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Automated procedure for determination of barbiturates in serum using the combined system of PrepStation and gas chromatography–mass spectrometry

Akira Namera[a,*], Mikio Yashiki[a], Yasumasa Iwasaki[a], Minako Ohtani[b], Tohru Kojima[a]

[a]*Department of Legal Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[b]*Department of Emergency and Critical Care Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*

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Abstract

A system of an automatic sample preparation procedure followed by on-line injection of the sample extract into a gas chromatograph-mass spectrometer (GC–MS) was developed for the simultaneous analysis of seven barbiturates in human serum. A sample clean-up was performed by a solid-phase extraction (SPE) on a C_{18} disposable cartridge. A SPE cartridge was preconditioned with methanol and 0.1 *M* phosphate buffer. After loading 1.5 ml of a diluted serum sample into the SPE cartridge, the cartridge was washed with 2.5 ml of methanol–water (1:9, v/v). Barbiturates were eluted with 1.0 ml of chloroform–isopropanol (3:1, v/v) from the cartridge. The eluate (1 μl) was injected into the GC–MS. The calibration curves, using an internal standard method, demonstrated a good linearity throughout the concentration range from 0.1 to 10 $\mu g\,ml^{-1}$ for all barbiturates extracted. The proposed method was applied to 27 clinical serum samples from three patients who were administrated secobarbital.

Keywords: Sample preparation; Automation; Barbiturates

1. Introduction

Barbiturates, benzodiazepines and other drugs are widely available in combination products to treat anxiety, gastrointestinal upset, pain, and sleep disorders. In Japan, barbiturate coma is one of investigational tools in the immediate management of hypoxic encepalopathy and an acceptable treatment of trauma-induced cerebral edema. 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 these cases, however, the diagnosis of brain death is neither difficult nor likely to be wrong [1,2]. 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 [3]. In addition, despite the development of new families of sedative and anticonvulsant drugs, barbiturates are still implicated in cases of intoxication [4] because barbiturates can be obtained in Japan without prescription. So, screening and confirmation of barbiturates in bodily

*Corresponding author.

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fluids are important for the investigation of poisoning and the monitoring of therapeutic drugs.

As instrumental approaches for the analysis of barbiturates in bodily fluids, immunoassay and many chromatographic methods have been published [5–13]. Rapid analysis commonly used for thin layer chromatography [7,8] and immunoassay [5,6]. The former is simple and inexpensive but its sensitivity is limited, while the latter is not suitable for quantitative analysis of drugs. Other chromatographic procedures have been applied for the determination of all common drugs. They require, however, time consuming sample preparations and are characterized by a low throughput.

In a previous paper [14], we reported an automated method for determination of barbiturates in urine using a PrepStation–GC–MS system. In this system, an analyst's interaction is confined to placing samples on the autosampler tray, all the following steps were effected automatically. Thus freeing analysts for other tasks and minimizing the potential for error. The present paper describes a fully automated PrepStation–GC–MS method for the simultaneous determination and quantification of seven barbiturates in human serum. The method employed liquid–solid extraction via extraction C_{18} cartridges. And finally, the proposed method was applied to 27 clinical samples from three patients who were administrated secobarbital.

2. Experimental

2.1. Materials and chemicals

Chloroform, isopropanol (IPA), acetonitrile, methanol, sodium dihydrogen phosphate, disodium hydrogen phosphate and trichloroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was purified and deionized using a Milli-Q Jr. reagent-grade water system (Nihon Millipore Kogyo, Japan). C_{18} SPE cartridges (300 mg, 13.5×7 mm I.D.) were purchased from Yokogawa Analytical Systems (Tokyo, Japan). Barbiturates used were free acid compounds. Secobarbital, thiamylal, and thiopental were supplied by Yoshitomi Pharmaceutical Industries (Osaka, Japan). Barbital, amobarbital, pentobarbital, phenobarbital, and allobarbital were extracted from commercial drugs and purified for use.

A drug free serum sample collected from a healthy adult male was used to make the barbiturate serum samples, and used as a control serum. Clinical serum samples collected from the Intensive Care Unit in Hiroshima University Hospital were kept frozen at −20°C until analyzed.

2.2. PrepStation–GC–MS system

The GC–MS used was a Hewlett Packard 5890 series II gas chromatograph-5971A mass selective detector, equipped with a 30 m×0.25 mm (I.D.) fused-silica capillary column (Hewlett Packard, HP-5MS, film thickness 0.25 μm). The column temperature was set at 100°C for 1 min, then programed from 100°C to 280°C at 15°C min^{-1} and held at 280°C for 3 min. The temperatures of the injection port and ion source were set at 250 and 280°C, respectively. Splitless injection mode was used. Helium with a flow-rate of 50 kPa was used as a carrier gas. The mass selective detector was operated in electron impact (EI) mode with 70 eV of electron energy, and a scan range from m/z 50 to m/z 550. All data was acquired in a scanning and a selected ion monitoring (SIM) mode.

Quantitation of seven barbiturates was performed on the following ions: m/z 156 for barbital, amobarbital and pentobarbital, m/z 167 for allobarbital (I.S.), m/z 168 for secobarbital, m/z 172 for thiopental, m/z 184 for thiamylal, and m/z 204 for phenobarbital.

This fully automated analytical system was described in the previous paper [14].

2.3. Automatic sample preparation

A serum sample (0.17 ml) were transferred manually into a vial. All the following steps were effected automatically. A phosphate buffer (0.1 *M*, pH 6.8, 1.63 ml) and 50 μl of allobarbital (0.1 mg ml^{-1}), as an internal standard, was dispensed in the vial containing a serum sample. The vial was mixed for 10 s. The sample (1.5 ml) containing a buffer and an internal standard was loaded into a SPE cartridge, which was activated by washing with 2.5 ml of methanol and 5.0 ml of phosphate buffer (0.1 *M*, pH

6.8), where barbiturates were retained. The cartridge was then washed with 2.5 ml of methanol–water (1:9, v/v) and dried with a nitrogen gas for 10 min. Barbiturates were eluted with 1.0 ml of chloroform–IPA (3:1, v/v) from the washed cartridge. And finally, 1 μl of the eluate was injected into the GC–MS for analysis.

2.4. Recovery, linearity and repeatability

To determine extraction recovery, standard serum samples spiked with seven barbiturates at the concentration of 5.0 $\mu g\,ml^{-1}$ were prepared and analyzed using the above procedure ($n=6$). Extraction recovery was evaluated by comparing the peak area of seven barbiturates in the spiked serum with that obtained after injection of a known amount of standards.

To determine linearities, standard serum samples spiked with seven barbiturates at the concentrations of 0.01–20 $\mu g\,ml^{-1}$ were prepared and analyzed using the above procedure ($n=6$). The calibration curve was obtained by plotting the peak area ratio between seven barbiturates and allobarbital (I.S.).

Repeatability was evaluated by analyzing aliquots from a serum sample spiked at the concentrations of 0.50 and 5.0 $\mu g\,ml^{-1}$ of seven barbiturates on the same day (intra-day repeatability, $n=6$) and the six consecutive days (inter-day repeatability).

2.5. Influence of serum protein

For investigation of the influence of the serum protein, serum samples were deproteinized by trichloroacetic acid or acetonitrile. One ml of serum sample was mixed with 100 μl of trichloroacetic acid (0.1 *M*) or acetonitrile. After vortex-mixing for 30 s and centrifugation at 7 000×*g* for 5 min, the clear supernatant (0.17 ml) was transferred to a vial. The following steps were performed above procedure.

2.6. Conventional method

To compare with the conventional method, serum samples were extracted with a Extrelut-Florisil column and were analyzed by GC–MS. The procedure was reported by Kojima et al. [3].

3. Results and discussion

3.1. Recovery of barbiturates

The reconstructed SIM chromatograms are shown in Fig. 1. No impurity peak overlapped the peak of these barbiturates and allobarbital (I.S.). The recovery of seven barbiturates is shown in Table 1. The recovery of barbiturates from serum was 89–110%. The coefficients of variation for 0.5 and 5.0 $\mu g\,ml^{-1}$ of seven barbiturates in serum were 1.2 to 2.8% and 1.7 to 2.3%, respectively.

Methods for simultaneous and quantitative analysis of barbiturates in blood or serum using a liquid–liquid or a solid-phase extraction have also been published [3,10–12]. Recoveries of barbiturates were 80.6–103% by the liquid–liquid extraction [10], 71.8–90% by the solid-phase extraction with Bond Elute C_{18} [11,12], and 87–152% by the Extlerut

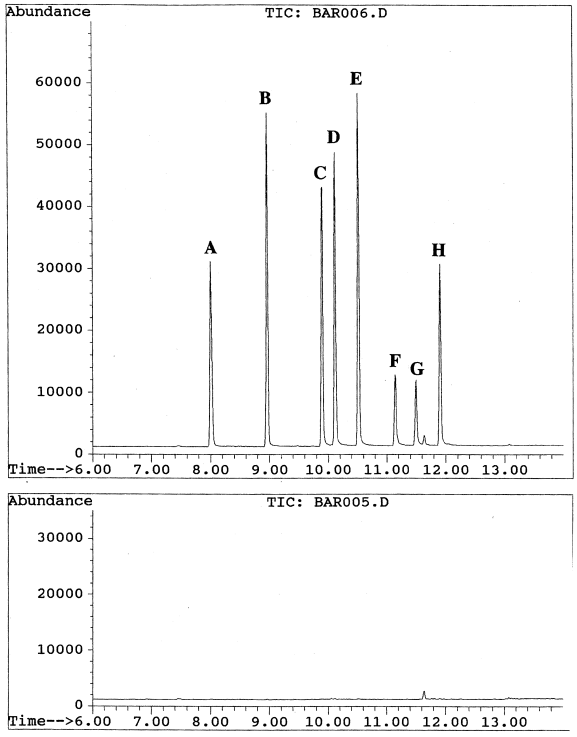

Fig. 1. The reconstructed SIM chromatograms of seven barbiturates from a serum. Top: 5.0 $\mu g\,ml^{-1}$ spiked barbiturates in serum, Bottom: serum blank. Peaks; A: barbital, B: allobarbital (I.S.), C: amobarbital, D: pentobarbital, E: secobarbital, F: thiopental, G: thiamylal, H: phenobarbital.

Table 1
Intra-day and inter-day repeatability ($n=6$)

Drugs	Concentration added ($\mu g\ ml^{-1}$)	Concentration found			
		Intra-day		Inter-day	
		Mean±S.D. ($\mu g\ ml^{-1}$)	C.V. (%)	Mean±S.D. ($\mu g\ ml^{-1}$)	C.V. (%)
Barbital	0.50	0.45±0.008	1.8	0.46±0.013	2.8
	5.00	4.66±0.09	2.0	4.78±0.11	2.4
Amobarbital	0.50	0.51±0.006	1.2	0.50±0.012	2.4
	5.00	5.40±0.10	1.9	5.50±0.12	2.2
Pentobarbital	0.50	0.50±0.005	1.2	0.48±0.012	2.6
	5.00	4.54±0.08	1.8	4.81±0.18	3.7
Secobarbital	0.50	0.48±0.006	1.3	0.49±0.009	2.0
	5.00	4.62±0.10	2.3	4.83±0.16	3.2
Thiopental	0.50	0.50±0.006	1.2	0.49±0.030	6.2
	5.00	5.45±0.18	2.2	4.86±0.31	6.3
Thiamylal	0.50	0.47±0.013	2.7	0.46±0.028	6.1
	5.00	5.00±0.11	2.2	4.58±0.23	5.0
Phenobarbital	0.50	0.48±0.013	2.8	0.47±0.014	3.0
	5.00	4.77±0.08	1.7	4.95±0.15	2.9

column extraction [3]. The recovery and coefficient of variation in the present method, however, compared favorably with these conventional methods.

Although high-performance liquid chromatography with multiwavelength detection (REMEDi) is used for drugs monitoring or analyzing in many laboratories, good results were not obtained in the detection and identification of opiates or barbiturates [15,16]. According to the manual, acidic drugs can be misidentified in the REMEDi system. Therefore, the PrepStation system is useful for detection and identification of barbiturates.

3.2. SPE Cartridge wash

In order to investigate the effect of a washing solvent, the cartridge was washed with 2.5 ml of deionized water or methanol–water (1:9, 1:2, 1:1, v/v). When the cartridge was washed with deionized water, the recovery was good, but many impurity peaks appeared in SIM. The barbiturates were likely lost in the elution solvent containing a higher percentage of methanol. Washing with 2.5 ml of methanol–water (1:9, v/v) was found to be adequate to remove interferences from the cartridge without affecting barbiturate recovery.

3.3. Analytical data

The analytical data were shown in Table 2. There was linear relationship between 0.1 and 10 $\mu g\ ml^{-1}$. The correlation coefficients of the calibration curves were 0.994 to 0.999. The limits of detection in serum were 0.10 $\mu g\ ml^{-1}$ for amobarbital, pentobarbital,

Table 2
Characteristics of the quantitation methods

Drugs	Range of linearity[a] ($\mu g\ ml^{-1}$)	Correlation coefficient (r^2)	Limit of detection ($\mu g\ ml^{-1}$)
Barbital	0.20–10	0.999	0.20
Amobarbital	0.10–10	0.999	0.10
Pentobarbital	0.10–10	0.999	0.10
Secobarbital	0.10–10	0.999	0.10
Thiopental	0.20–10	0.994	0.20
Thiamylal	0.20–10	0.994	0.20
Phenobarbital	0.10–10	0.999	0.10

[a] Six data points in duplicate.

secobarbital, and phenobarbital and 0.20 μg ml^{-1} for barbital, thiopental, and thiamylal, respectively. The limits of detection for the published methods were 0.5 μg ml^{-1} for liquid–liquid extraction [10], 0.1 μg ml^{-1} for GC or GC–MS [3,10,11], 0.05 μg ml^{-1} for HPLC [9]. The quantification limits of the proposed method are adequate for forensic and clinical analysis. In fatal intoxications of barbiturates, the concentrations in blood or plasma were reported to be 1–5 μg ml^{-1}, which can sufficiently be measured by our present method.

Inter-day repeatability for data analysis of seven barbiturates at two different serum concentrations are summarized in Table 1. Inter-day repeatability ranged from 2.0 to 6.2% for 0.5 μg ml^{-1} and 2.2 to 6.3% for 5.0 μg ml^{-1}, respectively.

3.4. Influence of serum protein

As thin teflon tubes and needles are used in the PrepStation, it is possible to give a good accuracy and measure a little volume. However, if small precipitations were contaminated in a sample solution, the needle and the tube were clogged. A sample solution and organic solvents flowed in same tubes in the system. If a serum sample was mixed with organic solvents, serum proteins were precipitated into the tube and the tube was clogged. In addition, if serum proteins were precipitated in the SPE cartridge for the solid-phase extraction step, elutant could not flow through the cartridge. For investigation of the serum protein, serum sample was deproteinized with trichloroacetic acid or acetonitrile.

When the serum was deproteinized by trichloroacetic acid or acetonitrile, the recovery of barbiturates was 55–70%. It seems that some part of barbiturates were taken in the precipitate of protein in the deproteinized step. When the serum sample was diluted with a buffer, the system worked without a problem. In order to operate easily, detailed conditions were not further examined using trichloroacetic acid or acetonitrile. Therefore, the serum sample was only diluted with a buffer.

3.5. Application to clinical samples

The proposed method was applied to 27 serum samples from three patients who were administrated a large amount of secobarbital, and secobarbital concentrations were monitored. In the cases which the concentration of serum samples was out of the linear range, the same serum samples were diluted with drug-free serum and then re-analyzed. The typical SIM chromatogram and the mass spectrum are shown in Fig. 2. The SIM chromatograms were very clear in all clinical samples. Mean serum concentration-time curves and dosages of secobarbital are depicted in Fig. 3. The half life periods of secobarbital in serum have been reported to be 19–34 h [17,18], similar values were obtained in this experiments,.

The result of the proposed method was compared with the conventional method using 27 serum samples from three patients who were administrated a large amount of secobarbital. The diagram are shown in Fig. 4. The correlation value was 0.967. The

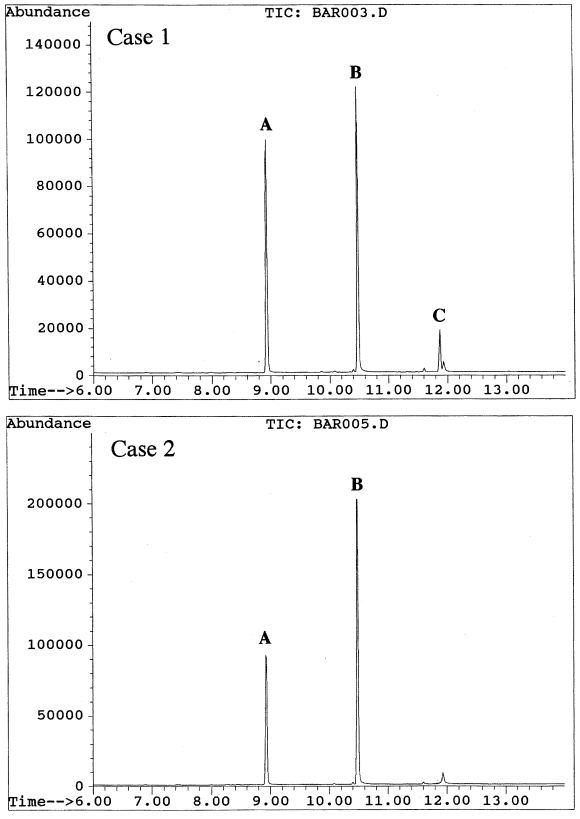

Fig. 2. The reconstructed SIM chromatograms of secobarbital for the two different clinical cases. Peaks; A: allobarbital (I.S.), B: secobarbital, C: phenobarbital. Case 1: status epileptics (13.0 μg ml^{-1} secobarbital, 2.09 μg ml^{-1} phenobarbital), Case 2: head injury (25.8 μg ml^{-1} secobarbital).

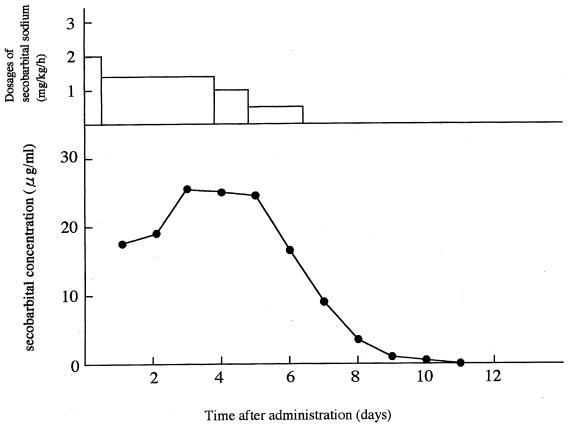

Fig. 3. Dosages of secobarbital sodium and serum concentration of secobarbital in the patient who was administrated secobarbital for status epileptics.

results of the PrepStation method were a good agreement with that of conventional method.

4. Conclusion

The PrepStation–GC–MS system has fully automated the simultaneous analysis of barbiturates in human serum. Clean extracts were obtained from human serum with good recovery. This system is capable of continuous preparation of samples, thus freeing analysts from mundane tasks and minimizing the opportunities for error. The PrepStation system was demonstrated to be a reliable, accurate, precise and cost-effective alternative to manual techniques.

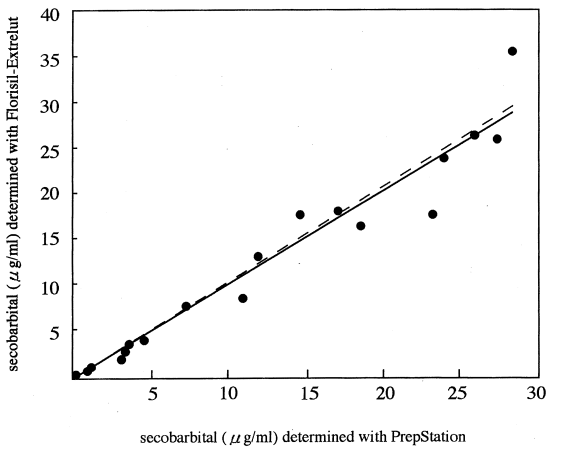

Fig. 4. The regression for secobarbital concentration in serum samples determined with PrepStation method versus the conventional Extrelut-Florisil method. - - - -: line of quality, ——: linear regression of the serum samples. $y=1.02x+0.023$, $r^2=0.967$.

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