Review

LC Packing Materials for Pharmaceutical and Biomedical Analysis

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Abstract

The author has prepared novel liquid chromatography (LC) packing materials for pharmaceutical and biomedical analysis. Those include LC packing materials for direct serum injection assays of drugs and their metabolites, LC packing materials for resolution of enantiomeric drugs, and uniformly sized molecularly imprinted polymers for drugs and their metabolites. This review article deals with the preparation of those materials and the pharmaceutical and biomedical applications of them in recognition of The Society of Chromatographic Sciences Award.

Keywords: LC stationary phase; direct serum injection assay, restricted access media; chiral stationary phase; protein; molecularly imprinted polymers

1. Introduction

Many liquid chromatography (LC) packing materials have been prepared since the introduction of HPLC. One type is for general purposes such as octadecyl-silyl silicas. The other type is for specific purposes such as chiral stationary phases (CSPs) and restricted access media (RAM). The author has prepared LC packing materials for direct serum injection assays of drugs and their metabolites, LC packing materials for resolution of enantiomeric drugs, and uniformly sized molecularly imprinted polymers (MIPs) for drugs and their metabolites. This review article deals with the preparation of those materials and the pharmaceutical and biomedical applications of them in recognition of The Society of Chromatographic Sciences Award.

2. Restricted Access Media

For the LC determination of drugs and their metabolites in serum or plasma, tedious and time-consuming pretreatment procedures such as the removal of proteins by precipitation, liquid-liquid extraction or solid phase extraction (SPE) have been required in the past. Recently, direct serum injection analysis of drugs in biological fluids by HPLC has been explored. One of the direct serum injection methods is the use of RAM. Many RAM have been prepared and used for enrichment and pretreatment of the analytes in proteinaceous samples by HPLC [1]. With RAM large molecules such as proteins are eluted in the void volume without destructive accumulation because of restricted access to some surfaces, while allowing small molecules such as drugs and their metabolites to reach the hydrophobic, ion-exchange or affinity sites and to be separated. We prepared two RAM, internal-surface reversed-phase (ISRP) and mixed functional phase (MFP) materials.

2.1. Internal surface reversed-phase

The ISRP silica materials, produced from porous silica gels with 8 nm average pore diameter, have hydrophilic exterior and hydrophobic interior surfaces, as shown in Figure 1. Hagestam and Pinkerton [2] prepared an ISRP support from covalently modified glycerylpropyl (i.e. diol) phases by attachment of the tripeptide glycine-L-phenylalanine-L-phenylalanine (GFF), bound via the amino groups to the glycerylpropyl phases. The phenylalanine moieties were then cleaved from the external surface of the silica with carboxypeptidase A [2] which is too large to enter the small pores. After this enzymatic treatment, the glycerylpropyl-glycine phases remained on the external surfaces, while the internal surfaces remained uncleaved. Thus, the external and internal surfaces consisted of hydrophilic and hydrophobic phases, respectively. Further, on the negatively charged GFF, analytes were retained by a unique combination of mechanisms; π-electron interactions and weak cation-exchange properties [3]. This GFF ISRP material, which is commercially available, had disadvantages in that it could not retain certain classes of hydrophilic drugs such as the ampho-

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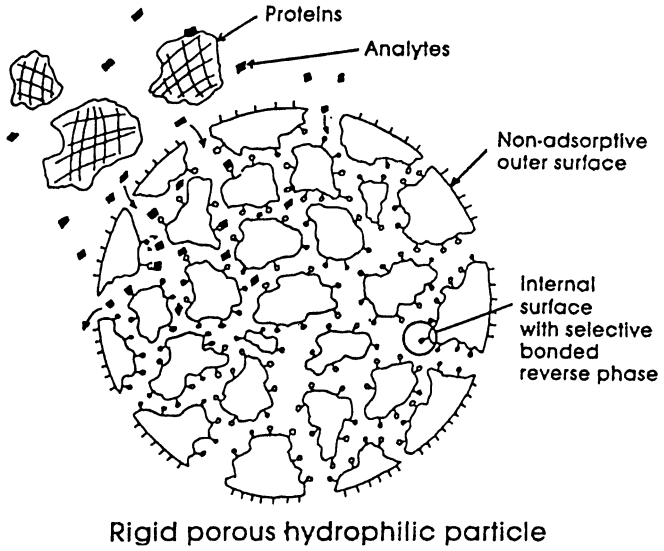

Figure 1. Schematic representation of an internal-surface reversed-phase (ISRP) material. Proteins do not adsorb on the hydrophilic exterior surfaces and do not penetrate into the hydrophobic interior surfaces, while analytes can reach the interior surfaces and be separated.

teric drugs, cephalosporins and penicillins, the recommended eluent pH range is limited to 6.0-7.5, and it is difficult to obtain the desired batch-to-batch reproducibility of its properties. We designed a new ISRP support having *N*-octanoylaminopropyl and *N*-(2,3-dihydroxypropyl)aminopropyl groups on the internal and external surfaces by using a novel enzyme, polymyxin acylase [4]. This enzyme, a monomer (with molecular mass 62,000), has an affinity for long fatty acid groups and can cleave acyl groups of C2 to C16, phenyl and substituted phenyl residues [5]. The neutral ISRP support prepared by us is more effective than that designed by Hagestam and Pinkerton, when separating hydrophilic drugs (Figure 2) [4] and when an eluent pH of 3 to 7 is employed [6]. Further, the neutral ISRP support prepared by us was applied to direct serum injection assays of anticonvulsant drugs and methylxanthine derivatives combined with a column-switching technique [7].

2.2. Mixed functional phase (MFP)

We prepared MFP materials for direct serum injection assays of achiral [8-12] and chiral [13, 14] drugs, having a β-cyclodextrin (CD)-diol phase and a hydrophobic (phenyl, butyl or octyl)-diol phase bonded to porous silica particles. The MFP materials were synthesized from porous silicas in three steps: introduction of hydrophobic or β-CD groups, introduction of 3-glycidoxypropyl groups, and hydrolysis of the oxirane ring to diol groups. The MFP materials having hydrophobic-diol phases were prepared from starting silica materials with average pore diameters of 6-10 nm.

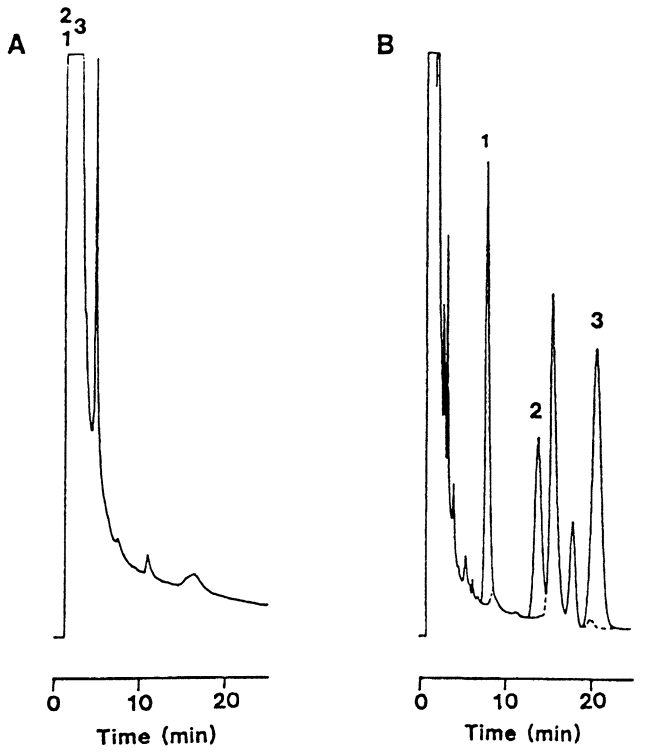

Figure 2. Separation of cephalosporins in human serum by direct injection onto the ISRP materials designed by (A) Hagestam and Pinkerton, and (B) us. Peak assignments: 1 = cefotaxime (10 μg mL^{-1}); 2 = cefmenoxime (10 μg mL^{-1}); 3 = cefamandol (20 μg mL^{-1}). HPLC conditions: eluent, 100 mM phosphate buffer-acetonitrile (12:1, v/v) (final pH 6.9); flow-rate, 0.8 mL min^{-1}; detection, 254 nm; injection volume, 20 μL. Dotted lines indicate serum blank.

Serum proteins were completely recovered from them at the first injection by changing the balance of hydrophobic and hydrophilic phases. The obtained MFP materials could tolerate over 500 injections of a 20-μL serum sample with no change of back pressure and column efficiency. The MFP packing materials having β-CD-diol groups had been prepared using an 8-nm pore size silica. Bovine serum albumin (BSA) was eluted from the material only bonded with β-CD as a tailing peak with protein recovery of 90%. However, from the β-CD-diol material BSA was eluted with a protein recovery of almost 100% [13]. This is due to low surface coverage of the material bonded only with β-CD. The β-CD-diol material can be used for over 400 injections of a 20-μL serum sample. Figure 3 shows chromatograms of direct injection analysis of chlorpheniramine in human serum on the β-CD-diol packings [13].

It was found that protein-based CSPs worked as MFP materials because a protein, especially a glycoprotein, has hydrophilic external surfaces [15]. Ovomucoid-based CSP could be used for di-

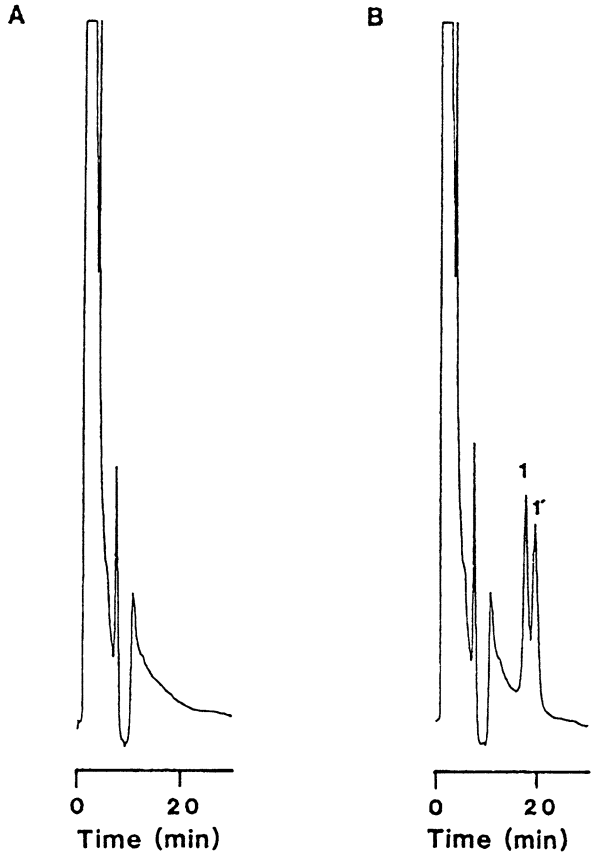

Figure 3. Chromatograms of (A) control serum and (B) serum spiked with chlorpheniramine by direct injection on the β-CD-diol silica. Peaks 1 and 1' are *(R,S)*-chlorpheniramine (25 μg mL^{-1}). Chromatographic conditions are as follows: eluent, 99 mM NaH_2PO_4 + 1 mM H_3PO_4-acetonitrile (6:1, v/v) (final pH 4.3); flow-rate, 0.6 mL min^{-1}; detection, 264 nm; injection volume, 20 μL.

rect serum injection assays of chiral drugs [15].

3. Protein-based chiral stationary phases

A protein and glycoprotein, respectively, consist of amino acids, and amino acids and sugars, both of which are chiral. Thus, all proteins have the ability to discriminate a chiral molecule. However, only a limited number of proteins have been investigated as HPLC CSPs. Protein-based CSPs are of special interest because of their unique, enantioselective properties and because they are suitable for separating a wide range of enantiomeric forms [16-18]. The advantages of protein-based CSPs generally include the use of an aqueous mobile phase, as for reversed-phase HPLC, enantioselectivity for a wide range of compounds and direct analysis without derivatization. The disadvantages have included low capacity, lack of column ruggedness and limited understanding of the chiral recognition mechanism. A few trials were made to overcome these

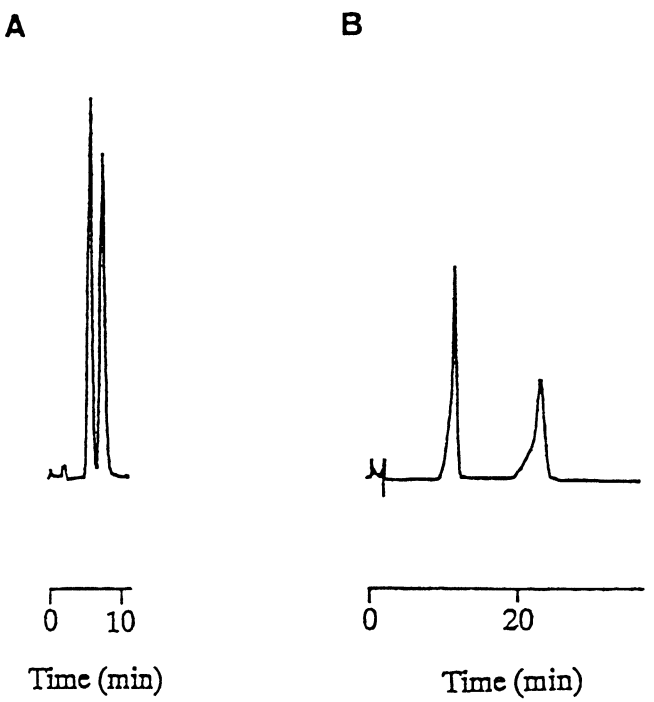

Figure 4. Chromatograms of benzoin on the BSA (A) and BSA-fragment (B) columns.
HPLC conditions: column, 2.1 mm i.d. × 100 mm; eluent, 50 mM phosphate buffer (pH 7.5)-1-propanol (96:4, v/v); column temperature, 25 ℃; flow-rate, 0.2 mL min^{-1}; detection, 254 nm; injected amount, 0.1 μg each.

disadvantages of protein-based CSPs as described below.

3.1. Chiral stationary phases based on serum albumin fragments

The BSA fragment columns obtained by other investigators could have less capacity and enantioselectivity, and be less stable than the intact BSA column. We isolated the BSA fragment of molecular mass 35,236 estimated by electrospray ionization mass spectra [19, 20]. The estimated molecular mass was in good agreement with the predicted molecular mass 35,234 of the half-cystinyl BSA fragment, which is ascribable to amino acid sequences 1-307 having eight disulfide bonds and one half-cystinyl bond in the 34th. Next, the intact and fragmented BSA were bound to *N,N'*-disuccinimidyl carbonate (DSC)-activated aminopropyl-silica gels. The bound amounts of the BSA fragment were 2.2-2.7 times more than that of the intact BSA. Chiral resolution of 2-arylpropionic acid derivatives, benzodiazepines, warfarin and benzoin was attained with the BSA fragment columns. Figure 4 shows chromatograms of benzoin on the BSA and BSA-fragment columns. The BSA fragment columns gave higher enantioselectivity for lorazepam and benzoin because of the larger bound amounts, and lower enantioselectivity for other compounds tested, compared with the BSA column. The lower enantioselectivity might be due to changes

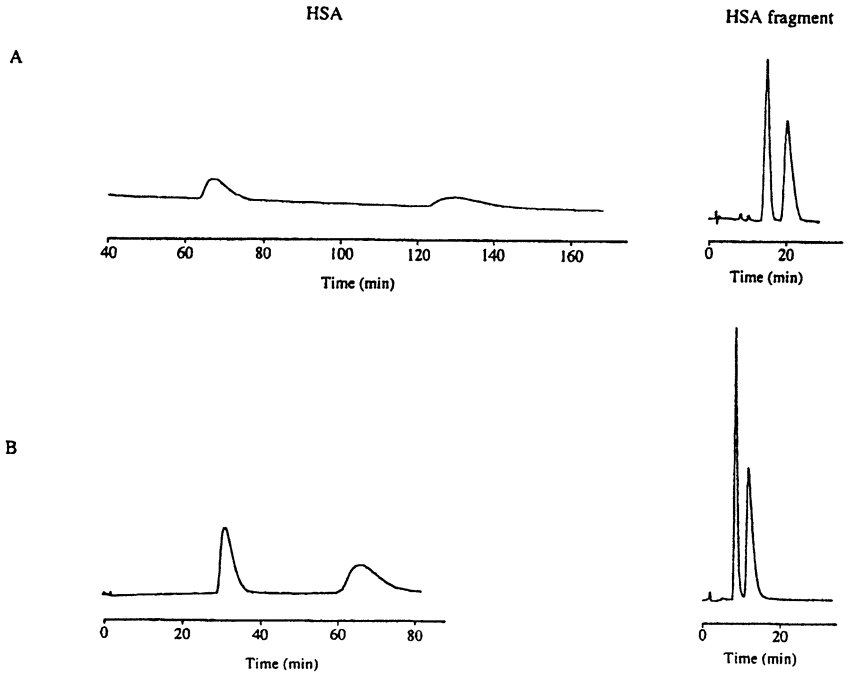

Figure 5. Chromatograms of pranoprofen (A) and warfarin (B) on HSA and HSA-FG 75 columns.
HPLC conditions: column size, 2.0 mm i.d. × 100 mm; eluent, 50 mM sodium phosphate buffer (pH 7.5)-1-propanol (94:6, v/v); flow rate, 0.2 mL min^{-1}; detection, 210 nm; temperature, 25 ℃; loaded amount, 100 ng.

in the globular structure of the BSA fragment and/or changes in the local environment around the binding sites.

Similarly, a human serum albumin (HSA) fragment was isolated by size-exclusion chromatography following peptic digestion of HSA [21]. Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectra indicated three $(M + H)^+$ ion peaks, whose molecular masses were estimated to be 34,816, 29,178 and 44,134. The molecular masses estimated were in good agreement with the predicted molecular weights of amino acid sequences 1-308, 49-308 and 1-387. The fragment protein, whose molecular mass was about 35,000, was mainly observed. Next, the intact and fragmented HSA were bound to DSC-activated aminopropyl-silica gels. Though the retentivity and enantioselectivity of all solutes tested were lower on the HSA fragment column than the HSA column, benzodiazepine (oxazepam), 2-arylpropionic acid derivatives (ibuprofen, ketoprofen, pranoprofen and flurbiprofen) and warfarin were still enantioseparated on the HSA fragment column. However, no enantioseparation of benzoin was attained. As reported by He and Carter [22], primary binding sites for benzodiazepines and 2-arylpropionic acid derivatives are located in the subdomain IIIA, while that of warfarin in the subdomain IIA. It is interesting that benzodiazepine (oxazepam) and 2-arylpropionic acid derivatives (ibuprofen, ketoprofen, pranoprofen and flurbiprofen) participate in enantioselective binding interaction with the *N*-terminal half of HSA, despite the fact that the primary binding sites of these compounds are on the other half of HSA. Figure 5 shows the enantioseparations of pranoprofen and warfarin on the HSA and HSA fragment columns. Though the HSA column showed higher enantioselectivity than the HSA fragment column, the enantioseparations of pranoprofen and warfarin were attained with a shorter analysis time on the HSA fragment column. This could be due to removal of the non-specific binding sites of HSA, changes in the globular structure of the HSA fragment and/or changes in the local environment around the binding sites [21]. Further, the HSA fragment column was as stable as the intact HSA column for repetitive injection of samples. These results suggest that the HSA fragment column could be useful for the enantioseparation of solutes having strong affinities for HSA.

3.2. α_1-acid glycoprotein

Since the molecular mass of α_1-acid glycoprotein (AGP) from human plasma ranges from 38,800 to 48,000, it is generally assumed to be 40,000 [23]. Recently, we reported that the average molecular mass of AGP could be ca. 33,000 by MALDI-TOF mass spectra, and that the sugar content of AGP could be estimated to be 34% [24]. There is no precise information on the chiral recognition

sites and mechanism of AGP because of the lack of information on the tertiary structure. Further, the role of sugar moieties in enantioselective bindings by AGP has not been investigated precisely. We prepared partially deglycosylated (pd)-AGP by removal of a sugar moiety of AGP by treatment with *N*-glycosidase [24]. The average molecular mass of pd-AGP was estimated to be ca. 30,600 by MALDI-TOF mass spectrometry. Figure 6 A and B shows chromatograms of ethotoin enantiomers on the AGP and pd-AGP columns, respectively. The retentivity and enantioselectivity of the neutral, acidic and basic solutes tested on the pd-AGP column were significantly or not significantly larger in most solutes than those on the native AGP column. A possible explanation is that by cleavage of a sugar chain(s), pd-AGP could become more hydrophobic than AGP, and/or that a solute could be easily accessible to the specific and/or non-specific binding sites of pd-AGP [24]. Further study is required to clarify the chiral recognition mechanism of AGP and the role of the sugar moiety on chiral recognition.

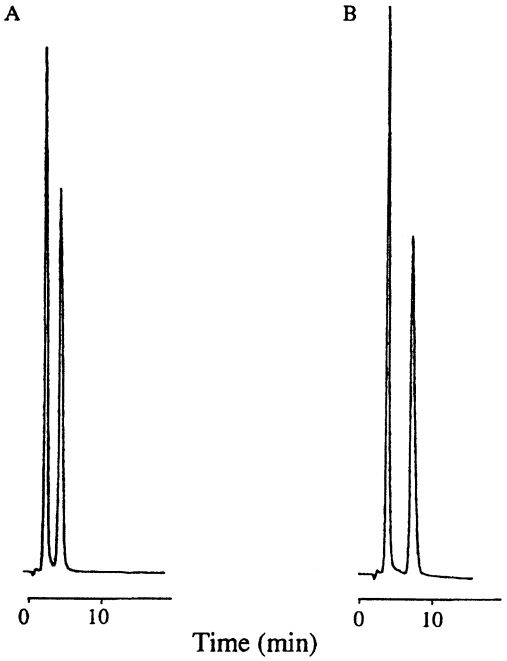

Figure 6. Chromatograms of ethotoin enantiomers on AGP (A) and pd-AGP (B) columns.
HPLC conditions: column size, 2.0 mm i.d. × 100 mm; eluent 10 mM phosphate buffer (pH 5.0); flow-rate, 0.2 mL min^{-1}; detection, 210 nm; temperature, 25 ℃; loaded amount, 100 ng.

3.3. Ovomucoid and ovoglycoprotein

CSPs based on ovomucoid from chicken egg whites (OMCHI) were developed by Miwa et al. [25]. The OMCHI column can resolve a wide range of acidic, basic and neutral enantiomers.

Various ovomucoids such as ovomucoid from turkey egg whites (OMTKY) and OMCHI exist as three tandem, independent domains. In order to gain information regarding the enantioselective recognition mechanism of ovomucoid proteins, it is of interest to know whether chiral recognition is expressed by all three domains, by only one domain, or by a combination of domains. Each domain and combination domains, first and second, second and third domains, were isolated, purified and characterized [26]. Further, columns were made with purified OMTKY and OMCHI domains to test chiral recognition properties [25, 26]. The third domain of OMTKY and OMCHI consisted of glycosylated (OMTKY 3S and OMCHI3S) and unglycosylated domains (OMTKY3 and OMCHI3). The third domains of the OMTKY and OMCHI domains were found to be enantioselective for at least two classes of compounds, benzodiazepines and 2-arylpropionic acid derivatives as shown in Tables 1 and 2, respectively. Glycosylation of the third domain did not affect chiral recognition. Further, the chiral recognition mechanism of the OMTKY3 was elucidated by using NMR measurements, molecular modeling and computational chemistry [26]. Figure 7 illustrates the binding orientations of the enantiomers of U-80413, which is one of 2-arylpropionic acid derivatives, in each of the two surface regions identified on the OMTKY3 [26]. The first group of amino acids, Val 6, Arg 21, Pro 22, Leu 23, Ly 34 and Phe 53, is on the left, while the second group, Val 41, Val 42, Leu, 48 and Lue 50, is on the right. The tubular structure represents the protein backbone, and side chains of selected amino acid residues (Arg 21, Lys 34 and Phe 53) are shown. The peptide

Table 1. Retention factors and enantioseparation factors of benzodiazepines and 2-arylpropionic acid derivatives on OMTKY domain columns[a].

	OMTKY3[b]		OMTKY3S		OMTKY2		OMTKY1	
	k_1	α	k_1	α	k_1	α	k_1	α
Clorazepate	1. 53	1.14	1.57	1.12	1.33	1.00	0. 86	1.00
Lorazepam	2.00	1.12	1.94	1.08	1.65	1.00	1.73	1.00
Pranoprofen	3.14	1.04	3.34	1.00	2.75	1.00	1.86	1.00
U-80,413	3.14	1.10	3.08	1.05	2.03	1.00	1.40	1.00

[a]HPLC conditions: column, 2.0 mm i.d. x 100 mm; eluent, 20 mM phosphate buffer (pH 6.9)/2-propanol = 97/3 (V/V); flow rate, 0.1 mL min^{-1}; column temperature, ambient.
[b]OMTKY3, unglycosylated third domain of OMTKY; OMTKY3S, glycosylated third domain of OMTKY; OMTKY2, second domain of OMTKY; OMTKY1, first domain of OMTKY.

Table 2. Retention factors and enantioseparation factors of benzodiazepines and 2-arylpropionic acid derivatives on OGCHI domain colunms.

	OGCHI 3		OGCHI 3S		OGCHI 2	
	k_1	α	k_1	α	k_1	α
Clorazepate	2.63	1.00	2.12	1.00	2.34	1.00
Lorazepam	3.19	1.00	2.77	1.00	2.68	1.00
Pranoprofen	6.28	1.00	5.24	1.00	-	-
U-80,413	4.48	1.05	4.29	1.06	2.34	1.00

[a]HPLC conditions: column, 2.0 mm i.d. x 150 mm; eluent, 20 mM phosphate buffer (pH 6.8)/2-propanol = 99/1 (V/V); flow rate, 0.1 mL min^{-1}; column temperature, ambient.
[b]OMCHI3, unglycosylated third domain of OMCHI; OMCHI3S, glycosylated third domain of OMCHI; OMCHI2, second domain of OMCHI.

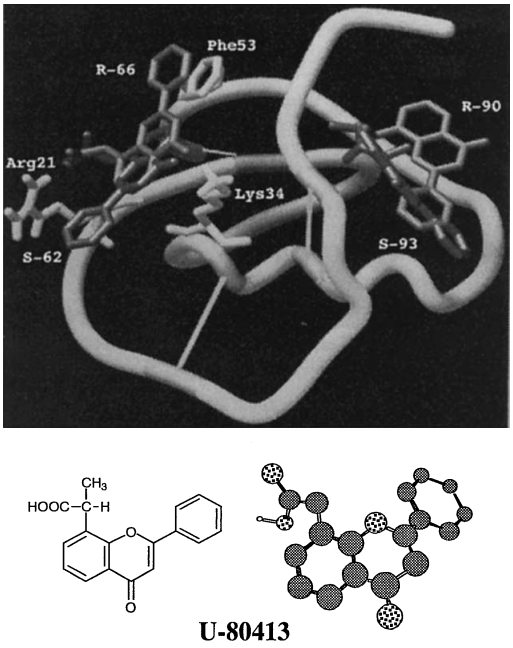

Figure 7. Molecular modeling simulation of U-80413 enantiomers bound to OMTKY3.
Ligands are labeled according to their *R* or *S* chirality and numbered according to their position among the 100 lowest energy minimized binding orientations.

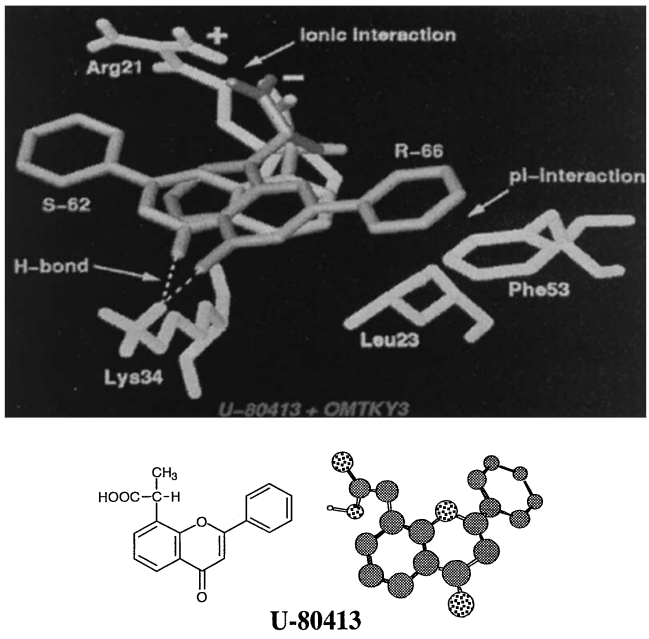

Figure 8. Enantiomers of U-80413 in enantioselective binding site of OMTKY3.

strand is wrapped around an α-helix and held in place by three disulfide bonds. The *N*-terminal is at the top right and the C-terminal is in the back. The dockings in the second site to the right were of higher energy and without apparent points of interaction that could produce chiral recognition. This site could account for the nonspecific binding site. The selected, specific binding model for each of the *(R)*- and *(S)*-U-80413 with OMTKY3 is shown in Figure 8 [26]. One can see similarities and differences in orientation and intermolecular interactions between the *(R)*- and *(S)*-U-80413. The carboxyl groups of each enantiomer engage in electrostatic interactions with the positive charge on Arg 21. The carbonyl group on U-80413's central ring share a hydrogen bond with the NH_3^+ group of Lys 34. The distinguishing difference between the enantiomers is the proximity of the phenyl group of *(R)*-U-80413 and Phe 53. However, neither the first, second nor a combination domain of OMTKY gave appreciable chiral recognition ability [26].

Recently, we isolated and characterized a new protein from chicken egg whites [27, 28]. It was termed ovoglycoprotein from chicken egg whites (OGCHI). In addition, it was found that 10% of OGCHI was included in crude OMCHI preparations [28]. Moreover, OMCHI and OGCHI columns were made from isolated, pure proteins and were compared with regard to chiral recognition abilities. It was found [28] that the chiral recognition ability of OMCHI reported previously [25] came from OGCHI, and that OMCHI had no chiral recognition ability. Further, it was found that OGCHI is preferentially bound to DSC-activated aminopropyl-silica gels compared with OMCHI, even though the average molecular mass of OGCHI and OMCHI are 30,000 and 27,000, respectively [29, 30]. This is why CSPs based on crude OMCHI, which are now

commercially available, show moderate chiral recognition ability.

The effects of the sugar moieties of OGCHI on the enantioseparations of various solutes were investigated. The retentive and enantioselective properties of the OGCHI, asialo OGCHI and asialo-agalacto OGCHI columns were compared. Generally, removal of a sialic acid or sialic acid-galactose group of OGCHI resulted in no change or an increase of the retention factors and enantioseparation factors of solutes tested. It was concluded that sialic acid and sialic acid-galactose groups of OGCHI did not participate in chiral discrimination of the solutes tested [31]. Moreover, pd-OGCHI and completely deglycosylated OGCHI (cd-OGCHI) were obtained by treatments of OGCHI with *N*-glycosidase, and a mixture of endoglycosidase and *N*-glycosidase, respectively [32, 33]. The average molecular masses of pd-OGCHI and cd-OGCHI were estimated to be about 28,400 and 21,400, respectively. It was found that the pd-OGCHI column showed excellent chiral recognition abilities comparable to the OGCHI column, and that the retentivity and enantioselectivity of basic solutes tested on the pd-OGCHI column were higher than those on the OGCHI column, while those of acidic solutes on the pd-OGCHI column were lower. Further, cd-OGCHI still showed chiral recognition abilities for various solutes tested. These results revealed that the chiral recognition site(s) for OGCHI existed on the protein domain of OGCHI.

OGCHI was bound to aminopropyl-silica gels via an amino or carboxyl group(s) of OGCHI [34]. The OGCHI materials prepared via a carboxyl group(s) of OGCHI are suitable for chiral resolution of acidic solutes, and those via an amino group(s) of OGCHI are suitable for chiral resolution of basic solutes. It was suggested that electrostatic interaction between an amino or carboxyl group of OGCHI and a charged solute should play an important role in chiral recognition of the solute [34]. Further, chiral recognition properties of OGCHI and ovoglycoprotein from Japanese quail egg whites (OGJPQ) were compared [35]. The average molecular masses of OGCHI and OGJPQ were estimated to be about 30,000 and 27,400. The OGCHI column is suitable for chiral resolution of basic compounds, while the OGJPQ column is suitable for that of acidic compounds. With regard to chiral resolution of neutral compounds, it is dependent on the compound to be resolved which column could be suitable. The results obtained revealed that chiral recognition of various solutes should be efficiently attained by using both columns complementarily.

3.4. Chiral stationary phases based on other proteins

CSPs based on lysozyme [36] and pepsin [37] were developed

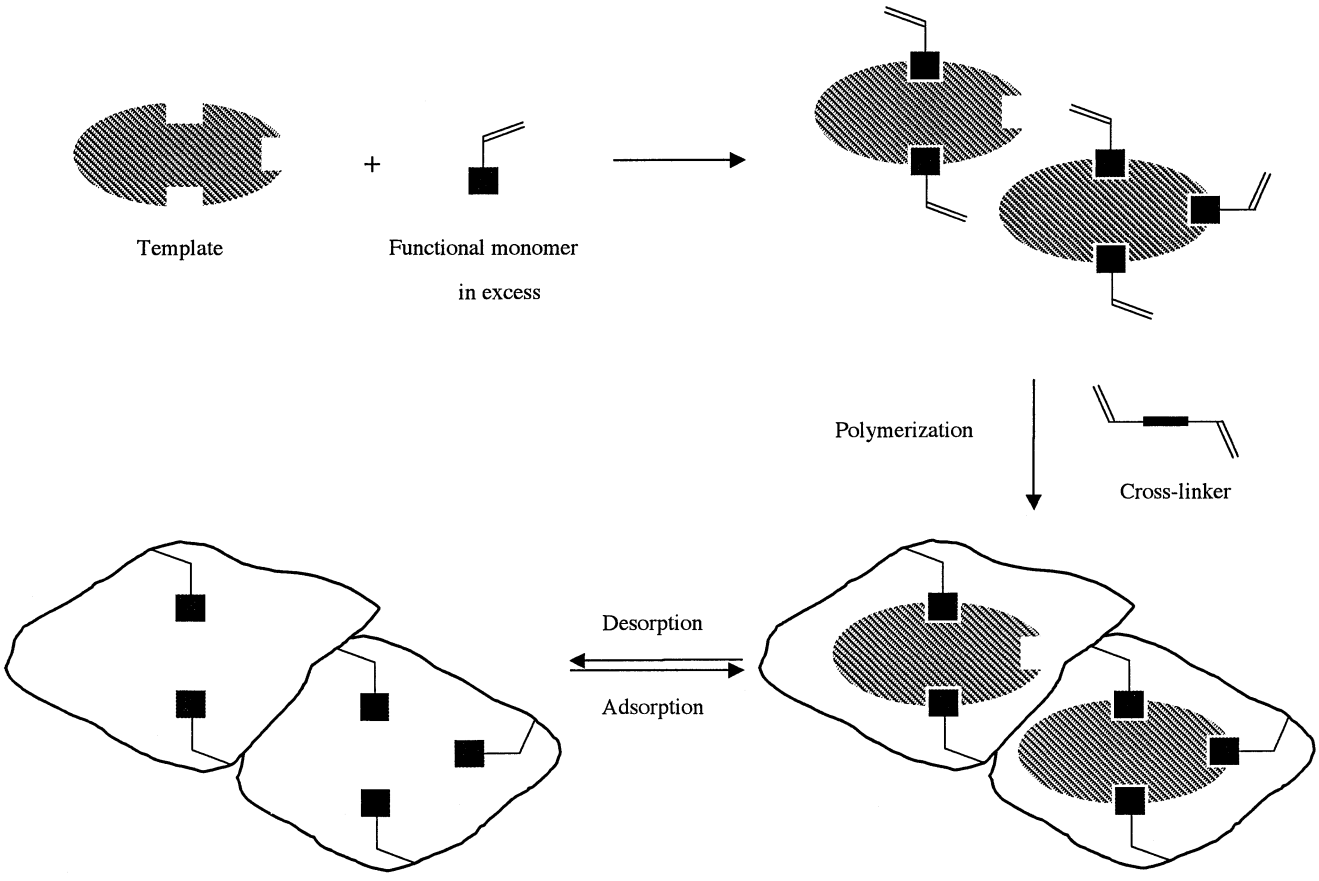

Figure 9. Schematic diagram of preparation of non-covalent MIPs.

by us. By using a mixture of phosphate buffer and organic modifier as an eluent, basic and uncharged enantiomers were resolved on both CSPs, while no resolution of acidic enantiomers was observed. Further, the pepsin and OMCHI were mixed-immobilized onto the same porous aminopropyl-silica gels [38]. The retentive and enantioselective properties of the mixed-protein-based CSP were compared with those of pepsin- and OMCHI-based CSPs. The pepsin-OMCHI-based CSP showed a similar enantioselectivity with the pepsin-based CSP because OMCHI has no enantioselectivity. In addition, the pepsin-OMCHI-based CSP was more stable than the pepsin-based CSP for repetitive injections of samples and continuous flow of an eluent. The pepsin-OMCHI-based CSP was further stabilized by crosslinking with glutaraldehyde [38].

4. Uniformly sized molecularly imprinted polymers

Since molecular imprinting techniques can afford complementary binding site(s) for a template molecule (Figure 9), the MIPs are used for chromatographic separations, SPEs, membranes, antibody-mimics and sensors for the purpose of specific recognition of the target molecule [39, 40]. Usually, non-aqueous bulk polymerization methods have been utilized to obtain MIPs. The disadvantage of the method is that the obtained block polymers had to be crushed, ground and sieved to produce packing materials. The MIPs obtained are unsuitable for HPLC packing materials owing to random shape and size distribution. Uniformly sized MIPs have been prepared through a combination of a typical multi-step swelling and polymerization method and molecular imprinting technique [41, 42]. The advantages of the method are as follows: it is easy to prepare uniformly sized and monodispersed particles, easy to perform in situ modification, and suitable for preparing HPLC packing materials. We have prepared uniformly sized MIPs for basic and acidic drug enantiomers, and applied the obtained MIPs for resolution of drug enantiomers by HPLC.

Recently, selective enrichment and pretreatment of analytes in complex matrixes have been attained with SPE based on MIPs. The SPE based on MIPs has been generally carried out by an off-line mode. We have prepared a RAM-MIP, a uniformly sized MIP selectively modified with a hydrophilic external layer, through a combination of molecular imprinting and hydrophilic surface modification techniques. Further, the obtained RAM-MIP for 2-arylpropionic acid derivatives was applied for the direct serum injection assays of the drug by a column-switching system, consisting of a RAM-MIP and a conventional C18-silica column.

4.1. Chiral separation using molecularly imprinted polymers

We prepared uniformly sized MIPs for *(S)*-propranolol [43, 44] and *(S)*-chlorpheniramine [45] by a two-step swelling and polymerization method using methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) as the functional monomer and crosslinker, respectively. The retentive and enantioselective properties of these two drugs and their structurally related compounds on the MIPs were evaluated using an aqueous eluent. Hydrophobic and ionic interactions could mainly be responsible for the retention and enantioseparation of propranolol and chlorpheniramine in an aqueous eluent. The MIP showed the highest recognition for the template molecules and slight recognition for their structurally related compounds. Further, the MIPs for 2-arylpropionic acid derivatives, *(S)*-naproxen [46-48] and *(S)*-ibuprofen [49] were prepared using 4-vinylpyridine (4-VPy) and EDMA as the functional monomer and crosslinker, respectively, by a two-step swelling and polymerization method. The obtained MIP for *(S)*-naproxen, evaluated in aqueous eluent, gave similar enantioselectivity for naproxen, compared with that prepared by the bulk polymerization method and evaluated in an organic eluent [47]. The baseline resolution of naproxen enantiomers was attained by optimization of the preparation method of the uniformly sized MIP and separation pa-

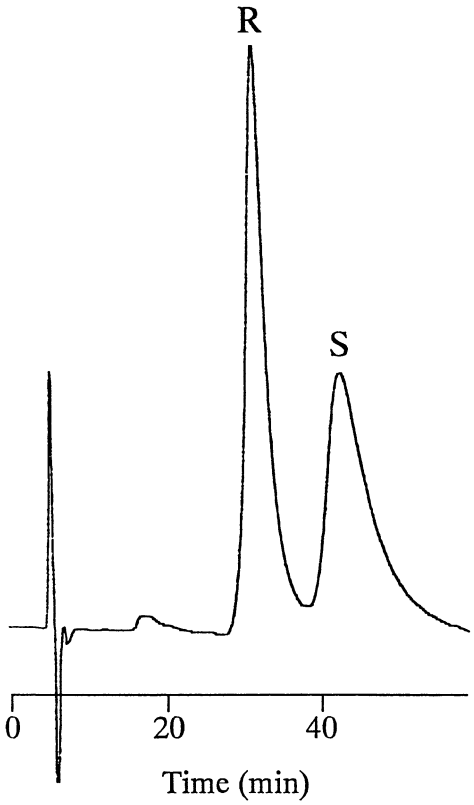

Figure 10. Separation of naproxen enantiomers on the MIP for *(S)*-naproxen.
HPLC conditions: column size, 4.6 mm i.d. × 100 mm; column temperature, 70 ℃; eluent, 20 mM phosphate buffer (pH 3.2)/acetonitrile = 50:50 (v/v); detection, 220 nm; flow rate, 0.2 mL min^{-1}. Loaded amount, 250 ng.

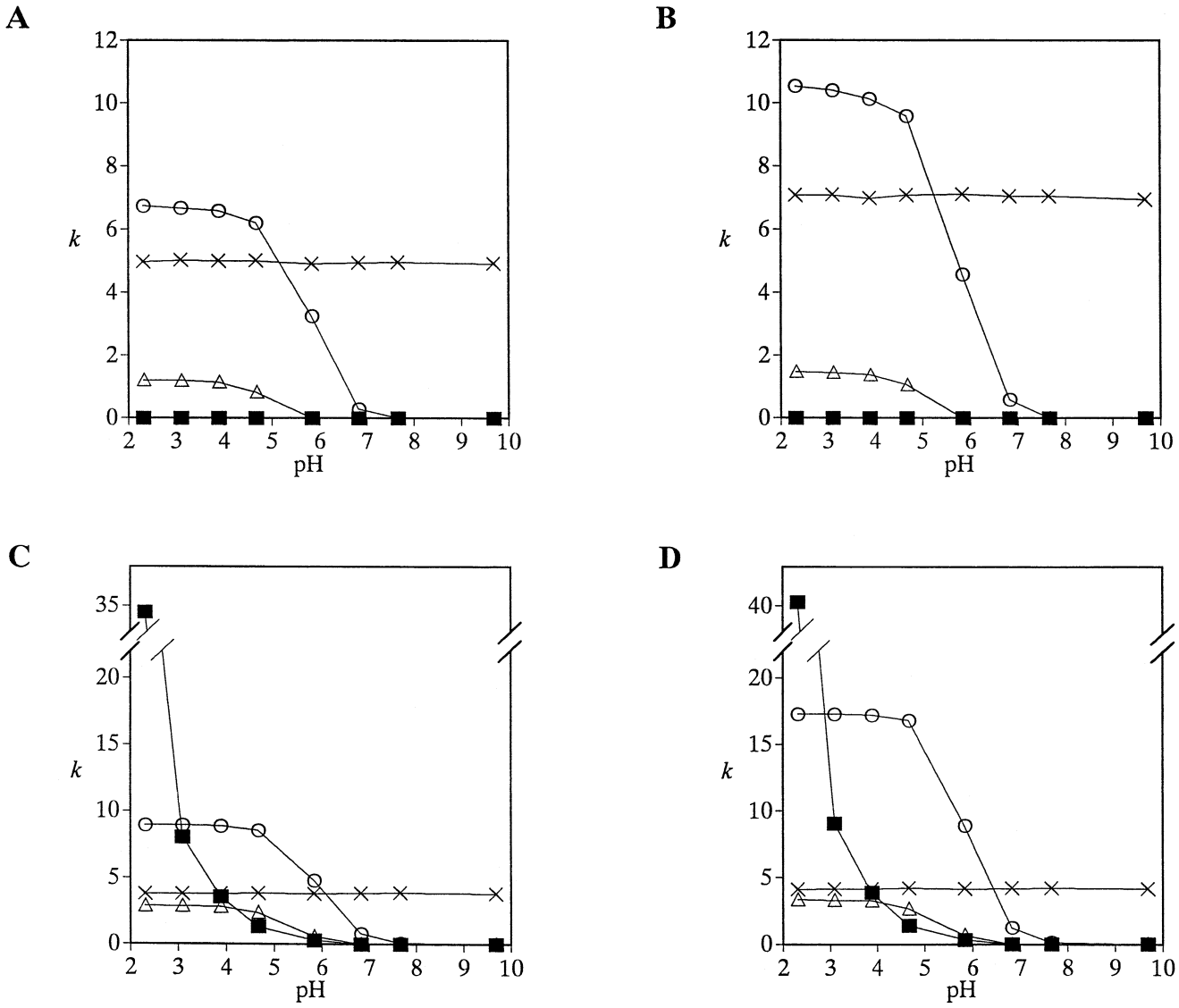

Figure 11. Effect of eluent pH on the retention properties of *(S)*-ibuprofen, benzoic acid, benzenesulfonic acid and benzene on non-imprinted EDMA (A), styrene-EDMA (B) and 4-VPy-EDMA (C), and *(S)*-ibuprofen imprinted 4-VPy-EDMA (D) materials.
Keys: ○-○, *(S)*-ibuprofen; △-△, benzoic acid; ■-■, benzenesulfonic acid; ×-×, benzene. HPLC conditions: column size, 4.6 mm i.d. × 100 mm; column temperature, 25 ℃; eluent, 20 mM phosphoric acid and/or sodium phosphate/acetonitrile = 60:40 (v/v); detection, 220 nm; flow rate, 1.0 mL min^{-1}. Loaded amount, 250 ng.

rameters such as flow rate and column temperature (Figure 10) [48].

The retentive and enantioselective mechanism of the MIP for *(S)*-ibuprofen was precisely examined in aqueous media. Figure 11, parts A, B, C and D, shows the effects of eluent pH on the retention properties of *(S)*-ibuprofen, benzoic acid, benzenesulfonic acid and benzene on non-imprinted EDMA, styrene-EDMA and 4-VPy-EDMA, and *(S)*-ibuprofen imprinted 4-VPy-EDMA materials, respectively [49]. Since the first two polymers had no ionizable groups in the polymer backbone, solutes were mainly retained with hydrophobic interactions. Benzenesulfonic acid was not retained on the EDMA and styrene-EDMA materials among the eluent pHs tested. It is thought that the apparent pKa value of benzenesulfonic acid is < 2.3. On the other hand, benzenesulfonic acid was retained on the non-imprinted and *(S)*-ibuprofen imprinted 4-VPy-EDMA materials by decreasing the eluent pH. The retention of benzenesulfonic acid was due to ionic interactions of sulfonyl groups of benzenesulfonic acid with the positively charged 4-VPy-EDMA materials. It was reported that the apparent pKa value of the pyridinyl group of the matrix was ~ 4.7 [50]. However, the retention data of bezenesulfonic acid suggested the shift of the average apparent pKa value of 4-VPy-EDMA materials to < 3. Benzoic acid was

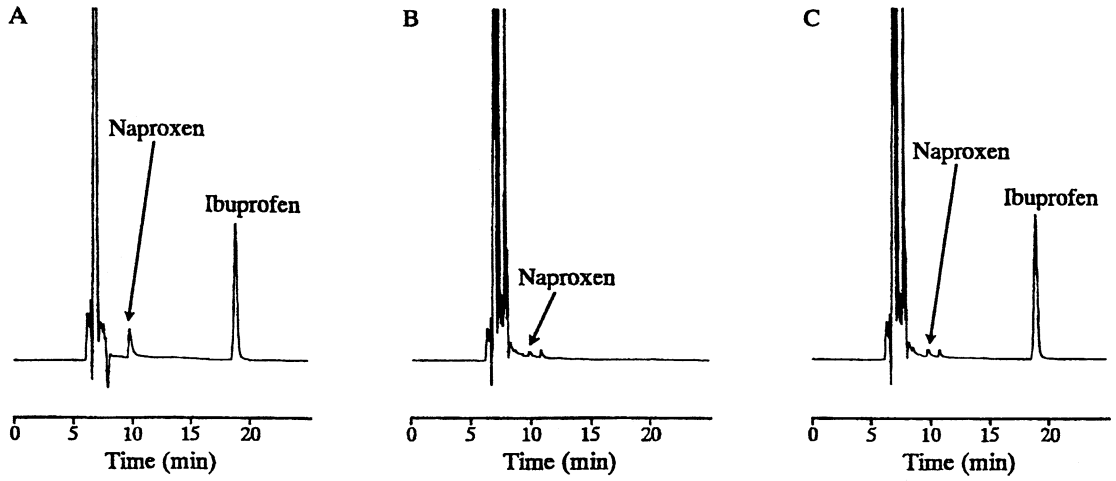

Figure 12. Chromatograms of standard ibuprofen sample (A), control plasma sample (B), and control plasma sample spiked with ibuprofen (C) using column-switching techniques.
HPLC conditions; Precolumn, RAM-MIP for *(S)*-ibuprofen (10 mm × 4.0 mm i.d); analytical column, Cosmosil 5C18-MS (150 mm × 4.6 mm i.d.); eluent for pretreatment, 20 mM phosphoric acid-acetonitrile (78:22 (v/v), pH 2.24) at 1.0 mL min^{-1} for 5 min; eluent for analysis, 20 mM sodium phosphate buffer-acetonitrile (75:25 (v/v), pH 7.34) at 1.0 mL min^{-1}; detection, UV absorbance at 223 nm. Ibuprofen concentration is 5.0 μg mL^{-1} in (A) and (C).

more retained by hydrogen bonding interaction with a pyridinyl group on the non-imprinted and *(S)*-ibuprofen imprinted 4-VPy-EDMA materials than the EDMA and styrene-EDMA materials. The retention factor of *(S)*-ibuprofen increased with the order EDMA, 4-VPy-EDMA, styrene-EDMA and *(S)*-ibuprofen imprinted 4-VPy-EDMA materials. *(S)*-Ibuprofen could be retained mainly with hydrophobic interactions with EDMA and styrene-EDMA materials, and hydrophobic and hydrogen bonding interactions with 4-VPy-EDMA materials. Further, *(S)*-ibuprofen was more retained by the molecular imprinting effect on the *(S)*-ibuprofen imprinted 4-VPy-EDMA materials than the non-imprinted ones.

4.2. Restricted access media-molecularly imprinted polymers

We have prepared a RAM-MIP material, a uniform-sized MIP for *(S)*-naproxen selectively modified with a hydrophilic external layer, through a combination of molecular imprinting and hydrophilic surface modification techniques [51, 52]. Further, we prepared RAM-MIP materials for *(S)*-naproxen and -ibuprofen, and tried to apply the respective RAM-MIP for direct serum injection assays of the drug by column-switching system, consisting of the RAM-MIP material and a conventional C18-silica column. However, leakage of the imprint molecule prevented accurate and precise assays of the drug. Thus, the RAM-MIP material for *(S)*-naproxen was applied for the assays of ibuprofen in rat plasma [52]. Figure 12, parts A, B and C, shows chromatograms of standard ibuprofen sample (5.0 μg mL^{-1}), control plasma sample and control plasma sample spiked with 5.0 μg mL^{-1} of ibuprofen, respectively, using column-switching techniques. Figure 12 illustrates the fact that ibuprofen is separated from the ordinary components of plasma samples, and that ibuprofen is almost completely recovered from the serum samples. Further, *(S)*-naproxen, imprint species, appeared on a chromatogram. However, it was completely separated from ibuprofen on a C18 column.

The developed method should have wide applicability for the determination of a drug in biological fluids.

5. Conclusions

The author has prepared LC packing materials for direct serum injection assays of drugs and their metabolites, LC packing materials for resolution of enantiomeric drugs, and uniformly sized MIPs for drugs and their metabolites. These materials could be useful for specific purposes; for the assays of drugs and their metabolites and resolution of enantiomeric drugs. However, we require further studies to clarify the chiral recognition site(s) and mechanism on OGCHI. In addition, MIPs for hydrophilic compounds, proteins and peptides are the targets to be pursued. In the future, we will need further LC packing materials for high-performance separations as well as for selective separations.

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