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Uniform-Sized Molecularly Imprinted Polymers for 2-Arylpropionic Acid Derivatives Selectively Modified with Hydrophilic External Layer and Their Applications to Direct Serum Injection Analysis

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Uniform-sized molecularly imprinted polymers (MIPs) for (*S*)-naproxen and -ibuprofen selectively modified with hydrophilic external layer, restricted access media (RAM)-MIPs, have been prepared. First, the MIP for (*S*)-naproxen or -ibuprofen was prepared using 4-vinylpyridine and ethylene glycol dimethacrylate as a functional monomer and cross-linker, respectively, by a multistep swelling and thermal polymerization method. Next, a 1:1 mixture of glycerol monomethacrylate and glycerol dimethacrylate was used for hydrophilic surface modification, and it was added directly to the MIP for (*S*)-naproxen or -ibuprofen 4 h after the start of molecular imprinting. The obtained RAM-MIP material for (*S*)-naproxen or -ibuprofen was applied for direct serum injection assays of the drug by a column-switching system, consisting of a RAM-MIP material and conventional C18-silica column. However, leakage of the imprint molecule prevented accurate and precise assays of the drug. This problem has been overcome by using the RAM-MIP for (*S*)-naproxen for the assays of ibuprofen in rat plasma. The optimized column-switching system was applied successfully to the assay of ibuprofen in rat plasma after oral administration.

Recently, selective enrichment and pretreatment of analytes in complex matrixes have been attained with solid-phase extraction (SPE) based on molecularly imprinted polymers (MIPs).[1,2] This technique has been used for various drugs and their metabolites such as pentamidine,[3] sameridine,[4] propranolol,[5] tamoxifen,[6] hydroxycoumarin,[7] darifenacin,[8] and theophylline.[9,10] The drawbacks of SPE based on MIPs include the requirement of precipitation of proteins for drugs in proteinaceous samples and the effect of the bleed of an imprint molecule from the MIP on accuracy and precision of the assay in the case of ultratrace bioanalysis. Thus, SPE based on MIPs was generally carried out by off-line mode. With regard to leakage of an imprint molecule from the MIP, it could be due to remainder of a trace amount of the imprint molecule in the resultant MIP. This problem has been overcome by imprinting a structurally related analogue and combining with chromatographic separations.[4,5,8,11]

On the other hand, a lot of restricted access media (RAM) have been prepared and used for enrichment and pretreatment of the analytes in proteinaceous samples by HPLC.[12−14] With RAM materials, 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 be separated. Recently, we prepared a RAM-MIP material, a uniform-sized MIP for (*S*)-naproxen selectively modified with hydrophilic external layer, through a combination of molecular imprinting and hydrophilic surface modification techniques.[15] Further, we preliminarily showed the applicability of the obtained RAM-MIP material to direct serum injection assay of (*S*)-naproxen. In this study, 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 a 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. This paper involves evaluation of the RAM-MIP materials for (*S*)-naproxen and -ibuprofen and application of the RAM-MIP material for (*S*)-naproxen to the assays of ibuprofen in rat plasma.

EXPERIMENTAL SECTION

Materials. Ethylene glycol dimethacrylate (EDMA) and 4-vinylpyridine (4-VPY) were purchased from Tokyo Chemical

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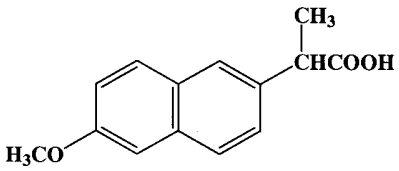
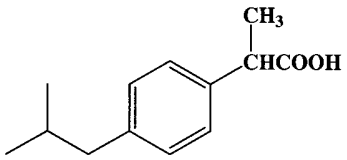


Naproxen



Ibuprofen

Figure 1. Structures of naproxen and ibuprofen.

Industry (Tokyo, Japan) and Wako Pure Chemical Industry (Osaka, Japan), respectively. Both monomers were purified by general distillation techniques in vacuo to remove the polymerization inhibitor. 2,2′-Azobis(2,4-dimethylvaleronitrile) (V-65), potassium peroxodisulfate, and bovine serum albumin (BSA) were purchased from Nacalai Tesque (Kyoto, Japan) and used without further purification. Glycerol monomethacrylate (GMMA) and glycerol dimethacrylate (GDMA) were gifts from Fuso Chemical (Osaka, Japan). (*S*)-(+)-Naproxen and racemic naproxen were purchased from Tokyo Chemical Industry. (*S*)-(+)-Ibuprofen and racemic ibuprofen were purchased from Aldrich Chemical (Milwaukee, WI). The structures of naproxen and ibuprofen are shown in Figure 1. Other reagents and solvents of an 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 eluent and the sample solution.

Preparation of RAM-MIPs. The RAM-MIPs for (*S*)-naproxen (RAM-MIP-N) and -ibuprofen (RAM-MIP-I) were prepared by a multistep swelling and polymerization method followed by hydrophilic surface modification techniques, as reported previously.[15] Similarly, nonimprinted, surface modified polymers (RAM) were prepared for comparison.

A water dispersion of the uniformly sized, polystyrene seed particles (0.497 g mL^{-1}), 0.17 mL, was admixed with a microemulsion prepared from 0.48 mL of dibutyl phthalate as activating solvent,[16] 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 oil microdroplets were completely disappeared. To the swollen particles, a microemulsion prepared from 0.375 g of V-65, 4 mL of toluene, 12.5 mL of water, and 10 mL of 4.8% poly(vinyl alcohol) solution was added. This second-step swelling was carried out at room temperature for 2 h with stirring at 125 rpm. To the dispersion of swollen particles, a dispersion of 6 mL of EDMA, 6 mmol of 4-VPY, 12.5 mL of water, and 10 mL of 4.8% poly(vinyl alcohol) solution was added. This third-step swelling was carried out at room temperature for 2 h with stirring at 125 rpm. When the template molecule was added, 2 mmol of (*S*)-naproxen or -ibuprofen was admixed with the monomers utilized to prepare the dispersion for the third-step swelling. After the third-step swelling was completed, the polymerization procedure was started at 50 °C under argon atmosphere with slow stirring. After 4 h of polymerization, the hydrophilic monomers (0.5 mL of GMMA and 0.5 mL of GDMA), with 0.02 g of potassium peroxodisulfate, were added to the polymerizing materials. After a further 20 h of stirring at 70 °C, the dispersion of polymerized particles was poured into 250 mL of methanol and the supernatant was discarded after sedimentation of the particles. The polymer particles were redispersed into methanol, and the supernatant was again discarded after sedimentation. This procedure was repeated three times in methanol, once in water, and twice in tetrahydrofuran (THF). The resulting 5−6-μm polymer particles were collected using a membrane filter, washed with THF and then with acetone, and finally dried at room temperature.

The prepared materials were packed into a stainless steel column (l00 mm × 4.6 mm i.d. or 10 mm × 4.0 mm i.d.) by a slurry technique using methanol as the slurry medium to evaluate their chromatographic characteristics.

Evaluation of RAM-MIPs. Scanning electron micrographs (SEMs) were performed on the MIP and RAM-MIP for (*S*)-naproxen using an S-4300 instrument (Hitachi, Tokyo, Japan).

The HPLC system used was composed of a PU-980 pump, a UV-970 spectrophotometer (both from Japan Spectroscopic Co., Tokyo, Japan), a Rheodyne 7125 injector with a 20-μL loop (Cotati, CA), and a C-R6A integrator (Shimadzu, Kyoto, Japan). The flow rate was maintained at 1.0 mL min^{-1}. Detection was performed at 223 nm. Retention factors were calculated from the equation $k = (t_R - t_0)/t_0$, where t_R and t_0 are retention times of retained and unretained solutes, respectively. The retention time of unretained solute, t_0, was measured by injecting acetone. The enantioseparation factor is calculated from the equation $\alpha = k_S/k_R$, where k_R and k_S are the retention factors of the first and second eluted enantiomers, respectively. The selectivity factor is calculated from the equation $S = k_{RAM-MIP}/k_{RAM}$, where $k_{RAM-MIP}$ and k_{RAM} are the retention factors on the RAM-MIP and RAM, respectively. All separations were carried out at 25 °C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The eluents were prepared by using phosphoric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, and acetonitrile.

Recovery of BSA from RAM-MIPs. The recovery of BSA from the RAM and RAM-MIPs was calculated based on the peak area of BSA sample (1 mg) by taking the area obtained without a column as 100%.

Application of RAM-MIP for Direct Serum Injection Assay of Ibuprofen. Column-Switching Procedure. With addition to the HPLC system described above, an LC-10AD pump (Shimadzu, Kyoto, Japan) and a six-port switching valve (Analchem, Luton, U.K.) were used. The precolumn packed with RAM-MIP-N (10 mm × 4.0 mm i.d.) was equilibrated with 20 mM phosphoric acid−acetonitrile (78:22 (v/v), pH 2.24) (eluent A), and a 20-μL aliquot of a serum sample was loaded. The precolumn was washed for 5 min with the eluent A at a flow rate of 1.0 mL min^{-1} to remove proteinaceous components and ordinary plasma components. Then the six-port switching valve was actuated, and ibuprofen retained on the precolumn was swept to the analytical column (Cosmosil 5C18-MS, 150 mm × 4.6 mm i.d.) in the back-flush mode by 20 mM sodium phosphate buffer−acetonitrile (75:25

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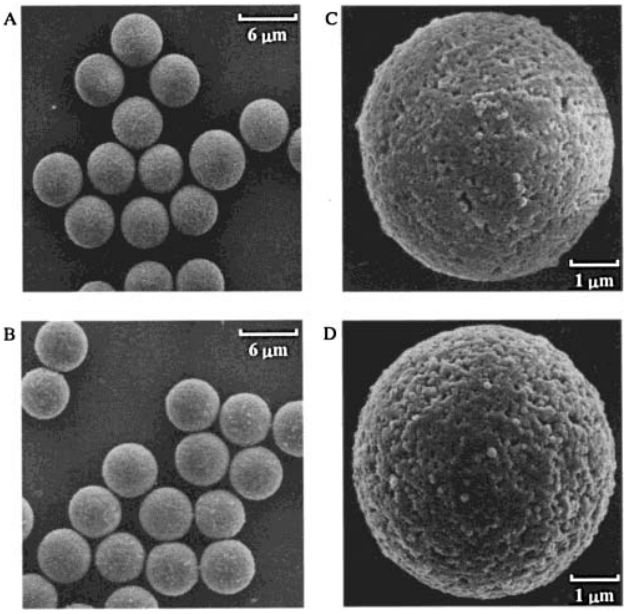

Figure 2. Scanning electron micrographs of the MIP (A, C) and RAM-MIP (B, D) for (*S*)-naproxen: (A, B) 2000× magnification; (C, D) 10000× magnification.

Table 1. Retention, Enantioseparation, and Selectivity Factors of Naproxen and Ibuprofen on RAM-MIP-N and -I Materials[a]

solute	RAM k	RAM-MIP-N k_S	RAM-MIP-N α	RAM-MIP-N S[b]	RAM-MIP-I k_S	RAM-MIP-I α	RAM-MIP-I S	eluent
naproxen	3.61	15.84	1.62	4.39	5.35	1.00	1.48	1
	2.11	8.35	1.49	3.96	3.71	1.00	1.76	2
ibuprofen	3.41	5.45	1.00	1.60	6.09	1.17	1.79	1
	2.24	3.78	1.00	1.69	4.42	1.08	1.97	2

[a] HPLC conditions: column size, 100 mm × 4.6 mm i.d.; flow rate, 1.0 mL min^{-1}; column temperature, 25 °C; loaded amount, 250 ng. Eluents: eluent 1, 20 mM phosphate buffer (pH 3.20)/CH_3CN = 50:50 (v/v); eluent 2, 20 mM phosphate buffer (pH 5.08)/CH_3CN = 50:50 (v/v). [b] S is the selectivity factor, $k_{RAM-MIP}/k_{RAM}$

(v/v), pH 7.34) (eluent B) at a flow rate of 1.0 mL min^{-1}. The precolumn and analytical column, respectively, were operated at ambient temperature and at 30 °C using a water bath (Thermo Minder Lt-100, Taitec). The precolumn was switched back after 2 min and equilibrated with eluent A. Ibuprofen was separated on the analytical column with eluent B.

Method Validation. The intra- and interday validation data were obtained with the assay of rat plasma samples spiked with ibuprofen over five and three replicates, respectively. For calibration standards, the plasma samples were prepared at varied concentrations from 0.2 to 50 μg mL^{-1} ibuprofen and assessed by five replicate determinations at each concentration.

Sample Preparation. Under urethan anesthesia, ibuprofen (10 mg kg^{-1}) was administered orally to a male Sprague−Dawley rat; 200 μL of blood sample was withdrawn from an abdominal vein at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, and 8 h after the administration. The collected blood sample was immediately transferred into a 1.5-mL polypropylene tube containing disodium ethylenediaminetetraacetic acid at a final concentration of 1 mg mL^{-1}. The plasma sample was separated by centrifugation (1500*g*

Table 2. Recovery (%) of BSA from RAM and RAM-MIPs[a]

material	eluent pH 7.1	eluent pH 3.4
RAM	104.3 ± 2.0[b]	107.5 ± 1.8
RAM-MIP−N	101.4 ± 3.4	101.9 ± 1.2
RAM-MIP−I	104.1 ± 2.2	109.7 ± 1.5

[a] HPLC conditions: column, 100 mm × 4.6 mm i.d.; eluent, 50 mM phosphate buffer/CH_3CN = 90:10 (v/v); flow rate, 1.0 mL min^{-1}; detection, UV absorbance at 280 nm. [b] Average ± SD.

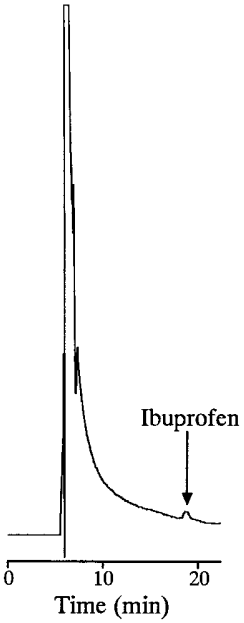

Figure 3. Chromatogram obtained with a 20-μL injection of water using column-switching techniques. Precolumn, RAM-MIP-I (10 mm × 4.0 mm i.d); analytical column, Cosmosil 5C18-MS (150 mm × 4.6 mm i.d.); eluent for pretreatment, 20 mM phosphoric acid−acetonitrile (78:22 (v/v), pH 2.24) at 1.0 mL min^{-1} 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.

for 10 min) from the blood and stored at −20 °C until analysis. The plasma was filtered through a 0.45-μm membrane filter, and a 20-μL portion of the sample was injected onto the precolumn.

RESULTS AND DISCUSSION

Preparation and Evaluation of RAM-MIPs. We prepared a RAM-MIP material for (*S*)-naproxen (RAM-MIP-N), a uniform-sized MIP for (*S*)-naproxen selectively modified with hydrophilic external layer.[15] First, the MIP for (*S*)-naproxen was prepared using 4-VPY and EDMA as a functional monomer and cross-linker, respectively, by a multistep swelling and thermal polymerization method. Next, a 1:1 mixture of GMMA and GDMA was used for hydrophilic surface modification, and it was added directly to the MIP for (*S*)-naproxen 4 h after the start of molecular imprinting. Similarly, we prepared a RAM-MIP material for (*S*)-ibuprofen, RAM-MIP-I, and nonimprinted, surface-modified material, RAM. Figure 2 shows SEMs of the MIP and RAM- MIP for (*S*)-naproxen. The good size uniformity was obtained before and after hydro-

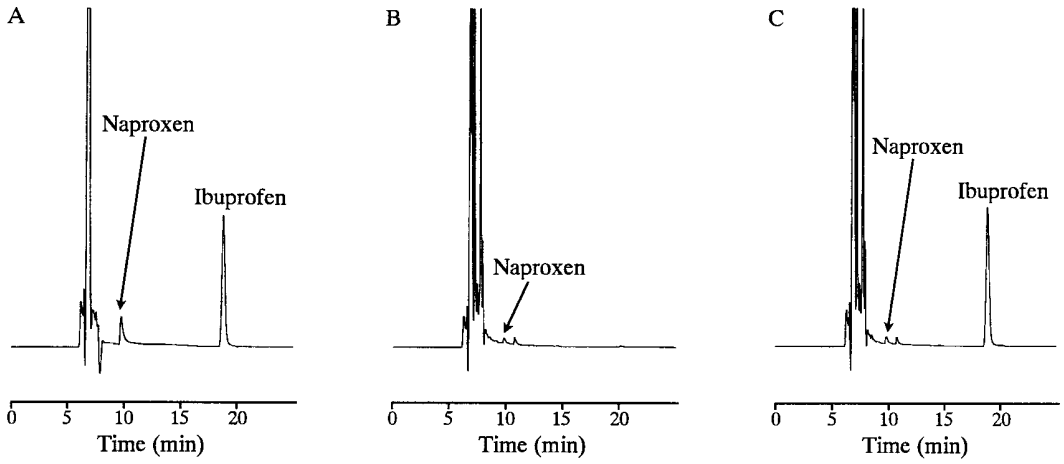

Figure 4. Chromatograms of standard ibuprofen sample (A), control plasma sample (B), and control plasma sample spiked with ibuprofen (C) using column-switching techniques. HPLC conditions as in Figure 3. Ibuprofen concentration is 5.0 μg mL^{-1} in (A) and (C).

philic surface modification. Moreover, the outward appearances were relatively similar to each other. These observations strongly suggest that the added GMMA and GDMA could be adsorbed and incorporated on the surface of the base particles. Further, the results obtained revealed that the RAM-MIP could be applicable to direct serum injection assays of (*S*)-naproxen. In this study, we tried to apply the RAM-MIPs for (*S*)-naproxen and -ibuprofen to direct serum injection assays of them.

Table 1 shows the retention, enantioseparation, and selectivity factors of naproxen and ibuprofen on RAM-MIP-N and RAM-MIP-I materials. The RAM-MIPs, RAM-MIP-N and -I, could resolve racemic naproxen and ibuprofen, respectively. These results revealed that the chiral recognition sites of (*S*)-naproxen and -ibuprofen remained unchanged with hydrophilic surface modification. The RAM-MIP-N retained (*S*)-naproxen selectively and (*S*)-ibuprofen moderately, while the selectivity factor obtained for (*S*)-ibuprofen on the RAM-MIP-N was similar to that obtained on the RAM-MIP-I.

Recovery of BSA from RAM-MIPs. Table 2 shows the recovery of BSA from RAM, RAM-MIP-N, and RAM-MIP-I after injection of 1 mg of BSA using a mixture of phosphate buffer and acetonitrile as an eluent. After hydrophilic surface modification of the nonimprinted and imprinted materials, BSA was almost completely recovered from all the materials. The results described above reveal that the MIPs for (*S*)-naproxen and -ibuprofen are selectively modified with hydrophilic external layer and that direct serum injection assays of these drugs could be attained using the RAM-MIPs.

Application of RAM-MIP for Direct Serum Injection Assays of Ibuprofen. Column-Switching Procedure. We tried to apply the RAM-MIP-N and -I to the direct serum injection assays of (*S*)-naproxen and -ibuprofen, respectively, using column-switching techniques. The method includes adsorption of racemic naproxen and ibuprofen on the respective MIPs, RAM-MIP-N and -I, stepwise desorption of the (*R*)- and (*S*)-forms, and analysis of the respective enantiomer on a C18 column. However, the respective enantiomer was not individually desorbed from the MIPs. Next, we tried to apply the RAM-MIP-I to the direct serum injection assays of racemic ibuprofen. Figure 3 shows a chromatogram obtained with a 20-μL injection of water using RAM-MIP-I and C18 columns as a precolumn and analytical column, respectively. A peak at 18.8 min appeared with no injection of ibuprofen. This result suggests leakage of the imprint species from the precolumn. It has been reported that even thorough wash of the imprinted materials results in appearance of a peak corresponding to the imprint species.[4,5,8,11] These results suggest that neither RAM-MIP-I could be used for the assays of ibuprofen as a precolumn nor RAM-MIP-N for the assays of naproxen.

Table 3. Intraday and Interday Precision and Accuracy Data for Ibuprofen Assays in Rat Plasma

concentration (μg mL^{-1})			
added	measured[a]	RSD[b] (%)	accuracy[c] (% deviation)
intraday (n = 5)			
0.2	0.199 ± 0.010	5.0	−0.4
5.0	4.74 ± 0.09	1.8	−5.3
50.0	51.6 ± 0.2	0.4	3.2
interday (n = 3)			
0.2	0.201 ± 0.007	3.5	0.4
5.0	4.65 ± 0.10	2.1	−7.0
50.0	51.4 ± 1.5	2.9	2.7

[a] Average ± SD. [b] RSD, relative standard deviation. [c] Accuracy: % deviation = [(concentration measured − concentration added)/concentration added] × 100.

As described above, the RAM-MIP-N recognized ibuprofen moderately. We tried to use the RAM-MIP-N for selective adsorption of ibuprofen in biological samples. The effect of eluent pH on the separation of ibuprofen enantiomers on the RAM-MIP-N was examined. Similar retentivity was obtained with eluent pH between 2.4 and 4.8. The retention of ibuprofen could be due to hydrophobic and hydrogen-bonding interactions between uncharged ibuprofen and 4-VPY-*co*-EDMA polymers.[17] On the other hand, with an increase in the eluent pH, the retention of ibuprofen was decreased because of dissociation of ibuprofen. Thus, ibuprofen was adsorbed onto the RAM-MIP-N using an acidic eluent and desorbed using an neutral eluent. Optimal eluents selected were 20 mM phosphoric acid−acetonitrile (78:22 (v/v), pH 2.24) for pretreatment, and 20 mM sodium phosphate buffer−acetonitrile (75:25 (v/v), pH 7.34) for analysis.

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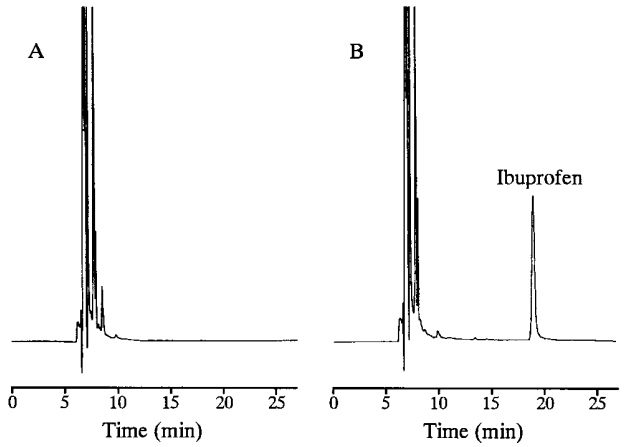

Figure 5. Chromatograms of rat plasma samples before (A) and 2 h after an oral administration of ibuprofen (B) using column-switching techniques. HPLC conditions as in Figure 3. Ibuprofen concentration is estimated to be 5.7 μg mL^{-} in (B).

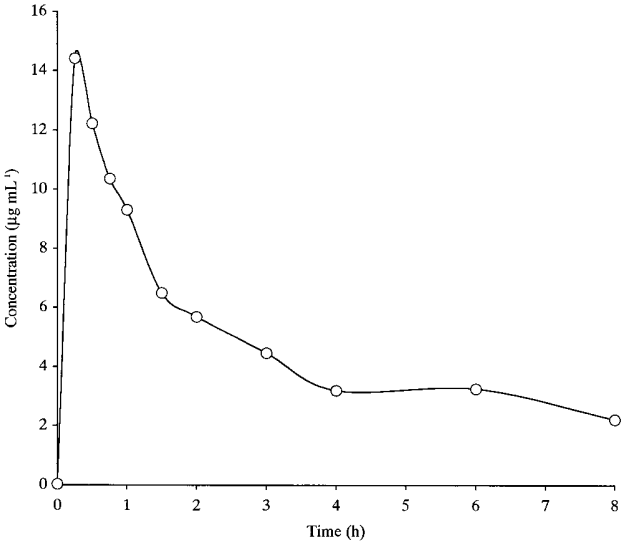

Figure 6. Time course data of the ibuprofen concentration in rat plasma after oral administration.

When a RAM material, polymer-coated mixed-functional silica,[18] was used under the same conditions instead of the RAM-MIP-N, ibuprofen was eluted from the precolumn. The RAM-MIP-N materials could be used for ~500 repetitive injections of a 20-μL plasma sample without a decrease in column efficiency or increase in back pressure. However, the limit of use has yet to be determined. The merits of the RAM-MIP materials are that selective enrichment and pretreatment are attained, that eluent pH range is wider compared with silica-based RAM, and that the materials could be washed by alkaline eluent in order to elute proteinaceous components.

Method Validation. Figure 4, 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 4 illustrates the fact that ibuprofen is separated from ordinary components of plasma samples and that ibuprofen is almost completely recovered from the serum samples. Further, (*S*)-naproxn, imprint species, appeared on a chromatogram. However, it was completely separated from ibuprofen on a C18 column. Table 3 shows the intra- and interday precision and accuracy data of ibuprofen assays in rat plasma samples. The relative standard deviation (RSD) of the ibuprofen assay was highly reproducible, as less than 5%, while the absolute percentage deviations ranged from 0.4 to 7.0%. The calibration graph, constructed from peak area versus ibuprofen concentration, was linear with a correlation coefficient of 0.999 over the concentration ranges 0.2−50 μg mL^{-1}. The quantitation limit was 0.2 μg mL^{-1}with a 20-μL injection, as less than 2% in RSD. The detection limit was 0.05 μg mL^{-1} at a signal-to-noise ratio of 3 with a 20-μL injection.

Assays of Ibuprofen after Oral Administration. The optimized method was applied to the assays of ibuprofen after the oral administration. Figure 5, parts A and B, shows chromatograms of rat plasma samples before and 2 h after an oral administration of ibuprofen (10 mg kg^{-1}), respectively. Figure 6 shows time course data of the ibuprofen concentration in rat plasma after oral administration. The obtained result agrees well with those reported previously.[19]

CONCLUSIONS

We prepared RAM-MIP materials, uniform-sized MIPs for 2-arylpropionic acid derivatives selectively modified with hydrophilic external layer, through a combination of molecular imprinting and hydrophilic surface modification techniques. The obtained RAM-MIP materials could be used for direct serum injection assays of the drug by column-switching techniques. Further, leakage of the imprint molecule from the RAM-MIP was prevented by molecular imprinting of a structurally related analogue of an analyte of interest. The proposed method could have wide applicability for affinity-based extraction of drugs in biological fluids.

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