Original

Study of a Capillary LC System using Temperature−Responsive Polymer−Modified Packing Materials

Masahito Kanezawa[1], Eri Ayano[1], Hideko Kanazawa[1†], Yoshikatsu Akiyama[2] and Teruo Okano[2]

[1] *Kyoritsu University of Pharmacy, 1−5−30 Shibakoen, Minato−ku, Tokyo 105−8512, Japan.*

[2] *Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8−1 Kawadacho, Shinjyuku−ku, Tokyo 162−8666, Japan*

Abstract

We have developed a new method of HPLC packing materials modified with a temperature−responsive polymer, poly (*N*−isopropylacrylamide) (PNIPAAm). The surface properties and functions of the stationary phases are controlled by the external temperature. We could separate various pharmaceuticals, peptides and proteins by using only the aqueous mobile phase. In this study, we developed a capillary chromatographic system using packed PNIPAAm modified silica beads. We demonstrated the separation of steroids and phenylthiohydantoins (PTH)−amino acids on a capillary column by temperature control. The retention times of the analytes increased with increasing the temperature, and temperature−responsive elution behavior was observed. The result suggested that we can separate a trace amount of samples used in the pharmaceutical and biomedical fields by only changing the temperature in this system.

Key words: poly (*N*−isopropylacrylamide) (PNIPAAm); capillary column; temperature−responsive chromatography; steroid; PTH−amino acid.

1. INTRODUCTION

The needs of biotechnology for high−resolution purification and analytical technology have spurred new methods for chromatographic systems, concerning both columns and equipment.

In the case of the separation of peptides, proteins, and other biological molecules, and for maintaining viable cells, it is frequently necessary to avoid the use of organic solvents in the mobile phase, because these can cause sample denaturation. Complete avoidance of organic solvents is also advantageous for environmental reasons. Reversed−phase chromatography (RPC) using chemically modified stationary phases is generally faster and easier than other modes of high−performance liquid chromatography (HPLC), and consequently, has achieved very wide popularity. The retention and selectivity in RPC are controlled primarily by changing the polarity of the mobile phase. The use of organic solvents is necessary to prevent excessively long retention times with conventional reverse−phase columns.

Stimuli−responsive polymers, which change their structure and physical properties in response to external signals, comprise new materials with interesting applications in biomaterial science and technology. Such polymers that are responsive to changes in the pH [1], temperature, and light have been widely utilized for drug−delivery systems [2, 3], cell culture substrates [4, 5], and bioconjugates [6]. However, there have been few reports on the use of these stimulus−responsive polymers in chromatographic separations. Temperature seems to be one of the most attractive tools for such stimuli. There are technical difficulties associated with solvent−gradient elution in capillary columns. Temperature programming is used in microcolumns and capillary columns [7−10]. According to these reports, temperature change has been used as a

[†]**To whom correspondence should be addressed:**
Hideko KANAZAWA, Ph.D.
Department of Physical Chemistry, Kyoritsu University of Pharmacy 1−5−30 Shibakoen, Minato−ku, Tokyo, JAPAN
Phone: +81−3−5400−2657
Fax: +81−3−5400−1378
E−mail: kanazawa−hd@kyoritsu−ph.ac.jp

technique for changing the mobile−phase properties, for reducing the solvent viscosity, and for increasing the diffusion coefficient. However, in our system, changing the temperature strongly affects the properties and surface functions of the stationary phase, rather than the mobile phase. Also packed capillary columns are expected to be, due to their relatively low heat capacity and small diameter, more suitable for temperature programming than conventional HPLC columns[11].

Poly (*N*−isopropylacrylamide) (PNIPAAm) has the sharpest phase transition in the class of thermosensitive *N*−alkyl acrylamide polymers. This feature makes it the most suitable for studies and practical applications. PNIPAAm exhibits a thermally reversible soluble−insoluble change in response to temperature changes across a lower critical solution temperature (LCST) at 32℃ in aqueous solutions. Polymer chains of NIPAAm hydrate to form expanded structures below the LCST. At higher temperature than LCST, however, the chains form compact structures by dehydration. The remarkable phase transition of PNIPAAm in aqueous media is due to rapid hydration and dehydration changes of the polymer chain.

In this study, we prepared PNIPAAm−modified silica as column packing materials. Using capillary columns packed with PNIPAAm−modified silica, we demonstrated the analysis of steroids and phenylthiohydantoins (PTH)−amino acids using only an aqueous solution as a mobile phase without an organic solvent.

2. EXPERIMENTAL

2.1. Chemicals

N−isopropylacrylamide (NIPAAm) was kindly provided by KOHJIN (Tokyo, Japan) and was purified by recrystallization from hexane and dried at room temperature *in vacuo.* 3−Mercaptopropionic acid (MPA), 2, 2'−azobisisobutyronitrile (AIBN), *N, N*−dimethylformamide (DMF), ethyl acetate and dioxane were purchased from Wako Pure Chemicals (Tokyo, Japan). *N, N'*−dicyclohexylcarbodiimide (DCC) and *N*−hydroxysuccinimide were obtained from Wako Pure Chemicals. (Aminopropyl) silica (averaged diameter of 3μm, pore size 120Å) was purchased from Nishio Kogyo (Tokyo, Japan). HPLC−grade uracil and hydrocortisone were purchased from Wako Pure Chemical. Dexamethasone, testosterone and prednisolone were of HPLC grade and purchased from Sigma (St. Louis, MO, USA). PTH−amino acids were obtained from Wako Pure Chemicals. 6β−Hydroxytestosterone was purchased from Sumitomo Chemical (Tokyo, Japan).

Water was distilled and passed through a Milli−Q purification system (Millipore, Bedford, MA, USA).

2.2. Temperature−responsive polymer

We synthesized carboxyl semitelechelic polymers of NIPAAm by radical telomerization using MPA as a chain−transfer agent. PNIPAAm−grafted silica was used as a packing material. The immobilization reaction involved the formation of an amide bond between the carboxylic acid end group of PNIPAAm and surface amino groups on the aminopropyl silica support, using activated ester−amine coupling. This immobilization strategy is the same as that reported previously [12].

2.3. Instrumentation

The polymer−modified silica support was packed into a stainless−steel capillary column (250 mm × 0.5 mm i.d.) and a PEEKsil™ tube (100 mm × 0.15 mm i.d.; Upchurch Scientific, WA, USA) by a Capillary column packer (Nano baume; Western Fluids, CA, USA) at 5 MPa (8 hr at room temperature).

A Model MP 710 plunger pump controller for the constant−flow mode (GL Sciences Inc. Tokyo, Japan) was used as the eluent delivery system. The liquid chromatograph embodied a Model C 4 −1344−.1 internal sample injector with an injection volume of 100 nl (Valco Instruments, Houston, TX, USA), a Model MU 701 UV detector (GL Sciences Inc.) and an EZChrom Elite data processor (GL Sciences Inc.). The packed capillary column was housed in a model MO 706 column oven (GL Sciences Inc.). Milli−Q grade water was used as the mobile phase. The elution behaviors of the samples were recorded at a flow−rate of 1 ~ 5μL/min at various temperatures.

3. RESULTS AND DISCUSSION

Temperature−responsive chromatographic analysis of steroids and PTH−amino acids on a stainless−steel capillary column.

Using a capillary column packed with PNIPAAm modified silica connected to an HPLC system, the separation of steroids was carried out by changing the temperature. Milli−Q water was used as the sole mobile phase. Figure 1 shows typical chromatograms of steroids on a PNIPAAm terminally−grafted silica column packed into a stainless−steel capillary (250 mm × 0.5 mm i.d.). The retention times of hydrocortisone and dexamethasone were 14.87 and 18.07 min at 10℃, respectively, and 20.79 and 28.71 min at 50℃, respectively. With increasing the temperature, increased interactions between the solutes and the PNIPAAm terminally−grafted surfaces of the stationary phases were observed. The results indicated that a surface modified with PNIPAAm of the stationary phase exhibited temperature−controlled hydrophilic−hydrophobic changes. The surface was hydrophilic at a lower temperature and hydrophobic at an elevated temperature. The surface properties and functions of the stationary phases enabled to control by an external temperature; thus, the separation selectivity and retention of the solute were controlled by changes in the column temperatures

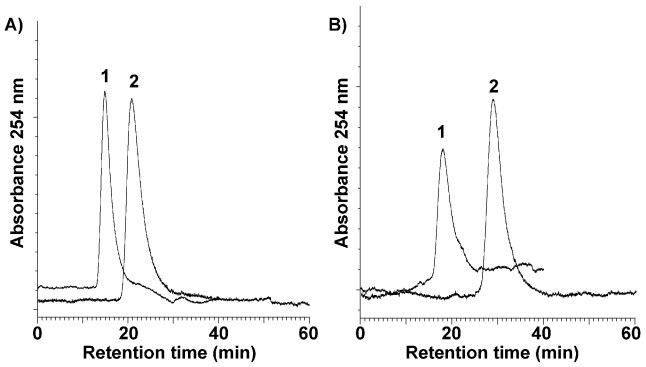

Figure 1. Chromatograms of steroids at two temperatures. Column, 250 mm × 0.5 mmi.d., packed with PNIPAAm−modified silica; flow rate, 5.0 μL/min; eluent, Milli−Q water; Samples; (A) hydrocortisone (100 μg/mL); (B) dexamethasone (60 μg/mL), column temperature; (1) 10℃ and (2) 50℃; detection, UV, 254 nm, injection volume, 0.1 μL.

Table 1. Comparison with conventional column and capillary column.

	Injection volume (μL)	Flow rate (mL/min)	Cloumn volume (mm^3)
Conventional column (4.6 ϕ × 150 mm)	10	1	2492
Capillary column (05 ϕ × 250 mm)	0.1	0.002	49
PEEKsil™ tubing capillary column (0.15 ϕ × 100 mm)	0.1	0.001	1.77

without any change in the eluent with the capillary column.

Table 1 gives a comparison with the conventional column and capillary column. The sample injection volume was reduced to one −hundredth, the flow rate was reduced to one−to−five−hundredth and the retention time increased by five times in the case of analyzing dexamethasone. By using a capillary column, the consumptions of the eluent and the sample injection volume could be achieved to be one−hundredth that using the conventional column.

We previously demonstrated that a hydrophobic interaction between steroids and PNIPAAm grafted surfaces was readily modulated by the temperature using conventional−size columns. As previously reported, the retention of steroids exhibits a linear relationship with the log *P* values (the partition coefficients in 1−octanol/water system). The log *P* values for each steroid are 1.61 for hydrocortisone, 1.62 for prednisolone, and 1.83 for dexamethasone. In this study, similar results were obtained; the more hydrophobic steroids showed longer retention times.

In order to compare the effects of the temperature in the reverse−phase chromatography of steroids with a packed capillary column, the retention factors (*k*) were measured at column temperatures from 10 to 50℃ in 10℃ intervals; *k* can be expressed as

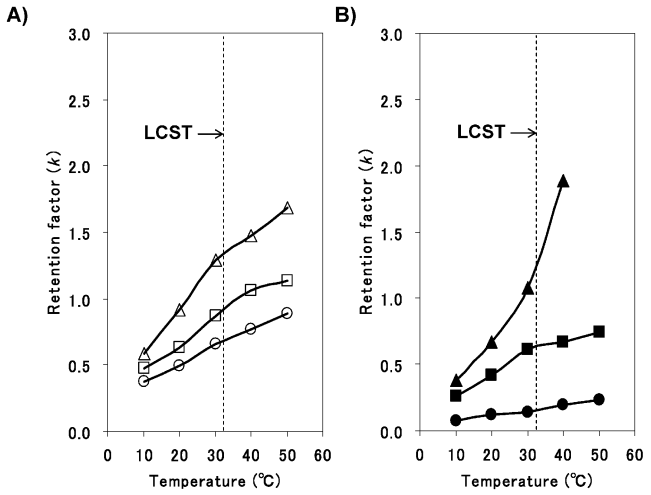

Figure 2. Retention factors of (A) three steroids and (B) three PTH−amino acids on a capillary column packed with PNIPAAm−modified silica with Milli−Q water used as a mobile phase. Flow rate, 2.0 μL/min; injection volume, 0.1 μL; detection, UV, 254 nm. Samples: (A) ○, hydrocortisone (20 μg/mL); □, prednisolone (32 μg/mL);△, dexamethasone (36 μg/mL); (B) ●, PTH−glycine (50 μg/mL); ■, PTH−valine (24 μg/mL); ▲, PTH −leucine (14 μg/mL).

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

Where t_R is the retention time of steroids and t_0 is the retention time of Uracil. Uracil was used as an inert tracer for measuring the mobile−phase hold−up times. Figure 2 (A) shows the elution behavior of steroids in Milli−Q water over a variety of column temperatures. With increasing temperature, increased retention factors were observed, despite a reduced back−pressure in the column. It is noteworthy that the retention of steroids showed large changes above the LCST of PNIPAAm. This implies that the transition of hydrophilic−hydrophobic surface properties at the LCST causes this anomalous retention behavior of the steroids. Figure 2 (B) shows the elution behavior of PTH−amino acids. The chromatographic conditions are the same as those in Figure 2 (A).

Figure 3 shows van't Hoff plots for steroids (A) and PTH−amino acids on the capillary column packed with PNIPAAm−modified silica. A nonlinear relationship between the reciprocal temperature (1/T) and ln *k* values is apparent for each steroid and PTH−amino acid. This provides evidence for the polymer phase transition on the stationary−phase surface. Generally, these plots should be linear for conventional chromatographic processes on commercially available reversed−phase columns under conditions where the retention mechanisms do not change. On the temperature −responsive polymer−modified column, however, deviations from linearity between the ln *k* values and the reciprocal temperature

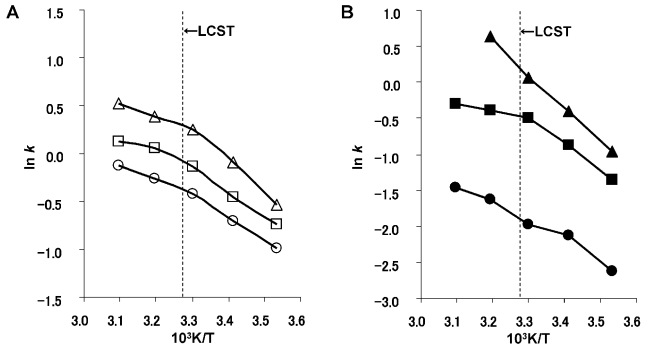

Figure 3. Van't Hoff plots of (A) three steroids and (B) three PTH−amino acids on the capillary column. The chromatographic conditions are the same as those in Figure 2. Samples: (A) ○, hydrocortisone; □, prednisolone;△, dexamethasone; (B) ●, PTH−glycine; ■, PTH−valine; ▲, PTH−leucine.

were observed. These deviations are consistent with the known phase transition of the grafted polymer stationary phase. Additionally, the slope of the van't Hoff plots on the packed with PNIPAAm−modified silica capillary column was negative, which is opposite to that seen for conventional chromatography. This provides additional evidence that the interaction between steroids or PTH−amino acids and temperature−responsive surfaces becomes stronger at elevated temperatures.

The results of our studies indicate that the driving force for retention in this system is hydrophobic interactions between the solute molecules and polymer chains on the surface. At temperatures higher than LCST, PNIPAAm−grafted surfaces exhibited hydrophobic properties and the sensitivity to hydrophobicity of the solutes was remarkably increased. In the case of being the column packed into capillary with a PNIPAAm−modified silica, the analysis of steroids and PTH−amino acids could be achieved with Milli−Q water as the mobile phase by controlling the external temperature.

Temperature−responsive chromatographic analysis of steroids and PTH−amino acids on a PEEKsil™ capillary column.

Figure 4 shows typical chromatograms of steroids. The separation of hydrocortisone and testosterone were achieved by changing the temperature with Milli−Q water as a mobile phase using a PEEKsil™ capillary column (100 mm × 0.15 mm i.d.) packed with PNIPAAm modified silica. In reversed−phase HPLC using an ODS column, the retention times should decrease with increasing temperature. However, the opposite behavior of the retarded retention times was observed with increasing temperature using a PNIPAAm modified capillary column. It was considered that a hydrophobic interaction exists between steroids and the PNIPAAm−modified silica.

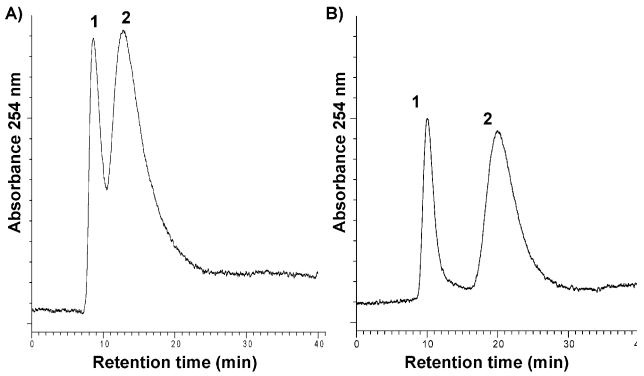

Figure 4. Chromatograms of steroids on a PEEKsil™ capillary column (100 mm × 0.15 mm i.d.) packed with PNIPAAm−modified silica.
Flow rate, 1.0 μL/min; eluent, Milli−Q water; temperature, (A) 10℃ and (B) 50℃; solutes: 1, hydrocortisone (10 μg/mL); 2, testosterone (15 μg/mL); detection, UV, 254 nm; injection volume, 0.1 μL.

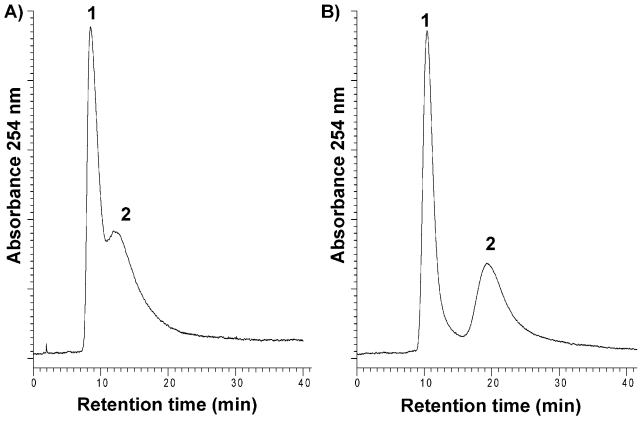

Figure 5. Chromatograms of testosterone and 6β−hydroxytestosterone at two temperatures. Column, 100 mm × 0.15 mm, packed with PNIPAAm−modified silica; flow rate, 1.0 μL/min; eluent, Milli−Q water; temperature, (A) 10 ℃ and (B) 50℃; solutes: 1, 6β−hydroxytestosterone (50 μg/mL); 2, testosterone (22.5 μg/mL); detection, UV, 254 nm; injection volume, 0.1 μL.

The oxidative metabolism of drugs in the liver is catalyzed by substrate−specific or selective cytochrome P 450 (CYP), a superfamily of haemoproteins that catalyze the metabolism of a large number of clinically important drugs [13−15]. Hepatic drug oxidation is a major source of interindividual variations in drug pharmacokinetics and therapeutic response. Testosterone is widely used as a probe to measure the activity of CYP 3 A 4, an important member of the cytochrome P 450 superfamily of drug−metabolizing enzymes. Testosterone undergoes oxidative metabolism to one major metabolite, β−hydroxytestosterone, in humans, a pathway which seems to be mediated almost exclusively by CYP 3 A isoforms. Figure 5 shows chromatograms of 6 β−hydroxytestosterone and

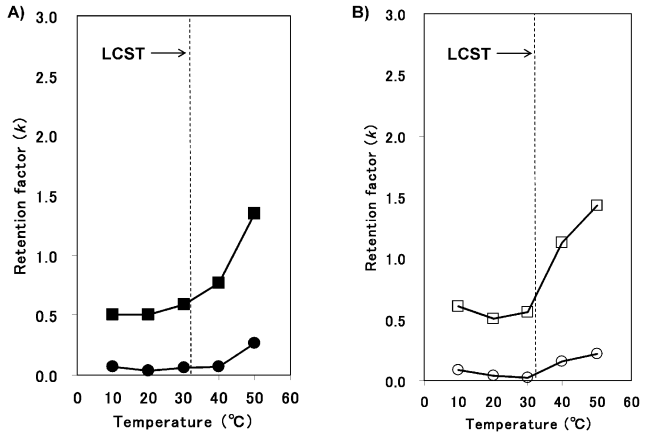

Figure 6. Retention factors of (A) hydrocortisone and testosterone; (B) 6β−hydroxytestosterone and testosterone on a PEEKsil™ tubing capillary column packed with PNIPAAm−modified silica with Milli−Q water used as a mobile phase. Flow rate, 1.0 μL/min; injection volume, 0.1 μL; detection, UV, 254 nm. Samples: (A) ●, hydrocortisone (10 μg/mL); ■, testosterone (15 μg/mL); (B), ○, 6 β−hydroxytestosterone (50 μg/mL); □, testosterone (22.5 μg/mL).

testosterone obtained at 10℃ and 50℃. For 6 β−hydroxytestosterone with lower hydrophobicity, the retention time hardly changed at 10℃ and 50℃. However, for testosterone with a higher hydrophobicity, the retention time was increased too much, and influenced the hydrophobic interaction at 50℃.

Figure 6 shows temperature−dependent retention profiles for steroids on a PNIPAAm modified capillary column. The stationary phase showed a greater affinity for steroids at a higher temperature (50℃) compared with those at a lower temperature (10℃). These observations would be due to a temperature−responsive conformational change of the NIPAAm polymer.

4. Conclusion

PNIPAAm−modified silica exhibits temperature−controlled changes in the hydrophilic−hydrophobic surface properties in aqueous systems. By using a capillary column packed with PNIPAAm−modified silica, it was observed that increasing the temperature increased the retention times of the analytes. This is the first report concerning a temperature−responsive chromatographic system using a PNIPAAm−modified silica capillary column. A system for controlling the function and properties of the HPLC stationary phases simply by changing the temperature, and furthermore decreasing consumption of the eluent, solutes and packing materials would be very useful.

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