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Automated preparation and analysis of barbiturates in human urine using the combined system of PrepStation and gas chromatography–mass spectrometry

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Abstract

A system for an automatic sample preparation procedure followed by on-line injection of the sample extract into a gas chromatography–mass spectrometry (GC–MS) system was developed for the simultaneous analysis of seven barbiturates in human urine. 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 a 1.5 ml volume of a urine 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 a GC–MS system. The calibration curves, using an internal standard method, demonstrated a good linearity throughout the concentration range from 0.02 to 10 μg/ml for all barbiturates extracted. The proposed method was applied to several clinical cases. The total analysis time for 20 samples was approximately 14 h. © 1998 Elsevier Science B.V.

Keywords: Barbiturates

1. Introduction

The development of modern, reliable and computer-controlled analytical instrumentation has enabled scientists to do unattended analytical work overnight. Although automated systems for liquid chromatography or gas chromatography [1–3] and automated sample preparation instruments [4–6] have been developed, most sample preparations have almost invariably been carried out manually and analyzed by automated analytical instruments, for there has been difficulty combining the automated analytical instrument and the automated sample preparation instrument.

Recently, Hewlett–Packard introduced a system called 'PrepStation', which is comprised of a SPE module for analyte enrichment, desorption with an organic solvent and a loading/transfer-to-GC module [7]. In the integrated system, the concentrated sample extracts are transferred on-line from the SPE unit to the autosampler of the GC system. And finally, an aliquot is injected into the GC–MS system. The total

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set-up should be simple and essentially requires no optimization. In this system, an analyst's interaction is confined to placing samples on the autosampler tray, thus freeing analysts for other tasks and minimizing the potential for error. For determination of the presence of micropollutants in environmental samples and of drugs in urine, some totally automated methods using PrepStation have been published [8–10].

Barbiturates are one of the largest groups of the sedative–hypnotic drugs. In Japan, they are widely used for the treatment of insomnia, anxiety and convulsive disorders as well as for anaesthetic and preanaesthetic medication.

In a previous paper [11], we reported a rapid method for detecting barbiturates in serum. The present paper describes a fully automated PrepStation–GC–MS method for the simultaneous determination and quantification of seven barbiturates in human urine. The method employed liquid–solid extraction via extraction C_{18} cartridges. And finally, the proposed method was applied to 9 clinical cases.

2. Experimental

2.1. Materials and chemicals

Chloroform, dichloromethane, methanol, isopropanol (IPA), sodium dihydrogen phosphate and disodium hydrogen phosphate 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 (13.5×7 mm I.D.) were purchased from Yokogawa Analytical Systems (Tokyo, Japan). The 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 urine sample collected from a healthy adult male was used to make the barbiturate urine samples, and used as a control urine. Clinical urine 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 chromatography-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 programmed from 100°C to 280°C at 15°C/min 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 full-scan mode and 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 consists of three components: PrepStation HP-7686 (a sample preparation device), HP-7673 (an auto injector) and GC–MS. Control of the instrumentation was effected through preinstalled PrepStation software. This software consists of two components. One is PrepStation which is used to crate/edit the sample preparation and controls the operation of the PrepStation module, the other is 'Bench Supervisor' which acts as the overseer of the automated system.

2.3. Automatic sample preparation

A urine sample (1.7 ml) and 20 μg of allobarbital, as an internal standard, were transferred manually into a vial. All the following steps were effected automatically. A 1.5-ml volume of the sample containing the 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). 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 system for analysis.

2.4. Recovery, linearity and repeatability

To determine extraction recovery, standard urine samples spiked with seven barbiturates at the concentration of 5.0 μg/ml were prepared and analyzed using the above procedure. Extraction recovery was evaluated by comparing the peak area of seven barbiturates in the spiked urine with that obtained after injection of a known amount of standards.

To determine linearities, standard urine samples spiked with seven barbiturates at the concentrations of 0.01–20 μg/ml were prepared and analyzed using the above procedure. The calibration curve was obtained by plotting the peak area ratio between seven barbiturates and allobarbital (I.S.).

Repeatability was evaluated by analysing aliquots from a urine sample spiked at the concentrations of 0.20 and 5.0 μg/ml of seven barbiturates on the seven consecutive days (inter-day repeatability).

3. Results and discussion

3.1. Recovery of barbiturates

The typical total ion chromatogram (TIC) and extracted-ion chromatograms are shown in Fig. 1. The small peak presented in the chromatogram of the urine extract was due to caffeine in the urine. 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 urine eluted with chloroform–IPA (3:1, v/v) was slightly higher than that of dichloromethane or methanol, and had smaller coefficients of variation. The coefficients of variation for 5.0 μg/ml of the seven barbiturates in urine were 2.1 to 6.2%. The recovery of thiopental and thiamylal was slightly lower than that of the other barbiturates. Sennello and Kohn [12] pointed out that peroxide present in the solvent reacted with thiopental to produce an artifact. Yashiki et al. [13] reported that peroxide in the solvent should be removed to get high recovery of thiopental and thiamylal from the biological materials.

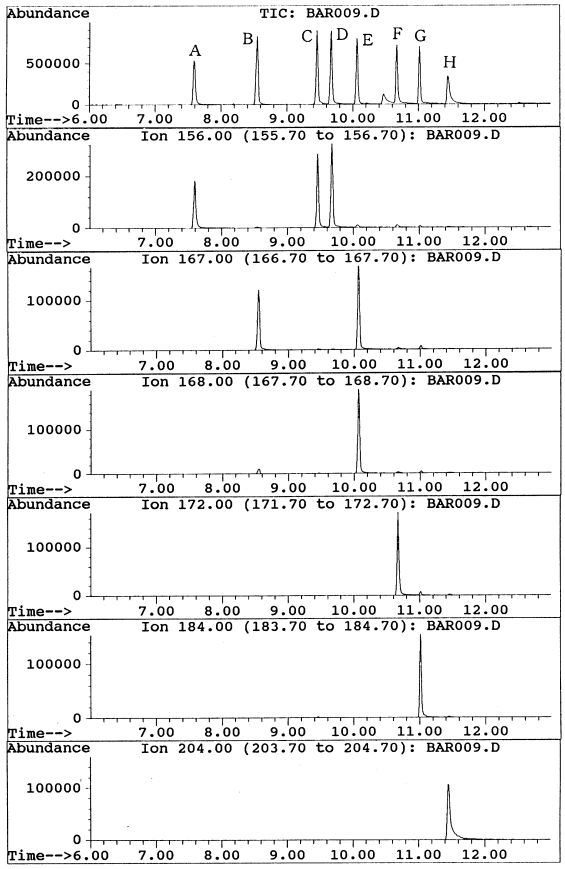

Fig. 1. Total ion chromatogram and extracted-ion chromatograms of seven barbiturates (5.0 μg/ml spiked in urine). Peaks: A=barbital, B=allobarbital (I.S.), C=amobarbital, D=pentobarbital, E=secobarbital, F=thiopental, G=thiamylal, H=phenobarbital.

A method for simultaneous and quantitative analysis of barbiturates using a liquid–liquid or a solid-phase extraction has also been published [14–16]. The recovery in the present method, however, compared favourably with these conventional methods in the range of 72–100%. Therefore the condition for elution with chloroform–IPA (3:1, v/v) was adopted.

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

Table 1
Extraction recovery of barbiturates from urine (n=5)

Drugs	Dichloromethane		Chloroform–IPA[a] (3:1)		Methanol	
	Recovery (%)	C.V.[b] (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Barbital	89	3.2	92	3.5	107	8.3
Amobarbital	88	2.4	96	2.5	85	8.4
Pentobarbital	88	2.4	104	2.6	88	9.3
Secobarbital	88	2.1	95	2.9	84	9.3
Thiopental	72	4.0	83	2.0	84	4.8
Thiamylal	68	4.1	81	2.6	81	3.6
Phenobarbital	92	6.2	102	2.7	105	8.4

[a] IPA: isopropanol.
[b] C.V.: coefficient of variation.

peaks appeared in full scan and SIM. The barbiturates were likely lost in the elution solvent containing a higher percentage of methanol. When the cartridge was washed with methanol–water (1:1, v/v), barbital and phenobarbital were most likely lost from the cartridge. It may be due to rapid desorption with methanol in the washing solvent. 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. Effect of urine pH on recovery

The effect of urine pH for extraction recovery at pH 5, 7 and 9 were investigated. Acetic acid and ammonium hydroxide were used to prepare the different buffers needed to adjust the sample pH and conditioning solvent. The recoveries are shown in Fig. 2. The recovery of seven barbiturates was over 80% at least for pH 5–7.

The recovery of amobarbital, pentobarbital, seco-

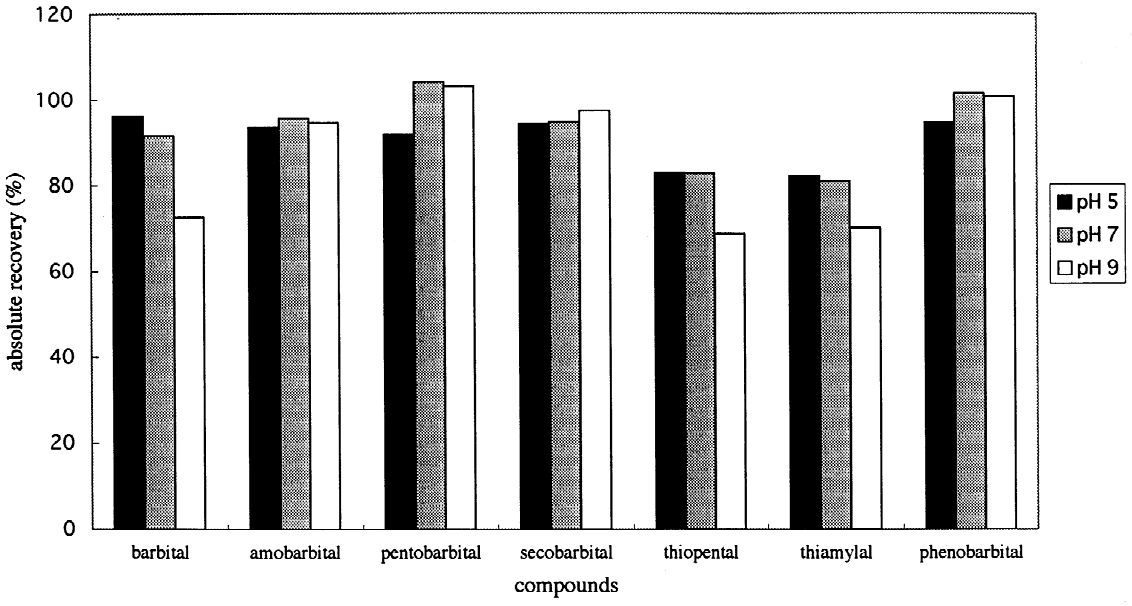

Fig. 2. Relationship between the recovery of barbiturates and the urine pH value.

barbital and phenobarbital from urine was independent of urine pH. But barbital, thiopental and thiamylal showed lower recovery at pH 9. At this pH, these three barbiturates behave as charged compounds, which have a low affinity for the hydrophobic solvent. In our laboratory, the pH values of more than 300 clinical urine samples were investigated, there are 91% of the samples between pH 5.0 to 7.9 [17]. Therefore, no pH conditioning was required in the urine samples.

3.4. Analytical data

The analytical data are shown in Table 2. There was a linear relationship between 0.20 and 10 μg/ml for scan mode, 0.02 and 1.0 μg/ml for SIM mode, respectively. The correlation coefficients of the calibration curves were 0.996 to 0.999. The limits of detection for barbiturates in urine were 0.10 to 0.20 μg/ml for scan mode and 0.02 to 0.05 μg/ml for SIM mode, respectively.

Inter-day repeatability for data analysis of seven barbiturates at two different urine concentrations are summarized in Table 3. Inter-day repeatability ranged from 2.4 to 4.3% for 5.0 μg/ml and 1.6 to 4.2% for 0.20 μg/ml (Table 3).

Table 3
Inter-day repeatability

Drugs	Concentration added (μg/ml)	Concentration found	
		Mean±S.D. (μg/ml)	C.V. (%)
Barbital	0.20	0.19±0.003	1.6
	5.00	4.60±0.20	4.3
Amobarbital	0.20	0.19±0.008	4.2
	5.00	4.85±0.16	3.3
Pentobarbital	0.20	0.20±0.005	2.5
	5.00	5.10±0.17	3.3
Secobarbital	0.20	0.19±0.004	2.1
	5.00	4.74±0.15	3.2
Thiopental	0.20	0.17±0.007	4.1
	5.00	4.12±0.10	2.4
Thiamylal	0.20	0.17±0.003	1.8
	5.00	4.08±0.12	2.9
Phenobarbital	0.20	0.21±0.006	2.9
	5.00	5.06+0.18	3.6

3.5. Clinical cases

The proposed method was applied to 9 clinical cases. Barbital, amobarbital, pentobarbital, secobarbital, thiopental, thiamylal or phenobarbital were detected in the clinical samples. The concentration of barbiturates in urine is shown in Table 4. The TIC was very clear in all clinical samples. The TIC and

Table 2
Characteristics of the quantitation methods

Drugs	Range of linearity[a] (μg/ml)	Correlation coefficient (r^2)	Limit of detection (μg/ml)
Barbital[b]	0.20–10	0.999	0.20
	0.05–1.0	0.998	0.05
Amobarbital	0.20–10	0.999	0.10
	0.02–1.0	0.999	0.02
Pentobarbital	0.20–10	0.999	0.10
	0.02–1.0	0.998	0.02
Secobarbital	0.20–10	0.999	0.10
	0.02–1.0	0.998	0.02
Thiopental	0.20–10	0.999	0.20
	0.05–1.0	0.996	0.02
Thiamylal	0.20–10	0.999	0.20
	0.05–1.0	0.996	0.02
Phenobarbital	0.20–10	0.999	0.20
	0.02–1.0	0.999	0.02

[a] Six data points in duplicate.
[b] Upper row: scan mode, lower row: SIM mode.

Table 4
Results of clinical cases

Entry	Age	Sex[a]	Barbiturate concentration[b] (μg/ml)			
			Mean (C.V., %)			
1	48	M	secobarbital	0.9 (6.3)	thiamylal	2.8 (3.7)
2	53	F	secobarbital	21 (2.9)		
3	25	F	phenobarbital	14 (3.7)		
4	33	M	barbital	8.6 (5.3)		
5	38	M	amobarbital	2.5 (4.8)		
6	23	M	pentobarbital	7.3 (5.4)	thiopental	8.3 (3.7)
7	51	M	pentobarbital	6.3 (4.3)	thiopental	5.2 (3.9)
8	24	F	pentobarbital	0.2 (7.1)	thiopental	0.4 (6.1)
9	31	M	secobarbital	0.2 (6.9)	thiamylal	2.4 (4.4)

[a] M=male, F=female.
[b] The sample was analyzed twice per patient.

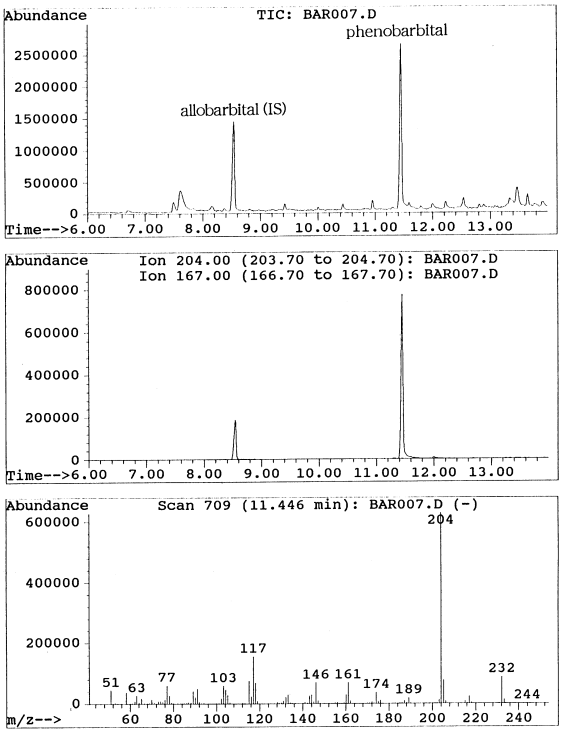

Fig. 3. Total ion chromatogram, extracted-ion chromatogram and EI-mass spectrum of phenobarbital of the clinical case (entry No. 3). Top: Total ion chromatogram of the sample. Middle: Extracted-ion chromatogram of the clinical sample. Bottom: EI–mass spectrum of the peak at 11.446 min for the total ion chromatogram of the sample.

mass spectrum in the urine sample of the patient (No. 3) are shown in Fig. 3.

Both thiamylal and secobarbital were identified in the urine samples (No. 1 and 9), when thiamylal was administered as the parent barbiturate, because secobarbital was one of the main metabolites of thiamylal. Similar situations were observed in the urine (No. 6, 7 and 8) when thiopental was administered as the parent barbiturate. The biotransformation of thiobarbiturates has been investigated well in humans, and it is known that 10–25% of a dose is excreted in the urine as a desulfurated product such as secobarbital or phenobarbital and a side-chain oxidated product [18,19].

4. Conclusion

The PrepStation-GC–MS system has fully automated the simultaneous analysis of barbiturates in human urine. Clean extracts were obtained from human urine 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 has demonstrated that it can be a reliable, accurate, precise and cost-effective alternative to manual techniques.

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