Original

Preparation of Packing Materials for Temperature-Responsive Chromatography. Modification of Silica Surface with Poly (*N*-isopropylacrylamide)

Kazuo Yamamoto[1], Hideko Kanazawa[1]*, Yoshikazu Matsushima[1], Nobuharu Takai[2], Akihiko Kikuchi[3] and Teruo Okano[3]

[1] *Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan*

[2] *Faculty of Science and Engineering, Tokyo Denki University, Saitama 350-0394, Japan*

[3] *Institute of Biomedical Engineering, Tokyo Women's Medical University3), 8-1 Kawadacho, Shinju-ku, Tokyo 162-8666, Japan.*

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Abstract

Poly (*N*-isopropylacrylamide) (PIPAAm) with a carboxyl terminal was coupled with aminopropylsilica for preparation of packing materials of temperature-responsive chromatography. Several coupling methods were examined. The best results were obtained by the method, in which an active ester of the terminal carboxyl with succinimide was coupled in 1,4-dioxane. Scanning electron micrographs showed similar external appearances for both PIPAAm modified and unmodified silica, indicating the particle and pore sizes were essentially the same. With the packing material, five steroids and benzene were well separated with water as a sole mobile phase at a column temperature of 50˚C, while they were poorly separated at 15˚C, which is lower than the lower critical solution temperature of PIPAAm (32˚C).

Key words : Poly (*N*-isopropylacrylamide); temperature-responsive chromatography; packing material.

Introduction

Poly(*N*-isopropylacrylamide) (PIPAAm) exhibits thermally reversible soluble-insoluble changes in aqueous solution in response to temperature changes across a lower critical solution temperature (LCST) at 32˚C. The polymer chains show an expanded conformation in water below the LCST due to strong hydration and change to compact forms above the LCST by dehydration.

PIPAAm hydrogel has been utilized for drug delivery systems (1-3), and cell culture substrate (4-6). PIPAAm has also been utilized in thermoresponsive bioconjugates (7-12), which can be applied in reversible bioreactor systems. We have recently developed a new chromatography system based on PIPAAm (13) grafted on the surface of the packing materials.

There have been many reports on polymer-immobilization methods on material surfaces. However they are not satisfactory for preparation of the packing materials for HPLC columns, because of low polymer density on the surfaces. In an effort to obtain packing materials for temperature-responsive chromatography, we tried various methods to immobilize the polymer on silica surface. Most successful method obtained to date is to prepare PIPAAm with a carboxyl group on one terminal and to couple it with the surface of aminopropylsilica. However, some materials obtained by the method showed poor temperature-responsive properties. We assumed that the properties may be dependent on the conditions of coupling reaction

*Corresponding author

and examined the chromatographic behaviors of packing materials prepared under various experimental conditions. In this report, we present the results of the investigation.

Experimental

Materials

N-Isopropylacrylamide (IPAAm; Kodak, Rochester, NY) was purified by recrystallization from a toluene-hexane mixture and dried in vacuo at room temperature. 3-Mercaptopropionic acid (MPA; Wako Pure Chemicals, Osaka, Japan) was distilled under reduced pressure and a fraction boiling at 95˚C (5 mmHg) was used.

2,2'-Azobis [isobutyronitrile] (AIBN), *N*, *N*-dimethylformamide (DMF), ethyl acetate (EtOAc) and 1,4-dioxane were obtained from Wako Pure Chemicals and purified by the conventional methods. *N*, *N'*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (SuOH) and 4-dimethylaminopyridine (DMAP) were purchased from Wako Pure Chemicals and aminopropylsilica (averaged diameter of 5 μm, pore size 12 nm) from Nishio Kogyo (Tokyo, Japan). Sulfosuccinimidyl-4-*O*-(4,4'-dimethoxytrityl)butyrate (s-SDTB) was obtained from Pierce (Rockford, IL). 1-Ethyl-3-[3-(dimethylamino) propyl]carbodiimide hydrochloride (EDC) was obtained from Dojindo Laboratories (Kumamoto, Japan). Coomassie Brilliant Blue G-250 (CBB) was obtained from Bio-Lad Laboratories(Tokyo, Japan). Milli-Q grade water was used for sample solutions. Other reagents and solvents were obtained from commercial sources and used without further purification.

Phase Transition Measurements

The LCST of IPAAm polymers were determined by change of optical transmittance. The transmittances at 500 nm of IPAAm polymer solutions (5 mg/mL) were measured at various temperatures using a Shimadzu, UV-240 spectrophotometer. The temperature of observation cell was controlled with a deviation of ± 0.02 ˚C with a LAUDA RC20 waterbath.

Differential Scanning Calorimetry (DSC) thermogram of PIPAAm (8.9mg) was obtained using a heating rate of 2.5 $Kmin^{-1}$ with a METTER TA3000 SYSTEM.

Polymerization Procedure

Semitelechelic *N*-isopropylacrylamide (IPAAm) polymer was prepared by radical polymerization of IPAAm in DMF (14). AIBN and MPA were used as an initiator and a chain transfer agent, respectively. IPAAm was dissolved in DMF and AIBN and MPA were added. The reaction mixture was degassed by subjecting it to freeze-thaw cycles and introduced into an ample, which was sealed under reduced pressure. The reaction was then carried out at 70˚C for 12h. After evaporation of the solvent, the reaction mixture was poured into diethylether and precipitated polymer was collected. The polymer with a carboxyl terminal thus obtained was further purified by repeated precipitation from DMF solution with addition of diethylether.

Coupling of IPAAm polymer to aminopropylsilica

Seven packing materials were prepared by coupling the polymer with a carboxyl terminal to aminopropylsilica under various reaction conditions. The coupling methods are grouped into dehydration condensation method using EDC (material 1,2) and activated ester method (material 3-7). Materials prepared were washed three times with 50mL of water and 50mL of methanol consecutively, dried at room temperature in vacuo and packed into columns.

Material 1

IPAAm polymer with a carboxyl terminal (1.0 g) was macerated in 30mL of water. To the completely dissolved solution, 0.5g of aminopropylsilica was added and after addition of 0.2g of EDC, the polymer solution was incubated at 4˚C. This process was repeated further three times with 30-min intervals. The total amount of EDC added was 1.0g. The reaction mixture was incubated for 17 h at 4˚C.

Material 2

The pH of the solution of the polymer, aminopropylsilica and EDC was adjusted to 3.0 with 0.1M HCl and was maintained at 3.0 during the reaction. The solution was incubated in the same way as in material 1.

Substitution reactions of IPAAm polymer

The terminal carboxyl group of the IPAAm polymer was activated by coupling with SuOH using DCC in dry ethyl acetate. A mixture of the polymer, SuOH and DCC in molar ratio of 1:2.5:2.5 was kept at 0˚C for 2 h, then at 25˚C for 12h. After precipitated dicyclohexylurea was removed by filtration, the mixture was concentrated by evaporation. The activated polymer was recovered by precipitation from dry diethylether and used for preparation of material 3-7.

Material 3

To a solution of 1.0g of the activated polymer in 30 mL of cold water was added 0.5g of aminopropylsilica. The condensation reaction between the active ester and the amino group was allowed to proceed at 25˚C for 17 h with gentle shaking.

Material 4

The pH of the solution of the polymer and aminopropylsilica was adjusted to 8.5 with 50mM $NaHCO_3$ and was maintained at 8.5 during the reaction. The reaction was allowed to proceed by incubation in the same way as in material 3.

Material 5

To a solution of 1.0 g of the activated polymer in 30 mL of dry-DMF was added 0.5g of aminopropylsilica. The condensation reaction was allowed to proceed at 25˚C for 17 h with gentle shaking.

Material 6

The activated polymer (1.0 g) and DMAP (1.0 g) were macerated in 30 mL of dry-DMF. After the activated polymer was completely dissolved, 0.5 g of aminopropylsilica was added to the solution, which was incubated in the same way as in material 5.

Material 7

To a solution of 1.0g of the activated polymer in 30mL of dioxane was added 0.5g of aminopropylsilica. The condensation reaction was allowed to proceed at 25˚C for 17h with gentle shaking.

Glycidol End-Capping Reaction

Glycidol (15 mL, 226 mmol) was allowed to react with PIPAAm-modified silica (3g) in 1,4-dioxane at room temperature for 24h. After the end capping, PIPAAm modified packing material was washed with H_2O and methanol. The protocols used in the *N*-acetyl and glycidol end-capping reaction were based on the method of the previous reports (15,16).

Recovery of peptides from the PIPAAm modified silica support

The recovery of peptides from the PIPAAm modified silica support was examined by the CBB method (15). The recovery was calculated from the absorbance ratio at 595 nm with and without column.

Quantification of Amino Group on the Support

The amount of amino group on silica supports was determined by spectrophotometric method using s-SDTB (17). The dry support (~1 mg) was immersed into 5mL of 50mM sodium bicarbonate buffer (pH8.5) in a centrifuge tube. One milliliter of 0.1 mM s-SDTB solution was added to the tube, and the support was resuspended. The suspension was vigorously shaken at 25˚C for 30 min. After removal of the supernatant, the support was washed with water four times. A weighed support (W, mg) was treated with perchloric acid solution to liberate dimethoxytrityl cation. Absorbance at 498nm (Δ_{498}) was measured on a spectrophotometer (Shimadzu, UV-240). The amount of amino group on the support (Q μmol/g of solid support) is then calculated from the following equation:

$$Q=14.3\Delta_{498}V/W$$

where V expresses the volume of perchloric acid solution.

RESULTS AND DISCUSSION

Phase transition of PIPAAm

The DSC thermograms of PIPAAm showed a sharp endothermic peak at 32˚C. The phase transition was also observed in the temperature dependence of optical transmittance of PIPAAm polymer solutions. The LCST of PIPAAm used in this study was 32˚C.

Coupling of IPAAm polymer to aminopropylsilica

On PIPAAm-terminally grafted surfaces, we have previously observed a drastic and reversible surface hydrophilic/hydrophobic property alteration due to rapid changes in polymer hydration state around the polymer's transition temperature (18). The temperature-responsive surface property changes of terminal grafted surfaces were rapid and significant, suggesting that conformational freedom for PIPAAm graft chains (19) influence polymer dehydration and hydrogen bonding with water molecules. In this study, seven different experimental methods were employed for introduction of PIPAAm with a carboxyl terminal into aminopropylsilica. They are grouped into dehydration condensation method and activated ester method.

The amount of amino group on the silica supports was estimated by a spectrophotometric method using s-SDTB (17). The amounts on the aminopropylsilica used for preparation of materials 1-6 and material 7 were 210 μmol/g and 331 μmol/g of solid support, respectively. The residual amounts of amino group on the polymer grafted silica were 34~165 μmol/g of solid support. These indicate that 21~83% of the amino group on aminopropylsilica was bound PIPAAm molecules. Table 1 summarizes the results, which indicate that the activated ester method was superior to the dehydration condensation method judging from the introduction ratio.

Water soluble carbodiimide (WSC) has been used as a condensing agent in peptide syntheses. Gewehr et al. (20) employed the WSC method for preparation of PIPAAm modified packing materials. They examined gel permeation chromatography using porous glass beads modified with the IPAAm polymers. Grafted PIPAAm was used to control pore size by changing column temperature. Only slight changes in solute retention were observed on their column. This may be due to the fact that the introduction ratio of PIPAAm

Table 1. Preparation methods and Properties of the Packing Materials.

Material	Condensation method	Solvent	Residual amount of amino group (μmol/g)	Introduction ratio of PIPAAm (%)
1	EDC [a)]	H_2O	165 [c)]	22
2	EDC [a)]	H_2O(pH3)	144 [c)]	32
3	s-PIPAAm [b)]	H_2O	48 [c)]	77
4	s-PIPAAm [b)]	50mM $NaHCO_3$	85 [c)]	60
5	s-PIPAAm [b)]	dry-DMF	34 [c)]	84
6	s-PIPAAm [b)]	DMAP+dry-DMF	39 [c)]	81
7	s-PIPAAm [b)]	1,4-dioxane	64 [d)]	81

a) Dehydration condensation using EDC.
b) Reaction of activated ester of PIPAAm with SuOH and aminopropylsilica.
c) Amino group on the silica support was 210 μmol/g.
d) Amino group on the silica support was 331 μmol/g.

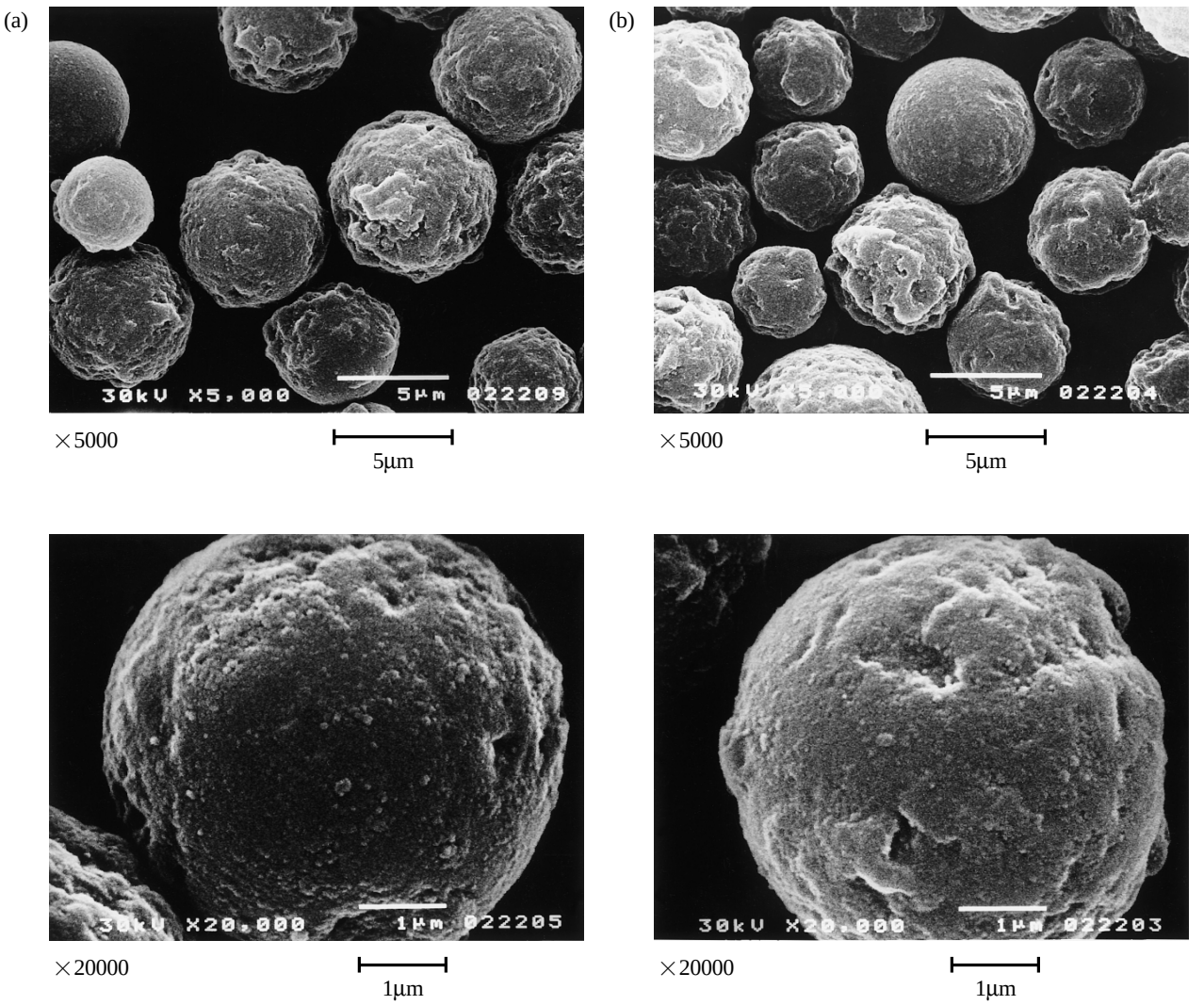

Figure 1. SEM of PIPAAm modified silicagel and aminopropylsilica
(a) unmodified aminopropylsilica, (b) PIPAAm modified silica.

molecules on the surface as well as inside surfaces of the porous beads was too low to alter the solute diffusion through the pores. The present results indicate that the WSC method gave lower introduction ratio of PIPAAm than the activated ester method. In the reactions with WSC in aqueous media, the activated species may be subject to hydrolysis, which lower the overall yield (21).

It was reported that addition of N-hydroxysulfosuccinimide to WSC mediated coupling reactions of peptides and proteins greatly enhanced the yield (21). Then we examined the active ester coupling method and tried to find the optimum conditions for higher introduction rate of PIPAAm. Formation of an active ester of the carboxy terminal of PIPAAm with SuOH was confirmed by means of IR and UV spectroscopy. The IR spectrum of the activated ester of PIPAAm showed the absorption at 1780 cm^{-1} assignable to the C=O stretching mode of succinimidyl group. The UV absorption spectra of aqueous solutions of PIPAAm and the activated ester showed no absorption band at the wavelengths longer than 250 nm. By the addition of ammonia solution, the band at 260 nm appeared in the solution of the activated ester and not in that of PIPAAm. The band is assignable to succinimidyl anion.

Use of DMAP as a catalyst for the coupling did not significantly improve the yield as shown by the results of material 6. Although the use of nonaqueous solvents such as DMF and 1,4-dioxane did not affect the yield, the procedure of solvent removal from reaction mixtures was much easier in 1,4-dioxane than in water and DMF. In conclusion, the active ester method in 1,4-dioxane solvent (material 7) was most suitable for the introduction the polymer to the aminopropylsilica.

Characteristics of the Prepared Particles

Scanning electron micrographs (SEM) of the modified silica particles (material 7) are shown in the Fig. 1 with those of the unmodified silica particles. Both the PIPAAm-modified and the unmodified silica showed similar external appearances. These indicate that the particle and pore sizes were essentially the same in both modified and unmodified silica and the induced polymer had negligible volume on the surface.

Effect of end-capping of the subsurface amino groups

For the preparation of the temperature-responsive packings, aminopropyl silica was used as a base support. After the coupling of large-size PIPAAm molecules to slica surface, residual aminopropyl groups are present and they sometimes adsorb biomolecules, such as protein. Recently, we found that residual surface amines are primary cause of the chemical instability of silica surface. Thus the elimination of residual surface amines are necessary. End capping of PIPAAm modified surface was examined by using acetyl chloride. However the end-capping reaction resulted in loss of thermosensitivity. The increased retention time of the solute was observed at low temperature. It may be caused to increase hydrophobicity of silica surface. Consequently, we end-capped the PIPAAm modified surface by glycidol.

The amount of residual amino groups on the PIPAAm-modified silica support (material 7) estimated by the spectrophotometric method using s-SDTB was 64.2 μmol/g solid support. After the end capping, the amount decreased to 34.3 μmol/g solid support.

To evaluate the effect of the end capping, elution behaviors of steroids with a variety of column temperatures were examined using water as a mobile phase. With both capped and noncapped columns, the same elution profiles of the mixture of steroids were obtained (shown in Figure 2).

The recoveries of insulin chains A and B and β-endorphin 1-27 fragment with the non-end capped column were 99.8, 74.9 and 87.8 %, respectively. The recoveries of the three peptides were 96.4, 87.0 and 90 %, with the capped column. The results indicated that glycidol end capping was effective for improving recoveries of more hydrophobic proteins such as chymotripsinogen (22).

Temperature-Responsive Chromatography

Materials prepared were packed into stainless steel columns and the columns were evaluated with a mixture of benzene, hydrocortisone, prednisolone, dexamethasone, hydrocortisone acetate, and testosterone. HPLC chromatograms were taken at varying column temperatures with water as a sole mobile phase.

With the columns packed with material 1 and 2, the analytes in the mixture were not separated at any temperature. The columns packed with material 4 gave only poorly separated chromatograms at 50˚C and unseparated chromatograms at lower temperatures. By using the column of materials 5, 6 and 7, excellent separation of the analytes was achieved at an elevated temperature and typical temperature-responsive elution behaviors were observed. The retention of steroids on column packed with material 3 is lower than that on column packed with material 7. It might be caused to deference of amount of amino group on used silica support. However, when the activated ester amine coupling reaction is carried out in an aqueous media, the active species should be subject to hydrolysis. Thus we used material 7 for temperature responsive chromatography.

Fig. 2 shows the chromatograms obtained at 15˚C, 30˚C and 50˚C using the column of material 7. The retention of hydrophobic

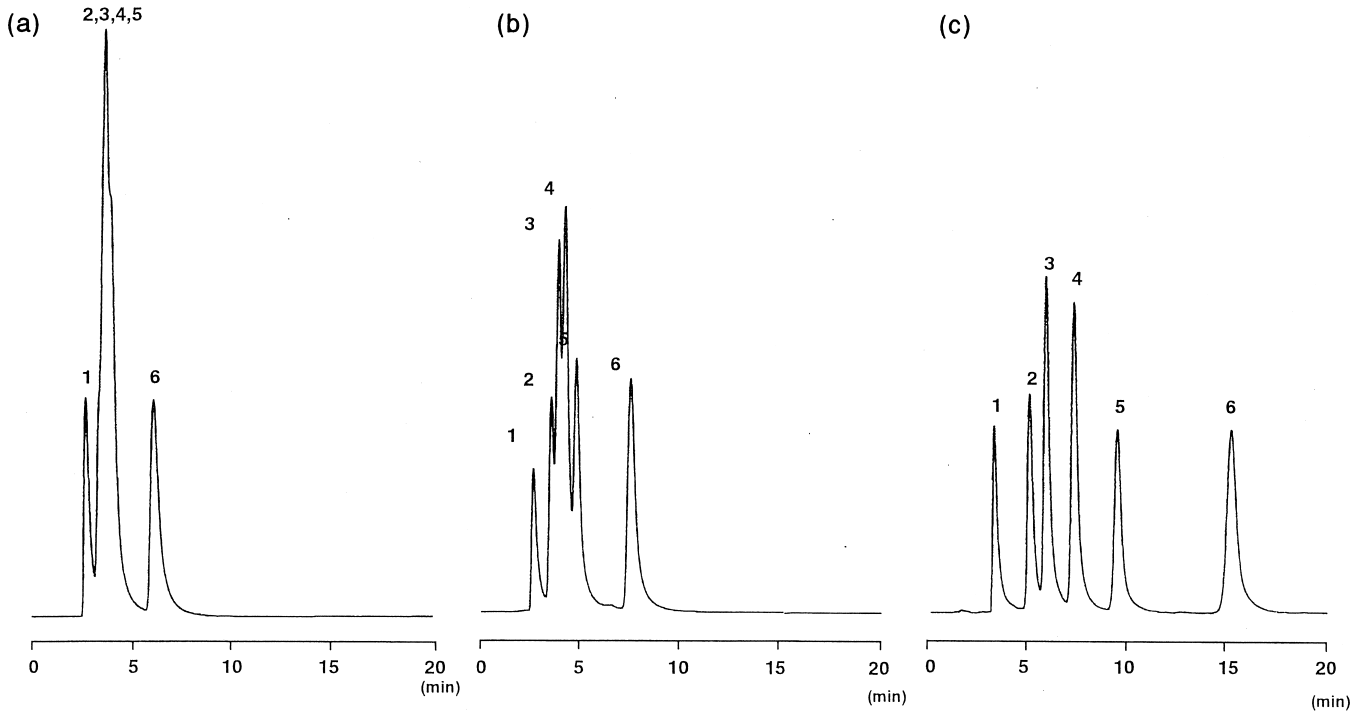

Figure 2. Chromatograms of a mixture of 5 steroids and benzene at various temperature. Column temperature:(a)15˚C, (b) 30˚C , (c) 50˚C. Peak No.1: benzene, 2: hydrocortisone, 3: prednisolone, 4: dexamethasone, 5: hydrocortisone acetate, 6: testosterone. Eluent : water, flow-rate: 1.0 mL/min, detection: UV 254 nm.

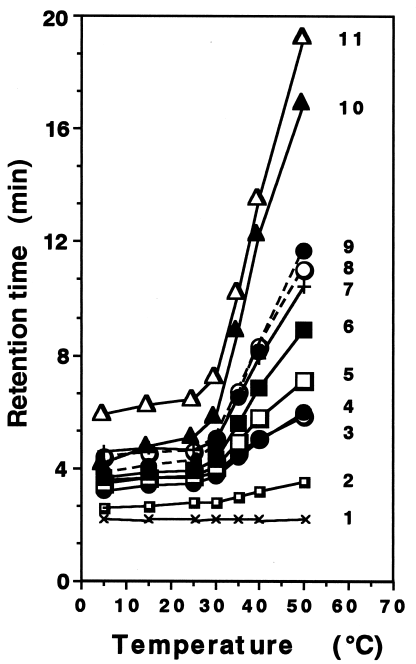

Figure 3. Temperature dependence of retention times of various steroids.
1: methanol, 2:benzene, 3:cortisone, 4:hydrocortisone, 5: prednisolone, 6:dexamethasone, 7: naphthalene, 8: cortisone acetate, 9: hydrocortisone acetate, 10: estriol, 11: testosterone.

steroids remarkably increased, as the column temperature was raised. At temperatures lower than LCST (32˚C), the steroids were not chromatographically resolved except for testosterone, the most hydrophobic steroid in the mixture. The excellent resolution of the steroids was achieved at 50˚C.

As shown in Fig. 3, the retention times of hydrophobic substances including steroids increased with increasing temperature. The elution profile was strongly affected by temperature. Changes in the retention times for hydrophobic steroids were larger than those of their hydrophilic counter parts and benzene. On the temperature-responsive column, the variation of the retention times with changes in temperature clearly demonstrated a reversed tendency compared to ordinary HPLC columns.

Generally, adsorption of molecules on surfaces and viscosity of mobile phases were decreased, while the solubility was increased at an elevated temperature. Due to these properties, the retention times decrease with increasing temperature for a normal chromatographic process. However, in the PIPAAm-modified columns, the opposite behavior was observed with increasing temperature in spite of decreased backpressure of column. It is noteworthy that the retention of steroids shows large changes above the LCST of PIPAAm. This implies that the transition of hydrophilic/hydrophobic surface proper-

ties at LCST causes this anomalous retention behavior of the steroids.

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