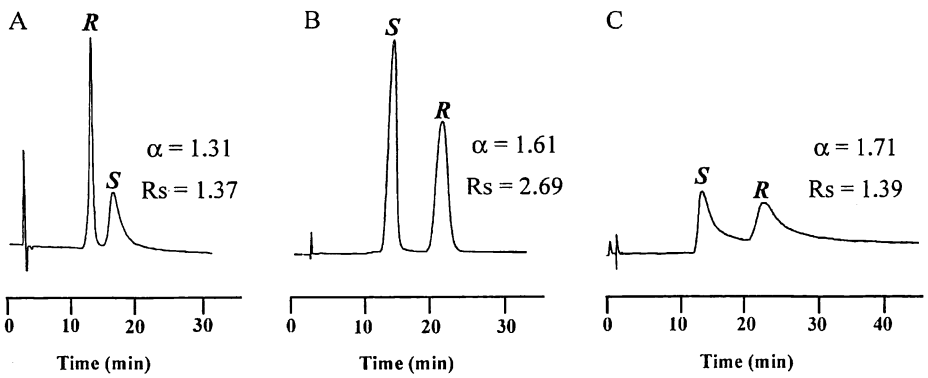

Figure 7. Comparison of enantioseparation of nilvadipine on MIP 8 (A) with OVM (B) and α_1-AGP (C) columns. HPLC conditions for (A): column size, 100 mm × 4.6 mm i.d.; column temperature, 70 °C; mobile phase, 20 mM sodium phosphate buffer (pH 4.0)–acetonitrile (55:45, v/v); flow rate, 0.5 mL min^{-1}. HPLC conditions for (B): column size, 150 mm × 4.6 mm i.d.; column temperature, 25 °C; mobile phase, 10 mM sodium phosphate buffer (pH 4.7)–ethanol (80:20, v/v); flow rate, 1.0 mL min^{-1}. HPLC conditions for (C): column size, 100 mm × 4.0 mm i.d.; column temperature, 25 °C; mobile phase, 30 mM sodium phosphate buffer (pH 6.5)–2-propanol (92.5:7.5, v/v); flow rate, 0.9 mL min^{-1}. For all experiments, the loaded amount was 0.5 μg and the detection wavelength was 236 nm.

MIPs 5–8). Note that the retention factor of the first-eluted enantiomer, (*R*)-nilvadipine, is almost the same between the latter two MIPs, while the second-eluted enantiomer is retained more on MIP 8. As a result, MIP 8 gave the highest enantioselectivity. It was reported that the choice of porogen has affected both the porosity of MIPs and swelling properties.[29] We prepared MIPs 8–10 using toluene, chloroform, and cyclohexanol as the porogen. Though all MIPs showed enantioselectivity for nilvadipine, MIP 8 gave the highest enantioselectivity among the MIPs (data not shown). In the following experiments, we used MIP 8.

Effects of Column Temperature and Flow Rate on Enantioseparation of Nilvadipine on (*S*)-Nilvadipine-Imprinted 4-VPY-*co*-EDMA Polymers. Table 6 shows the effect of column temperature on the separation of nilvadipine enantiomers on MIP 8. With an increase of column temperature, the higher number of theoretical plates could be obtained, but the enantioseparation factor became lower. As a compromise, the highest resolution was achieved at a column temperature of 40 °C. Figure 6 shows the effect of flow rate on the separation of nilvadipine enantiomers. With a decrease of flow rate, higher enantioselectivity and resolution could be obtained despite a longer retention time. By optimizing chromatographic conditions such as column temperature and flow rate, the baseline separation of nilvadipine enantiomers could be achieved with a short analysis time.

Table 6. Effect of Column Temperature on the Separation of Nilvadipine Enantiomers on MIP 8[a]

column temp (°C)	nilvadipine					
	k_R	k_S	α	Rs	N_R	N_S
25	11.1	17.5	1.57	1.45	1904	126
30	10.1	15.7	1.55	1.45	2071	131
40	8.53	12.8	1.49	1.51	2205	150
50	7.14	10.2	1.43	1.49	2232	166
60	5.92	8.05	1.36	1.44	2429	243
70	4.87	6.36	1.31	1.37	2724	324

[a] HPLC conditions as in Figure 4 except that the mobile phase used was a mixture of 20 mM phosphate buffer (pH 4.0)–acetonitrile (55:45, v/v) and the flow rate was 0.5 mL min^{-1}.

Comparison of (*S*)-Nilvadipine-Imprinted 4-VPY-*co*-EDMA Polymers with OVM and α_1-AGP Columns. As shown in Figure 7, the enantioseparation of nilvadipine on MIP 8 was compared with those on commercially available chiral stationary phases base on a protein, i.e., OVM or α_1-AGP. The second-eluted enantiomer on MIP 8 was (*S*)-nilvadipine, while the elution order was reversed on OVM and α_1-AGP columns. The number of

(29) Spivak, D. A.; Shea, K. J. *Anal. Chim. Acta* **2001**, *435*, 65–74.

theoretical plates of the first-eluted enantiomer on MIP 8 was higher than those on the two protein-based columns, while that of the second-eluted enantiomer on MIP 8 was between the OVM and α_1-AGP columns. Furthermore, resolution of nilvadipine enantiomers on MIP 8 was lower than that on the OVM column but was similar to that on the α_1-AGP column. The baseline separation of nilvadipine enantiomers could be attained on the artificial, (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers with a short analysis time and with a column efficiency comparable to chiral stationary phases based on commercially available, naturally occurring OVM and α_1-AGP. This is the first report of a chiral MIP offering column efficiency and resolution for a target compound comparable to commercially available chiral stationary phases.

In addition, the (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers were stable against changes of mobile-phase composition (pH, organic modifier content), column temperature, and flow rate. There were almost no changes of retention and enantioseparation factors of nilvadipine throughout the experiments described above.

Application of (*S*)-Nilvadipine-Imprinted 4-VPY-*co*-EDMA Polymers to Purity Test of (*S*)-Nilvadipine. Panels A and B of Figure 8 show chromatograms of a racemic mixture of nilvadipine enantiomers and (*S*)-nilvadipine, respectively. Using MIP 8, 0.025% of the (*R*)-antipode could be separated and determined precisely with a relative standard deviation of 5.4%.

CONCLUSIONS

We prepared uniformly sized MIPs for (*S*)-nilvadipine using MAA, TFMAA, 2-VPY, or 4-VPY as a functional monomer and EDMA as a cross-linker. The prepared (*S*)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers showed the highest molecular recognition ability for nilvadipine among the MIPs prepared. ^{1}H NMR and molecular modeling studies suggested a one-to-one hydrogen-bonding-based complex formation of (*S*)-nilvadipine with 4-VPY in chloroform. According to the results obtained, not only molecular shape recognition but also hydrophobic and hydrogen-bonding interactions can play important roles in the retention and chiral recognition of nilvadipine on the 4-VPY-*co*-EDMA polymers in hydroorganic mobile phases. By optimizing chromatographic conditions such as column temperature and flow rate, the baseline separation of nilvadipine enantiomers was attained with a short analysis time and with a column efficiency comparable to commercially available chiral stationary phases based on a protein, i.e., OVM or α_1-AGP. The MIPs for (*S*)-nilvadipine should be useful as the chiral stationary phase for analytical and preparative purposes.

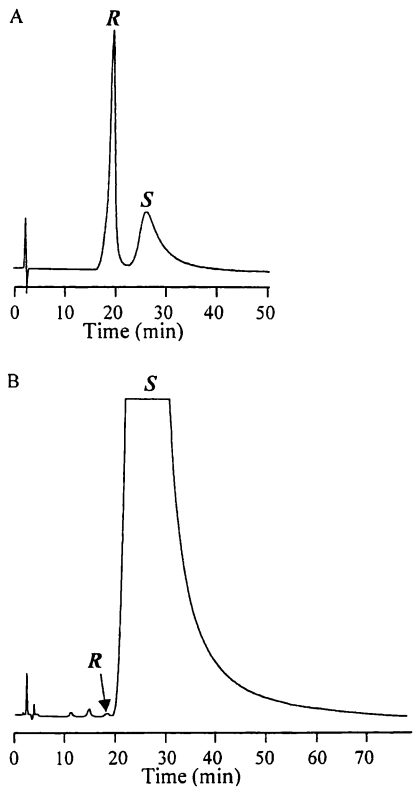

Figure 8. Chromatograms of a racemic mixture of nilvadipine enantiomers (A) and (*S*)-nilvadipine (B) on MIP 8. HPLC conditions: column size, 100 mm × 4.6 mm i.d.; column temperature, 50 °C; mobile phase, H_2O–acetonitrile (55:45, v/v); flow rate, 0.5 mL min^{-1}; detection, 236 nm.

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