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 butalbit. 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 butalbit 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 butalbit (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 Tsoukal-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 Winak 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 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 distribution 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 butalbit). 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, Hen-goo, Mid Glamorgan, U.K.), sodium hydroxide (Merck, Darmstadt, Germany), sodium dihydrogen phosphate (JT Baker...
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 Turrax, Ica Labor Technik, Milan, Italy), sonicator (Bramson 1210, Bramson, Damburg, CO), centrifuge (ALC 4218, ALC, Milan, Italy), vortex mixer (Falc, Bergamo, Italy), Analyticchem 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 from 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 supernatant 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.

Preparation 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 interference, 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 temperature under a slow stream of nitrogen, and then reconstituted with 100 μL of ethyl acetate.

Derivatization

Extracts were methylated directly on columns with Methelute obtaining a “flash methylation” (dimethyl-derivatives 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
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 derivatives (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 1.

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.

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
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.

Conclusions

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.

References


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