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Open tubular capillary columns with basic templates made by the generalized preparation protocol in capillary electrochromatography chiral separation and template structural effects on chiral separation capability

Shabi Abbas Zaidi, Seung Mi Lee, Won Jo Cheong*

Department of Chemistry, Nano Fine Center, and Institute of Basic Research, Inha University, 253 Yonghyung-Dong, Nam-ku, Incheon 402-751, South Korea

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ABSTRACT

Some open tubular (OT) molecule imprinted polymer (MIP) silica capillary columns have been prepared using atenolol, sulpiride, methyl benzylamine (MBA) and (1-naphthyl)-ethylamine (NEA) as templates by the pre-established generalized preparation protocol. The four MIP thin layers of different templates showed quite different morphologies. The racemic selectivity of each MIP column for the template enantiomers was optimized by changing eluent composition and pH. The template structural effects on chiral separation performance have been examined. This work verifies the versatility of the generalized preparation protocol for OT-MIP silica capillary columns by extending its boundary toward templates with basic functional group moieties. This study is the very first report to demonstrate a generalized MIP preparation protocol that is valid for both acidic and basic templates. The chiral separation performances of atenolol and sulpiride by the MIPs of this study were found better than or comparable to those of atenolol and sulpiride obtained by non-MIP separation techniques and those of some basic template enantiomers obtained by MIP based techniques.

1. Introduction

Pharmaceuticals often exhibit chiral structures and are most widely used as racemic mixtures. The pharmacological activity of these drugs, however, resides predominantly in the S-enantiomer and there are some side effects related to the R-enantiomer [1]. Such difference in pharmacological and pharmacokinetic effects between the two enantiomers has driven a strong demand for effective methods of enantioselective separation.

Molecular imprinting technology provides a way to synthesize the polymer with cavities of a template and the polymer is subsequently capable of selectively rebinding the template [2–4]. Molecular imprinted polymers (MIPs) have attracted significant interest in separation science because of their unique pre-determinative selectivities and practical abilities for template recognition. In the last two decades, this technique has been employed in variety of applications such as pharmaceutical study, environmental chemistry and chromatographic separation as well as isolation of drugs, toxins, pesticides and food components [5–7]. Although MIPs have shown a great potential in HPLC based analyses, the use of MIP coupled with CEC is still trying to make its niche in analytical science [8–10]. MIP–CEC studies have been well introduced in a review article [11]. Monodispersed spherical MIP particles as chromatographic media [12] and MIPs as media for sample preparation [13] have also been extensively reviewed in the recent literature.

Owing to the versatility and the potential that can be achieved, the future of this technique is promising. The basic concepts of MIP preparation are rather simple and easy. However, to obtain a highly efficient MIP requires tedious optimization in the formulation of polymerization mixture and reaction conditions via synthesis and evaluation of various polymers in extensive experiments. Furthermore, a very different MIP preparation protocol is generally required to resolve a new pair of enantiomers. The entire procedure is time consuming and needs to be generalized. In a particular study, a computational method (MIP dialing) using combinatorial screening and molecular modeling was proposed as a general procedure for fast preparation of a specific MIP [14]. In another report, the cross selectivity MIP synthesized using only one imprint showed chiral separation for several pairs of β-blockers [15].

Recently, we demonstrated a generalized preparation protocol for several pairs of non-steroidal anti inflammatory drugs (NSAIDs) by fabricating short OT-MIP CEC columns [16]. In one of our earlier reports, similar MIP preparation protocol was employed to acquire long open tubular molecularly imprinted columns (OT-MIP) in capillary, which offered exceptionally high chiral separation efficiencies for ketoprofen enantiomers [17]. We have

* Corresponding author. Tel.: +82 328607673; fax: +82 328675604.
E-mail address: wjcheong@inha.ac.kr (W.J. Cheong).

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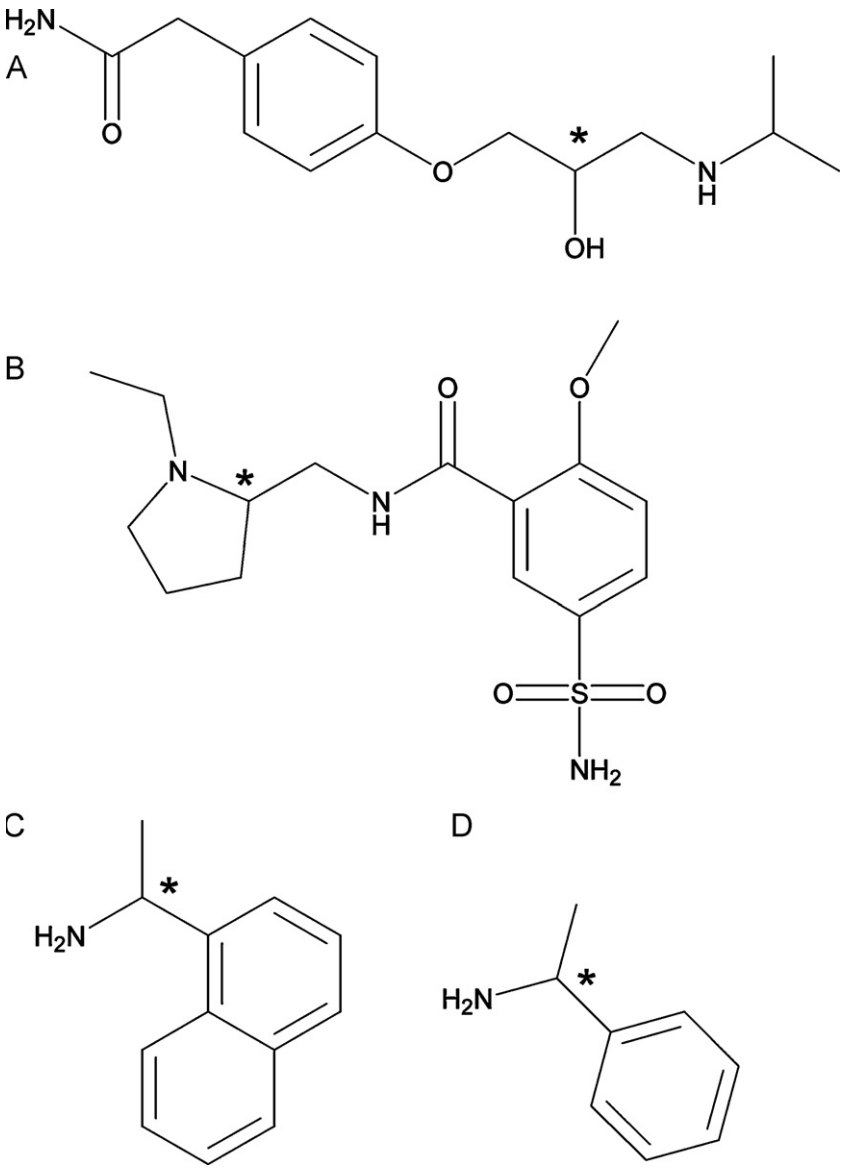


Fig. 1. Molecular structures of the templates employed in this study. (A) Atenolol, (B) sulpiride, (C) (1-naphthyl)-ethylamine, and (D) methyl benzylamine.

further extended the generalized protocol to some additional acidic templates and camphor derivatives [18,19]. However, the real generalized MIP preparation protocol should be valid for a variety of templates of a wide range of different chemical properties including typically opposing subgroups such as acidic vs. basic templates.

Thus, synthesis of MIPs with basic templates and their application in chiral separation have been explored in this work. The basic templates used in this study are atenolol, sulpiride, methyl benzylamine (MBA) and (1-naphthyl)-ethylamine (NEA). Atenolol is a β-blocker agent being used in the treatment of angina pectoris, hypertension, arrhythmias and in several cardiovascular disorders while sulpiride is a selective dopamine D_2 antagonist with antipsychotic and antidepressant activity. Several chromatographic separation procedures were employed for chiral separation of racemic atenolol, including HPLC separation on a Chiralcel OD column in normal phase mode [20], CE separation with dual cylodextrin additives composed of β-cyclodextrin and β-cyclodextrin–glucose [21], derivatization with a fluorogenic reagent followed by separation on a Chrialcel OJ-R column in reversed phase mode [22] to show excellent detection sensitivity, and HPLC separation on a Chiralpak AD-H column in normal phase mode with a small amount of ethanesulfonic acid incorporated in the eluent bringing up dramatically improved chromatographic resolution [23].

Chiral separation of sulpiride has been carried out by various chromatographic techniques such as CE with 2% sulfated β-cyclodextrin added to the mobile phase as a chiral selector [24], HPLC with a chiral cellobiohydrolase (CBH) column [25], and CE based on partial filling technique [26].

In addition to the above well known basic drugs, two other structurally different basic templates, methyl benzylamine (MBA) and (1-naphthyl)-ethylamine (NEA), were included in the group of basic templates to investigate the effects of template structure on the chiral recognition capability of the resultant MIPs.

This work handles the significant task of fabricating and evaluating OT-MIP capillary columns with basic templates using the generalized preparation protocol previously developed for acidic templates. The molecular structures of the basic templates employed in this study are given in Fig. 1.

2. Experimental

2.1. Chemicals and materials

Fused silica capillaries (50 μm ID, 365 μm OD) were purchased from Alltech (Deerfield, IL, USA). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), γ-methacryloxypropyl trimethoxysilane (γ-MAPS), 4-styrenesulfonic acid (4-SSA), sodium bihydrogen phosphate, sodium hydroxide, R-enantiomer of atenolol and S-enantiomers of sulpiride, methyl benzylamine (MBA) and (1-naphthyl)-ethylamine (NEA), and racemic analytes were obtained from Sigma–Aldrich (St. Louis, MO, USA). Azobisisobutyronitrile (AIBN) was purchased from Junsei Chemical (Tokyo, Japan). HPLC grade acetonitrile and water were obtained from SK Chemicals (Ulsan, Korea). All of the reagents except for 4-styrenesulfonic acid (sodium salt hydrate) were used as received. The acidification of 4-SSA was carried out as introduced in our previous report [4].

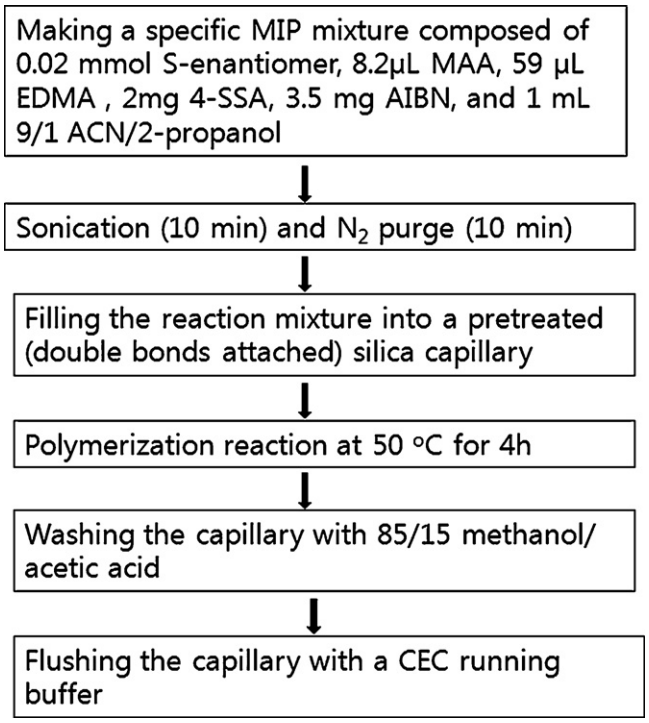
2.2. Instrumentation

Electrochromatographic experiments were performed with an Agilent (Waldbronn, Germany) HP^{3D} CE system with a diode array detector and the Chemstation data processing software was used. The electrolytes were prepared by mixing 50 mM NaH_2PO_4 and NaOH to the desired pH in the range of 6.0–10.0. The buffers were then added to acetonitrile in the desired proportions at the time of analysis. All analytical samples were made to a concentration of 1.0 mg/mL by dissolving in the mobile phase and further diluted. All the samples and eluents were filtered through a 0.2 μm cellulose membrane before analysis. Samples were injected hydrodynamically for 7 s under a pressure of 6 mbar. The detection wavelength was set to 214 nm. All the separations were carried out at a constant CE voltage of 25 kV and a temperature of 25 °C throughout. Each newly installed OT-MIP column was flushed with running the eluent for about 1 h to acquire the stable baseline and flushed with the same eluent for 5 min between consecutive analyses. The effective length of all the MIP columns was 28 cm (total length: 36.4 cm).

2.3. Fabrication of open tubular S-enantiomer MIP capillary columns with basic template molecules

The fused silica capillary was modified according to the procedure published elsewhere [17]. Briefly, the silica capillary was treated with 1 M NaOH, washed with water, 0.1 M HCl, water, and acetone in sequence, and dried under a flow of N_2. A solution composed of 4 μL γ-MAPS in 1 mL of 6 mM acetic acid was filled in the capillary for 6 h and the capillary was flushed thoroughly with methanol and dried under a nitrogen flow.

A thin film of each MIP was fabricated inside the capillary as reported in the previous study [16] except that the various templates were replaced with the same molar amount of R-enantiomer of atenolol and S-enantiomer of sulpiride, methyl benzylamine and (1-naphthyl)-ethylamine. Each capillary was filled with the degassed mixture composed of template molecule (0.020 mmol), MAA (8.2 μL), EDMA (59 μL), 4-SSA (2 mg) and AIBN (3.5 mg) dissolved in 1 mL 90/10 (v/v) acetonitrile/2-propanol, by a syringe installed with a 0.2 μm syringe filter and the capillary ends were sealed. Then the capillary was kept in a water bath at 50 °C for 4 h. After polymerization reaction, the capillary was taken out and immediately rinsed extensively with methanol–acetic acid (85/15, v/v %) and acetonitrile in sequence. A small piece of capillary tip was cut for SEM characterization. A detection window was created at a distance of 8.4 cm from the outlet end of each OT-MIP capillary column by burning the polyimide coating. The capillary column was then installed in the instrument for analysis. At least three replicate runs, were carried out on different days to check reproducibility.



Scheme 1. The schematic of the general preparation protocol of MIP OT-CEC capillary columns.

3. Result and discussion

We had demonstrated that the template molecular structure influences the morphology of the resultant MIP and its chiral separation performance in the previous studies. We also paid a special attention to examine how the properties of the MIP were influenced by variation of template molecular structure in this study where basic templates were incorporated. In addition, systematic investigation was carried out for optimization of the chiral separation performance of each MIP column by varying the chromatographic parameters such as eluent composition and pH.

The schematic of the general OT MIP preparation protocol is given in Scheme 1. The superior performance of the MIP capillary columns prepared by this protocol may be based on the choice of proper capillary dimension, the specific and rather dilute polymerization mixture enabling formation of a rugged and porous MIP layer with a proper thickness, and secured optimization in CEC separation [17].

3.1. Morphological architecture of OT-MIP columns

We successfully prepared open tubular MIP columns with the basic templates. R-enantiomer of atenolol and S-enantiomer of sulpiride, MBA and NEA were used as the templates. Each template has at least an aromatic ring and an amine group (Fig. 1). The SEM photos of the MIP layers formed on the capillary inner wall with various templates are compared in Fig. 2(A–F). Some SEM pictures of Fig. 2(E and F) were adopted from our earlier work [16] for the comparison purpose. As shown in Fig. 2, some variation was observed in the surface morphology of each MIP layer. The surface morphology and molecular recognition capability of MIP are known to be affected by choice of a porogenic solvent [27–29]. It is also known

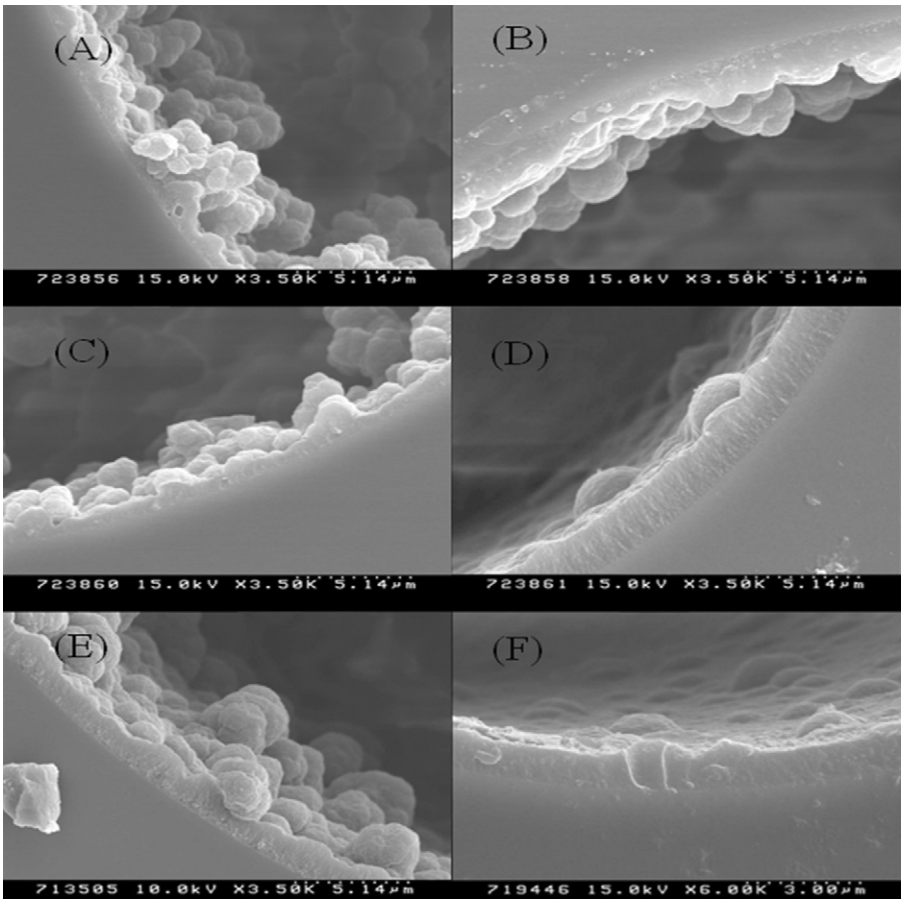


Fig. 2. SEM photographs of the cross sectional views of various OT-MIP columns with different templates. (A) R-atenolol, (B) S-sulpiride, (C) S-NEA, (D) S-MBA, (E) S-ketoprofen, (F) S-methylsuccinic acid.

that choice of template molecule plays an important role in determining the surface morphology, too [16,30,31]. Detailed theories on how the surface morphology of MIP is influenced by choice of porogen and/or template, however, have not been yet established.

The SEM photos of atenolol, sulpiride, and NEA (Fig. 2A–C) indicate the quite good rugged (uneven) morphology that resulted in agreeable separation performance, while the SEM photo of MBA (Fig. 2D) shows minimum ruggedness and porosity resulting in poor chiral separation in all eluent.

Rugged and porous surface morphology with proper layer thickness (1–5 μm) is required for good separation efficiency [4,16,17]. Rugged and porous surface secures fast mass transfer kinetics in the retention process resulting in narrow peak bandwidths.

Quantitative MIP thickness reproducibility is somewhat hard to describe since the surface is highly rugged and it is difficult to monitor all the cross-sectional appearances of the MIP layer along the column length. It may be indirectly monitored by measuring the reproducibility of retention times and separation resolution for columns of different batches. It was examined for a selected MIP (atenolol) and the reproducibility was at least better than 8% for the three batches of MIP columns.

Table 1
Summary of the chromatographic data for the OT-MIPs columns under optimized conditions.

MIP template (analyte)	Eluent[a]	N_1/m[b]	N_2/m[c]	Resolution
Atenolol[d]	70/30, pH 9.0	87,100 ± 1200	21,200 ± 400	3.6 ± 0.06
Atenolol[e]	70/30, pH 10.0	187,000 ± 1900	54,000 ± 2100	2.6 ± 0.05
Sulpiride	80/20, pH 9.0	188,000 ± 2100	60,200 ± 1000	3.0 ± 0.05
NEA	80/20, pH 9.0	36,700 ± 900	25,600 ± 550	1.2 ± 0.04
MBA	80/20, pH 9.0	–	–	–
Methylsuccinic acid[f]	92/08, pH 3.5	–	–	–
Ketoprofen[g]	70/30, pH 3.5	156,000 ± 5000	40,000 ± 1200	10.5 ± 0.21
Ketoprofen[h]	92/08, pH 3.5	817,000 ± 22,000	324,000 ± 15,600	3.2 ± 0.06

[a] Mixing volume ratio of acetonitrile vs. 50 mM NaH_2PO_4 (v/v %) and buffer pH.
[b] Number of theoretical plates/meter for the non-template enantiomer.
[c] Number of theoretical plates/meter for the template enantiomer.
[d] Optimized for the best separation resolution.
[e] Optimized for the best separation efficiency.
[f] Poor separation (resolution <0.5). Data from Ref. [13].
[g] Data from Ref. [4]. Optimized for the best separation resolution.
[h] Data from Ref. [14]. Optimized for the best separation efficiency.

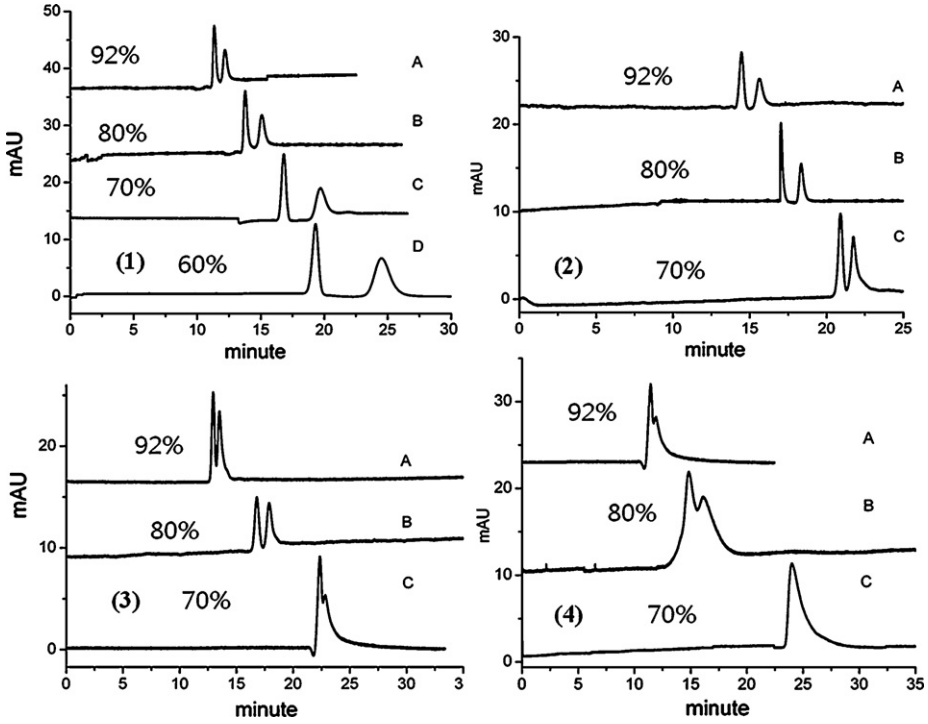


Fig. 3. Effect of eluent ACN content on MIP chiral separation of R- and S-enantiomers. (1) Atenolol, (2) sulpiride, (3) (1-naphthyl)-ethylamine, (4) methyl benzylamine. The first peak is S-enantiomer for (1), and R-enantiomer for (2–4). Experimental conditions: applied voltage: 25 kV, temperature: 25 °C, injection: 7 mbar 6 s, detection wavelength: 214 nm. Mobile phase: pH: 7.0 for (1) and pH 9.0 for (2–4). Acetonitrile compositions: (A) 92%, (B) 80%, (C) 70%, (D) 60%.

Details of template structural effects on morphology and chiral separation will be discussed later.

3.2. Optimization of chiral separation of each MIP column

Optimization was carried out to achieve the best chromatographic resolution. Fig. 3 shows the optimization of acetonitrile (ACN) composition for the four MIP columns prepared in this study, and Fig. 4, the optimization of pH. The optimization results are summarized in Table 1. The results of ketoprofen and methylsuccinic acid are adopted from Refs. [4,16,17].

3.2.1. Optimization of ACN composition

Optimization was first conducted for the atenolol MIP. In its optimization procedure, pH 7.0 was used (Fig. 3-1). However, the optimum pH was not 7.0 but 9.0 in the next step of optimization (Fig. 4-1). It seemed that the optimum pH should be higher than pH 7.0 for basic templates. Thus, pH 9.0 was used in the optimization procedure of ACN composition for the rest MIP columns.

As shown in Fig. 3, retention times of template enantiomers decreased with increase of acetonitrile composition in the eluent (D → C → B → A: 60 → 70 → 80 → 92%) owing to increase of electroosmotic flow and decrease of hydrophobic interaction between the stationary phase and the solute. Acetonitrile was mixed with 50 mM NaH_2PO_4–Na_2HPO_4 buffer adjusted at pH 7.0 or 9.0. Upon increase of acetonitrile content from 70% to 92%, the retention of both enantiomers decreased, and the two peaks tended to be congested. On the other hand, extended decrease of acetonitrile content usually caused band broadening owing to increased hydrophobic interaction and retention. There should be a compromised composition where the two effects (peak congestion and band broadening) are delicately suppressed to give the optimized resolution. The optimum resolution was obtained at 70% ACN (Fig. 3-1-C) for the atenolol MIP.

80% ACN was found the optimum eluent composition for the sulpiride MIP (Fig. 3-2-B), the NEA MIP (Fig. 3-3-B), and the MBA MIP (Fig. 3-4-B). The variations of elution patterns for them were somewhat different from that of the atenolol MIP. Upon extended decrease of ACN content, not only band broadening but also peak congestion was observed.

Note that a very poor chiral resolution of MBA enantiomers was obtained for the S-MBA MIP at any organic modifier content (Fig. 3-4) and at any pH (Fig. 4-4). MBA is structurally similar to NEA except for a substituent, that is, a phenyl ring for MBA vs. a naphthyl ring for NEA (Fig. 1). Based on the structures of both templates, the aromatic ring should offer π–π interaction and steric hindrance to fulfill the 3 points interaction rule in chiral recognition. It seems that a phenyl ring does not efficiently carry out such a task while a naphthyl ring does.

Briefly, the optimum acetonitrile composition was chosen and used for the next pH optimization as follows: 70% for atenolol, and 80% for sulpiride, NEA and MBA.

3.2.2. Optimization of pH

Optimization of pH was conducted by varying the pH in the range of 6.0–10 considering the fact that all the studied compounds have their pK_a values in the range of 9.0–10.0 [32–38]. Atenolol has a pK_a value of ca. 9.5 [33–36]. There are two pK_a values for sulpiride. According to Ref. [34], pK_{a1} is 9.1 and pK_{a2}, 10.19 while pK_{a1} is 8.9 and pK_{a2}, 10.9 according to Ref. [38]. On the other hand, only one pK_a value of 9.12 was reported for sulpiride in another resource [37]. The pK_{a2} value for sulpiride is not clearly defined although it seems to be related to phenylsulfonamide group. It was not considered in this study since it is beyond 10, and a pH over 10 is hardly employed in silica capillary based columns. There has been no pK_a data available for NEA and MBA in the literature. Instead, the pK_a value of benzylamine was found 9.33, and the pK_a value of 3-phenylpropylamine, 10.16 [32]. The pK_a values of NEA and MBA seem to be similar and approximately midway between the

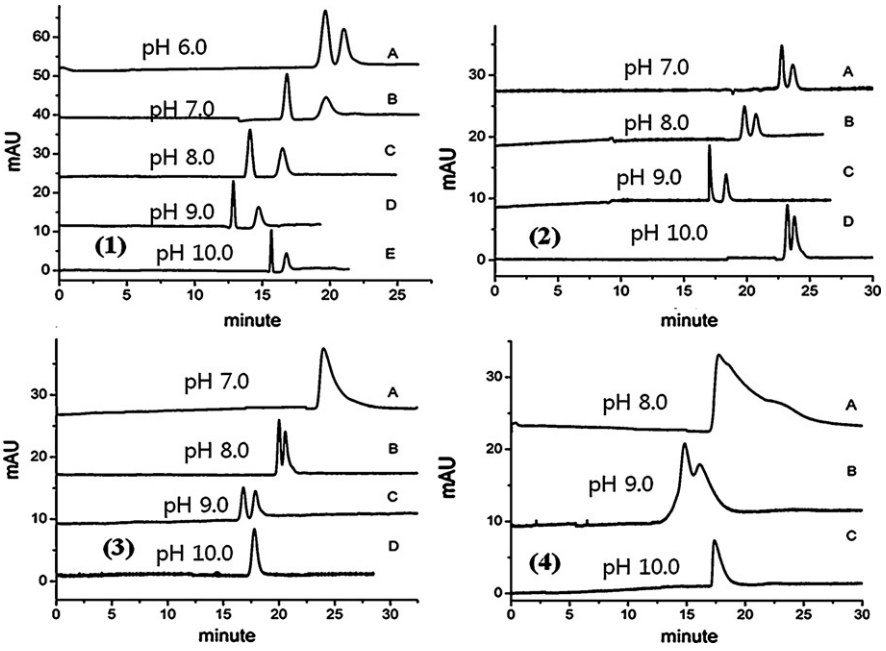


Fig. 4. Effect of eluent pH content on MIP chiral separation of R- and S-enantiomers. (1) Atenolol, (2) sulpiride, (3) (1-naphthyl)-ethylamine, (4) methyl benzylamine. The first peak is S-enantiomer for (1), and R-enantiomer for (2–4). Experimental conditions: applied voltage: 25 kV, temperature: 25 °C, injection: 7 mbar 6 s, detection wavelength: 214 nm. Mobile phase: 70/30 (v/v %) ACN/50 mM NaH_2PO_4 for (1), and 80/20 (v/v %) ACN/50 mM NaH_2PO_4 for (2–4). pH: (A) 6.0, (B) 7.0, (C) 8.0, (D) 9.0, and (E) 10.0 for (1), (A) 7.0, (B) 8.0, (C) 9.0 and (D) 10.0 for (2 and 3), and (A) 8.0, (B) 9.0 and (C) 10.0 for (4).

pK_a values of benzylamine and 3-phenylpropylamine, that is, ca. 9.6.

As shown in Fig. 4, migration times of template enantiomers decreased with increase of pH (A → B → C → D: 6.0 → 7.0 → 8.0 → 9.0). In the pH range of 6.0–10.0, there will be virtually no change in the charge density of MIP surface as all the acidic (MMA and 4-SSA) moieties will be almost completely dissociated. Thus there will be no virtual change in electro-osmotic flow (EOF) in this pH range.

Thus, the variation of analyte migration time with respect to pH is primarily owing to the change of analyte charge density. A basic template will be positively charged, and the charge density will be increased with decrease of pH. There will be electrostatic attraction between the negatively charged MIP and the positively charged analyte. With decrease of pH from 9 to lower values, such interaction got stronger and analyte migration time was increased as shown in Fig. 4.

There is another factor that influences the solute migration. Owing to the applied potential, the solute mobility is increased and its migration time should be decreased with decrease of pH. The solute–MIP electrostatic attraction effect overrided the solute mobility effect, however, thus the overall solute migration time was increased with decrease of pH from 9 to lower values. On the other hand, it seems that the solute mobility effect became more significant when the pH was increased from 9 to 10 since the solute migration time was rather increased as shown in Fig. 4. At pH 10, the number of neutral solute is greater than the number of protonated solute, and the positive contribution of the solute mobility effect (lowered + charge density) to solute migration becomes more significant than the negative contribution owing to the decrease of the solute–MIP electrostatic attraction.

Nevertheless, a tendency of peak congestion was also observed at pH 10. Even collapse of the two template enantiomers into single peak was observed for the NEA MIP (Fig. 4-3-D) and the MBA MIP (Fig. 4-4-C) at pH 10.

The trend of chiral recognition of NEA enantiomers with respect to pH on the NEA MIP is worth close investigation. NEA is regarded to partially fulfill the 3 points interaction rule since a baseline separation was achieved at least under the optimized condition (Fig. 4-3-C). However, it is far from complete fulfillment of the 3 points interaction rule. Nevertheless, NEA is a typical base with only one amino group, and its behavior in chiral recognition trend with respect to pH is a prototype of the MIPs of base templates. Upon extended decrease of pH, collapse of the two NEA enantiomers into single peak (Fig. 4-3-A) was observed. As mentioned before, the electrostatic attraction between the MIP and the analyte gets stronger with decrease of pH. Such strong electrostatic interaction may cause severe peak broadening especially when there is only one functional (amino) group avoiding any interference from other functional groups. Note that the analyte migration time of Fig. 4-3-A could have been much greater if there were no positive mobility increase of analyte with decrease of pH.

Collapse of the two enantiomers into single peak was also observed upon extended increase of pH (Fig. 4-3-D). Taking the narrow bandwidth of the collapsed peak into account, the collapse was not owing to band broadening but primarily owing to loss of chiral recognition. The pH (10) was higher than pK_a, thus the analyte charge density was dramatically decreased and the analyte–MIP electrostatic interaction was also greatly reduced to cause loss of chiral selectivity. The above trend of chiral recognition with respect to pH was also observed for the MBA MIP (Fig. 4-4). The analyte migration time was either increased or maintained almost the same upon change of pH from 9.0 to 10.0 despite the dramatic decrease of the analyte–MIP interaction since the analyte mobility was also greatly decreased for the pH change. Based on the above observations, the analyte–MIP electrostatic interaction should not be too strong or too weak for good chiral selectivity. Thus, the optimum pH was close to pK_a where a half of the base analyte existed in the protonated form and the other half existed in the free base form as reported in the previous studies [39,40]. The pH of the final eluent was somewhat higher than the pH of the buffer before being mixed

Table 2
Chiral separation of atenolol and sulpiride reported in the literature.

Analyte	Separation mode	Stationary phase or mobile phase additive	Eluent	N^a	Rs[b]	Ref.[c]
Atenolol	NP-HPLC	Chiralcel-OD	75:25:0.1 Hexane/ethanol/diethyl amine	–[e]	~2	[20]
Atenolol	CE	β-CD and a β-CD derivative as additives	50 mM Tris–H_3PO_4 buffer pH 2.5	–	2.77	[21]
Atenolol[d]	RP-HPLC	Chiralcel OJ-R	100% methanol	–	2.20	[22]
Atenolol	NP-HPLC	Chiralpak AD-H	85:15:0.1 Hexane/ethanol/ethane sulfonic acid	–	2.35	[23]
Sulpiride	CE	2% sulfated β-CD as an additive	40/75/885 2-propanol/1.25 mM EDTA/0.01 M phosphate buffer pH 7.0	–	2.14	[24]
Sulpiride	CE (partial filling technique)	α_1-Acid glycoprotein (α_1-AGP) as pseudo stationary phase	50 mM phosphate buffer pH 6.0	–	1.8–3.0[f]	[26]

[a] Number of theoretical plates.
[b] Resolution.
[c] Quoted reference.
[d] Atenolol derivatized with a fluorogenic reagent was analyzed.
[e] Not reported.
[f] Variant depending upon the vendor of α_1-AGP.

with acetonitrile, thus the optimum pH (measured before addition of ACN) was actually somewhat smaller than the template pK_a [4]. The optimum pH was found 9.0 for all the MIPs used in this study.

The chromatographic data obtained under the optimized conditions were summarized in Table 1. Optimization was carried out for the best separation resolution, and the best separation efficiency was also observed under the same optimized conditions except for the atenolol MIP. Thus, the data of optimization for the best separation efficiency was also included for the atenolol MIP in Table 1.

3.3. *The effects of structure and functional groups of MIP template on chiral separation performance*

One of the scopes of this study has been fulfilled by the successful fabrication of MIP layers. The generalized preparation protocol we developed in the previous studies [16,17] is truly a general one to form an open tubular thin layer of MIP by tailoring a variety of templates with acidic as well as basic functionalities. The other scope of this study is to examine the relationship between the template molecular structure and the chiral separation performance of the resulting MIP.

Intrinsic chiral susceptibility of the MIP template is another important requirement to achieve good enantioseparation with a MIP column in addition to formation of a thin rugged MIP layer. The template should satisfy the 3-points interaction rule to secure efficient differential chiral recognition. That is, at least three simultaneous interactions are required at the three different substituents (including steric hindrance) near the chiral center. Enantiomers with marked asymmetric carbon having substituents that vary greatly in polarity and size can be separated easily [41].

Thus, the chiral center should be accompanied by enough functional groups to enable at least three different interactions. Such interactions may be hydrogen bonding, pi-pi interaction, steric hindrance, electrostatic interaction, and hydrophobic interaction, etc. In our previous report, we have demonstrated that profen drugs (for example, ketoprofen) fulfill this requirement [16]. Profen drugs are 2-phenylpropionic acids with a large substituent (either electron donating or withdrawing) on the phenyl ring securing favorable intrinsic chiral susceptibility and we could obtain quite high chiral separation efficiencies and resolutions for the profen drug MIPs. Interestingly, the MIP fabricated with a template of good intrinsic chiral susceptibility showed a good morphology (severely rugged and porous) to yield a good chiral separation performance as well [16].

Taking the template molecular structures into account (Fig. 1), we can note that atenolol and sulpiride look certainly to fulfill the 3 points interaction rule for chiral recognition and that NEA and MBA are short of such requirements. The shortage of qualification for chiral recognition is more severe for MBA than for NEA. The NEA MIP offered a resolution of 1.2 under the optimized condition (Fig. 4-3-C) while a baseline separation has never been achieved in any eluent for the MBA MIP (Fig. 4-4). The reason for the better chiral susceptibility of NEA over MBA is easy to explain. MBA contains a phenyl ring (Fig. 1C) inducing limited π–π and steric hindrance interactions while NEA has a naphthyl ring (Fig. 1D) enabling stronger π–π and steric hindrance interactions.

Quite good chiral separation was obtained for the atenolol and sulpiride MIPs and moderate separation, for the NEA MIP, but the quality was still quite inferior to that of the ketoprofen MIP (Table 1). It is worth to note that each template used in this study is subject to strong attractive electrostatic interaction with the MIP causing band broadening. It seems to contribute to degrading of chiral separation performance.

Another factor of lessening chiral recognition capability for atenolol and sulpiride is the possibility of intra-molecular hydrogen bonding. They have functional groups that are subject to form intra-molecular hydrogen bonding around the chiral center (Fig. 1A and B) by conformation leading to weakening of the template–MIP molecular interaction, which will certainly degrade chiral recognition ability.

The empirical rule that the MIP fabricated with a template of good intrinsic chiral susceptibility shows a good morphology and a good chiral separation performance [16] has been verified in this study again.

The morphology of the atenolol MIP (Fig. 2A) and sulpiride MIP (Fig. 2B) were comparable to that of the ketoprofen MIP (Fig. 2E), then their chiral separation performances were also partially comparable to that of the ketoprofen MIP with some inferiority (Table 1).

The morphology of the MBA MIP was with minimized ruggedness (Fig. 2D), then its chiral separation capability was also severely degraded (Figs. 3-4 and 4-4). Its morphology was similar to that of the methylsuccinic acid MIP (Fig. 2F) that showed very poor chiral separation performance [16].

The morphology of the NEA MIP (Fig. 2C) was midway of the two extreme cases, then its chiral separation performance was better than that of the MBA MIP but inferior to those of the atenolol and sulpiride MIPs (Table 1 and Figs. 3 and 4).

Table 3
Chiral separation of pairs of template enantiomers by MIP based techniques with a basic template reported in the literature.

Template	Mode	Stationary phase or pseudo stationary phase	Eluent	N^a	Rs[b]	Ref.[c]
Zolmitriptan	CEC	Ion liquid mediated silica based hybrid monolith	70/30 ACN/50 mM Tris pH 5.4	–[g]	4.26	[42]
Propranolol	CEC	MIP nanoparticles[d]	20/80 ACN/10 mM phosphate buffer pH 7.0	25,000–60,000	~1.0	[43]
Propranolol	CEC	MIP microparticles[d]	90/10 ACN/25 mM phosphate buffer pH 3.5	–	Poor[h]	[44]
Propranolol	CEC	Short MIP monolith	80/20 ACN/2 M acetate buffer pH 3.0	–	Poor	[45]
Ephedrine	HPLC	Packed with ground MIP	80/20 DCM[e]/acetic acid or 95/5 DCM/$BuNH_2$[f]	–	Poor	[46]
Ephedrine	HPLC	Packed with ground MIP	Chloroform with some modifier	–	Poor	[47]
Chlorpheniramine	HPLC	Packed with uniform sized MIP particles	70/30 ACN/50 mM phosphate buffer pH 6.0	350–890	0.83	[48]
Chlorpheniramine	HPLC	Packed with uniform sized MIP particles	70/30 ACN/phosphate buffer pH 3.2	–	0.96	[49]

[a] Number of theoretical plates/m.
[b] Resolution.
[c] Quoted reference.
[d] Partial filling technique.
[e] Dichloromethane.
[f] n-Butylamine.
[g] Not given.
[h] Not given and quite less than 1.0 based on the chromatograms.

3.4. Comparison of chiral separation performances of this study with those reported in the literature

There has been no report in the literature so far for chiral separation of the enantiomer pairs of this study by MIP based techniques. The literature data of chiral separation of atenolol and sulpiride by non-MIP techniques have been summarized in Table 2. Only chromatographic resolution values were reported in these references. The separation resolution data of this study for atenolol and sulpiride (Table 1) are either better or comparable to the literature data (Table 2).

The above evidences certainly support the novelty of the MIPs prepared by the protocol of this study. However, the above comparison does not guarantee the novelty of the MIP preparation protocol of this study over the other MIP preparation methods. Direct comparison is impossible between the MIPs prepared for the same template by different methods since there has been no report for MIP based chiral separation of the enantiomer pairs of this study. Thus, the literature data of MIP based chiral separation of basic analytes (template enantiomers) have been collected instead in Table 3. The separation performances shown in Table 3 are generally quite poor. The data of only one case [42] is somewhat better than ours. Otherwise, the resolution values were less than 1 and the chromatographic peaks were rather broad. Novel separation of resolution 4.26 was obtained for chiral separation of zolmitriptan with a silica based hybrid monolith CEC capillary column prepared by formation of an ionic liquid mediated MIP [42]. The novelty (separation performance) of our MIP columns (Table 1) for atenolol and sulpiride is comparable to that of the above sophisticated hybrid MIP column for zolmitriptan.

The novel results of this study are primarily owing to the superiority of OT-CEC columns in analytical separation. First of all, CEC combines the partitioning effect of HPLC and high separation efficiency of CE to take advantage of both systems. Especially, OT-CEC MIP columns are easy to prepare, free of bubble formation, and easy to wash for removal of template molecules [4]. In addition, eluent optimization is easily realized without any restriction since there is negligible pressure drop across the OT-CEC column.

3.5. Significance of complete validation of the generalized preparation protocol of OT MIP

In many literature studies of MIP based chiral separation, only a single eluent is used for comparison of variant MIPs prepared for a given template. Such research strategy should be altered since optimization of eleunt is essential as demonstrated in our studies and the optimized eluents may be quite different for various MIPs prepared with different reaction mixtures even for the same template.

It will require a huge amount of time to develop a useful MIP for chiral separation of a new pair of enantiomers if optimization is mandatory in both MIP formation and chromatographic elution. Thus, foundation of a generalized preparation protocol regardless of the types of template will reduce such burden dramatically. Now we have demonstrated the validity of the general preparation protocol for acidic as well as basic templates. Extension of the general MIP preparation protocol toward neutral templates is under way and some success has been already attained.

It should be noted that the above generalized MIP preparation protocol is valid only for fabrication of OT-CEC MIP columns. Our similar trials for generalization in preparation of bulk monolithic MIPs or precipitated MIP particles have failed. We suspect that in our protocol the formation of porous rugged MIP layer is activated only from the inner surface of capillary and the polymer matrix is grown gradually thereon in a well controlled and structured network. This pattern is likely to be solid and consistent regardless of choice of template. On the other hand, in bulk or precipitation MIP formation, polymerization seems to occur randomly and the polymer is likely to grow in scattered directions and in an ill-organized network. A general MIP preparation protocol seems to be difficult to be realized in such a situation.

In addition, some empirical rules of eluent optimization based on our studies are summarized as follows for the OT-MIP CEC columns prepared by the generalized protocol. The eluent composed of acetonitrile and an aqueous buffer (either acetate or phosphate) will be useful and the optimized acetonitrile composition is in the range of 70–92% (v/v). The optimized buffer pH is 3–5 for weak acid templates, 2–3 for strong acid templates, 6–8 for neutral templates, and ca. 9 for basic templates. The above description may help reduce efforts and time in eluent optimization.

Briefly we confirmed that the generalized MIP preparation protocol is effective for not only acidic but also basic templates as long as a template with good chiral recognition susceptibility is employed in fabrication of MIP.

4. Conclusion

We have successfully extended the application of the generalized MIP preparation protocol to the basic templates. We also demonstrated the template structural effects on separation per-

formance of the resultant MIP. It was confirmed that good MIP morphology and chiral separation performance are obtained for the OT-MIP column if the three points interaction rule is fulfilled regardless of the chemical type of template. Subtle variations of the template molecular structures have been found to influence chiral recognition ability of the MIP.

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