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Determination of amobarbital and phenobarbital in serum by gas chromatography–mass spectrometry with addition of formic acid to the solvent

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ABSTRACT

A rapid and accurate method for quantification of amobarbital and phenobarbital was developed using gas chromatography–mass spectrometry (GC–MS) without derivatization. Though the compounds measured without derivatization showed low sensitivity because of adsorption, addition of 3% formic acid to the solvent improved the sensitivity for the analytes. Taking account of matrix effect, solid-phase and liquid–liquid extraction from serum were examined. The correlation coefficients of the calibration curves were 0.9995 or better, and the accuracy and precision of intraday and interday assays were in line with Food and Drug Administration (FDA) criteria.

1. Introduction

Barbiturates have been widely used as sedative hypnotics and some poisoning cases have been reported [1,2]. Analytical methods for barbiturates include gas chromatography (GC)-flame ionization detection [3–5], GC-nitrogen phosphorus detection [6], GC–mass spectrometry (MS) [7–12], liquid chromatography-ultraviolet (LC-UV) detection [13,14], LC–MS [15,16] and capillary electrophoresis-UV [17–19]. GC–MS has merits due to high resolution and precise retention times with sharp peaks; however, the sensitivity of GC–MS for barbiturates is compromised by adsorption at its NH group. To overcome this problem, derivatization prior to injection is widely used, but this procedure is time-consuming [7,10,12]. On-column methylation is a rapid and sensitive method [6], but we have encountered incomplete reaction due to obstruction by the extracted matrix. In addition, phenobarbital cannot be distinguished from mephobarbital after methylation.

Adsorption of barbiturates is reduced by addition of formic acid to the carrier gas for GC using a packed column [3–5]. Current capillary columns give less adsorption than a packed column, but addition of formic acid to the carrier gas has a negative influence on the capillary column and MS. We have reported that theophylline is adsorbed to the glass wool packed into the inlet liner, and that this effect is reduced by acetic acid [20]; therefore, we expected that barbiturates would also be mainly adsorbed to the glass wool. A preliminary study in our laboratory suggested that addition of a low concentration of carboxylic acid to the solvent (ethyl acetate) reduced adsorption and had negligible effects on the column and MS. Accordingly, we have examined amobarbital and phenobarbital (as a representative of barbiturate) analyses in serum with addition of a carboxylic acid to the solvent.

First, the carboxylic acid (formic acid or acetic acid) and the inlet temperature (250 °C or 300 °C) were optimized and the appropriate carboxylic acid concentration was determined, using amobarbital and phenobarbital standards. Under the optimized conditions, calibration curves were generated for quantification of amobarbital and phenobarbital extracted from serum, with pentobarbital used as an internal standard (IS). The accuracy and precision of this method were examined using criteria in the Food and Drug Administration (FDA) guidance [21]. The matrix extracted from serum might improve the sensitivity of the assay for barbiturates, since it occupies active sites on the glass wool, and therefore we compared solid-phase extraction (SPE) and liquid–liquid extraction (LLE), since these methods produce different matrices.

2. Experimental

2.1. Chemicals

Phenobarbital was purchased from Sigma–Aldrich (St. Louis, MO, USA), amobarbital was obtained from Nippon Shinyaku (Kyoto, Japan), and pentobarbital sodium was purchased from Tokyo

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Kasei Kogyo (Tokyo, Japan). Formic acid, acetic acid, ethyl acetate, methanol, sodium acetate trihydrate and diethyl ether were purchased from Wako Pure Chemical Industries (Osaka, Japan). Normal human serum was obtained from Chemicon International (Temecula, CA, USA). Ultra-pure water was prepared using a Milli-Q purification system (Millipore, Tokyo, Japan). Formic acid and acetic acid were LC–MS grade, and all other chemicals were analytical reagent grade. Bond Elut Certify II (200 mg, 3 cm^3) extraction cartridges were purchased from Varian (Harbor City, CA, USA).

Stock standard solutions of phenobarbital and amobarbital were prepared in methanol at concentrations of 1 mg/mL, and pentobarbital (sodium) was prepared in water at 1 mg/mL. Working standard solutions were prepared by dilution of these stock solutions to adequate concentrations. These standard solutions were stored at 4 °C.

2.2. Instrumentation and GC–MS conditions

GC–MS analysis was conducted using an HP 5973 mass-selective detector interfaced to an HP 6890 gas chromatograph with an HP 7673C autosampler (Agilent Technologies, USA). Chromatographic separation was achieved on a J&W DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), using high-purity helium (99.9999%) as carrier gas at a flow rate of 1.0 mL/min. The column oven temperature was programmed from 60 °C (1-min hold) to 180 °C at 10 °C/min, and from 180 °C to 300 °C (3-min hold) at 20 °C/min. A deactivated taper-liner with glass wool (Agilent, USA) was used as the inlet, and was kept at 300 °C in splitless mode for 1 min. The injection volume was 1.0 μL. Ionization was achieved in electron impact mode at 70 eV, and the transfer line, ion source and quadrupole temperature were 280 °C, 230 °C and 150 °C, respectively. MS was performed using selected ion monitoring (SIM) and scan mode, with SIM set to *m*/*z* 156.0, 141.0 for 5–16 min and *m*/*z* 232.0, 204.0, 117.0 for 16–22 min, and scan set to *m*/*z* 50.0–500.0. The solvent delay was 5 min.

2.3. Standard sample preparation

Volumes of 10 μL of amobarbital and 20 μL of phenobarbital working solution (100 μg/mL) were transferred to glass test tubes and dried under a stream of nitrogen at 40 °C. The dried residues were redissolved in 100 μL of 0, 1, 2, 3 and 4% formic acid or acetic acid in ethyl acetate, respectively. A 1-μL aliquot of the solution was injected into the GC–MS at an inlet temperature of 300 °C and the SIM ions were monitored. The 0–4% samples were measured in sequence and it was repeated four times. Similarly, the standard samples dissolved in 0–4% formic acid–ethyl acetate were measured by GC–MS at 250 °C. Between samples, 1 μL of 2% formic acid or acetic acid–ethyl acetate blank solution was injected into the GC–MS to prevent carry-over of amobarbital and phenobarbital. After one analysis sequence (0–4% × 4), the inlet liner with glass wool was exchanged for a new one.

2.4. Serum sample extraction procedure

Solid-phase extraction (SPE) was performed according to the procedure described by Pocci et al. [11], with a few modifications. Appropriate amounts of amobarbital and phenobarbital working solutions (1, 10 and 100 μg/mL) were transferred to a glass test tube and dried under a stream of nitrogen at 40 °C. Volumes of

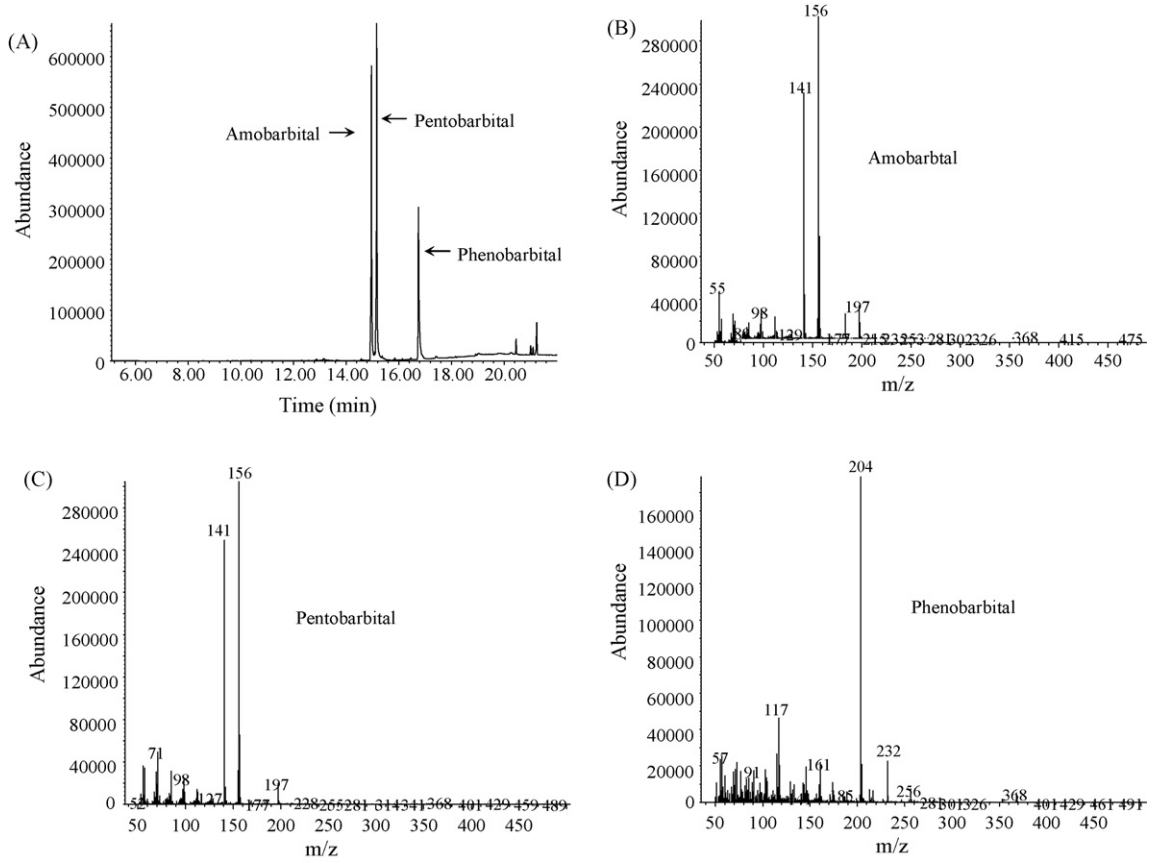

Fig. 1. SIM chromatogram of amobarbital, pentobarbital and phenobarbital (0.5 μg/0.5 mL) (A), and the mass spectra (scan) of amobarbital (B), pentobarbital (C) and phenobarbital (D). The analytes were extracted from serum using SPE and dissolved in 3% formic acid–ethyl acetate.

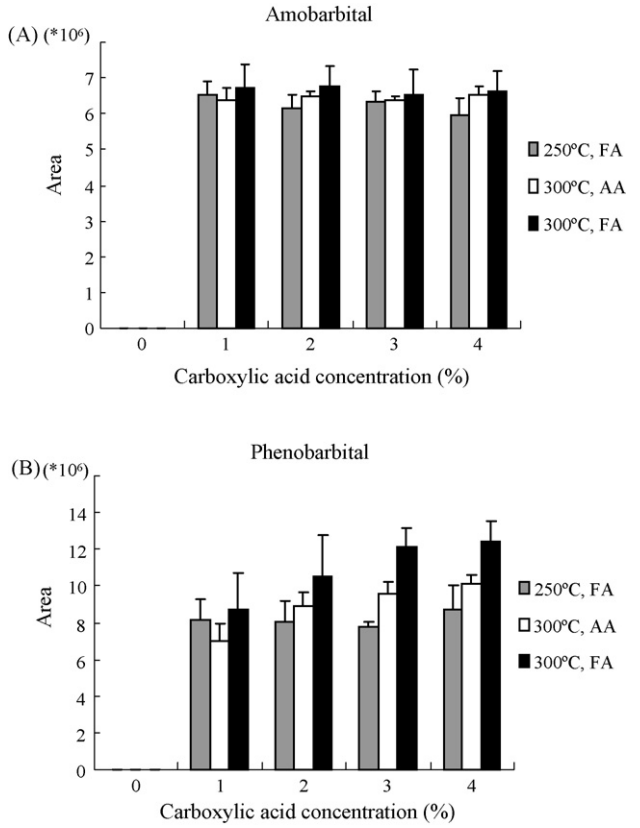

Fig. 2. The peak area (mean ± SD; $n = 4$) of 10 ng (on-column) amobarbital (A) and 20 ng (on-column) phenobarbital (B) dissolved in 0, 1, 2, 3 and 4% formic acid (FA) or acetic acid (AA)–ethyl acetate at an inlet temperature of 300 °C or 250 °C.

40 μL of pentobarbital solution (10 μg/mL: IS) and 0.5 mL of blank serum were added to the tube and vortex mixed, followed by addition of 2 mL of 0.1 M sodium acetate–acetic acid buffer (pH 7.0). The mixed sample was loaded into a Bond Elut Certify II cartridge pre-conditioned with 2 mL of methanol followed by 2 mL of 0.1 M sodium acetate-acetic acid buffer (pH 7.0). The cartridge was washed with 3 mL of 0.1 M sodium acetate–acetic acid buffer (pH 7.0) and dried under full vacuum for 5 min with Vac-Elut™ (Varian, USA). The cartridge was further washed with 2 mL of hexane–ethyl acetate (95:5, v/v). Analytes were eluted with 2 mL of hexane–ethyl acetate (75:25, v/v) and the eluate was evaporated to dryness under a stream of nitrogen at 40 °C. The dried residue was reconstituted in 100 μL of 3% formic acid–ethyl acetate and 1 μL of the sample was injected into the GC–MS.

Liquid–liquid extraction (LLE) was performed using the procedure described by Terada et al. [6], with a few modifications. Mixture of barbiturates and serum were prepared as described above, and 2 mL of 0.2 M sodium acetate–acetic acid buffer (pH 6.0) and 6 mL of ethyl acetate–diethyl ether (1:1, v/v) were added. The contents were shaken for 10 min and centrifuged for 10 min (1000 × *g*, 4 °C). The organic layer was transferred to a glass test tube and evaporated to dryness under a stream of nitrogen at 40 °C. The dried residue was reconstituted in 100 μL of 3% formic acid–ethyl acetate and 1 μL of the sample was injected into the GC–MS.

A serum sample diluted to 2 or 10 folds was extracted using SPE or LLE. Twofolds dilution was performed by adding 0.25 mL of water to 0.25 mL of serum spiked with 1 μg of amobarbital, 4 μg of phenobarbital and 0.4 μg of pentobarbital (IS), and 10 folds dilution was performed by adding 0.45 mL of water to 0.05 mL of serum spiked with 1 μg of amobarbital, 4 μg of phenobarbital and 0.4 μg of pentobarbital (IS). The following extraction procedures were the same as described above.

2.5. Matrix sample preparation

After a blank serum (0.5 mL) was extracted using SPE or LLE described above (two samples for each), 10 μL of amobarbital and 20 μL of phenobarbital working solution (100 μg/mL) were added to the extracted matrix, and dried under a stream of nitrogen at 40 °C. The residues were reconstituted in 100 μL of 0 or 3% formic acid–ethyl acetate, respectively. No matrix sample with amobarbital and phenobarbital of same amount was dissolved in 3% formic acid–ethyl acetate, and the standard sample and four matrix samples (0 and 3% formic acid × SPE and LLE) were measured in sequence at an inlet temperature of 300 °C. Between samples, 1 μL of 2% formic acid–ethyl acetate blank solution was injected into the GC–MS to prevent carry-over.

3. Results

3.1. Mass spectrum and retention time

To confirm mass spectrum and retention time, SIM chromatogram of amobarbital, pentobarbital and phenobarbital (0.5 μg/0.5 mL) and respective mass spectra (scan) were shown in

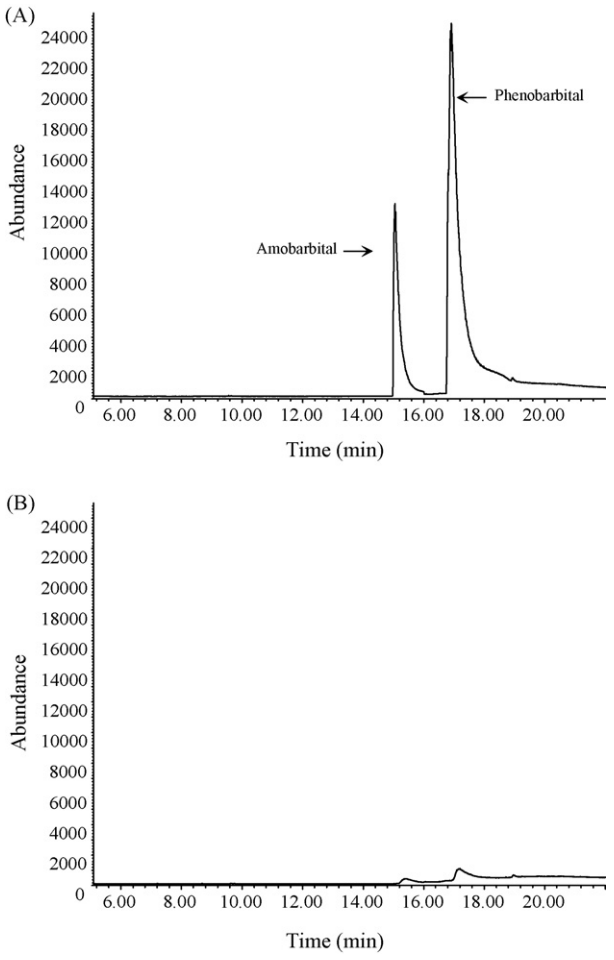

Fig. 3. SIM chromatograms of 2% formic acid–ethyl acetate blank samples measured following standard samples (containing 1 μg of amobarbital and 2 μg of phenobarbital) dissolved in 0% formic acid–ethyl acetate (A) or 3% formic acid–ethyl acetate (B) at an inlet temperature of 300 °C.

Fig. 1. The analytes were extracted from serum using SPE and dissolved in 3% formic acid–ethyl acetate. On the basis of these results, intense ions were selected for SIM (described above), and the target ions for quantification were determined at m/z 156 for amobarbital and pentobarbital, and at m/z 204 for phenobarbital. The retention times of amobarbital, pentobarbital and phenobarbital were 14.97, 15.17 and 16.78 min, respectively.

3.2. Optimization of the carboxylic acid and inlet temperature

The response to amobarbital and phenobarbital was examined under three conditions (concentrations, formic acid or acetic acid, inlet temperatures). The response was represented as mean ± standard deviation (SD) of the peak area in Fig. 2 ($n = 4$). Neither amobarbital nor phenobarbital was detected in 0% carboxylic acid. Amobarbital was little affected in these conditions excluding 0% carboxylic acid (Fig. 2A). On the other hand, the response to phenobarbital increased with an increase in the carboxylic acid concentration and the inlet temperature, but there was little difference between 3 and 4% (Fig. 2B). The response to phenobarbital on the condition of 200 °C-formic acid was smaller than 250 °C-formic acid (data was not shown). From these results, subsequent experiments were conducted with 3% formic acid at an inlet temperature of 300 °C.

3.3. Carry-over test

Between all samples measured, 2% formic acid (or acetic acid)–ethyl acetate blank solution was injected to prevent carry-over. A carry-over was confirmed by measuring 2% formic

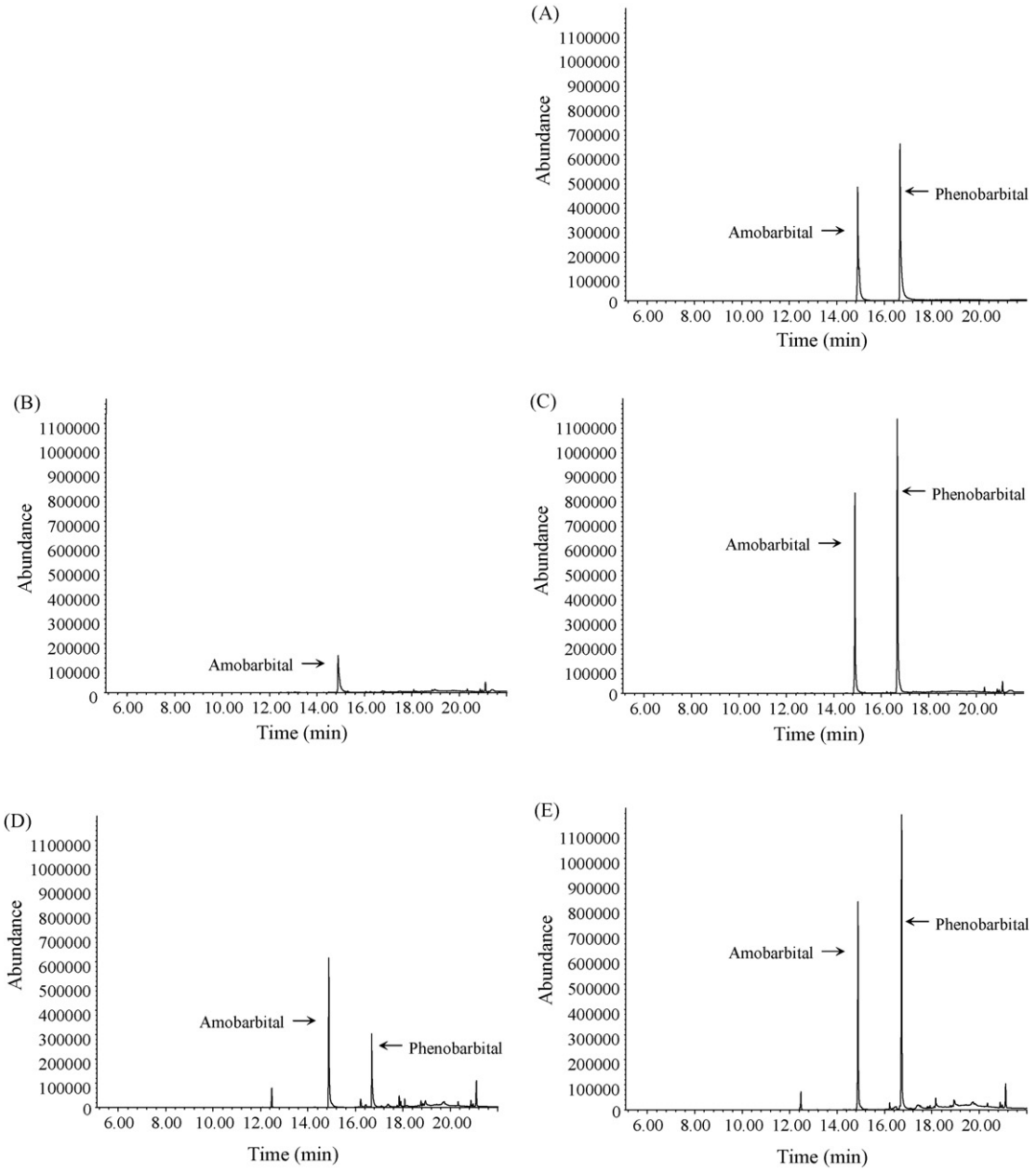

Fig. 4. SIM chromatograms of the standard sample dissolved in 3% formic acid–ethyl acetate (A), the serum sample extracted using SPE dissolved in 0% formic acid–ethyl acetate (B) or 3% formic acid–ethyl acetate (C), and the serum sample extracted using LLE dissolved in 0% formic acid–ethyl acetate (D) or 3% formic acid–ethyl acetate (E). All of these samples contained 1 μg of amobarbital and 2 μg of phenobarbital.

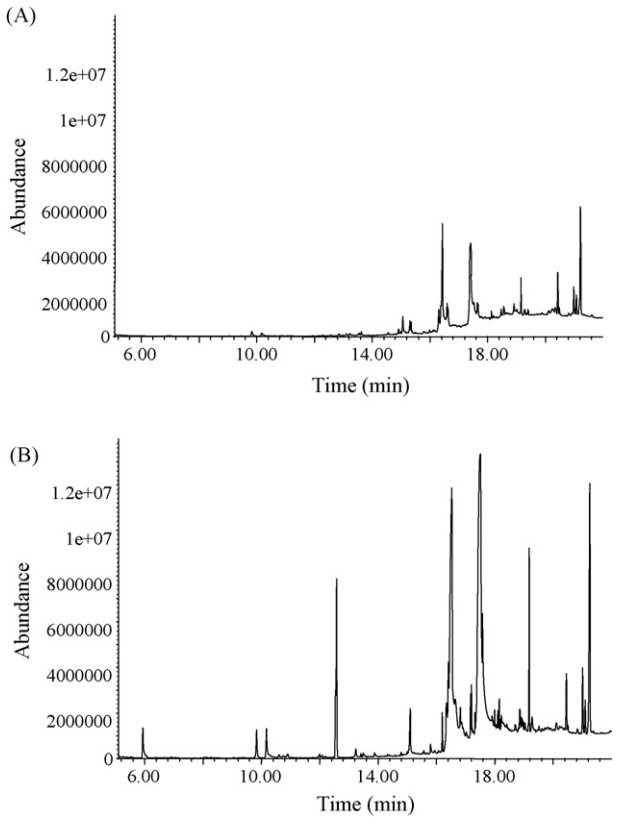

Fig. 5. Total ion chromatograms of blank serum extracts dissolved in 3% formic acid–ethyl acetate extracted using SPE (A) and LLE (B).

acid–ethyl acetate blank sample following standard sample (containing 1 μg of amobarbital and 2 μg of phenobarbital) dissolved in 0 or 3% formic acid–ethyl acetate at an inlet temperature of 300 °C (Fig. 3). Amobarbital and phenobarbital were detected in the blank sample following standard sample dissolved in 0% formic acid (Fig. 3A), while those were negligible following standard in 3% formic acid (Fig. 3B).

3.4. Matrix effect

To examine matrix effect, the standard sample was compared with the serum samples extracted using SPE and LLE. All of these samples contained 1 μg of amobarbital and 2 μg of phenobarbital. The standard sample was dissolved in 3% formic acid–ethyl acetate (it was not detected in 0% formic acid) and the extracted samples were dissolved in 0 or 3% formic acid–ethyl acetate, respectively (Fig. 4). In the samples dissolved in 3% formic acid, the extracts were higher response than the standard (Fig. 4A, C and E). In 0% formic acid, the response to LLE was higher than SPE (Fig. 4B and D), but there was little difference in 3% formic acid (Fig. 4C and E). The background noise for the blank samples extracted using SPE and LLE was shown in Fig. 5.

3.5. Calibration curves for amobarbital and phenobarbital extracted from serum

Analytes were extracted from serum samples spiked with amobarbital (0.1, 0.2, 0.4, 0.8, 1.4 and 2.0 μg/mL), phenobarbital (0.4, 0.8, 1.6, 3.0, 5.0 and 8.0 μg/mL) and pentobarbital (IS) using SPE and LLE. Calibration curves (concentration–peak area) were generated using the serum samples ($n = 6$). Regression equations, correlation coefficients (R^2), quantitation ranges, limits of detection (LOD: signal/noise ≥3), and limits of quantification (LOQ: signal/noise ≥10) are summarized in Table 1.

3.6. Accuracy, precision and recovery of amobarbital and phenobarbital extracted from serum

Accuracy (%bias) and precision (%CV: coefficient of variation) were calculated as follows [22]:

$$\%\text{bias} = \left[\frac{\text{measured value} - \text{true value}}{\text{true value}}\right] \times 100$$

$$\%\text{CV} = \left(\frac{\text{SD}}{\text{mean}}\right) \times 100$$

Intraday accuracy and precision were determined by five replicate analyses of quality control samples at low, medium, and high concentrations on the calibration curves. Interday accuracy and

Table 1
Equation, R^2, range, LOD and LOQ of amobarbital and phenobarbital extracted from serum by SPE and LLE

Compound	Extraction method	Equation[a] ($n = 6$)	R^2	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Amobarbital	SPE	$y = 1.1219x - 0.0233$	0.9998	0.1–2.0	0.04	0.1
	LLE	$y = 1.1060x - 0.0176$	0.9996	0.1–2.0	0.04	0.1
Phenobarbital	SPE	$y = 0.8652x - 0.0719$	0.9995	0.4–8.0	0.1	0.4
	LLE	$y = 0.9186x - 0.0592$	0.9996	0.4–8.0	0.1	0.4

[a] Equal weighting.
y: amobarbital or phenobarbital peak area/pentobarbital peak area.
x: concentration (μg/mL).

Table 2
Mean (μg/mL), accuracy (%bias), precision (%CV) and recovery (%) of amobarbital and phenobarbital at various concentrations (Conc.) using SPE

Compound	Conc. (μg/mL)	Intraday ($n = 5$)				Interday ($n = 15$)		
		Mean	Accuracy	Precision	Recovery	Mean	Accuracy	Precision
Amobarbital	0.1	0.099	−0.99	0.65	91.9	0.096	−3.70	2.57
	1.0	1.011	1.05	1.34	89.3	1.017	1.66	1.89
	2.0	1.934	−3.30	2.96	94.6	1.948	−2.62	3.21
Phenobarbital	0.4	0.421	5.27	1.17	89.6	0.419	4.73	3.43
	4.0	3.894	−2.65	1.45	92.4	3.708	−7.30	4.52
	8.0	7.391	−7.62	3.12	88.0	7.205	−9.93	3.88

Table 3
Mean (μg/mL), accuracy (%bias), precision (%CV) and recovery (%) of amobarbital and phenobarbital at various concentrations (Conc.) using LLE

Compound	Conc. (μg/mL)	Intraday (n = 5)				Interday (n = 15)		
		Mean	Accuracy	Precision	Recovery	Mean	Accuracy	Precision
Amobarbital	0.1	0.096	−3.78	2.84	92.1	0.097	−3.36	2.02
	1.0	1.068	6.85	1.18	95.3	1.078	7.82	1.23
	2.0	2.113	5.66	1.25	90.7	2.114	5.70	0.90
Phenobarbital	0.4	0.405	1.26	3.92	89.3	0.424	6.08	4.60
	4.0	4.101	2.52	2.88	97.8	4.087	2.17	3.12
	8.0	8.404	5.04	1.86	90.2	8.322	4.03	1.95

precision (n = 15) were obtained by three repetitions of the intraday assay. These experiments were performed with reference to FDA guidance [21]. The recovery was obtained by comparison of the peak area of analyte extracted from the spiked serum sample with the peak area of the same amount of standard added to the matrix extracted from blank serum. The matrix effect was counterbalanced by adding the standard to the extracted matrix. The mean concentration, accuracy, precision and recovery data for amobarbital and phenobarbital are summarized for SPE and LLE in Tables 2 and 3, respectively. The samples diluted to 2 and 10 folds were examined about the mean concentration, accuracy and precision (n = 5) for amobarbital and phenobarbital, which are summarized in Table 4.

4. Discussion

Analysis of amobarbital and phenobarbital was optimized by addition of 3% formic acid at an inlet temperature of 300 °C, without the need for derivatization. The difference between the responses with formic acid or acetic acid might be within the error caused by pre-deactivation on glass wool in the inlet liner (Fig. 2), but we chose formic acid for subsequent experiments since it showed a slightly larger response than with acetic acid and has a lower boiling point. In the presence of formic acid, the response to phenobarbital was much larger at an inlet temperature of 300 °C than at 250 °C (Fig. 2B). The optimum concentration of formic acid was determined to be 3%, similar to that for theophylline analysis [20]. More than 4% of carboxylic acid was not chosen since there was little difference between 3 and 4%.

We estimated that serum extract improves sensitivity for barbiturates and a different extraction matrix might alter the response. Since our purpose was to examine the effect of formic acid, established SPE and LLE procedures were used. These procedures have relatively low and high background noise, respectively (Fig. 5). The SPE described by Pocci et al. has reduced background noise because the extraction cartridge is washed by an organic solvent. The matrix effect clearly improved the sensitivity for amobarbital and phenobarbital (Fig. 4A, C and E). In the absence of formic acid, the different extraction method caused a different matrix effect (Fig. 4B and D), but the addition of 3% formic acid reduced the difference in the extraction methods (Fig. 4C and E). Furthermore, the serum extract with 3% formic acid was more sensitive than that without formic acid (Fig. 4B–E).

Calibration curves were constructed for the analytes and the accuracy and precision of the method were determined. Correlation coefficients were ≥0.9995 for all calibration curves, the LOD was 0.04 and 0.1 μg/mL for amobarbital and phenobarbital, respectively, and the LOQ was 0.1 and 0.4 μg/mL, respectively, for both SPE and LLE (Table 1). Since the therapeutic ranges are 1–5 μg/mL for amobarbital and 10–30 μg/mL for phenobarbital [23], these values indicate that this method is sufficiently sensitive to quantify amobarbital and phenobarbital. However, the range of the calibration curves is too narrow to be useful in a sample of high concentration. Therefore, the diluted samples were also examined. The accuracy and precision of amobarbital were within ±7.82 and 3.77%, respectively, and those of phenobarbital were within ±9.93 and 4.60%, respectively (Tables 2–4). The higher values for phenobarbital would be due to the physical and chemical properties of pentobarbital (IS) being more similar to amobarbital than phenobarbital. Samples obtained with either extraction method showed similar intraday and interday values. FDA guidance criteria recommend that accuracy and precision should both be within 15% (except at LLOQ < 20%) [21]. The data shown in Tables 2–4 are well within these criteria, indicating that addition of formic acid is effective for accurate analysis of amobarbital and phenobarbital.

Table 4
Mean (μg/mL), accuracy (%bias) and precision (%CV) of amobarbital and phenobarbital diluted to 2 and 10 folds using SPE and LLE (n = 5)

Compound	Conc. (μg/mL)	Extraction method	Mean	Accuracy	Precision
Amobarbital	4.0	SPE	3.813	−4.68	0.95
	4.0	LLE	4.211	5.26	2.72
	20.0	SPE	18.487	−7.57	3.77
	20.0	LLE	19.342	−3.29	0.56
Phenobarbital	16.0	SPE	16.025	0.16	2.67
	16.0	LLE	16.297	1.86	2.05
	80.0	SPE	79.781	−0.27	3.74
	80.0	LLE	78.076	−2.40	2.22

5. Conclusion

We demonstrated that addition of 3% formic acid to the solvent improved the sensitivity of amobarbital and phenobarbital detection by GC–MS. The method is rapid and accurate for serum samples using different extraction procedures, and is likely to be applicable in clinical and forensic practice.

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