A Thesis for the Degree of Ph.D. in Pharmaceutical Science

Development of the new separation methods for the drug analysis in the field of forensic toxicology

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Preface

Forensic toxicology is the field that gives medical and chemical clarification on legal issues concerning all poisoning and addiction. In addition to administrative research such as legal handling of toxic substances, analytical methods are also being studied for the purpose of giving a scientific evidence to prove the causes of poisoning and/or addiction.

Although many kinds of the causes have been known, drug is one of the serious causes and its addiction has been a worldwide problem. In Japan, methamphetamine abuse has been ongoing for over 50 years, and the number of arrests for violating the Stimulants Control Law has never fallen below 10 000 people per year for the past decade. Furthermore, psychoactive drugs are often abused and the addicts are frequently carried to a hospital because of their toxic symptoms.

It is important to accurately detect a drug as a cause from a biological sample of a drug abuser, even for punishing its addicts and for providing appropriate treatment. To aim the evolution of the means for drug detections, I developed two types of new separation methods. This thesis is constituted from the descriptions of the methods in each chapter.

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Chapter 1

The use of a temperature-responsive column for the direct analysis of drugs in serum by two-dimensional liquid chromatography

1.1 Introduction

In the pharmaceutical field, continued efforts are engaged in the development of rapid analytical methods with concomitant higher sensitivity and selectivity. High performance liquid chromatography (HPLC) can be considered as the technique of choice currently used for drug and metabolites analysis in biological fluids. However, biological matrices, such as serum and plasma, are complex mixtures incompatible with a direct injection into conventional HPLC supports [1]. Therefore, a sample preparation step is a key consideration for the development of reliable HPLC methods to measure therapeutic agents.

While many preparation techniques are reported, on-line preparation method, allowing the direct injections of biological matrices, is an attractive means to reduce the sample preparation time. Many types of pre-treatment columns which are used in HPLC or two-dimensional (2D) -HPLC configurations have been reported [2-11]. Among different supports, restricted access media (RAM) columns [2-6], such as internal surface reversed phase column, are known as popular extraction materials. This supports possess the property of excluding macromolecules while target analytes are retained by either hydrophobic or electrostatic interactions. Using RAM columns, multiple drugs can be analyzed simultaneously. However, due to the principle of the columns, foreign substances which cannot be passed through the column by the size exclusion mechanism are inevitably concentrated and eluted together with target drugs.

Hence, unnecessary peaks would be appeared in chromatograms derived from foreign substances and hinder the precise analysis.

In the viewpoint of reducing foreign substances sent to an analytical column, “heart-cutting” 2D-HPLC may be one of the effective methods in which only a drug eluted fraction from the first column can introduce into the second column using a column-switching valve [12-16]. Using this method, foreign substances entered to the second column would be little because introduced substances are limited to ones eluted with target drugs simultaneously from the first column. To take one example of many, the use of a size exclusion chromatography (SEC) column for a pre-treatment column has been reported [16]. SEC could be used to separate macromolecules and small molecules. The isolated small molecule was introduced to the analytical column by heart-cutting. Using SEC column, foreign substances contaminating the analytical column could be reduced owing to above mentions. However, when the analytes exist as multiple drugs and are eluted at different retention times in SEC, the introducing time of the drug eluted fraction may become longer and more foreign substances could be introduced to the analytical column. The differences of the retention times strongly depend on the size of target drugs and SEC carrier. Hence, it is difficult to control the drug eluted timings.

Here, I proposed a novel pre-treatment method to aim the conquest of above disadvantages. A temperature-responsive column [17-25] was newly used for a

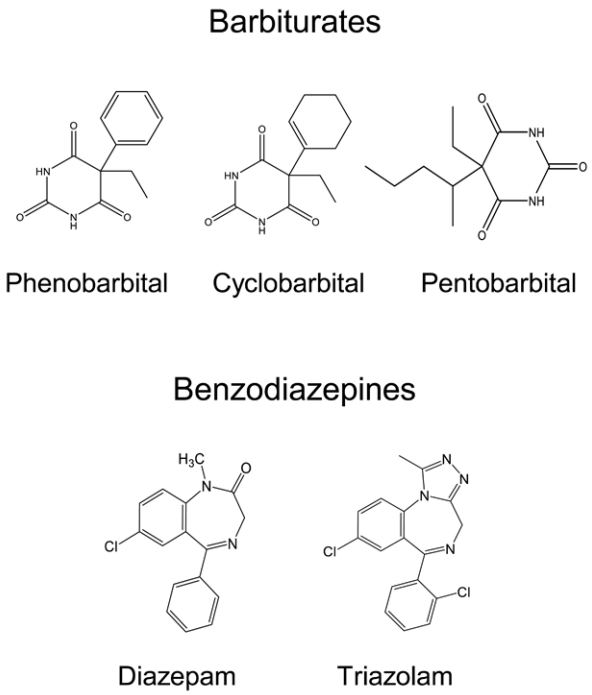

Fig. 1 Structures of barbiturates and benzodiazepines used in this study.

pre-treatment column in heart-cutting 2D-HPLC. The goal of this study was to analyze multiple drugs in serum directly and simultaneously, with a minimal introducing time of the drug eluted fraction for reducing the interferences from foreign substances. With the aim of using this method in real sample analysis, barbiturates and benzodiazepines were used as the analytes (Fig. 1). Since addictions of psychoactive drugs have been a serious problem [26], the analysis of these poisoning causes in biological fluids has been important. To prepare the temperature-responsive column, poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) [P(NIPAAm-*co*-BMA)] hydrogel was synthesized to modify the surface of the silica stationary phase. The

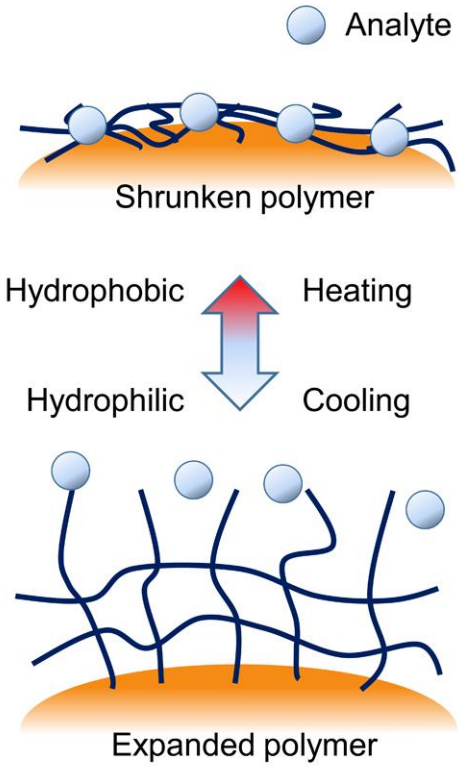

Fig. 2 Schematic illustration of the PNIPAAm-based hydrogel layer.

polymer-grafted surface exhibits temperature-regulated hydrophilic/hydrophobic characteristics as a result of polymer conformational changes (Fig. 2). Using this column with an aqueous mobile phase, the retention times of the analytes were changed with changing the column temperatures due to this surface property changes. Further, controlling the ionic strength of the eluent enables human serum albumin (HSA), which is a major component of serum, to pass through the column without retention [27]. From these features of the temperature-responsive column, controlling the column temperature has made it possible for the retention times of target drugs to be grouped

together, excluding HSA. These “grouped” drugs were introduced into the analytical column with the minimal introducing time. This study is the first to report the use of a temperature-responsive column for a pre-treatment column on 2D-HPLC.

1.2 Experimental

1.2.1 Chemicals

N-isopropylacrylamide (NIPAAm) was provided by KJ Chemicals Corporation (Tokyo, Japan). NIPAAm was purified by recrystallization from *n*-hexane and dried at 25 °C *in vacuo*. Butylmethacrylate (BMA), 4,4′-azobis(4-cyanovaleric acid) (V-501), *N*,*N*′-methylenebisacrylamide (MBAAm), *N*,*N*′-dimethylformamide (DMF), diazepam, triazolam, and HSA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclobarbital, pentobarbital, and phenobarbital were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was purchased from Peptide Institute, Inc. (Osaka, Japan). Aminopropyl silica beads (average diameter: 5 μm; pore size: 120 Å) were purchased from Nishio Industry (Tokyo, Japan). Human serum was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Other non-specifically listed chemicals were of analytical reagent grade.

1.2.2 Preparation of P(NIPAAm-*co*-BMA) hydrogel-modified silica

The synthesis of the polymer and the modification of aminopropyl silica were carried out according to literature procedure (Fig. 3) [19, 28]. An initiator, V-501 (3.50 g, 12.5 mmol), and a condensing agent, EEDQ (6.18 g, 25.0 mmol), were dissolved in 50 mL of DMF in a round-bottomed flask. N_2 gas was bubbled through the solution for 30 min. Next, 5.00 g of aminopropyl silica beads (5 μm in diameter) were immersed in the solution, and the mixture was degassed again for 30 min before starting the reaction. The reaction mixture was shaken for 6 h at 25 °C. The modified silica beads were washed with ethanol and dried *in vacuo* overnight. NIPAAm (4.67 g, 41.4 mmol), BMA (0.31 g, 2.18 mmol), and MBAAm (0.14 g, 0.88 mmol) were dissolved in 100 mL of ethanol in a round-bottomed flask. Subsequently, the initiator-immobilized silica beads (2.00 g) were added to the solution. N_2 gas was bubbled through the reaction mixture for 30 min, and the polymerization reaction was carried out for 5 h at 70 °C. The P(NIPAAm-*co*-BMA) hydrogel-modified silica beads were filtered, washed with

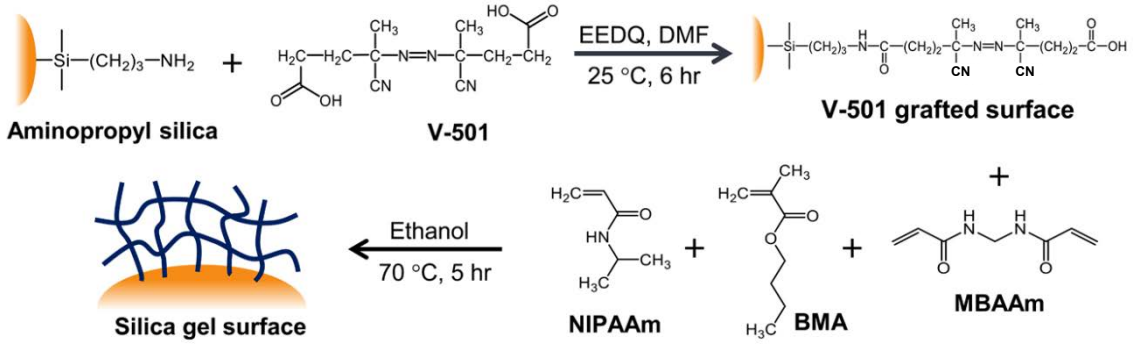

Fig. 3 Preparation scheme for the synthesis of the temperature-responsive hydrogel-modified silica.

methanol at 25 °C to remove any non-immobilized hydrogels, and dried *in vacuo* overnight. The polymer-grafted silica was then packed into a stainless-steel column.

1.2.3 Sample preparations

The HSA standard sample was prepared by dissolving HSA in deionized water at a concentration of 40 mg/mL. All drugs were dissolved in tetrahydrofuran at a concentration of 1.0 mg/mL as stock solutions and drug standard solutions with the desired concentrations were prepared by diluting the stock solutions with deionized water. For the preparation of serum samples, appropriate volumes of stock solutions were dried using N_2 gas purge, before dissolving the dried product in serum. All drug samples were prepared at a concentration of 30 μg/mL as individual compounds, except for the validation samples used in 1.3.4 section.

1.2.4 Instrumentation and analytical conditions

All experiments were performed with a Chromaster HPLC system (Hitachi, Tokyo, Japan) complete with an autosampler (model 5210), a column oven including a six-way switching valve (model 5310), an ultraviolet-visible (UV-VIS) detector (model 5420), a diode array detector (model 5430), and two mechanical pumps (model 5110). All detections were carried out at 220 nm.

The 2D-HPLC configuration used in this study is shown in Fig. 4. It includes the

P(NIPAAm-*co*-BMA) hydrogel-modified silica pre-treatment column (4.6 mm I.D. × 150 mm, 5 μm; for shortening: 4.6 mm I.D. × 50 mm, 5 μm). Meanwhile, the analytical column is a Hitachi LaChrom II C18 column (4.6 mm I.D. × 150 mm, 5 μm); for 1.3.6 section: a Shiseido CAPCELL PAK C18 column (2.0 mm I.D. × 100 mm, 3 μm). As shown in Fig. 4(A), a 10 μL sample was injected into the pre-treatment column and

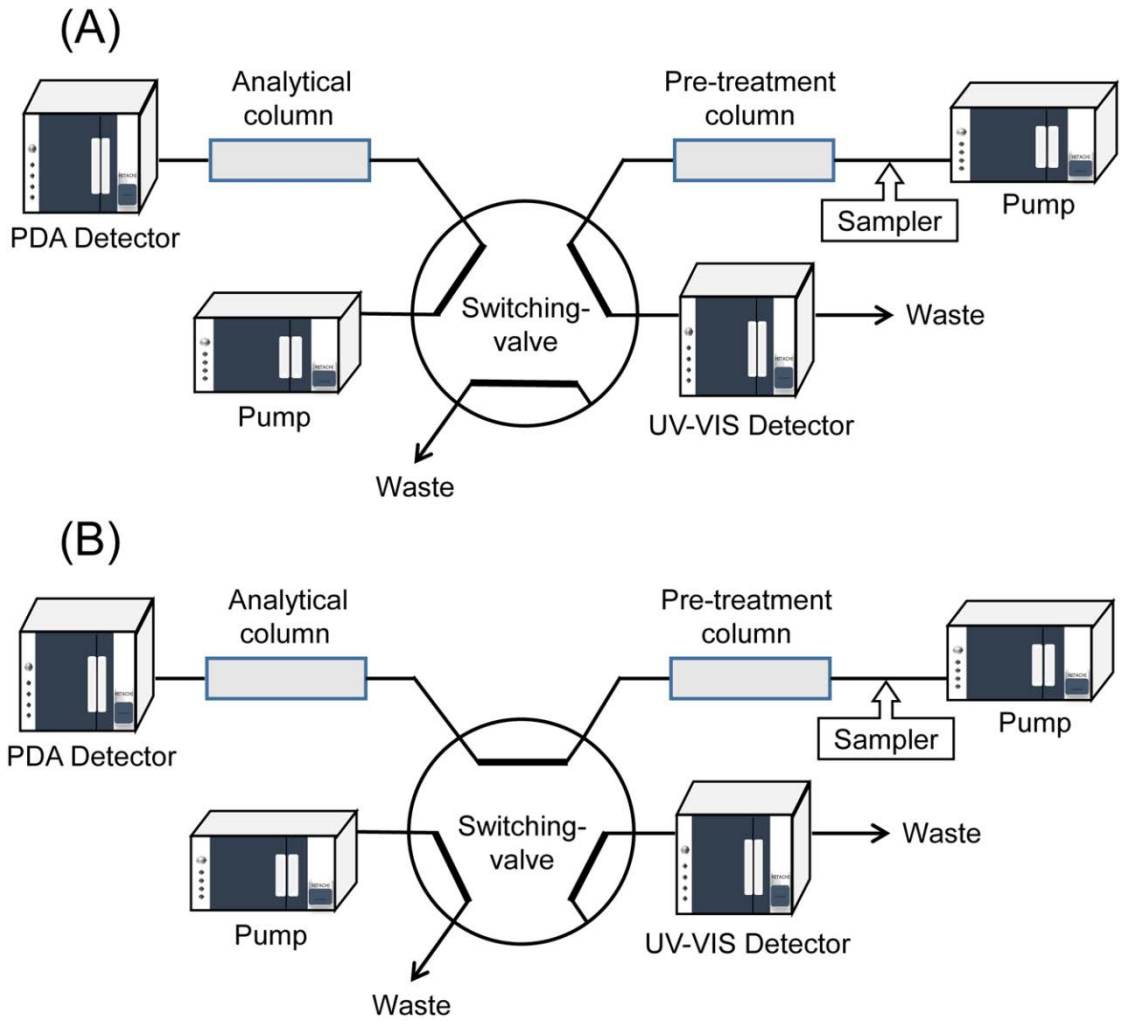

Fig. 4 Schematic representation of the 2D-HPLC configuration: (A) A disconnected system, separating the pre-treatment column from the analytical column, and (B) A connected system, via the use of a switching valve, for the analysis of multiple drugs.

eluted using a mobile phase of 10 mM ammonium acetate (pH 6.5) at a flow rate of 1.0 mL/min. The retention times of the analytes were monitored by UV-VIS detection, thus determining the valve switching times. The eluted drugs from the pre-treatment column were introduced into the analytical column by switching from valve (A) to (B). Thereafter, the valve was returned to its original position and a separation mobile phase of 10 mM ammonium acetate (pH 6.5)/acetonitrile (75:25 v/v for barbiturates and 60:40 v/v for benzodiazepines) was used at a flow rate of 1.0 mL/min. In some sections, analytical conditions were slightly modified from above ones in individual reasons. The modified conditions are described in the each section.

1.3 Results and discussion

1.3.1 Elution of HSA from the temperature-responsive column

It has been reported that the controlling of ion strength of the eluent makes it possible for HSA to pass the temperature-responsive column without the retention [27]. Eluents were investigated using water and 10 mM ammonium acetate (pH 6.5) with the temperature-responsive column at 40 °C. In the use of water (1.0 mL/min), HSA was retained on the hydrogel-modified surface for at least 30 min, before it was finally eluted from the temperature-responsive column. In contrast, in the use of 10 mM ammonium acetate, HSA was eluted immediately. Changing the column temperature from 10 to 40 °C did not affect the retention time of HSA. HSA, with an isoelectric

point at pH 4.7, is negatively charged around neutral pH. Therefore, it is likely that electrostatic interactions between HSA and the stationary phase were present when using water as an eluent, and HSA was eluted using 10 mM ammonium acetate because the salt weakens these interactions. To separate drugs from HSA effectively at the temperature-responsive column, it is desirable that the retention time of HSA gets far away from ones of drugs as possible. However, it is not to be desired that the elution of HSA is too late from drugs because HSA would interfere with next data in continuous analysis. Therefore, 10 mM ammonium acetate was chosen for use. HSA was eluted at earliest time in the all experiments.

1.3.2 Changes in the drugs' retention times with varying column temperatures

Barbiturates and benzodiazepines were analyzed using the temperature-responsive column. The standard solutions of these compounds were prepared and their retention times measured at different column temperatures. The retention times were altered by changing the column temperature. The plot of the retention times against column temperatures is shown in Fig. 5. At 40 °C, the benzodiazepines were eluting close to each other. In conventional reversed-phase HPLC, the retention times of all analytes typically decrease with increasing column temperature. Therefore, a convergence of retention times is not expected. In contrast, in the temperature-responsive column, the

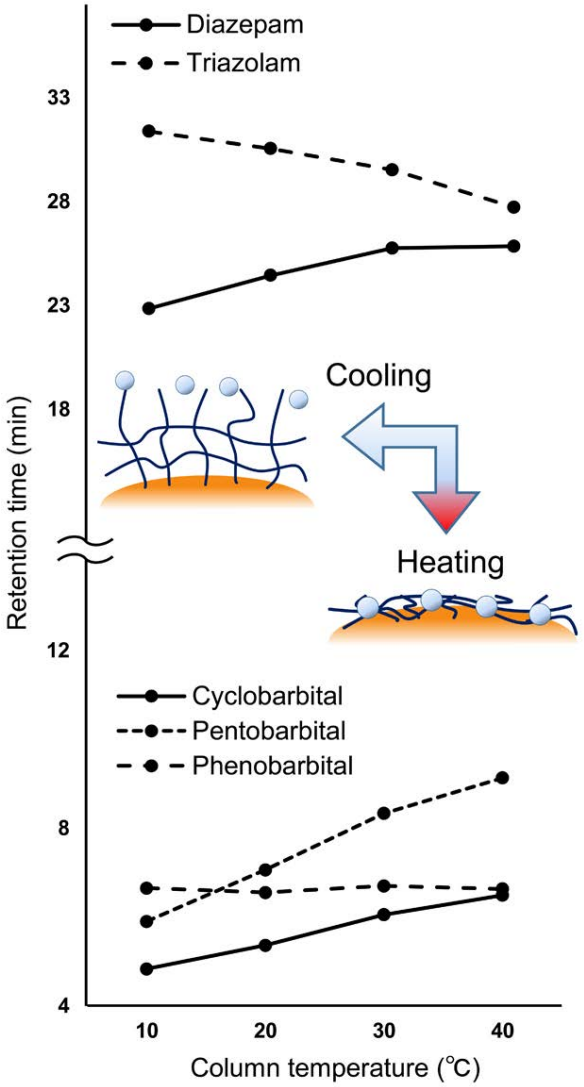

Fig. 5 Plot of the drugs' retention times against column temperatures in the temperature-responsive column.

stationary phase undergoes conformational changes, resulting in a change of surface property. It was easily found out that each retention time was changed with changing the column temperature, not to sole direction but with its own peculiar behavior. The direction and the degree of the changes on retention times vary by characteristics of the target molecules. Meanwhile, the introducing time of eluent from the pre-treatment

column to the analytical column must be shortest because foreign substances sent together should be little. Obviously, an optimal column temperature should result in relatively close retention times for the same group of drugs. For this reason, the column temperature was set at 40 °C for the benzodiazepines in the 2D-HPLC experiments. As for the barbiturates, cyclobarbital and phenobarbital were eluting close at 40 °C, while pentobarbital and phenobarbital were eluting close at 20 °C. Although the temperature that resulted in close all retention times did not appear in this condition, the column temperature was set at 30 °C for the barbiturates as an appropriate temperature tentatively and following experiments were performed.

1.3.3 Concentration of the analytes at the entrance of the ODS column

The introducing of analytes from the temperature-responsive column to the ODS column was occurred with the eluent of 10 mM ammonium acetate, a sole aqueous mobile phase. Therefore, the analytes were concentrated at the entrance of the ODS column because the analytes were retained by C18-phase and could not enter to the ODS column while introducing with a sole aqueous mobile phase. Figure 6 shows the chromatograms of phenobarbital, obtained via the exclusive use of the temperature-responsive column, as well as those from the 2D-HPLC analysis. To realize the change of peak shapes clearly, standard samples were measured at 30 °C. The

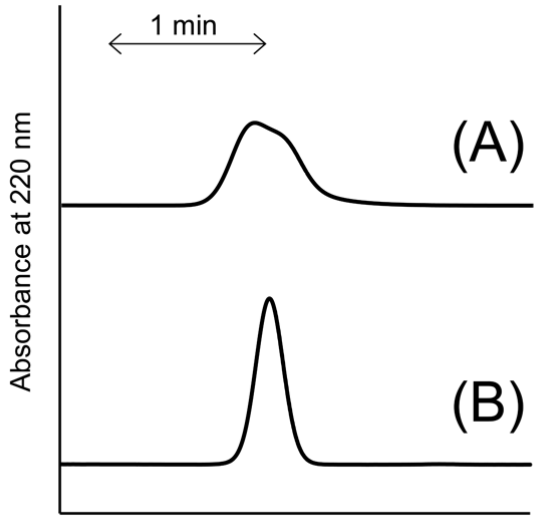

Fig. 6 Chromatograms of phenobarbital obtained using (A) only the temperature-responsive column, and (B) the 2D-HPLC analysis.

phenobarbital peak on the 2D-HPLC chromatogram was sharper than that of the temperature-responsive column. Furthermore, although a slight peak splitting was observed in Fig. 6(A), there was no splitting in Fig. 6(B). These results clearly indicated that the concentration of analyte at the ODS column was occurred. From this benefit, sharp peaks of drugs could be obtained using this 2D-HPLC system.

1.3.4 Analysis of drugs in serum

Serum samples laced with drugs were measured using the 2D-HPLC method. Figure 7(A) and (B) show the chromatograms of barbiturate and benzodiazepine samples that were obtained from an HPLC analysis, using only the temperature-responsive column. Peaks within close proximity were observed, along with the appearance of a shoulder

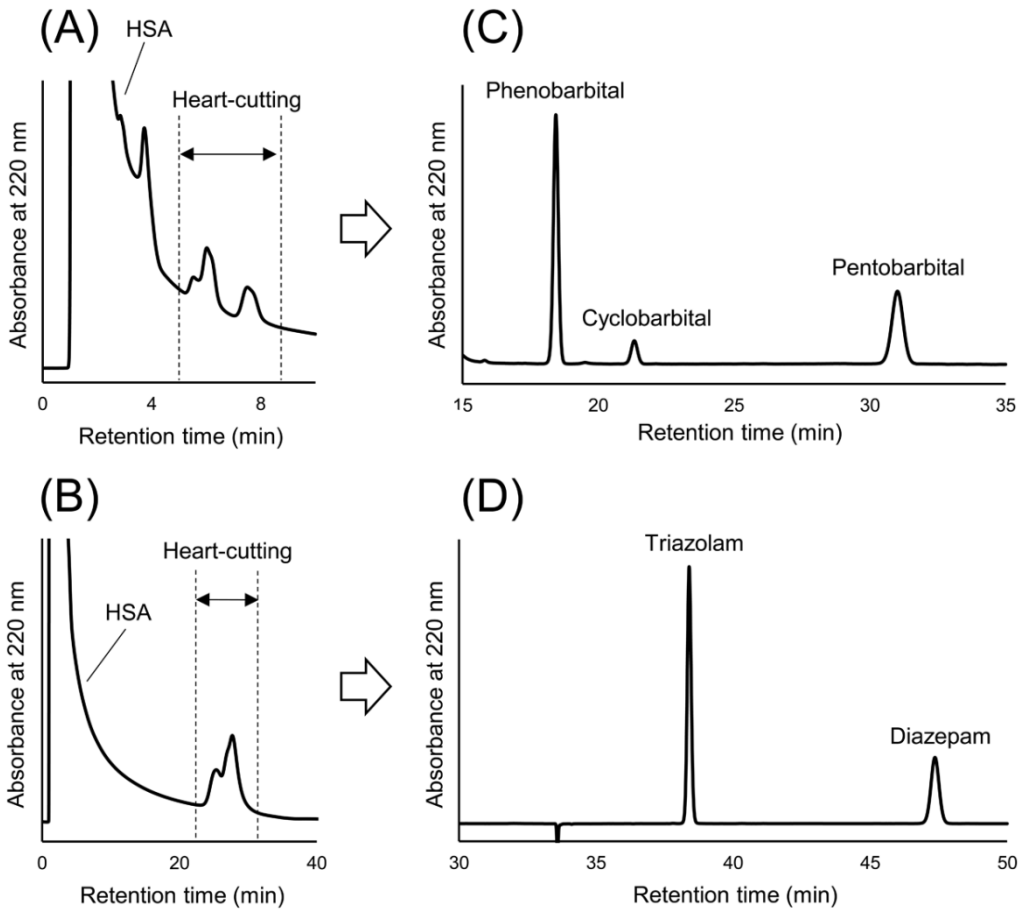

Fig. 7 Chromatograms of serum samples. Using only the temperature-responsive column: (A) the barbiturates, and (B) the benzodiazepines. Using the 2D-HPLC analysis: (C) the barbiturates, and (D) the benzodiazepines.

peak attributed to HSA. These eluted “fractions” (5.0-9.2 min for barbiturates and 23.5-31.5 min for benzodiazepines) were introduced into the ODS column via the use of a column-switching valve. Chromatograms obtained from the 2D-HPLC analysis are shown in Fig. 7(C) and (D). Clear peaks derived from the drugs were observed, and other obstructive peaks derived from foreign substances were scarcely observed. These results suggested that the 2D-HPLC method is superior in minimizing the contamination of the analytical (i.e., ODS) column and produces clear chromatograms, thus making an

Table 1 Repeatability of peak areas, linearity of calibration curves and recovery rates of each drug obtained via the 2D-HPLC analysis.

Compound	RSD of peak area (%) (n = 6)	R^2 at calibration curve (1, 3, 5, 10, 20 μg/mL)	Recovery rate (%)
Cyclobarbital	1.0	0.9999	93.3
Pentobarbital	1.9	0.9991	107.4
Phenobarbital	1.4	0.9999	100.4
Diazepam	0.3	0.9992	88.8
Triazolam	0.2	0.9997	95.5

accurate quantitative analysis possible. Table 1 shows the repeatability of the peak areas (each sample concentration: 10 μg/mL), the coefficient of determination (R^2) of the calibration curves (1, 3, 5, 10, and 20 μg/mL) and the recovery rates of each drug. These values were seemingly unaffected by the serum matrix, thus highlighting the potential of using 2D-HPLC analysis to quantitate drugs in serum without the risk of serum matrix interference.

The analytical run time could be reduced by shortening the temperature-responsive column from 150 to 50 mm. Figure 8 shows the chromatograms of the benzodiazepine mixture sample obtained from an HPLC analysis, using only the temperature-responsive column, as well as the corresponding 2D-HPLC method (heart-cutting time 9.5-12.5 min). Although both retention times were shortened compared with ones from 150 mm

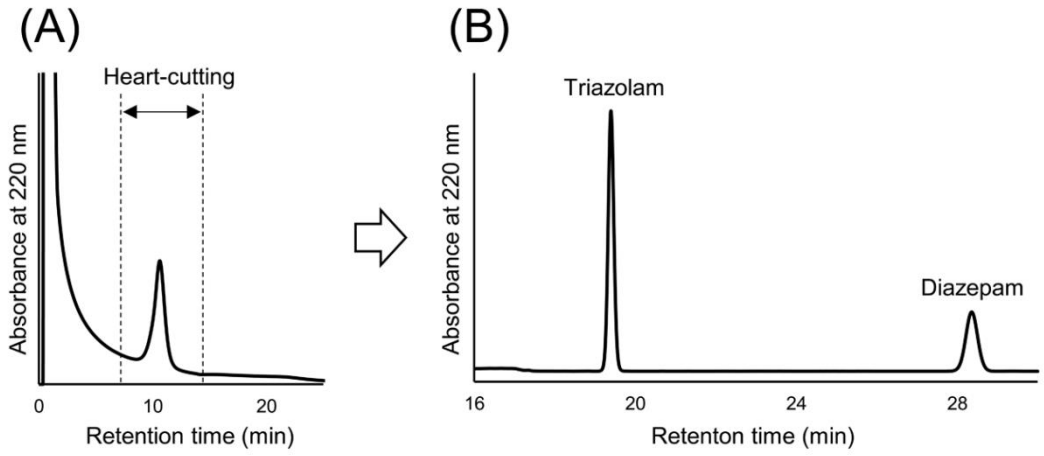

Fig. 8 Chromatograms of benzodiazepines obtained using: (A) only the temperature-responsive column, and (B) the 2D-HPLC analysis. The temperature-responsive column was 50 mm in length.

column, peak separation of the drugs in the 2D-HPLC chromatogram was kept enough. Moreover, the total analytical time was reduced to 30 min. In this way, a total analytical time could be easily regulated by changing the column length.

1.3.5 Large volume sample injection

In this 2D-HPLC system, the analytes accumulated at the entrance of the ODS column. Therefore, sharp peak signals of the drug analytes could be obtained when large volume sample was injected. At the setout, the sample volumes were increased from 10 μL to 500 μL with a mobile phase of 10 mM ammonium acetate. While a 500 μL sample was injected, the tailing of HSA peak was terrible and this peak was lain on the those of drug analytes. This tailing would be occurred for the injection with a large amount of

HSA. Some of the HSA could electrostatically interact with the stationary phase in 10 mM ammonium acetate and led to the serious tailing. To separate HSA from the stationary phase effectively, the concentration of the ammonium acetate was increased from 10 mM to 100 mM in this section. In the analysis with 100 mM ammonium acetate, the serious tailing of HSA peak disappeared and the peaks of drug analytes appeared with the separation from HSA as usual. A comparison of triazolam chromatograms obtained from various sample injection volumes in the 2D-HPLC analysis is shown in Fig. 9. When the sample volume was increased, the peak widths were almost unchanged but the peak heights became noticeably higher. Further, good linearity was observed on the correlation of the sample volumes against the peak areas (R^2 = 0.999). These results clearly indicated that the sample volume was easily increased to improve the detection sensitivity in this 2D-HPLC system.

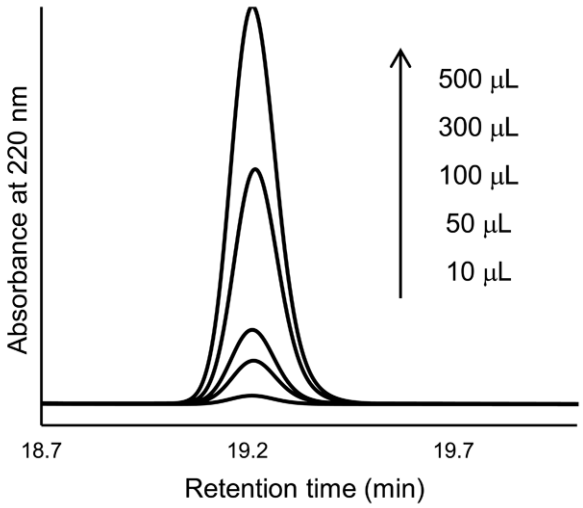

Fig. 9 Overlapping triazolam's chromatograms obtained from various sample volume injections in the 2D-HPLC analysis.

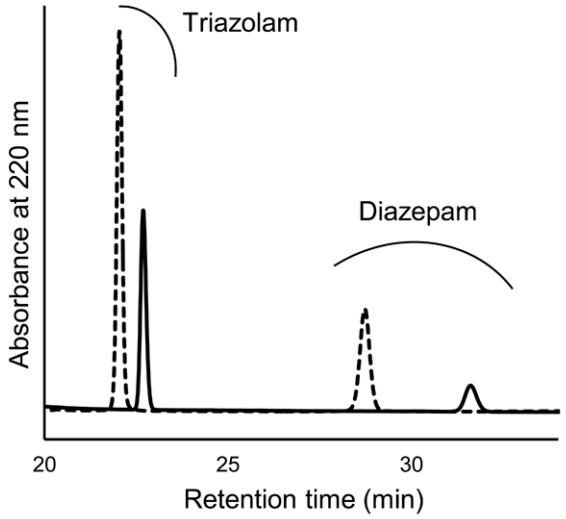

Fig. 10 A comparison of triazolam and diazepam chromatograms obtained from (solid line) 4.6 mm I.D. column and (dotted line) 2.0 mm I.D. column.

1.3.6 Using semi-micro column for the analytical column

Because the analytes accumulated at the entrance of the ODS column, it may be possible to decrease the diameter of the ODS column for improvement of sensitivity of drug analytes. Figure 10 shows a comparison of triazolam and diazepam chromatograms obtained from 4.6 mm I.D. column (150 mm, 5 μm) and 2.0 mm I.D. column (100 mm, 3 μm). In the analysis with 2.0 mm I.D. column, the flow rates were set at 0.8 mL/min in the first dimension and at 0.3 mL/min in the second dimension. The peak heights of drugs from 2.0 mm I.D. column were obviously higher than those from 4.6 mm I.D. column. These results could be explained by less axial dilutions of drug analytes at 2.0 mm I.D. column. Thus, the sensitivity of analytes can be readily increased by using narrow diameter column.

1.4 Conclusion

Temperature-responsive column was adopted for the pre-treatment column via the use of heart-cutting 2D-HPLC analysis. Barbiturates and benzodiazepines in human serum were directly analyzed by this method. Clear chromatograms were obtained with little signs of serum matrix-derived foreign substances. The repeatability of the peak areas and the linearity of the calibration curves as well as the recovery rates of each drug were satisfactory for the quantification of analytes. Further, the total analytical time could be reduced by shortening the temperature-responsive column, and the detection sensitivity could be easily increased by increasing the injected sample volume and using semi-micro column.

In this study, barbiturates and benzodiazepines were selected as analytes. It was demonstrated that multiple drugs from the same chemical group could be analyzed simultaneously (i.e., owing to the convergence of their retention times) by regulating the column temperature. However, under the reported analytical conditions, the concurrent analysis of these two types of drugs (benzodiazepines and barbiturates) is difficult because of not overlapping retention times. To address this issue, I am focusing on developing better temperature-responsive columns. It is known that properties of temperature-responsive columns can be changed and controlled by modifying the copolymerizing materials [18, 20-23]. In the future, I anticipate a feasible simultaneous analysis of multiple drugs as more suitable temperature-responsive columns become

increasingly available. The present 2D-HPLC method, which gives clear chromatograms of multiple drugs derived from biological samples, offers an effective way to acquire drug information in biological fluids.

Chapter 2

Development of the analytical method for methamphetamine in hair by chiral capillary electrophoresis: cation-selective exhaustive injection and sweeping cyclodextrin-modified micellar electrokinetic chromatography

2.1 Introduction

The abuse of drugs is a worldwide problem. In Japan, methamphetamine (MA) abuse has been ongoing for over 50 years, and the number of arrests for violating the Stimulants Control Law has never fallen below 10,000 people per year for the past decade [29].

To clamp down on MA abuse, it is important to arrest the abusers. For that reason, MA and its metabolite, such as amphetamine (AM), should be detected from the biological samples of the abuser [30]. While various types of biological samples have been analyzed, hair is known as a superior sample. Hair is a stable tissue and has the advantage over traditional matrices (e. g. blood or urine) of being able to confirm long-term exposure to drugs over a period of weeks to months [31]. However, unfortunately, the amounts of MA and AM in hair are rather little. Hence, high-sensitivity detectors and/or appropriate concentration techniques are generally needed in hair analysis. Besides, MA has an asymmetric carbon in its structure and thus exists two enantiomers, (*S*)-(+)-MA and (*R*)-(−)-MA. Almost all MA distributed in streets includes (*S*)-MA, because (*S*)-MA is 5-10 times more potent than (*R*)-MA with respect to its effect on the central nervous system [32]. On the other hand, it should be noted that some legal drugs are metabolized and change to (*R*)-MA. For example, selegiline, which is a drug for Parkinson's disease, is metabolized to (*R*)-MA, and the (*R*)-MA is further metabolized to (*R*)-AM. Therefore, chiral analysis of MA is important

for the determination of MA intake because it has been shown to be accurate [33].

Capillary electrophoresis (CE) has been recognized as a suitable technique for chiral analysis due to its advantages such as high separation efficiency, simplicity and versatility. The chiral CE analysis of trace amount of MA in hair has been an attractive challenge for analytical chemists. However, because of the short optical path-length and small sample volume, the detection sensitivity of CE is often insufficient for trace analytes. To overcome this shortcoming, numerous concentration techniques have been developed for use in conjunction with chiral CE [34-38]. Among them, the online concentration techniques have been known as an attractive means of reducing the sample preparation time and laborious sample handling. Various improvements to the online concentration strategies have been reported [39-49].

If chiral CE is applied to the hair analysis of MA, considerable concentration efficiencies must be needed. Meng *et al.* reported [50] that trace amounts of MA, AM and methylenedioxy-methamphetamine spiked to human hair could be detected by cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography (CSEI-sweeping MEKC), which is an achiral method. The concentration efficiencies were up to 1,000-fold. After seeing the result, I tried to develop a new online concentration technique for chiral CE based on CSEI-sweeping MEKC. In CSEI-sweeping MEKC [50-60], the sample is prepared in a low conductivity buffer (LCB). The capillary is first flushed with medium conductivity buffer (MCB),

then injected with a plug of high conductivity buffer (HCB). The cationic analytes are introduced into the capillary electrokinetically at normal-polarity; that is, the cathode is on the detector side. The high electric field strength in the sample zone leads to a higher rate of migration of analytes, resulting in analyte stacking at the LCB-HCB interface. Then, stacking cationic analytes are moved to the HCB zone with slower migration because of the low electric field strength. The analytes that cross the HCB-MCB interface and are injected into the MCB zone, can move back into the HCB zone to be swept. Hence, the sample ions injected into both the HCB zone and MCB zone can be concentrated. Finally, sodium dodecyl sulfate (SDS) micelles under a reversed electric field sweep the long sample zone into a sharp peak, and then the analytes are segregated in the MCB zone by the separation principle of MEKC.

In order to preserve all the concentration mechanisms of CSEI-sweeping MEKC, the addition of chiral selectors to both HCB and MCB seems ill-advised, since the extra interactions between analytes and chiral selectors could interfere with the effective sample concentration mechanisms. For that reason, I conceived the very simple idea of adding an anionic cyclodextrin (CD) as a chiral selector into the micellar buffer including SDS, without any other change of buffers in order to preserve the online concentration mechanism. In the present procedure, named CSEI-sweeping CD-modified MEKC, over 10,000-fold sensitivity increase in chiral CE was achieved. This technique was applied to the hair analysis of MA. To my best knowledge, this is

the first study reporting the combination of CSEI-sweeping MEKC with a chiral selector.

2.2 Experimental

2.2.1 Materials and chemicals

(*S*)-MA hydrochloride (HCl) was obtained from Dainippon Pharmaceutical (Osaka, Japan). Racemic amphetamine (AM) sulfate was obtained from Takeda Chemical Industries (Osaka, Japan). (1*R*,2*S*)-norephedrine (NEP) HCl was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Racemic ephedrine (EP) HCl was obtained from Fuji Chemical Industry Co., Ltd. (Toyama, Japan). (*S*)-AM was synthesized from (1*R*,2*S*)-NEP. Racemic MA was synthesized from Racemic EP. A 20 % aqueous solution of the anionic CD, highly sulfated *γ*-CD (HS-*γ*-CD), was obtained from Beckman Coulter Inc. (Fullerton, CA, USA). Deionized water treated by a Millipore Milli-Q System (Bedford, MA, USA) was used for preparations of all buffers and samples. All solutions were filtered through 0.45 μm membrane filters (GL Sciences, Tokyo, Japan). All other chemicals were of analytical reagent grade.

2.2.2 Preparations of solutions

Stock solutions of 0.5 M SDS and 0.5 M phosphate buffer (pH 2.7) were prepared. All buffers were prepared by mixing different portions of the stock solutions, a 20 %

HS-γ-CD solution and methanol with appropriate amount of deionized water. The pH was finally adjusted with 4 M phosphoric acid.

2.2.3 Apparatus

The experiments were carried out on an Agilent Capillary Electrophoresis system G1600A (Waldbronn, Germany) with a photodiode array detector. A fused-silica capillary (GL Sciences) of 64.5 cm in length (56 cm to detector) with an internal diameter of 50 μm was used. A temperature of 25 °C was maintained for all CE experiments. The detection wavelength was 195 nm.

2.2.4 Capillary conditioning

Before the first use, the capillary was rinsed with 1 M NaOH (30 min), followed by methanol (30 min) and water (30 min). To ensure repeatability, the capillary was flushed with 1 M NaOH (3 min), methanol (3 min), water (3 min), and the running buffer (6 min) between consecutive analyses. For the present method, nonmicellar buffer (MCB) was used to flush the capillary after rinsing with NaOH, methanol and water before each run.

2.2.5 CD-modified MEKC procedure

In the CD-modified MEKC analysis [61-64] for the comparison with the present

method, the running buffer was 20 mM SDS in 100 mM phosphate buffer at pH 2.7 with 20 % methanol. An HS-γ-CD solution was added to the running buffer at appropriate concentrations, and samples were measured using the capillary filled with the running buffer. A standard sample was prepared with racemic MA dissolved in water at 20 μg/mL. The conventional hydrodynamic injection of the sample was performed at 34.5 mbar x 3 s. Because the electroosmotic flow was significantly suppressed at pH 2.7, the net flow direction of cationic analytes was the same as that of negatively charged SDS micelles—i.e., towards the anode. Therefore, the separation was performed using a reversed-polarity mode at –18 kV.

2.2.6 CSEI-sweeping CD-modified MEKC

The present procedure was similar to the previously reported ones [50, 53] with a slight modification. At the beginning of each run, the capillary was conditioned with MCB (100 mM phosphate buffer at pH 2.7 with 20 % methanol). Then a plug of HCB without organic solvent (200 mM phosphate buffer at pH 2.7) was injected at 34.5 mbar x 4 min (15 % partial filling), followed by a short water plug (at 34.5 mbar x 5 s) at the inlet end. A standard sample was prepared with racemic MA dissolved in 1 mM phosphate buffer at 2 ng/mL. Phosphate buffer at a concentration of 1 mM has been associated with good repeatability of electrokinetic injections [53]. Using an electrokinetic injection at the anode (at +18 kV x 10 min), the cationic analytes were

introduced into the capillary through the water plug at high velocity and were then slowed down in HCB. Once the injection was finished, the buffers at both ends of the capillary were replaced with the micellar buffer (20 mM SDS in MCB). An HS-γ-CD solution was added to the micellar buffer at appropriate concentrations. The voltage was then switched to a negative polarity, thus permitting the entry of micelles and HS-γ-CD from the cathodic (negative) vial into the capillary for sweeping and enantioseparation. The concentration and the separation were performed in the reversed-polarity mode, –18 kV.

2.2.7 Full filling with HCB or MCB

To investigate the mechanism of CSEI-sweeping CD-modified MEKC, a capillary was filled with HCB or MCB, and the measurements were performed with a micellar buffer (20 mM SDS in MCB) at the ends of the capillary. An HS-γ-CD solution was added to the micellar buffer at appropriate concentrations. A standard sample was prepared with racemic MA dissolved in water at 60 μg/mL. The conventional hydrodynamic injection of the sample was performed at 34.5 mbar x 3 s, and then the separation was performed in the reversed-polarity mode at –18 kV.

2.2.8 Hair sample preparation

Blank hair sample was provided from a female, who has never taken illicit drugs, and

was obtained as a result of haircuts and submitted voluntarily. The hair from an MA user was collected from a Japanese male who had been arrested for ingesting MA. In preliminary qualitative and quantitative analyses by a previously reported method with gas chromatograph-mass spectrometer [65], it was revealed that the hair sample from this subject included ~3 ng/mg MA as well as its metabolite, AM. All hair samples were cut into ~5 mm long fragments. A sonicator was used to wash the cut samples with 1 % SDS for 1 min, then to wash them three times with water and methanol for 3 min each. The washed hairs were dried and weighed. A quantity of hair weighing 3 mg was then moved to test vials. The hairs were spiked with suitable amounts of racemic MA in the concentrations described below.

The extraction procedure was similar to that reported previously [50]. The hair samples were incubated in 1 mL of 1 M NaOH at 70 °C for 30 min. The hair digested under this condition was completely destroyed, and the drugs were released completely into the solution. Following digestion of the hair with NaOH, 2 mL of cyclohexane was added to the solution. The mixture was vortexed for 1 min, followed by centrifugation. The organic layer was transferred to a new vial, and a 50 μL aliquot of a mixture of concentrated hydrochloric acid-methanol (1 : 20) was added to prevent volatilization of the drugs. The organic solution was dried under a nitrogen stream at room temperature. The residue in the vial was dissolved in 250 μL of 1 mM phosphate buffer.

2.3 Results and discussion

2.3.1 Mechanism of CSEI-sweeping CD-modified MEKC

The predicted mechanism of the present procedure is shown in Fig. 11. Until sample injections, it is same as CSEI-sweeping MEKC. The vials at the ends of the capillary were set with the micellar buffer (including HS-γ-CD), and measurements were started in the reversed-polarity mode. Both SDS and HS-γ-CD were introduced to the capillary

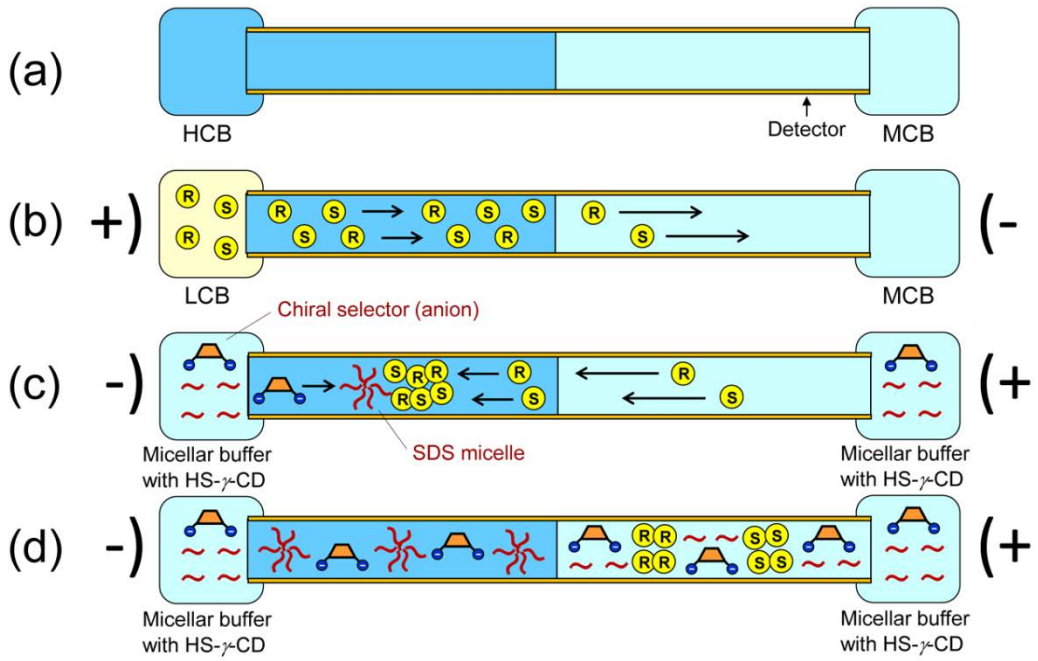

Fig. 11 The predicted mechanism of CSEI-sweeping CD-modified MEKC. (a) The capillary was filled with MCB, then partially filled with HCB. (b) Samples were injected by an electrokinetic injection with normal-polarity mode. (c) Both SDS and HS-γ-CD were introduced to the capillary with reversed-polarity mode. Because the migration of SDS micelles was faster than that of HS-γ-CD, the preceding SDS micelles swept the MA. (d) In MCB, the hydrophilicity was weakened by methanol, and the SDS micelles were mostly destroyed. Therefore, HS-γ-CD from the inlet vial could catch up with the swept MA, and allowed the separation of enantiomers by CD-modified MEKC.

with voltage impressing. The migration of SDS micelles was faster than that of HS-γ-CD. In HCB, the hydrophilicity of the solution was higher than that of MCB. Therefore, SDS constituted the micelles strongly, and the micelles and MA were highly bound. As a result, the preceding SDS micelles swept the MA that was previously concentrated by field enhancement. In MCB, the hydrophilicity was weakened by methanol, and the SDS micelles were mostly destroyed. The migration of swept drugs was via their affinity to the micelles. Consequently, HS-γ-CD from the inlet vial could catch up with the swept MA, and allowed the separation of enantiomers by CD-modified MEKC. Sweeping in HCB and CD-modified MEKC in MCB may have occurred sequentially, and online concentration and enantioseparation were effectively achieved.

2.3.2 Effect of anionic CD as a chiral selector

HS-γ-CD was gradually added to the running buffers of MEKC and the micellar buffers of CSEI-sweeping MEKC, then MA standard samples were measured by each method. The obtained electropherograms are shown in Fig. 12 (a), (b). In MEKC mode, MA signals were gradually separated with increasing HS-γ-CD concentrations. These separations could be explained by a CD-modified MEKC principle. MA was partitioned into the SDS micelles and the HS-γ-CD, and the enantioseparation was achieved. On the other hand, in CSEI-sweeping MEKC mode, MA signals were also separated with

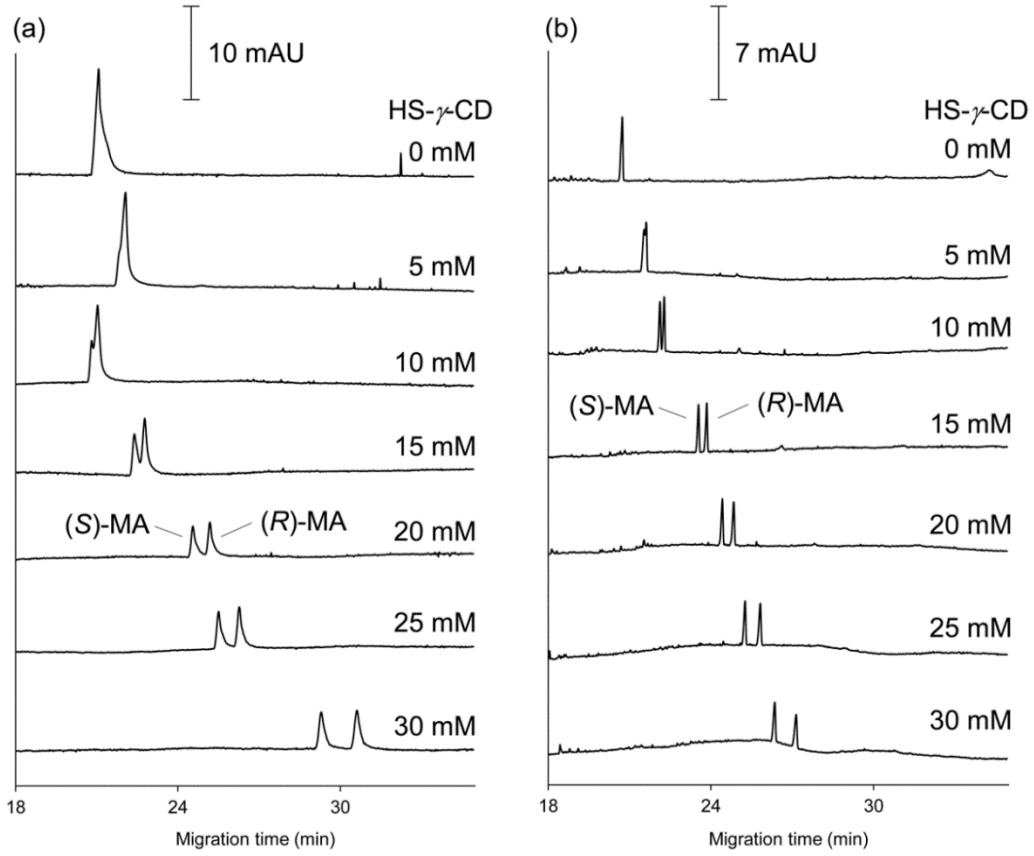

Fig. 12　Electropherograms obtained from (a) MEKC and (b) CSEI-sweeping MEKC. HS-γ-CD was gradually added to the running buffer and the micellar buffer.

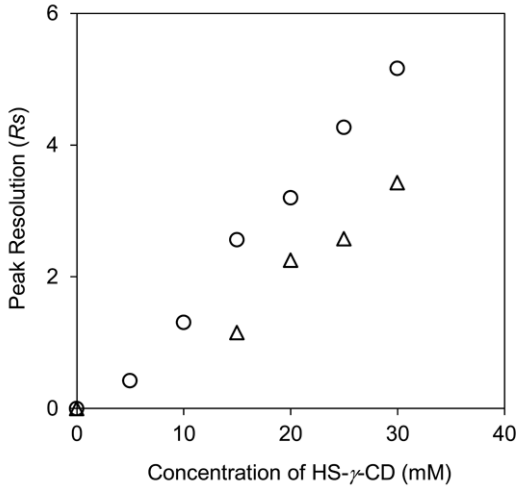

Fig. 13　A plot of peak resolutions on enantioseparation against the concentrations of HS-γ-CD from (△) MEKC and (○) CSEI-sweeping MEKC.

increasing HS-γ-CD concentrations. Moreover, the peak widths of MA were not broadened; that is, the online concentration mechanism remained constant. Figure 13 shows the peak resolutions on enantioseparation against the concentrations of HS-γ-CD. In both MEKC and CSEI-sweeping MEKC, the resolutions increased with increasing concentrations of HS-γ-CD. These results clearly indicate that HS-γ-CD affected the enantioseparation without extra interference, and the online concentration mechanism could be perfectly preserved in CSEI-sweeping CD-modified MEKC. With the conditions of Fig.12 (b), the repeatability, limits of detection (LODs; $S/N = 3$) and

Table 2 The repeatability, LODs and SEF obtained from CSEI-sweeping CD-modified MEKC measurements with 20 mM HS-γ-CD.

Compounds	(*S*)-MA	(*R*)-MA
RSD (%, $n = 8$)		
Migration time	0.5	0.5
Peak area	5.7	4.7
Peak height	3.8	4.2
LODs ($S/N = 3$, pg/mL)	77.9	88.8
SEF_{LOD}	13094	11149

SEF_{LOD} = Sensitivity Enhancement Factor
$= LOD_{\text{CD-modified MEKC}} / LOD_{\text{CSEI-sweeping CD-modified MEKC}}$

sensitivity enhancement factor (SEF) in terms of LODs were evaluated at 20 mM HS-γ-CD (Table 2). Repeatabilities of migration times (MT), peak heights and peak areas may be good enough for general analyses, and LODs were at the ppt levels. The SEF of (*S*)- and (*R*)-MA were achieved over 10,000-fold (LODs in normal CD-modified MEKC in my experiments; (*S*)-MA:1.02 μg/mL; (*R*)-MA: 0.99 μg/mL). It appeared that the LODs obtained from the normal CD-modified MEKC were slightly higher than those reported with the same procedure [62, 64]. The cause of this inferiority would be explained by the aging of a lamp and/or the degradation of a detector. Nevertheless, SEF must have been invariable and intrinsic values because all the measurements were performed with the same instrument. Sanchez-Lopez *et al.* reported in their review article [34] that Ma *et al.* used field-amplified sample stacking coupled with matrix removal by electroosmotic flow pump, and the authors found a sensitivity enhancement up to 3,000 times in chiral analysis when compared to hydrodynamically injected β-blockers [66]. This study was introduced as the most remarkable sensitivity improvement from all the works included in the review. It is clearly found from this report that the SEF values in my experiments must be arrestingly good.

2.3.3 Investigation of the mechanism

To verify the predicted mechanism of CSEI-sweeping CD-modified MEKC, a capillary was filled with only HCB or MCB, and the measurements were performed

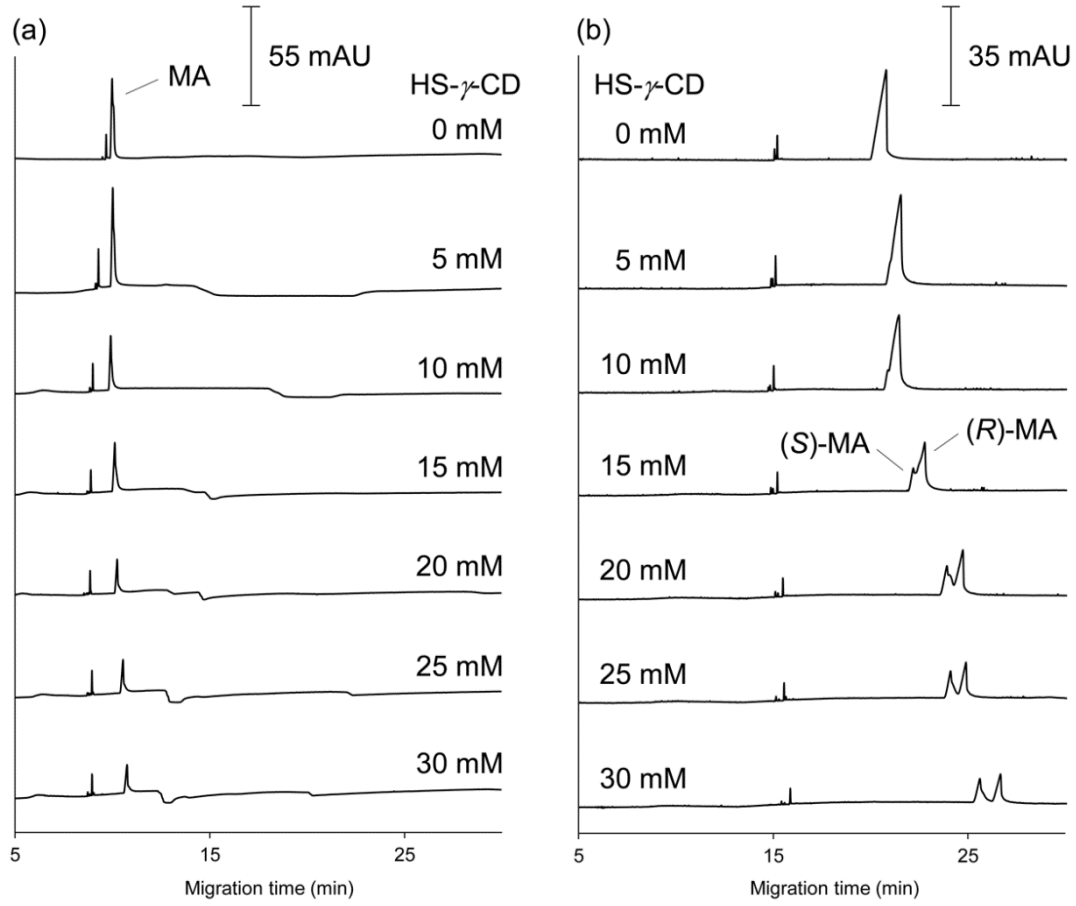

Fig. 14 Electropherograms obtained from the capillary filled with (a) HCB and (b) MCB. HS-γ-CD was gradually added to the micellar buffer.

with a micellar buffer (20 mM SDS in MCB) at the ends of the capillary (Fig. 14). An HS-γ-CD was gradually added to the micellar buffer. SDS and HS-γ-CD were introduced to the capillary with voltage impressing. When HCB filled the capillary, the MA peaks were not separated with increasing HS-γ-CD concentrations. On the other hand, when MCB filled the capillary, MA peaks were gradually separated and MTs of them were gradually retarded with increasing HS-γ-CD concentrations. In the comparisons of MTs between HCB and MCB, those from HCB were obviously faster

than those from MCB. From these results, it could be expected that the migration of SDS micelles was faster than that of HS-γ-CD. After sample injections, SDS micelles and HS-γ-CD migrated into the capillary. In HCB, SDS constituted the micelles strongly, and the micelles and MA were highly bound. As a result, the preceding SDS micelles swept the MA, thus their peaks were not separated with increasing HS-γ-CD concentrations. In MCB, the hydrophilicity was weakened by methanol, and the SDS micelles were mostly destroyed. Therefore, HS-γ-CD from the inlet vial could catch up with the swept MA, and allowed the separation by CD-modified MEKC. These results strongly supported the proposed mechanism in section 2.3.1.

2.3.4 Analysis of hair samples by CSEI-sweeping CD-modified MEKC

Blank hair and those spiked with racemic MA (0.2, 1.0 ng/mg) were pretreated and analyzed by the present method (Fig. 15). An HS-γ-CD concentration of 20 mM was chosen for these analyses, since this concentration was shown to provide sufficient enantioseparation in Fig. 12 (b). (*S*)- and (*R*)-MA peaks were clearly detected from the spiked hair samples (Fig. 15 (b), (c)) with enantioseparation. Concerning hair analyses, the Society of Hair Testing recommends that the cut-off concentration of MA be set at 0.2 ng/mg [31]. It was confirmed that the hair sample including the recommended cut-off concentration of MA could be analyzed by the present method using 3 mg

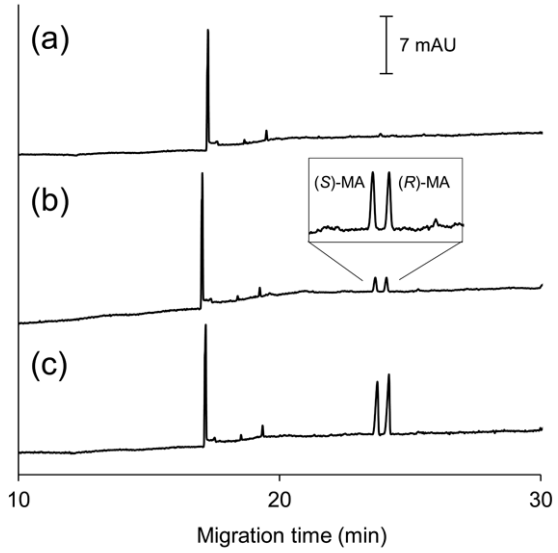

Fig. 15 Electropherograms obtained from (a) unspiked hair, (b) hair spiked with racemic MA at 0.2 ng/mg, and (c) hair spiked with racemic MA at 1.0 ng/mg.

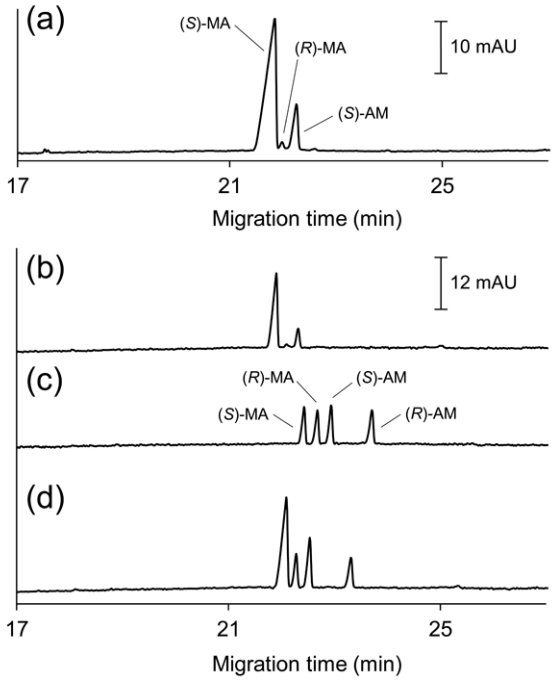

Fig. 16 Electropherograms obtained from (a) the hair of an MA user, (b) a 5-fold-diluted sample of (a), (c) an authentic standards solution of racemic MA and AM at 1 ng/mL as each enantiomer, and (d) a mixture of (b) and (c).

hair, this amount was used as the amount in a single sample in my experiments. The hair from an MA user was also analyzed (Fig. 16). MA and its metabolite, AM, were clearly detected with baseline enantioseparations. The MTs of MA and AM from the hair sample did not correspond to those from authentic standards, probably due to matrix effects (Fig. 16 (b), (c)). However, by adding the appropriate amounts of authentic standards to the hair sample, each peak could be assigned from the variations of peak areas (Fig. 16 (d)).

2.4 Conclusion

Effective online concentration and enantioseparation were simultaneously achieved by a slight modification of CSEI-sweeping MEKC. Simply by adding an anionic CD to the micellar buffer, sweeping in HCB and CD-modified MEKC in MCB may have occurred sequentially. The online concentration mechanism could be perfectly preserved, and an anionic CD affected the enantioseparation without extra interference. The present method was adapted to the analysis of a hair sample. Trace amounts of MA and its metabolite, AM, were clearly detected with enantioseparations.

Many reports have described the detection of trace amounts of analytes in various samples by CSEI-sweeping MEKC [50-60]. The usefulness of the CSEI-sweeping MEKC method has already been demonstrated. Therefore, the present technique, which was simply the CSEI-sweeping MEKC method with a slight modification, would give

an attractive approach that is applicable to almost any analytes for which CSEI-sweeping MEKC is applicable; all that is required is the selection of an appropriate anionic CD for addition to the micellar buffer.

2.5 Ethics

This study was approved by the ethics committee of Japanese association of forensic science and technology. An approved title: Development of the analytical method for methamphetamine in hair by chiral capillary electrophoresis (Authorization Number: 27M4).

Summary

A pre-treatment method, which was constructed by a 2D-HPLC system, was proposed for the direct analysis of drugs in human serum. A temperature-responsive column was used for a pre-treatment column. Using the developed method, high-quality chromatograms of multiple drugs were obtained without unwanted peaks from foreign substances.

A simple method that combines an online concentration technique with an enantioseparation technique for CE—namely, CSEI-sweeping CD-modified MEKC—was also proposed for the methamphetamine analysis in hair. The developed method achieved over 10 000-fold sensitivity increase compared to the LODs by the usual injection method. It was confirmed that the hair from the abuser could be analyzed by this method.

These proposed methods possess the superior points compared with the former ones. I sincerely hope that these methods contribute to the improvements of conventional methods used in inspection agencies.

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