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A rapid method for detecting barbiturates in serum using EI-SIM

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Abstract A simple and rapid method for analysis of barbiturates in serum has been developed. In order to extract and clean barbiturates in serum, a separation column packed with Extrelut and Florisil was used, and the eluate was directly analyzed by means of electron impact selected ion monitoring (EI-SIM). Selected ions used were base peak ions of 10 barbituartes, and the internal standard used was allobarbital or secobarbital. The calibration curves were linear over the range 0.5–5 ng. Extraction of replicate serum samples containing 20 μg/1.5 ml and 5 μg/1.5 ml resulted in a recovery of 87.2–105.2% and 81.6–104.6%, respectively, with the exception of phenobarbital, which was 151.9% and 172.1%, respectively. Secobarbital was also analyzed in the serum of 13 patients who had been given secobarbital intravenously. In 3 out of 10 cases, secobarbital levels greater than 1 μg/ml were detected more than 72 h after administration. This method seems to have possibilities for clinical use.

Key words Barbiturates · Serum analysis · GC/MS (EI-SIM) · Secobarbital therapy

Zusammenfassung Eine einfache und schnelle Methode zur Analyse von Barbituraten im Serum wurde entwickelt. Zur Probenaufarbeitung wurde eine Säulenextraktion mit Hilfe von Extrelut und Florisil angesetzt. Das Eluat wurde direkt analysiert mit Hilfe der GC/MS (EI-SIM). Als Ionen wurden die „base peaks" von 10 verschiedenen Barbituraten ausgewählt. Als interner Standard wurde Allobarbital oder Secobarbital verwendet. Innerhalb eines Bereiches von 0,5–5 ng wies die Eichkurve einen linearen Verlauf auf. Bei der Extraktion von gespeikten Serumproben, welche 20 μg/1,5 ml und 5 μg/1,5 ml enthielten, konnte eine Wiederfindungsrate von 87,2–105,2% und 81,6–104,6% gefunden werden. Phenobarbital stellt mit einer Wiederfindungsrate von 151,9% bzw. 172,1% eine Ausnahme dar. Die Secobarbital-Gehalte von 13 Patienten-Seren nach intravenöser Gabe von Secobarbital wurden analysiert. In 3 von 10 Fällen konnten Sekobarbital-Spiegel von mehr als 1 μg/ml bei mehr als 72 Stunden zurückliegender Secobarbital-Gabe nachgewiesen werden. Die Methode scheint eine Möglichkeit zur klinischen Barbiturat-Spiegel-Bestimmung zu bieten.

Schlüsselwörter Barbiturate · Serum-Analyse · GC/MS (EI-SIM) · Secobarbital-Therapie

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Introduction

Brain edema caused by head injuries, hypoxemia, and other diseases, frequently accompanies intracranial hypertension, making it necessary to administer a large amount of barbiturates to protect the brain. In Japan, brain death is not yet accepted as a definition of absolute death. However, according to the manual for the determination of brain death in the Hiroshima University School of Medicine [1], the diagnosis of brain death must be started 72 h after stopping barbiturate administration in a case where a patient has been given the usual amount of barbiturates. If a patient has been given a large amount of barbiturates, it is necessary to confirm that the patient is not affected by barbiturates by analyzing the concentration of barbiturates in blood.

An accurate, simple and rapid method for analysis of barbiturates in serum was developed, and secobarbital was analyzed in the serum of 13 patients who had been given secobarbital intravenously.

Materials and methods

Potassium hydrogen phosphate, ethyl acetate, diethyl ether, and other common chemicals of analytical grade were obtained from

Table 1 Time period and amount of secobarbital sodium given to patients, and secobarbital concentrations in serum

No.	Age/Sex	Diseases diagnosed	Secobarbital administration			Secobarbital concentration in serum (μg/ml)	
			Period (h)	Amount (mg/kg/h)	Total (mg/kg)	During administration	After stopping administration (h)
1	47 F	Head injuries[1]	81	0.5–5.0	85.5	5.87– 9.6	2.67/43
2	63 M	Status epilepticus	95	0.2–1.0	74.8	5.90–14.7	6.87/22, 3.33/46, 1.20/72, 0.60/96, 0.15/119
3	11 M	Head injuries	136.5	0.5–4.0	277.8	26.3 –55.0	17.3/3
4	33 F	Encephalitis	111	0.5–2.5	169.0	15.9 –35.5	7.4/23, 2.87/47, 0.87/71, 0.67/95
5	4 M	Drowning[2]	377	0.5–4.0	1.023.0	3.93–37.7	1.27/24, 0.47/48, 0.60/72, 0.33/96
6	20 F	Status epilepticus	162.5	0.5–2.0	165.0	2.87–19.5	2.93/1, 0.87/23, 0.60/47
7	73 F	Head injuries	33	1.0–2.0	48.0	9.00–15.9	8.67/22, 3.80/47, 0.93/94
8	44 F	Postoperative hydrocephalus	89.5	1.0–6.0	262.0	22.5 –46.2	29.5/6, 12.3/30, 3.73/54, 0.40/145, 0.20/169, 0.13/198, 0.13/217
9	43 F	Head injuries	188	0.5–5.0	317.0	33.5 –53.5	20.2/4, 5.20/30, 0.47/99
10	25 M	Head injuries	69	0.5–2.0	108.0	20.0 –26.9	5.07/21, 1.53/45, 0.27/93
11	25 F	Head injuries	303.5	0.5–3.0	593.8	14.8 –53.4	25.0/4, 12.2/25, 3.2/53, 0.47/76, 0.40/97, 0.33/123
12	69 M	Intraventricular hemorrhage	192	1.5–3.0	366.5	Unknown	33.7/24, 16.0/49, 4.43/71, 2.08/95, 0.78/120, 0.79/204, 0.06/216, 0.30/238
13	58 F	Cerebellar hemorrhage	276.7	0.5–4.0	651.3	34.9 –48.0	4.47/137

Age: Years, F: female, M: male

[1] Brain death was diagnosed
[2] Died in Intensive Care Unit

Wako. Sep-Pak C_{18} cartridges were purchased from Waters Associates, Extrelut (Art 11738) from Merck, Florisil (60–100 mesh) from Floridin, and Bond Elut Certify columns from Varian. Barbiturates used were free acid compounds, secobarbital, thiamylal and thiopental were supplied by Yoshitomi. Other barbiturates were extracted from commerical drugs and purified for use.

Standard barbiturate solutions were made by dissolving each barbiturate in ethyl acetate to a concentration of 1 mg/ml.

The internal standard solution used was a standard allobarbital solution, and when allobarbital was analyzed, a standard secobarbital solution was used.

The standard serum sample was PENTEX (Miles, human serum), which was refrigerated at 5°C until use.

The control serum sample was collected from a healthy adult male who had not been given any drugs, and kept at –20°C until use.

Serum samples were collected from 13 patients, who were hospitalized in the Intensive Care Unit in Hiroshima University Hospital, during and/or after the intravenous administration of secobarbital (Table 1). The samples were kept at –20°C until analysis.

Extraction cartridges and columns. Sep-Pak C_{18} cartridges were pretreated using the same procedure as reported by Suzuki et al. [2].

Bond Elut Certify columns were preconditioned using the same procedure reported by Chen et al. [3].

Extrelut columns were made by packing 2 g of Extrelut, which had been washed with diethyl ether and dried, into a glass tube (13 × 1 cm i.d.).

Extrelut-Florisil columns were made by packing 0.5 g of activated Florisil (105°C, 1 h) as a lower layer and 2 g of pretreated Extrelut as an upper layer into a glass tube (13 × 1 cm i.d.).

EI-SIM. A GC/MS (Hewlett Packard, GC 5890 II and MSD 5971A) equipped with a capillary column (HP-1 or Ultra-1, 12 m × 0.2 mm, film thickness 0.33 μm) was used. The temperatures of the injection port and the ion source were set at 250°C and 280°C, respectively. The column temperature was set at 50°C for 2 min, and then programed from 50°C to 250°C at 25°C/min. The ionization voltage was set at 70 eV. Helium with an inlet pressure of 5 psi was used as a carrier gas. Selected ions were base peak ions of 10 barbiturates: m/z 156 for barbital, amobarbital and pentobarbital, m/z 167 for allobarbital, m/z 168 for butalbital and secobarbital, m/z 172 for thiopental, m/z 184 for thiamylal, m/z 204 for phenobarbital, and m/z 218 for mephobarbital.

To establish a calibration curve an aliquot of each standard barbiturate solution was mixed with 20 μl of an internal standard and the solvent was removed in a nitrogen stream at room temperature. The residue was dissolved in 2 ml of ethyl acetate, and 1 μl of the solution was analyzed by EI-SIM.

Extraction procedure. When an Extrelut column or an Extrelut-Florisil column was used, serum samples (1.0 or 1.5 ml) were mixed with 0.25 ml of a saturated solution of potassium hydrogen phosphate, which after mixing for several seconds, were tansferred into an extraction column. The test tube was rinsed with several drops of water and added to the extraction column. After 20 μl of the internal standard solution was added to the extraction column, it was left at room temperature for 20 min. Ethyl acetate was passed through the column, 2.2 ml of the eluate was collected, and 1 μl of the eluate was analyzed by EI-SIM.

When a Sep-Pak C_{18} cartridge was used for extraction, the procedure reported by Suzuki et al. [2] was used. When a Bond Elut Certify column was used for extraction, the procedure reported by Chen et al. [3] was used.

Results

The calibration curves obtained by plotting the peak area ratios of each barbiturate to the internal standard against the amount of each barbiturate from 0.5 ng to 5 ng were straight lines passing through or near the origin and the correlation coefficients ranged from 1.000 to 0.915.

The calibration curves of 8 barbiturates, with the exception of thiamylal and phenobarbital, were also straight

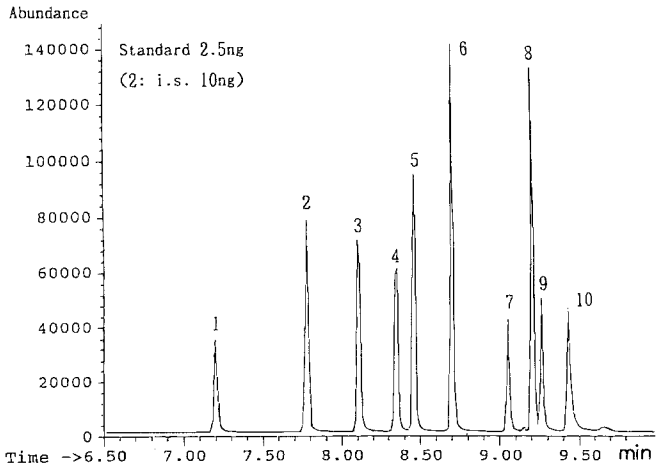

Fig. 1 Reconstructed SIM chromatograms of 10 barbiturates. 1: barbital, 2: allobarbital, 3: butalbital, 4: amobarbital, 5: pentobarbital, 6: secobarbital, 7: thiopental, 8: mephobarbital, 9: thiamylal, 10: phenobarbital

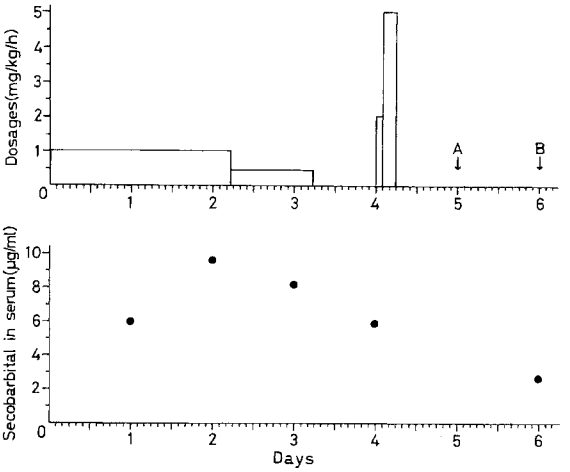

Fig. 2 Dosages of secobarbital sodium and secobarbital levels in the serum of case number 1. A: Diagnosis of brain death, first. B: Diagnosis of brain death, second

Table 2 Recoveries and deviations (%, $n = 6$)

Barbiturate	Added/1.5 ml serum	
	20 μg	5 μg
Barbital	105.2 ± 7.01	102.8 ± 5.28
Allobarbital	99.5 ± 2.06	97.2 ± 10.6
Butalbital	99.4 ± 1.30	97.3 ± 6.51
Amobarbital	95.5 ± 2.90	97.6 ± 8.89
Pentobarbital	101.8 ± 4.17	103.5 ± 8.65
Secobarbital	100.3 ± 2.08	104.6 ± 10.5
Thiopental	89.5 ± 7.08	87.1 ± 20.2
Mephobarbital	97.9 ± 9.25	102.2 ± 16.6
Thiamylal	87.2 ± 7.89	81.6 ± 19.7
Phenobarbital	151.9 ± 15.5	172.1 ± 22.6

lines passing through the origin within the range 0.5 ng–25 ng, and the correlation coefficients ranged from 0.999 to 0.992. The lower detection limit was around 0.25 ng. When 1.5 ml of serum sample was used, the lower detection limit was about 0.1 μg/ml.

Comparison of 4 extraction methods. Figure 1 shows the reconstructed EI-SIM chromatograms of 10 authentic barbiturates. Chromatograms of extracts of control serum samples without an internal standard using extraction cartridges and columns showed a peak at 9.4 min corresponding to phenobarbital, except when using an Extrelut-Florisil column. Therefore, an Extrelut-Florisil column was used as the extraction procedure for the following experiments.

When standard serum samples containing 20 μg or 5 μg of barbiturates were analyzed, the relative recoveries and the coefficient of variations shown in Table 2 were obtained. Recoveries of thiopental and thiamylal were low. When 5 μg of thiopental and thiamylal were added to the standard serum, deviations were more than twice as high as other barbiturates. Recoveries and deviations of phenobarbital were extremely high while in the other barbiturates, recoveries and deviations were good.

The half-life periods of secobarbital in serum after stopping secobarbital administration ranged from 17.1 to 30.1 h (average 22.0) in 10 cases.

The time course of secobarbital administration and the secobarbital concentration in the serum of case No. 1, where brain death was determined, is shown in Fig.2. The first diagnosis of brain death was performed 19 h after intravenous secobarbital administration had been stopped, and the second diagnosis of brain death was performed 24 h after the first diagnosis of brain death.

Discussion

When barbiturates are analyzed using packed columns, methylation of barbiturates [4] is necessary to avoid adsorption of barbiturates on the column. Recently, a weak polar capillary column coated with 5% phenyl and 95% methyl polysiloxane. DB-5 and CBP-5, was used for barbiturates analysis without methylation [5].

A non-polar capillary column coated with 100% dimethyl polysiloxane, HP-1 and Ultra-1, was used to analyze barbiturates together with other drugs.

Extrelut column extraction of dipterex, an organophosphorus pesticide, has been reported [6], and has been used for extraction of organophosphorus pesticides, amphetamines, drugs, and inflammable substances. Yashiki et al. developed an Extrelut-Florisil column extraction method for thiopental [7]. Recently, Suzuki et al. [2] reported a rapid extraction method for some barbiturates in plasma, whole blood and urine using a Sep-Pak C_{18} cartridge. Chen et al. [3] developed a systematic extraction method for drugs in plasma and urine using a Bond Elut Certify column.

When control serum samples were extracted using an Extrelut column, an Extrelut-Florisil column, a Sep-Pak

C_{18} cartridge, and a Bond Elut Certify column, a peak corresponding to phenobarbital was found in all the chromatograms except when using an Extrelut-Florisil column. This peak seems to come from palmitic acid. The preparation of an Extrelut-Florisil column was simple compared with that of a Sep-Pak C_{18} cartridge or a Bond Elut Certify column. The extraction procedure using a Sep-Pak C_{18} cartridge took about six times as long as the extraction using an Extrelut-Florisil column, and the extraction using a Bond Elut Certify column took almost the same time as the Extrelut-Florisil column. Our method, a combination of the Extrelut-Florisil column extraction and EI-SIM, took about 90 min to analyze 5 serum samples if the EI-SIM was ready for analysis. This method enabled an accurate, simple and rapid analysis of barbiturates in serum which seems to have possibilities for clinical use.

Recoveries of thiopental and thiamylal were slightly low. Peroxide in the solvent should be removed to get high recoveries of thiopental and thiamylal from the biological materials [7]. However, in order to simplify the analysis, peroxide in ethyl acetate for extraction was not removed in this experiment.

Recoveries and deviations of phenobarbital were extremely high. It seems that some biological substances such as palmitic acid disturb the analysis of phenobarbital by EI-SIM under the chosen conditions.

The half-life periods of secobarbital in plasma have been reported to be 19–34 h (mean 25) [8], and 23–29 h [9]. In this experiment, similar values were obtained.

In case number 1, the first diagnosis of brain death was performed 19 h after stopping secobarbital administration. This diagnosis of brain death did not follow the manual for the determination of brain death in the Hiroshima University School of Medicine [1] as it was used only to decide the plan of therapy.

According to Moffat [8], the therapeutic concentration of secobarbital in serum is in the range 2–10 μg/ml, and toxic effects are associated with blood concentrations greater than 8 μg/ml. In 276 reported fatalities attributed to secobarbital, blood concentrations ranged from 4 to 132 μg/ml (mean 30). According to Baselt [9], the therapeutic concentration of secobarbital in plasma ranges from 1 to 2 μg/ml, and the toxic (non-fatal) level from 3 to 67 μg/ml. A secobarbital serum level of more than 1 μg/ml seems to have some effect on the brain, and 72 h or more after the intravenous injection of secobarbital had been stopped, secobarbital concentrations in serum of not less than 1 μg/ml were detected in 3 cases out of 10. No references were found reporting the correlation between barbiturate concentrations in the brain and those in serum or blood. However, it seems to be necessary to know the blood level of barbiturates and the effect on the brain, if brain death is diagnosed in a case where a patient is given a large amount of barbiturates. According to Machata [10], the concentration of a drug with effects on the central nervous system should be kept below at least 1% of the therapeutic level.

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