Application of thermoresponsive HPLC to forensic toxicology: determination of barbiturates in human urine

SHORT COMMUNICATION

Application of thermoresponsive HPLC to forensic toxicology: determination of barbiturates in human urine

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Abstract A high-performance liquid chromatography (HPLC) method has been developed for the assays of five barbiturates in human urine using a new thermoresponsive polymer separation column, which is composed of *N*-isopropylacrylamide polymer. According to elevating the column temperature from 10 ^{o}C to 50 ^{o}C, five barbiturates, such as metharbital, primidone, phenobarbital, mephobarbital and pentobarbital, became well separated by this method. Five barbiturates showed good linearity in the range of 0.2-10 mg/ml. Good accuracy, precision and recoveries for these drugs were obtained at 1 and 5 μg/ml urine. The method with the new-type column seems to have high potential to be extensively used in forensic toxicology for analysis of many drugs and poisons by HPLC and HPLC-mass spectrometry (MS) (-MS).

Keywords Thermoresponsive polymer column · Temperature-responsive · Barbiturates · Phenobarbital · HPLC · Human urine

Introduction

Barbiturates are widely used as sedatives, hypnotics and antiepileptics, and sometimes cause death in suicide and accidents, because of their relatively narrow safe-dose ranges [1, 2]. The screening and determination of barbiturates in body fluids are very common task for forensic toxicologists.

Recently, a new thermoresponsive polymer, such as poly(*N*-isopropylacrylamide) (PIPAAm) (Fig. 1A) has been developed as packing material for high-performance liquid chromatography (HPLC) [3]. PIPAAm exhibits thermally reversible hydrophilic/hydrophobic phase transition on the surface of silica beads in aqueous solution across a lower critical solution temperature at about 32 ^{o}C (Fig. 1B). The separation of analytes can be controlled by adjusting the column temperature using only aqueous solutions as a mobile phase without any organic solvent. Using the thermoresponsive HPLC, some trials have been made to separate steroids [4], lysozyme [5] and peptides [6] from biochemical and pharmaceutical viewpoints.

In this study, we have first introduced the method into the forensic toxicology field, and achieved the separation of six barbiturates, such as metharbital, primidone, phenobarbital, mephobarbital, pentobarbital and secobarbital (internal standard, IS) using the thermoresponsive HPLC.

Materials and methods

Materials

Metharbital and primidone were obtained from Dainippon Pharmaceutical (Osaka, Japan); pentobarbital calcium, secobarbital sodium, mephobarbital and phenobarbital from Tanabe Pharmaceutical (Osaka, Japan), Yoshitomi Pharmaceutical (Osaka, Japan), Bayer (Leverkusen-Bayerwerk, Germany) and Fujinaga Pharmaceutical (Tokyo, Japan), respectively. Oasis HLB extraction cartridges were purchased from Waters (Milford, MA, USA). Other common chemicals used were of the highest purity commercially available. Pure water having a specific resistance of 17 MΩ cm was used for sample preparation and for the HPLC mobile phase.

Blank urine samples were collected from a healthy adult and kept frozen at -20 ^{o}C until analyzed. Barbiturate-positive urine samples obtained from male and female cadavers at forensic autopsies and stored at -80 ^{o}C were also used.

High-performance liquid chromatographic conditions

The analytical column was PIPAAm hydrogel-modified column (150 mm X 4.6 mm i.d.) (CellSeed, Tokyo, Japan). The column was connected to an HPLC system consisting of a pump (LC-10AD), a UV-VIS detector (SPD-10A) and an autosampler (SIL-20A) (Shimadzu, Kyoto, Japan). The column oven used was an Aqua Way Gradienter

(CellSeed). The mobile phase was 2.5 mM ammonium formate buffer adjusted to pH 7.5 with ammonium hydroxide. The elution was monitored at 215 nm at a flow rate of 1 ml/min at 50 °C.

Procedure

A 1-ml volume of urine containing barbiturate and secobarbital as IS (8 μg) was mixed with 1 ml of distilled water. The diluted urine sample was loaded onto a conditioned Oasis HLB cartridge. It was washed with 2 ml of 5% methanol in distilled water and 2 ml of 2% acetic acid in 25% methanol; the target compounds were eluted with 2 ml of 2% ammonium hydroxide in 35% methanol. The eluate was dried using a Savant SpeedVac system (Savant, Holbrook, NY, USA). The resultant residue was dissolved in 200 μl of mobile phase and a 20-μl volume was injected into the HPLC system by the autosampler.

Results and discussion

Separation of barbiturates with the thermoresponsive column

As shown in Fig. 2, the retention times and separation of barbiturates could be controlled only by changing the column temperature from 10 °C to 50 °C in the isocratic elution mode. At 10 °C, only metharbital and primidone could be separated from other

barbiturates; but the peaks of phenobarbital, mephobarbital, pentobarbital and secobarbital (IS) overlapped. According to elevating the column temperature, the retention times of mephobarbital, pentobarbital and secobarbital were increased and the overlapped peaks were separated into four peaks gradually. Therefore, we chose 50 ^{o}C for column temperature to detect the six barbiturates including IS.

Reliability of the method

Table 1 shows regression equations and correlation coefficients for five barbiturates spiked into human urine at concentrations of 0.2, 1, 5 and 10 μg/ml. All calibration curves exhibited good linearity in the range of 0.2-10 μg/ml with *r* values not smaller than 0.994. The limits of detection (single-to-noise ratio = 3) for metharbital, primidone, phenobarbital, mephobarbital and pentobarbital were 0.2, 0.2, 0.1, 0.1 and 0.1 μg/ml, respectively.

Intraday accuracy and coefficients of variation (CV), and recoveries for five barbiturates spiked into human urine at concentrations of 0.2, 1, and 5 μg/ml are shown in Table 2. The recoveries were calculated by comparing the peak areas obtained from the extracts of the spiked urine with those obtained by direct HPLC injection of nonextracted compounds dissolved in distilled water. Intraday accuracy of metharbital, primidone and mephobarbital at 0.2 μg/ml was not smaller than 135%; the CV values at this concentration were also relatively high for metharbital and primidone. The high accuracy may be due to the interference by impurity peaks of blank urine especially at

the low barbiturate concentration. At 1 and 5 μg/ml, however, both accuracy and CV values (precision) were within acceptable range. The recovery rates of barbiturates tested were generally satisfactory at all concentrations.

Application of the method to actual human samples

This method was applied to barbiturate measurements in actual cases. Both subjects had been suspected to ingest tablets containing phenobarbital. Figure 3 shows an HPLC chromatogram of urine extract from the male cadaver. As shown in the figure, phenobarbital could be detected from urine samples in actual cases. Its concentrations in urine samples from the male and female cadavers were 23.3 and 13.5 μg/ml, respectively.

Conclusions

In this report, we have presented an HPLC method for analysis of some barbiturates in human urine using a new type of separation columns. The property of this column changes according to different temperature; at 10 ^{o}C, the surface of column packing beads is hydrophilic and changes to hydrophobic ones according to the increase in column temperature up to 50 ^{o}C (Fig. 1B), which can control the retention time and separation of some drugs (Fig. 2). Such thermoresponsive polymer columns seem to have great potential in forensic toxicology; it may be usable for HPLC and for its

combination with mass spectrometry (MS) (-MS) to analyze a number of other drugs and poisons present in various matrices. This line of experiments is currently under way in our laboratories.

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Figure legends

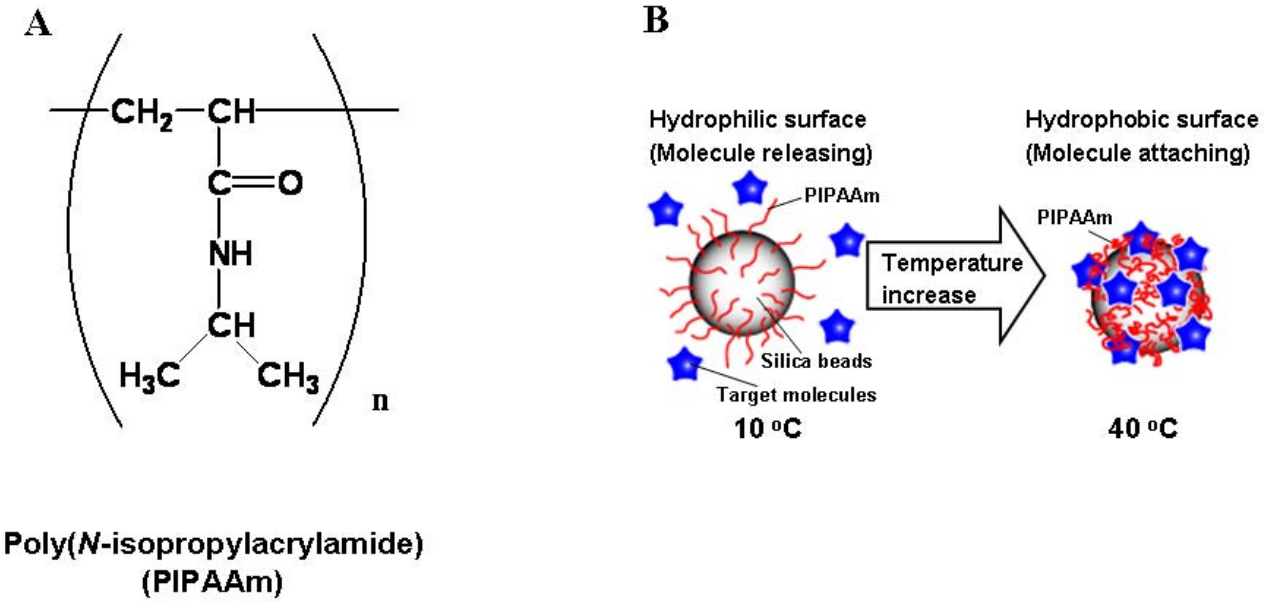

Fig. 1

Fig. 1A, B Structure of poly(*N*-isopropylacrylamide) (A) and schematic illustration of the thermoresponsive changes of the polymer on the surface of the silica beads provided by CellSeed with written permission (B)

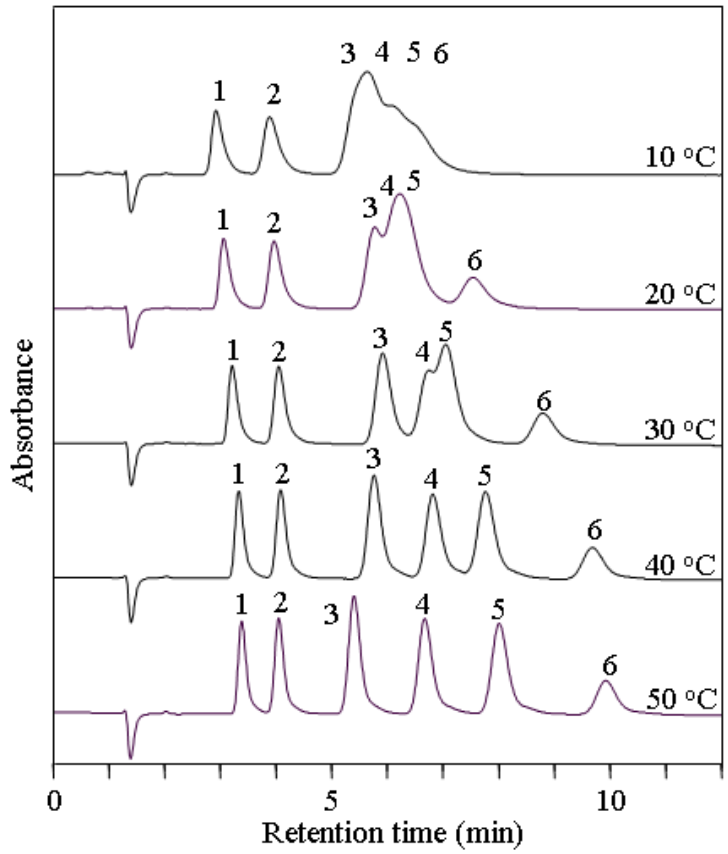

Fig. 2

Fig. 2 High-performance liquid chromatography (HPLC)-ultraviolet detection (UVD) chromatograms for six barbiturates (10 μg/ml each) with a poly(*N*-isopropylacrylamide) hydrogel-modified column at 10, 20, 30, 40 and 50 °C. The mobile phase was 2.5 mM ammonium formate buffer at pH 7.5. Peaks: 1, metharbital; 2, primidone; 3, phenobarbital; 4, mephobarbital; 5, pentobarbital; 6, secobarbital (internal standard, IS)

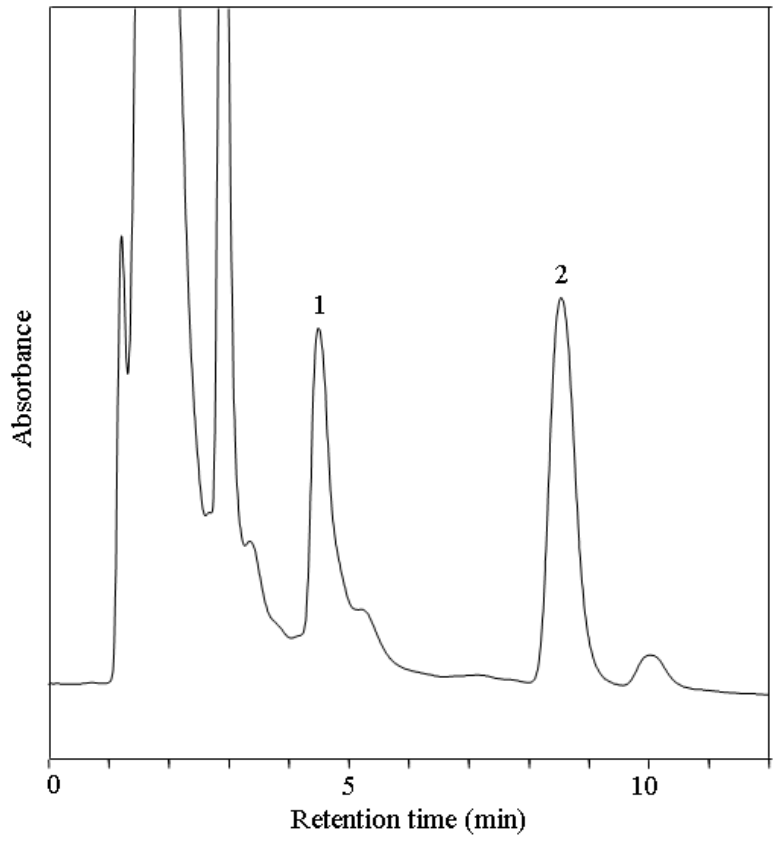

Fig. 3

Fig. 3 HPLC-UVD chromatogram of urine extract obtained from a male cadaver in an actual autopsy case measured by the present method. He had been suspected to ingest tablets containing phenobarbital. The cadaver urine was diluted 10-fold with blank urine before extraction, because the phenobarbital level was too high and beyond the calibration curve range. Peaks: 1, phenobarbital; 2, secobarbital (IS)

Table1 Regression equiations for five barbiturates spiked into human urine

Drug	Equation[a]	Correlation coefficient (r)
Metharbital	y[b] $= 0.117x + 0.0495$	0.994
Primidone	$y = 0.169x - 0.0385$	0.998
Phenobarbital	$y = 0.294x + 0.0456$	0.999
Mephobarbital	$y = 0.229x + 0.0427$	0.998
Pentobarbital	$y = 0.327x + 0.0535$	0.999

[a] Each equation was constructed by plotting 4 different concentrations (0.2-10 μg/ml)

[b] y is a peak area ratio of a barbiturate to secobarbital (8 μg/ml, internal standard)