Technical Note

Stability of Barbiturates in Fixed Tissues and Formalin Solutions

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Abstract

This study reports the results of the retention and quantitation of barbiturates in fresh tissue, in liver tissue fixed in formalin, and in the formalin solutions in which the same tissues were fixed for six months. Biological materials came from four cases of death due to phenobarbital and two due to butalbital. Results showed the presence of barbiturates in all studied materials. Mean recovery rates of phenobarbital in fixed liver were 57.11% and 30.84% in formalin from liver; and those of butalbital in fixed liver were 21.66% and 66.56% in formalin from liver. Total sums of recovery rates in formalin solutions and fixed tissues for phenobarbital (87.95%) and butalbital (88.22%) were comparable with those of the extraction efficiency of the method (90.2%) and indicate that these barbiturates have good stability even in biological specimens subjected to chemical fixation. This fact may be useful in qualitative evaluation of cases.

Introduction

The use of alternative matrices to plasma and urine in forensic toxicology is a field of increasing interest. In deceased persons, the range of specimens for drug analysis may be extended to solid tissues, usually stored in formalin solutions. This may be the case when, for some reason, fluids such blood and urine were not collected at the time of autopsy, and the toxicological interest of the case arises after burial and the only suitable materials from the body were formalin-fixed tissues collected for histopathological purposes. Some studies have analyzed drugs in formalin-fixed tissues (1–6), but to our knowledge, until now, only two studies, with differing results, concern the detection and quantitation of barbiturates in formalin-fixed tissues and formalin solutions (1,7). The stability of phenobarbital was assessed by Tsoukali-Papadoloulou (7) in formalin solutions in which brain samples had been preserved for a period of 5 months (10 µg/mL) and was indicated as only moderate (20%) by Winek et al. (1) in formalin

solutions and in fixed tissues after 28 days. The postmortem stability of barbiturates in unfixed biological tissues (liver) was also studied by Levine et al. (8) and in vitro in formaldehyde solutions by Gannett et al. (9). As barbiturates are widely used as sedatives, hypnotics, and antiepileptics, their use may result in accidental, suicidal, or homicidal death (10,11) and be of interest for the forensic toxicology laboratory. The distribuition of barbiturates in the bodies of people who died of barbiturate poisoning does not differ significantly from its distribution after therapeutic ingestion. Liver shows the highest concentration, generally followed by kidney, spleen, and brain (10,11). Barbiturates in biological materials are detected by means of classical ultraviolet-spectrophotometry, gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and high-performance liquid chromatography (12,13).

This study reports the results of the retention and quantitation of barbiturates in fresh liver tissue, liver tissue fixed in formalin, and the formalin solutions in which the same tissues had been fixed.

Materials

Toxicological analyses were performed on samples of fresh liver, formalin-fixed liver, and formalin solutions (pH 7, 10% buffered with 36mM sodium dihydrogen phosphate and 28mM sodium monohydrogen phosphate) in which samples were preserved. These biological materials came from six cases of death due to barbiturates (four phenobarbital, two butalbital). For each case, a sample of liver was taken at the time of autopsy, subdivided into two portions, the first (5 g) being analyzed immediately and the second (5 g) put in formalin. Tissue samples were preserved in formalin solutions for 6 months before analysis. Tissue weights, before and after formalin fixation, were checked and were not appreciably modified by preservation (5).

Analytical-grade chemicals and reagents were Isolute Certify Extraction columns (International Sorbent Technology, Hengoed, Mid Glamorgan, U.K.), sodium hydroxide (Merck, Darmstadt, Germany), sodium dihydrogen phosphate (JT Baker

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Chemical, BV, Beventer, Holland), sodium monohydrogen phosphate (J.T. Baker Chemical), methanol (Carlo Erba, Milan, Italy), hydrochloric acid (Carlo Erba), ammonium hydroxide (Sigma, St. Louis, MO), ethyl acetate (Baker, Phillipsburg, NJ), hexane (Baker) trimethylanilinium hydroxide (Methelute) (Pierce Biotechnology, Rockford, IL), 5-sulfosalicylic acid-2-hydrate (Riedel de Haen, Seelze, Germany), phenobarbital (Sigma),

butalbital (Sigma), and allobarbital (Sigma).

Equipment included a homogenizer (Ultra Tu rrax, Ica Labor Technik, Milan, Italy), sonicator (Bramson 1210, Bramson, Dambury, CO), centrifuge (ALC 4218, ALC, Milan, Italy), vortex mixer (Falc, Bergamo, Italy), Analytichem Vac Elut (Varian SPS 24, Varian, Bergen op Zoom, the Netherlands), temperature bath (Carlo Erba Thermostatic bath, Carlo Erba, Milan, Italy), and GC–MS (Fisons Instruments, MD 800-GC 8000, Milan, Italy).
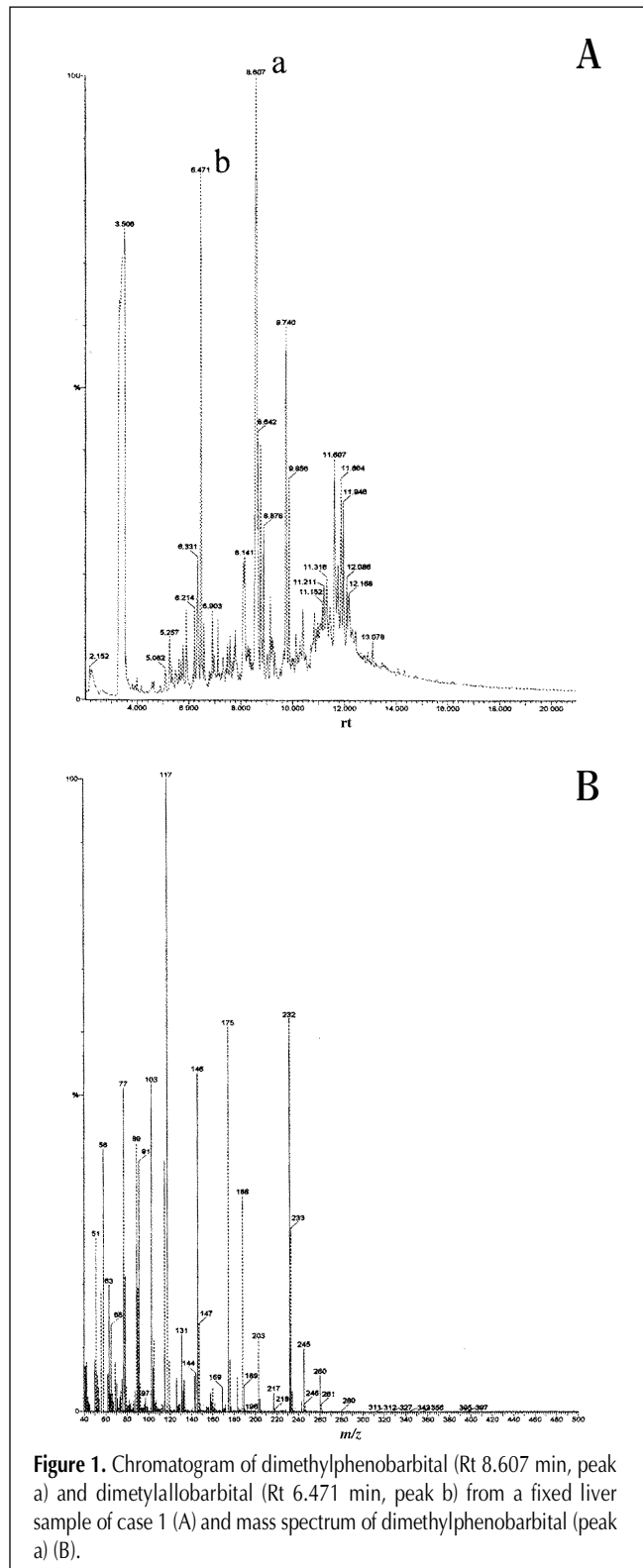
Methods

Preparation of samples

Tissues. Five grams of both fresh and formalin-fixed tissues f rom autopsy sources were separately crushed and homogenized in distilled water (1:1) and sonicated for 7–8 h. One milliliter of 0.1M HCl was added to homogenized solutions, followed by sulfosalicylic acid (about 1 g in total) for deproteinization. The solutions were centrifuged and the pH of the su pe rnatant adjusted to 5 with ammonium sulfate and NaOH (2M). One milliliter of allobarbital solution (1 mg/L in water) as internal standard and 2 mL of 0.1M phosphate buffer (pH 5) were added, and the solutions were centrifuged before the following phase of extraction.

Formalin solutions. The total amounts of solutions were evaporated to dryness at room temperature, and 5 mL of distilled water were added to the residue. One milliliter of allobarbital solution (1 mg/L in water) as internal standard and 2 mL of 0.1M phosphate buffer (pH 5) were added, and the solutions were centrifuged before the following phase of extraction.

Solid-phase extraction

Isolute Certify Extraction columns were used.

P reparation of columns. Columns were conditioned sequentially with 2 mL of methanol, 2 mL deionized water, and 2 mL of 0.1M phosphate buffer (pH 6).

Specimen application. Samples were slowly drawn through the columns under low vacuum at 1–2 mL/min.

Column rinsing. To elute interf e rences, columns were rinsed sequentially with 2 mL of 0.1M phosphate buffer (pH 6), and dried for 5 min under full vacuum. One milliliter 1M acetic acid was added and dried for 10 min under full vacuum, then 1 mL hexane was added, and vacuum was applied.

Elution of barbiturates. Analytes were eluted with 1 mL of a hexane/ethyl acetate solution (3:1); eluates were transferred to 3-mL silanized gradual tubes, evaporated to dryness at room temperatu re under a slow stream of nitrogen, and then re c o nstituted with 100 µL of ethyl acetate.

Derivatization

Extracts were methylated directly on columns with Methelute obtaining a "flash methylation" (dimethyl-derivates of barbiturates).

GC–MS analysis

The following conditions were applied: a wall-coated open-tubular capillary column (fused silica 30 m × 0.32 mm) was

Figure 1. Chromatogram of dimethylphenobarbital (Rt 8.607 min, peak a) and dimetylallobarbital (Rt 6.471 min, peak b) from a fixed liver sample of case 1 (A) and mass spectrum of dimethylphenobarbital (peak a) (B).

used; carrier gas was helium at a low flow rate of 1.5 mL/min. The temperature program was started at 80°C and increased to 310°C at 20°C/min. The injection volume was 1 μL (splitless mode). Electron impact (70 eV) was used for ionization. Mass spectra were recorded in the range m/z 70–500. Figure 1 shows the chromatogram and the mass spectrum of dimethylphenobarbital from a formalin-fixed liver sample.

Results

Phenobarbital and butalbital were identified by comparing their retention times with that of the internal standard allobarbital and the mass spectra of phenobarbital and butalbital standards, all as dimethyl derivates (dimethylphenobarbital m/z 117, 146, 175, and 232; dimethylbutalbital m/z 169, 195, 196, and 209; dimethylallobarbital, I.S., m/z 80, 110, 138, and 195). Quantitation was carried out by monitoring the abundances of m/z 232 ions (for dimethylphenobarbital), m/z 196 ions (dimethylbutalbital), and m/z 138 ions (dimethylallobarbital, I.S.). To verify the linearity of the detector and to calibrate the method, five calibrators were tested: 1.0, 5.0, 10, 20, and 30 mg/L or mg/kg (phenobarbital and butalbital in formalin solution and liver tissues previously tested to be negative). Linear regression analysis of the calibration data showed a correlation coefficient of 0.984. The limit of phenobarbital and butalbital detection was set at 0.05 mg/L, with a signal-to-noise ratio of 10. The variation coefficients between runs and days, retesting 5 calibrators (0.5 mg/L or mg/kg) 10 times each day for 10 days, were 4.1 (intra-assay for formalin solutions), 5.7 (interassay for formalin solutions), 5.4 (intra-assay for liver), and 6.1 (interassay for liver), respectively. The extraction efficiency of the method was assayed on four liver samples, previously tested to be negative, spiked with suitable amounts of internal standard and phenobarbital and butalbital to give a final concentration of 1 mg/kg. Recovery was 90.2%. Quantitative results for formalin-fixed liver samples and the formalin solutions in which they were preserved are listed in Table I.

The quantitative values of phenobarbital and butalbital obtained in fixed tissues and from the same tissues at the time of autopsy were quite different because detectable quantities were redistributed from the tissues into the formalin solution. In all cases, the amounts of butalbital in the formalin solutions in which liver was preserved were found to be higher than those recovered in the same fixed tissues. For phenobarbital, the distribution gave different results: in three cases, the prevalence was in fixed tissues, and in one case, it was in formalin solutions. Table II lists the mean amounts of phenobarbital and butalbital recovered from liver (both fixed tissues and formalin solutions in which the tissues were preserved), compared with the mean amounts from the same samples at the time of autopsy. For butalbital, the best recovery rates were found in formalin solutions (66.56%); for phenobarbital in fixed liver the value was 57.11%. The total sums of recovery rates in formalin solutions and fixed tissues for phenobarbital (87.95%) and butalbital (88.22%) were comparable with those of the extraction efficiency of the method (90.2%) and indicate that these barbiturates have good stability even in biological specimens subjected to chemical fixation. This fact may be useful in qualitative evaluation of cases.

These results confirm the experimental results of Tsoukali-Papadopoulou (7) and Gannett et al. (9), assuming the stability of barbiturates in formalin solutions buffered at pH 7.

Table I. Quantitative Results in Fresh Liver, Fixed Liver, and Formalin Solutions

Case	Liver at Time of Autopsy	Formalin Solution	Fixed Liver	Formalin + Fixed
1	phenobarbital 32 μg	phenobarbital 15 μg	phenobarbital 11 μg	phenobarbital 26 μg
2	phenobarbital 16 μg	phenobarbital 4 μg	phenobarbital 9 μg	phenobarbital 13 μg
3	phenobarbital 13 μg	phenobarbital 4 μg	phenobarbital 7 μg	phenobarbital 11 μg
4	phenobarbital 85 μg	phenobarbital 22 μg	phenobarbital 56 μg	phenobarbital 78 μg
5	butalbital 28 μg	butalbital 13 μg	butalbital 13 μg	butalbital 26 μg
6	butalbital 71 μg	butalbital 53 μg	butalbital 9 μg	butalbital 62 μg

Table II. Means of Quantitative Results for Phenobarbital and Butalbital and Rates of Recovery after Six Months

	Phenobarbital (n = 4)	Butalbital (n = 2)
Amount at time of autopsy (mean)	36.35 μg	49.17 μg
Amount in fixed tissues (mean)	20.76 μg	10.65 μg
Amount in formalin solutions (mean)	11.21 μg	32.73 μg
% of recovery in fixed tissues	57.11	21.66
% of recovery in formalin solutions	30.84	66.56
Total recovery (fixed tissues + formalin solutions)	87.95%	88.22%

Discussion

Forensic toxicologists are often required to perform analyses of embalmed tissues or specimens stored in formalin. Some substances have been detected in formalin-fixed tissues (1–7,14,15). The present study analyzed the presence of barbiturates in liver tissues fixed in formalin. Our results highlight the fact that the quantitative values of barbiturates obtained from fixed tissues and from the same tissues at the time of

autopsy are quite different, demonstrating leaching of the drug from the liver into the formalin solutions. This remarkable fact is probably due to the extraction capability of formalin solutions. Formaldehyde is a highly reactive chemical substance and has recently been shown to react with some drugs (14,15). Regarding barbiturates, it has been shown that, in vitro, although some compounds such as pentobarbital or secobarbital are not susceptible to substantial decomposition in the presence of formaldehyde, phenobarbital solutions slowly decompose in water up to pH 9.5 (9). Exceeding this value, formaldehyde significantly accelerates the decomposition rate of phenobarbital even 24 hours after contact between the substances. Some studies have compared the concentration of barbiturate in formalin-fixed tissues with that originally found after autopsy (1,10). It was concluded that significant amounts of barbiturates as phenobarbital could be identified in formalin-fixed tissues up to four weeks (1) and three years after formalin fixation (10). Moreover, both studies showed significant reductions (more than 63%) in the total amount (fixed liver tissues and formalin solution) of phenobarbital after 28 days (1) or 2 months (10). These significant reductions may be attributed to the effect of pH on the formaldehyde solutions—values that were not reported in the above studies. It is well-known that the postmortem stability of barbiturates in blood and tissues depends on pH (8). The pH values of our formaldehyde solutions were neutral—a fact that may explain our results, in which chemical interactions between phenobarbital and butalbital with formaldehyde could be excluded. In fact, as shown in Table II, the total amounts of drugs in formalin solutions and fixed tissues were comparable with those in fresh tissues. The absence of any differences indicates that barbiturates have good stability and do not react chemically with formaldehyde at this pH value. The result concerning butalbital is very interesting because there are no data in the literature about the stability of this barbiturate.

The detection of barbiturates again after 6 months indicates that it is feasible and reliable to use formalin-fixed tissues when blood is not available. Moreover, the absence of any differences in total amounts (tissues plus formaldehyde solutions) before and after fixation, due to the absence of chemical reactions between barbiturates and formaldehyde, may provide information about the concentrations of the substances at the initial moment of fixation or the embalming process. As phenobarbital exhibits minimal postmortem redistribution (10,11), this value may provide important information regarding the role played by the substance causing death.

Conclusions

The good stability of barbiturates in biological specimens subjected to chemical fixation allows fixed tissues to be used for the forensic toxicology diagnosis of barbiturate poisoning in cases in which toxicological analysis for barbiturates was not carried out at the time of autopsy. However, interpretation of quantitative data from fixed tissues requires considerable care. Formalin solutions in which the tissues were preserved can also be used for the same purpose. The double evaluation of barbiturates in fixed tissues and formalin solutions provides a good indication of the original quantity of barbiturates in the same tissues before fixation.

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