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Aqueous chromatographic system for the quantification of propofol in biological fluids using a temperature-responsive polymer modified stationary phase

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ABSTRACT

A new method for the quantitative analysis of monkey serum propofol, which is widely used as an anaesthetic agent, was developed by utilizing a temperature-responsive polymer of *N*-isopropylacrylamide (NIPAAm) and butyl methacrylate (BMA) as the stationary phase of HPLC–fluorescence detection. This poly(NIPAAm-*co*-BMA) copolymer undergoes a reversible phase transition from a hydrophilic to a hydrophobic microstructure when triggered by change in the temperature. Also this chromatographic system is possible to separate the analytes by using only water as a mobile phase. A pretreatment of the serum (80 μL) was only solid-phase extraction, and the recovery rate of propofol and internal standard was more than 77%, respectively. This method covered the calibration range from 0.5 μg/mL to 10 μg/mL and allowed a reproducible quantification of the serum propofol in administrated monkey serum. The intra- and inter-assay relative standard deviations were less than 14.1%. In addition, there was good relationship of the quantification values between the developed method and the widely used reversed-phase HPLC method. Our developed method has proven to be useful for a simple analysis of propofol in clinical practice, because the avoidance of complicated mobile phase preparation was possible, and only temperature changing could regulate the retention time of the analyte. In addition, by using water instead of fossil fuel, it is the ideal analytical method according to green chemistry.

1. Introduction

Poly(*N*-isopropylacrylamide) (PNIPAAm) exhibits thermally reversible soluble–insoluble changes in response to temperature changes across a lower critical solution temperature (LCST) at 32 °C in aqueous solution [1]. PNIPAAm undergoes a sharp phase separation and a coil–globule transition of the polymer chain at its LCST. In water, polymer chains of PNIPAAm show an expanded conformation below the LCST due to strong hydration, and a change to compact forms above the LCST by sudden dehydration. Based on these unique features, PNIPAAm has been utilized for drug delivery systems [2], cell culture dishes [3], cell sheets [4] and bioconjugates [5]. Furthermore, studies of the PNIPAAm-modified surface, such as, an evaluation of the intermolecular force between the PNIPAAm surface and protein are being actively carried out. Aside from these, we have been investigating PNIPAAm and related temperature-responsive polymers used to generate thermally responsible stationary phases in chromatographic systems [6–9]. Hydrophobic interactions between analytes and the PNIPAAm grafted silica stationary phase are driving forces for hydrophobic steroid separations [6]. A thermoresponsive hydrophobically modified copolymer of butyl methacrylate (BMA) and NIPAAm grafted silica was applied for the separation of phenyl thiohydantoin amino acid [7]. In addition, we separated ionic compounds, such as nucleotides by introducing an ionizable group into PNIPAAm stationary phases [8]. More recently, we achieved a successful separation of an enzyme without the loss of bioactivity [9]. Thermoresponsive modifiers on silica surfaces effectively modulated the separation efficiencies in these chromatography systems in a purely aqueous milieu.

As mentioned above, we developed a novel analytical method. In addition, because utilizing the mobile phase is only for aqueous solutions or water in our method, there is the specific advantage of applying it to therapeutic drug monitoring (TDM) in clinical practice or real-time drug level monitoring in an operating room. Because reversed-phase liquid chromatography (RPLC),

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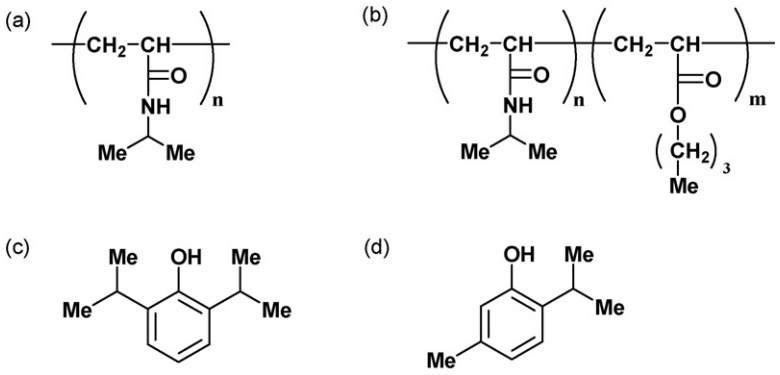

Fig. 1. The structure formula of PNIPAAm (a), poly(NIPAAm-co-BMA) copolymers (b), propofol (c) and thymol (d).

which is the most widely used HPLC technique in the quantification of pharmaceutical compounds, requires a large amount of organic solvent, there are some problems related to waste solutions and exposure of patients to harmful solvents. In this study, we applied temperature-responsive chromatography to the quantitative analysis of a pharmaceutical compound, propofol (2,6-diisopropylphenol) in a biological sample (Fig. 1). Propofol is an intravenous anesthetic that is used both for induction and maintenance of anesthesia [10,11]. Moreover, it is widely used during minor surgery of laboratory animals (rat, dog and monkey). Because a low propofol dosage causes intraoperative awaking, the monitoring of anesthetic drug levels during anesthesia or sedation is very important [12]. Though we developed some analytical methods utilizing LC–MS for determining of the propofol and related anesthetic agents [13–16], it is necessary to use a high concentration of the organic solvent for mobile phase. In the present paper, a new HPLC–fluorescence detection (HPLC–FL) method utilizing a temperature-responsive chromatographic system for the analysis of propofol is described. We also discussed the crucial difference between our developed method and the widely used RPLC one.

2. Experimental

2.1. Materials and chemicals

NIPAAm was kindly provided by Kohjin (Tokyo, Japan), and was purified by recrystallization from *n*-hexane and dried at 25 °C in vacuo. Propofol, thymol [internal standard (I.S.)] and BMA were purchased from Wako (Osaka, Japan). A stock solution of propofol was prepared as 100 μg/mL solutions in methanol. Subsequent dilutions were carried out with MeOH to prepare 800 ng/mL, 400 ng/mL, 200 ng/mL, 80 ng/mL and 40 ng/mL solutions. I.S. was dissolved in and diluted with MeOH to prepare a 400 ng/mL solution. Aminopropyl silica (average diameter of 5 μm; pore size, 12 nm) was purchased from Nishio Kogyo (Tokyo, Japan). High performance extraction disk cartridges [4115 (SD); Empore, St. Paul, MN, USA] were successively washed with MeOH (150 μL) and water (300 μL) prior to use. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All other reagents and solvent were of analytical grade.

2.2. Apparatus

HPLC–FL analysis was carried out using a Hitachi D-7000 controller equipped with L-7100 pump, L-2480 FL detector (excitation wavelength; 267 nm, emission wavelength; 310 nm). The column oven was an Aqua Way Gradienter product of Cellseed (Tokyo, Japan). The column was used at a flow rate of 1.0 mL/min and water was used as the mobile phase. The ^{1}H NMR spectra were obtained using a JEOL JNM-ECP600 spectrometer (600 MHz, Tokyo, Japan) and tetramethylsilane was used as the I.S.

2.3. Preparation of PNIPAAm-modified aminopropyl silica column

The synthesis of semitelechelic poly(NIPAAm-*co*-BMA) (IBc) copolymer and a modification of aminopropyl silica with the polymer were carried out as previously reported [7,17]. Poly(NIPAAm-*co*-BMA1%) (IBc-1%), poly(NIPAAm-*co*-BMA3%) (IBc-3%) and poly(NIPAAm-*co*-BMA5%) (IBc-5%) with the feed ratio of the NIPAAm/BMA molar ratio be fixed to 99:1, 97:3 or 95:5 were synthesized by radical polymerization with 2,2′-azobisisobutyronitrile (AIBN, radical initiator) and 3-mercaptopropionic acid (MPA, chain-transfer agent). The polymer-grafted silica bead support was packed into a stainless-steel column (150 × 4.6 mm I.D.).

2.4. Transmittance measurements

The LCSTs of IBc copolymers were determined by measuring the optical transmittance of a copolymer aqueous solution (0.5%, w/v). The transmittance changes at 500 nm of IBc copolymers were measured at various temperatures using a V-630 spectrophotometer (JASCO, Tokyo, Japan). The LCST was defined as the temperature at 50% optical transmittance of IBc copolymers solutions.

2.5. Pretreatment of monkey serum

Monkey (Japanese macaques, Macaca fsucata fsucata) sera were obtained from Primate Research Institute, Kyoto University and stored −80 °C. To the serum (80 μL) was added MeOH (10 μL) containing I.S. (400 ng). The mixture was purified using a 4115 (SD) cartridge. After washing with water (300 μL), the target analyte was eluted with MeOH (80 μL). 5 μL of which was subjected to HPLC–FL. An animal study was conducted under the guidelines provided by the Primate Research Institute, Kyoto University.

2.6. Calibration curve

The serum of monkey before the administration of propofol was used as the blank serum. The blank serum (80 μL) was spiked with propofol (40 ng, 80 ng, 200 ng, 400 ng and 800 ng each, corresponding to 0.5 μg/mL, 1.0 μg/mL, 2.5 μg/mL, 5.0 μg/mL, 10 μg/mL) and I.S. (400 ng), which was then pretreated and subjected to HPLC–FL. Calibration curves were constructed by plotting the peak area ratio (propofol/I.S., y) versus the concentration of propofol (μg/mL, x). LC conditions were as follows: column, IBc-3% modified column; column oven, 40 °C; mobile phase, H_2O; flow rate, 1.0 mL/min; FL detection, excitation wavelength; 267 nm, emission wavelength; 310 nm.

2.7. Recoveries of propofol and I.S. during pretreatment

A propofol spiked sample was prepared by the addition of propofol and I.S. (400 ng each) to the blank monkey serum. The spiked sample was pretreated as described in Section 2.5 and subjected to HPLC–FL. The percentage of recovery was calculated by comparing the peak area of pretreated samples with samples in which the same amount of compounds were diluted with the mobile phase and injected directly.

2.8. Assay precision

The intra-assay precision was assessed by determining two serum samples at different concentration levels ($n = 5$ for each sample). The inter-assay precision was assessed by determining these samples over a period of five days. The precision was determined as the relative standard deviation.

2.9. RPLC condition

RPLC condition was as follows: column, Inertsil ODS-3 (5 μm, 150 mm × 4.6 mm I.D. GL Science, Tokyo); column oven, 30 °C; mobile phase, H_2O–MeCN–H_3PO_4 (45:55:2, v/v/v); flow rate, 1.0 mL/min; FL detection, excitation wavelength; 267 nm, emission wavelength; 310 nm.

3. Result and discussion

3.1. Characterization of NIPAAm copolymers and their solubility changes in water

IBc copolymers with a single-terminal carboxyl group were synthesized by radical telomerization using MPA as a chain transfer agent (telogen) at 70 °C in *N,N*-dimethylformamide. Fig. 2 shows the structure of IBc copolymers and their composition were determined by ^{1}H NMR spectra in a $CDCl_3$ solution. The mole fraction of BMA in IBc was calculated by comparing the peak area of the singlet at 0.93 ppm arising from the terminal methyl group of the butyl side chains with the area of the singlet at 4.01 ppm, attributed to the resonance of the methine proton from isopropyl groups. Table 1 summarizes the results of the copolymerization of NIPAAm with BMA.

Fig. 3 shows the temperature-dependent optical transmittance changes for the IBc copolymer at (0.5%, w/v) aqueous solutions with various BMA compositions. The LCST values for IBc copolymers are also indicated in Table 1, which can be regulated by the amount of BMA. IBc copolymers all showed a lower LCST than that for homogeneous PNIPAAm. PNIPAAm exhibits its LCST at 32 °C, while LCSTs shift from 32 °C to 19.7 °C, along with an increase in the mole fraction of BMA over a range of 0.4–3.8 mol% in the copolymers. Phase transition of PNIPAAm depends on the balance between the hydrogen bond and the hydrophobic bond. In water PNIPAAm show an expand conformation below the LCST due to the hydrogen bond between amide group in the polymer and water molecule. In contrast above the LCST, PNIPAAm change to compact form by dehydration due to the isopropyl group in the polymer. For this reason, introduction of BMA which is hydrophobic molecule, into PNIPAAm enhances the hydrophobic property of copolymer and shifts the LCST to more low temperature [18].

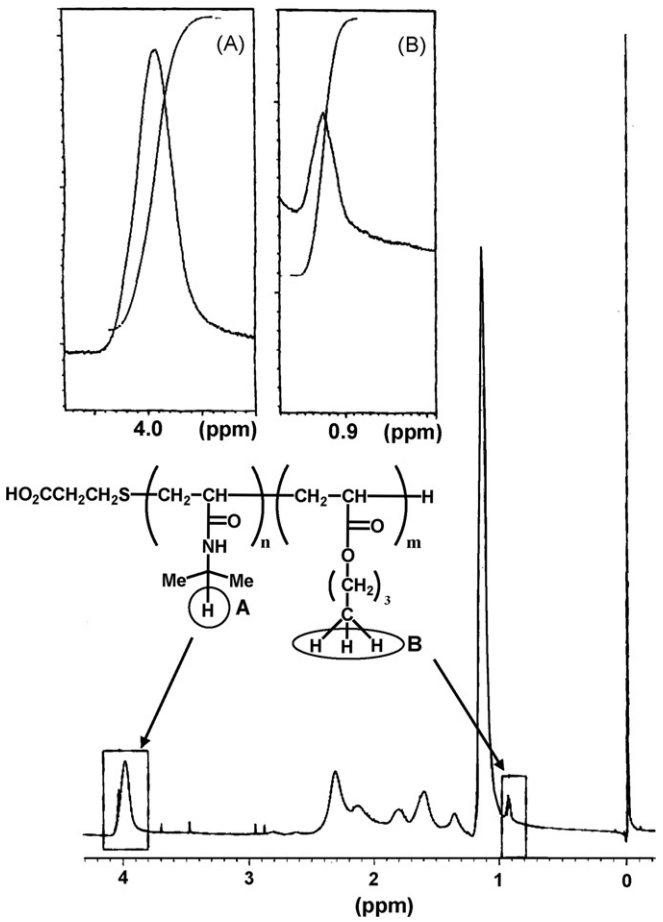

Fig. 2. The structure and the spectrum of ^{1}H NMR of copolymer of NIPAAm and BMA (IBc-5%). (A) Methane proton from isopropyl groups of NIPAAm. (B) Methyl proton from BMA. I.S.; tetramethylsilane. Solvent; deuterated chloroform.

Table 1
Properties of IBc-X copolymers having a semitelechelic carboxyl group.

	Mole fraction of BMA (mol%)		Mol wt[b]	LCST (°C)[c]
	In feed	Obsd[a]		
PNIPAAm	0	0	6134	31.8
IBc-1%	1.0	0.4	5263	30.3
IBc-3%	3.0	2.4	4100	24.6
IBc-5%	5.0	3.8	6696	19.7

[a] Determined by ^{1}H NMR analyses.
[b] Number-averaged molecular weight determined by end group titraton.
[c] LCST determined by transmittance measured at 500 nm.

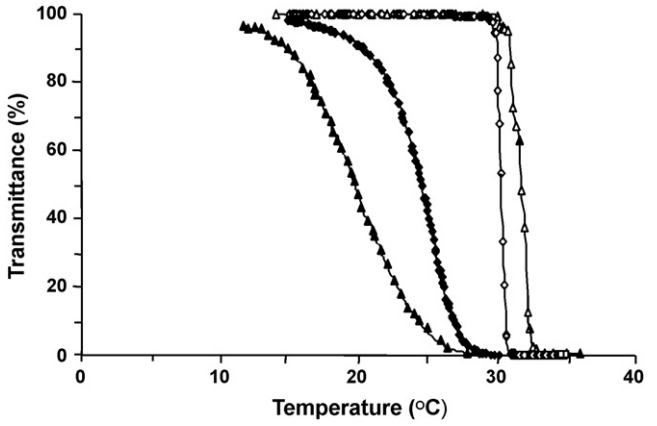

Fig. 3. Temperature-dependent optical transmittance changes for IBc copolymer solutions with various BMA composition: (△); PNIPAAm, (□); IBc-1%, (■); IBc-3%, (▲); IBc-5%.

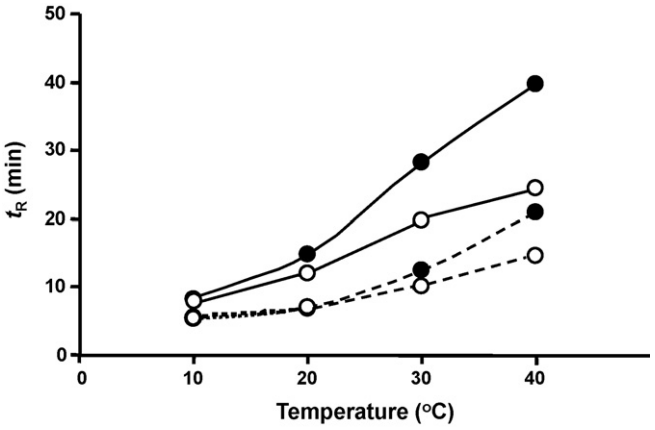

Fig. 4. Effect of a temperature change on the t_R of propofol (●); and I.S. (○); using IBc copolymer modified column, IBc-3% (dashed lines) and IBc-5% (solid lines). The t_0 is 2.1 min.

3.2. Temperature-responsive chromatography for propofol

For preparing temperature-responsive packing, aminopropyl silica was used as a base support. After conjugation of aminopropyl silica to PNIPAAm or IBc copolymer, prepared materials were packed into stainless-steel columns.

To establish the analytical method of propofol, we investigated the retention of propofol and IS on IBc copolymers modified columns. Fig. 4 shows the effect of temperature on the retention times (t_R values) of propofol and I.S. in temperature-responsive chromatography using an IBc-3% or IBc-5% copolymer modified column. The retarded retention times were observed with increasing temperature. With increasing temperature, an increased interaction between the solutes and IBc copolymer-grafted surfaces of the stationary phases was observed. It was considered that a hydrophobic interaction exists between the propofol and the IBc copolymer modified column. In contrast, in the common RPLC system, the opposite behavior of the decreased retention times was observed with increasing temperature. A temperature-dependent resolution was achieved using only water as a mobile phase on the IBc copolymer modified column. A drastic and reversible property alteration of IBc copolymer-terminally grafted surfaces from hydrophilic to hydrophobic is due to the rapid changes in polymer hydration state around the polymer's transition temperature. Below the LCST of both polymers, the t_R values of analytes were within 15 min and peak separations were difficult. On the other hand, above the LCST, though the t_Rs took more time, peak separations were successful, particularly at 40 °C. In this evaluation experiment, using all mobile phases are water and changing of temperature showed drastic effect on the retention and separation of analyte. In addition, temperature linear gradient was also successful to shorten an analysis time (Fig. 5) [19]. As the temperature changed, the surface property of the stationary phase switched from hydrophilic to hydrophobic. The retention on the polymer-modified stationary phase remarkably changed upon changing the temperature. With a single mobile phase of water, and by controlling the external temperature, it was possible to obtain an effect similar to the solvent gradient. A solvent gradient elution-like effect could be achieved with a single mobile phase by programmed temperature changes.

3.3. Determination of propofol in monkey serum

As mentioned in the introduction, propofol is a widely used intravenous agent for the induction and maintenance of anaesthesia in clinical practice. Several chromatographic methods, including HPLC [20,21], GC–MS [22] and LC–MS [23] have been reported for the dosage of propofol in biological fluids. Of them, HPLC–FL is the most suitable method regarding of specificity, sensitivity and economic in clinical practice.

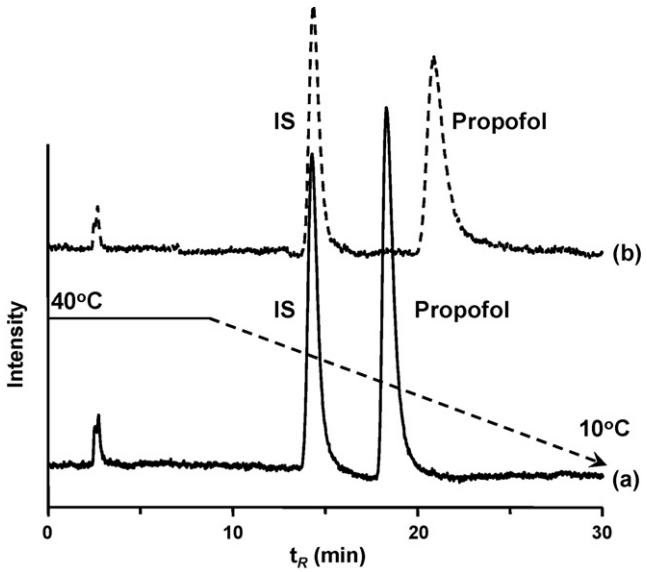

Fig. 5. Effect of a temperature change (linear gradient, a) on the t_R of propofol and I.S. using IBc-3% modified column from 40 °C to 10 °C (solid line). Dashed line (b) shows isocratic analysis. HPLC conditions (a): copolymer modified column, column temperature; 0–9 min (40 °C), 9–30 min (linear gradient, target temperature is 10 °C), (b): copolymer modified column, column temperature; 40 °C (isocratic).

Monkey serum was pretreated as described in Section 2.5. The recovery rate of propofol and I.S. during the pretreatment were 77.8 ± 6.1% and 86.3 ± 8.1% (mean ± SD, n = 5), respectively. The chromatogram shown in Fig. 6a was obtained from the serum of a non-administrated monkey subject, in which propofol were not detected, and that there was no interfering peak derived from the endogenous components at the elution position of propofol and I.S. Fig. 6b shows the chromatogram obtained from the propofol-administrated monkey, in which the peaks corresponding to the

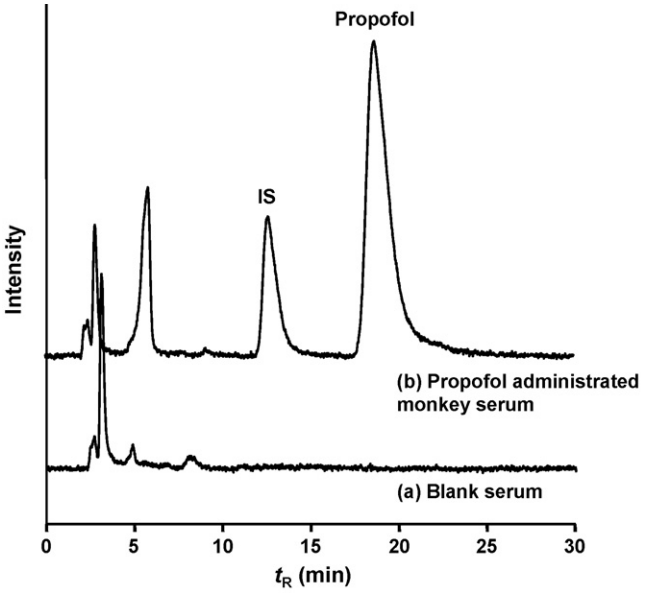

Fig. 6. Chromatograms of propofol and I.S. from a propofol-administrated monkey serum (a) and blank one. (b) HPLC condition: column, IBc-3% copolymer modified column; column oven, 40 °C; mobile phase, H_2O; flow rate, 1.0 mL/min; FL detection, excitation wavelength 267 nm, emission wavelength 310 nm.

Table 2
Intra- and inter-day variability of the assay.

	QC sample-1 (9.0 mg mL)	QC sample-2 (3.0 mg mL)
Intra-assay measured concentration[a]	9.75 ± 0.33	3.59 ± 0.11
RSD (%)	3.36	2.29
Inter-assay measured concentration	9.73 ± 0.57	3.13 ± 0.44
RSD (%)	5.84	14.11

[a] Mean ± SD (mg/mL, $n = 5$).

propofol and I.S. were clearly observed at 13.6 min and 19.5 min, respectively. The calibration curve was constructed by plotting the peak area ratios (propofol/I.S., y) versus the concentration (μg/mL, x) of propofol. The regression line obtained from the combination curves was $y = 1.91x - 0.14$ with a correlation coefficient (r) of 0.999 within the range of 0.5–10 μg/mL. The inter-assay ($n = 5$) RSD values were less than 14.1%, and good intra-assay ($n = 5$) values (less than 3.4%) were also obtained, as shown in Table 2. Fig. 7 shows the change in the serum propofol concentration obtained from a monkey who was single injected with propofol (8 mg/kg) intravenously. The developed method was successful for tracing the decline in the propofol level with time.

3.4. Comparison of temperature-responsive chromatography to RPLC

In this section, our developed analytical method is compared with the widely used RPLC one. In monkey, the clinically effective propofol concentration range is regarded as 3–8 μg/mL. We used propofol contained sera obtained from a monkey which received intravenous bolus dose of 5 mg/kg followed by continuous infusion at 40 mg kg^{-1} h^{-1} for 10 min, 20 mg kg^{-1} h^{-1} for 10 min and 15 mg kg^{-1} h^{-1} for 100 min, as analytical samples. A comparison of the results obtained by our developed method with the RPLC method showed Fig. 8. Though the temperature-responsive chromatography method estimated a slightly higher value than RPLC, a good relation was observed between both methods. These results show that our developed method has possibilities in practical applications in clinical practice. The comparison of the column properties was summarized in Table 3. In our developed column, theoretical plate numbers (N) and symmetry factor (S) were slightly inferior to conventional RPLC column. Because our column is "handmade" one, and not as the marketed product which is available extreme pressure in the packing. To improve the performance, we will use the smaller particle-size aminopropyl silica (less than 3 μm) or create the dense polymer brushes on silica bead surfaces by precise polymerization [24]. The back pressure of our column is same as RPLC column. If only H_2O was used as the mobile phase in conventional RPLC columns, their back pressures become extremely high.

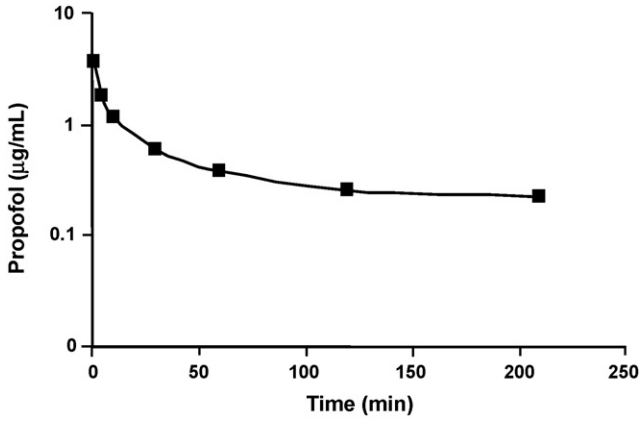

Fig. 7. Degradation curve of monkey serum propofol level after single injection of propofol (8 mg/kg) intravenously.

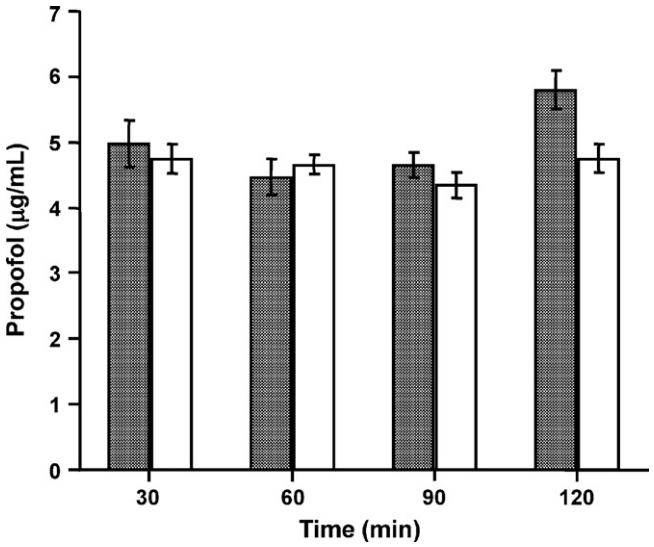

Fig. 8. Comparison of quantitative values of the propofol level in monkey serum obtained from temperature-responsive chromatography (grey bars) and RPLC (white bars). The number of subjects tested is each five.

Table 3
Comparison of column properties.

Column	Analyte	N	S	Back pressure (MPa)
IBc-3% modified column[a]	Propofol	4307	1.25	5.5
	I.S.	6416	1.21	
Inertsil ODS-3 column	Propofol	14679	0.94	5.7
	I.S.	8880	0.98	

[a] HPLC condition was described in the caption of Fig. 3.

4. Conclusion

In this study, we developed a new quantification method of propofol in monkey serum. Good separation was achieved between propofol and the interfering peak derived from the endogenous components by only changing the temperature. This is a first example of temperature-responsive chromatography to the quantitative analysis of a pharmaceutical compound in a biological sample. Our developed method covered the calibration range from 0.5 μg/mL to 10 μg/mL, and was able to detect a decline of the propofol level with time after single administration to a monkey. In addition, there was a good relationship of the quantification values between the developed method and widely used RPLC method. Because our developed method used only water as the mobile phase, there are some advantages compared with the reported RPLC method. First, the avoidance of complicated mobile phase preparation was possible, and only temperature changing could regulate t_R of the analyte. Second, our method is useful for conducting TDM in clinical practice because using the organic solvent has major issues of waste solution and exposure of patients to harmful solvents. In addition, by using water instead of fossil fuel, it is the ideal analytical method according to green chemistry. In the future, we expect that temperature-responsive chromatography will be widely used in clinical practice and industries.

Acknowledgements

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