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CASE REPORT

TOXICOLOGY

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Identification and Quantification of Phenobarbital in a Mummified Body 10 Years After Death

ABSTRACT: This article reports the determination of phenobarbital in the mummified body of a 56-year-old man found completely mummified 10 years after his death. When alive, he was being treated for epilepsy with phenobarbital, and the recent analyses, performed with both immunochemical techniques and gas chromatography with mass spectrometry (GC-MS), have revealed the presence of this substance in various tissues: the mean content of barbiturate in the mummified liver tissue was 93 μg/g, 216 μg/g in the heart, 17 μg/g in the lungs, 12 μg/g in muscles, and 31 μg/g in the skin. Preliminary screening tests with immunochemical techniques to evaluate the presence of other drugs were also performed. The sample resulted negative for all substances tested. Phenobarbital can be identified and quantified thanks to its excellent chemical stability and a hypothesis of what the concentrations in the fresh tissue could have been has also been reported.

KEYWORDS: forensic science, forensic toxicology, phenobarbital, mummified body, immunochemical analyses, gas chromatography-mass spectrometry, chemical stability

Barbiturates and other prescription drugs, such as hypnotics and sedatives, are widely used to treat epilepsy. The risk of accidental or intentional poisoning remains high, although the abuse of barbiturates is currently low compared with other classes of abused drugs. Several cases of fatal intoxication have been reported in previous studies, together with an evaluation of the concentrations of barbiturate that have been found in postmortem biological specimens (fluids or tissues) (1–9).

Some other studies have reported the stability of some barbiturates in blood or liver samples taken during autopsy and stored at different temperatures (10), or in biological samples coming from four cases of death because of phenobarbital overdose and in two cases where butalbital was the cause of death. In this study, the stability of barbiturates was evaluated comparing their concentrations in fresh liver tissue and in those founded in liver tissue fixed in formalin even after 6 months of storage. Results concerning the stability of phenobarbital and butalbital in formalin solution have also been given (11).

Our study reports the discovery of phenobarbital in several tissues (liver, heart, lung, muscle, and skin) taken from a mummified body and, according to our search in the literature, a similar case has never been reported.

Case Report

The body of a 56-year-old man who had been missing for more than 10 years was found completely mummified inside a brick hut, not far from his home. The body was in a prone position, leaning against the left wall of the hut and wearing the same clothes he had on the last time he was seen alive. Three empty boxes of Gardenale® (phenobarbital, 100 mg/tablet, 20 tablets each box—a drug he took in a daily dose of 200 mg for epilepsy—were found near him. The autopsy revealed no signs of trauma on the mummified remains, and some specimens of different tissues were taken for toxicological investigations.

The purpose of this study was to check the persistence of the barbiturate phenobarbital in mummified bodies. For this, we used the same methods used for its identification and quantification in fresh tissues, making just simple changes in the preliminary stages.

Materials and Methods

FPIA Analysis

A preliminary screening test for cocaine, opiates, methadone, amphetamines, cannabinoids, benzodiazepines, barbiturates, and tricyclic antidepressants was performed on a sample of mummified liver tissue. The sample (0.65 g) was rehydrated with 3 mL of H_2O in a warm ultrasonic bath (37°C), homogenized, deproteinized with 3 mL of 10% (w/v) trichloroacetic acid, centrifuged at 1006 × *g* for 5 min, filtered and diluted with phosphate buffer (1:1 v/v) before FPIA analysis. The sample resulted highly positive for barbiturates and negative for all the other substances tested, so the investigations were extended (only for barbiturates) to other tissues taken during autopsy and which, after extraction, were analyzed using gas chromatography with mass spectrometry (GC-MS).

Solid-Phase Extraction Prior to GC-MS Analysis

The samples were dried at 50°C for 48 h to a constant weight, and the same procedure was performed on similar tissues taken from fresh cadavers in order to use a certain amount of dry weight of each tissue to relate to the corresponding amount of fresh tissue.

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No appreciable weight losses were observed. Different portions of liver (0.15 g), heart (0.10 g), lung (0.16 g), muscle (0.11 g), and skin (0.15 g) were rehydrated with 3 mL of water in a warm ultrasonic bath (37°C). The material was then homogenized in water with hexobarbital as internal standard (1 μg/mL). Finally, the samples were deproteinized with trichloroacetic acid 10% (w/v) and centrifuged (1006 × *g* for 5 min).

The supernatant was extracted using a Clean Screen© extraction column (ZSDAU020, 200 mg; UCT Inc. Bristol, PA) according to the specific method recommended by the manufacturer for barbiturates. Briefly, the column was first conditioned with 1 × 3 mL of CH_3OH, 1 × 3 mL H_2O, and 1 × 1 mL 100 mM phosphate buffer prior to loading the sample onto the column. The column was then washed with 1 × 3 mL H_2O and 1 × 1 mL of acetic acid 1.0 M and then dried. After a further washing with 1 × 2 mL of hexane, the barbiturates were eluted with 1 × 3 mL of hexane/ethyl acetate (1/1).

The eluate was dried under a gentle stream of nitrogen and reconstituted with 100 μl of ethyl acetate with formic acid (3% v/v), as recommended by Saka et al. (12), so that it was possible

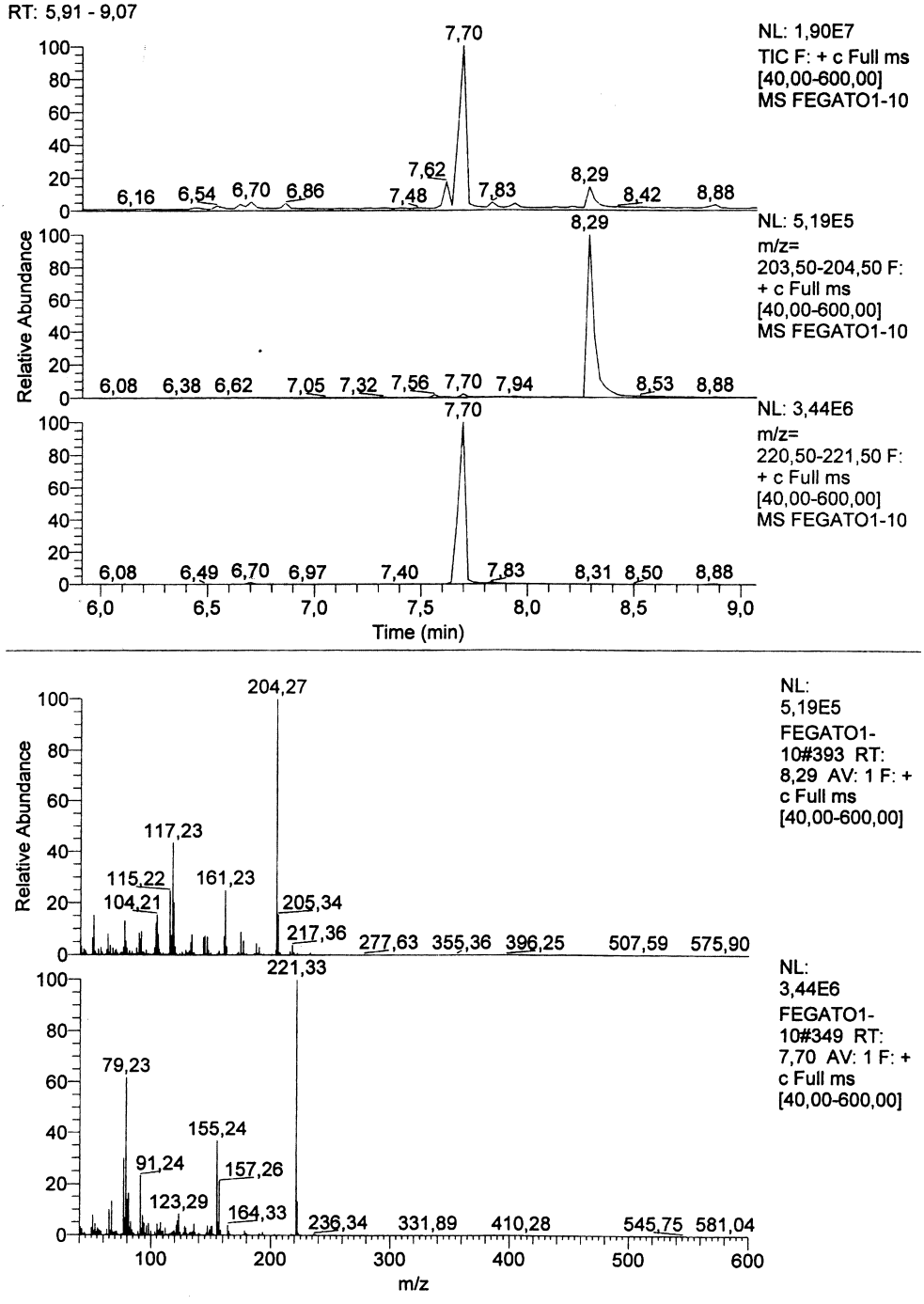

FIG. 1—*Identification of phenobarbital (retention time = 8.29 min) and internal standard hexobarbital (retention time = 7.70 min) in the sample of mummified liver.*

to analyze the phenobarbital by GC-MS without any derivatization step. A similar operation was performed on a blank sample of liver (previously tested in FPIA): increasing concentrations of phenobarbital (0.2, 0.5, 1, and 2 μg/mL) together with an internal standard (hexobarbital, 1 μg/mL) were added, after homogenization and deproteinization of the liver, to prepare the appropriate calibration curve. Each point was repeated three times.

GC-MS Analysis

Instrumentation: Focus gas chromatograph equipped with a SGE-BP1 (15 m × 0.5 mm i.d.; Restek Corp., Bellafonte, PA) column and a Polaris Q ion trap MS-MS detector (Thermo Finnigan, Waltham, MA).
Analytical conditions:
Temperature: from 230 to 290°C with increments of 10°C/min.
Total run time: 15 min.
Retention time for phenobarbital: 8.29 min.
Retention time for hexobarbital (internal standard): 7.70 min.

Results

The qualitative analysis confirmed the presence of phenobarbital in all five samples analyzed and, as reported in Fig. 1 for liver tissue, both phenobarbital and hexobarbital showed a symmetrical peak, allowing a good quantification procedure. The linearity of the calibration curve was also acceptable ($Y = 0.568146 \times X$, $R^2 = 0.9536$). Mass spectra showed peaks corresponding both to phenobarbital (204 *m/z*) and to the internal standard (hexobarbital, 221 *m/z*), and the acquisition of the full mass spectrum of the molecule by GC-MS led to a perfect identification by the NIST library supplied with the instrument (Fig. 1). The results of quantitative analyses on the weight of the dried samples, calculated as the mean of three replicas, are reported in Table 1.

In order to work out the antemortem values of the drug, samples of the same kind of tissues from cadavers undergoing autopsy investigations were dried (50°C for 48 h). The reduction in weight for each tissue was as follows: liver 78%, heart 81%, lung 74%, muscle 81%, and skin 81%. Using these values, the probable concentrations of phenobarbital as the cause of death may be in the range shown in Table 2.

TABLE 1—*Mean concentrations of phenobarbital in mummified tissues.*

Sample	Phenobarbital (μg/g)
Liver	93 ± 4
Heart	216 ± 9
Lung	17 ± 0.8
Muscle	12 ± 0.7
Skin	31 ± 1.8

TABLE 2—*Hypothetical concentrations of phenobarbital in fresh tissue.*

Sample	Phenobarbital (μg/g)
Liver	1.19
Heart	2.70
Lung	0.23
Muscle	0.15
Skin	0.38

Discussion

This study shows that phenobarbital can be not only detected by the immunochemical screening but also identified and quantified by GC-MS in mummified tissues even 10 years after the death of the subject, thanks to its excellent chemical stability. Although currently there are no reports showing the presence or stability of barbiturates in mummified tissue, previous studies have already demonstrated the good stability of phenobarbital and other barbiturates in biological samples. Levine et al. (10) evaluated the stability of five barbiturates (amobarbital, butabarbital, pentobarbital, phenobarbital, and secobarbital) and thiopental in blood, serum, and liver stored at 4°C and at room temperature (25–27°C) for a period of 3 months; their results showed that over 75% of the barbiturates originally present were detected at the end of the period.

A different study on the stability of barbiturates was performed by Cingolani et al. (11). In their article, they reported the results of the retention and quantification of barbiturates (phenobarbital and butalbital) in fresh tissue, in liver tissue fixed in formalin, and in formalin solution in which the same tissues had been fixed for 6 months. The total sums of recovery rates in the formalin solution and fixed tissues for phenobarbital and butalbital (87.95% and 88%, respectively) were comparable with those of the extraction efficiency of the method (90%). These values indicate that phenobarbital and butalbital have good stability even in biological samples that have undergone chemical treatment.

Some other authors evaluated the concentration of different barbiturates in biological samples from several autopsy cases. Ferrara et al. (9) examined the plasma, urine, brain, lung, liver, and kidney specimens (as fresh tissues) coming from five cases of death owing to intoxication caused by the association of flurazepam with phenobarbital.

The values found for phenobarbital in liver were usually higher than in other tissues and ranged from 24 to 89 μg/g. High concentrations of phenobarbital were also found in kidney (16–61 μg/g), brain (6–45 μg/g), and plasma (7–80 μg/mL). Both lung and urine showed very low levels.

A comparative study for barbiturates (and some other drugs) on different postmortem matrices has been published by Ziminski et al. (13). These authors quantified the presence of amobarbital, pentobarbital, phenobarbital, and secobarbital in vitreous humor, blood, brain, liver, and kidney, obtaining values ranging from 17 to 63 μg/g in liver, 5–27 μg/g in brain, 15–28 μg/g in kidney, 4–25 μg/mL in blood, and 2–22 μg/mL in vitreous humor.

A more recent (1997) fatal case involving ethanol and phenobarbital intoxication has been described by Isobe et al. (7), where the levels of phenobarbital in blood, urine, and stomach contents were 65, 20, and 501 μg/mL, respectively.

As reported above, our case deals with a mummified body and it may be difficult to correlate the concentrations revealed in his organs with those found in fresh tissue; moreover, no data are available about this in the literature. We have tried to estimate the concentration of phenobarbital just after the death by considering the water content that could had been present in the live subject in the various organs examined and in light of that found in the same fresh tissues taken from other subjects.

Furthermore, the body was found lying in a brick hut that had been subjected to over 10 years of adverse weather conditions (extreme cold in winter and intense heat in summer). Despite this, phenobarbital was detected and quantified in several mummified tissues. The mean barbiturate content in mummified liver tissue was 93 μg/g, 216 μg/g in heart, 17 μg/g in lung, 12 μg/g in muscle, and 31 μg/g in skin (Table 1), and according to the water

content found in the analogous fresh organs and reported above, the hypothetical concentration of phenobarbital in fresh postmortem tissues could have been those reported in Table 2.

In this specific case, the quantitative results obtained from the study require some considerations: the values found (compared to those in fresh tissue) were lower than those found and reported in cases of acute intoxication, such as those mentioned above, but were certainly higher than those reached with the therapeutic administration of barbiturates in subjects (14).

Moreover, because the presence of phenobarbital was detected in the sample of skin as well, it can be hypothesized that because the drug does not accumulate physiologically at this level, the phenomena of postmortem diffusion and redistribution took place besides the loss of liquids.

All these considerations may justify a scenario where the real concentration of phenobarbital at the moment of death could had been certainly much higher than that found in the mummified body 10 years after. As highlighted in this case, molecules with good chemical stability remain unchanged inside mummified bodies for a very long time, and this can be very useful in studies dealing with intoxication or lifestyles applied to anthropology and paleopathology.

References

1. Curry AS, Sunshine J. The liver/blood ratio in cases of barbiturates poisoning. Toxicol Appl Pharmacol 1960;5:602–6.
2. Robinson AE. Critical survey of barbiturates and allied poisoning. Med Sci Law 1967;7:17–22.
3. Felby S, Olsen J. Comparative studies of postmortem barbiturate and meprobamate in vitreous humor, blood and liver. J Forensic Sci 1969;14:507–14.
4. Gillet R, Warburton FG. Barbiturate blood levels found at necroscopy in proved cases of acute barbiturate poisoning. J Clin Pathol 1970;23: 435–9.
5. De Zorzi C, Lopez A. *La distribuzione dei derivati barbiturici nei tessuti e nei liquidi biologici in 30 casi di avvelenamento mortale*. Scritti in Onore di Cesare Gerin 1974;2:180–95.
6. De Ferrari F, Gambaro V, Marozzi E. *Avvelenamenti da barbiturici. Considerazioni su 91 casi*. Zacchia 1976;12:268–87.
7. Isobe E, Tsukamoto S, Kanegae T, Muto T, Matsumura Y, Hirose M, et al. A fatal case involving ethanol and phenobarbital intoxication. Nihon Univ J Med 1997;39:225–9.
8. Yamamoto K, Yamamoto Y, Hattori H, Ueda M. An unusual case of fatal poisoning by phenobarbital overdosage. Jpn J Leg Med 1985;39:27–9.
9. Ferrara SD, Tedeschi L, Marigo M, Castagna F. Concentration of phenobarbital, flurazepam and flurazepam metabolites in autopsy cases. J Forensic Sci 1979;24:61–9.
10. Levine BS, Blanke RV, Valentour JC. Postmortem stability of barbiturates in blood and tissue. J Forensic Sci 1984;29:131–8.
11. Cingolani M, Cippitelli M, Froldi R, Tassoni G, Mirtella D. Stability of barbiturates in fixed tissue and formalin solutions. J Anal Toxicol 2005;29:205–8.
12. Saka K, Uemura K, Shintani-Ishida K, Yoshida K. Determination of amobarbital and phenobarbital in serum by gas chromatography–mass spectrometry with addition of formic acid to the solvent. J Chromatogr B Biomed Sci Appl 2008;869:9–15.
13. Ziminski KR, Wemyss CT, Bidanset JH, Manning TJ, Lukash L. Comparative study of postmortem barbiturates, methadone, and morphine in vitreous humor, blood, and tissue. J Forensic Sci 1984;29:903–9.
14. Lewis RJ, Jonson RD, Southern TL, Canfield DV. Distribution of butalbital in postmortem tissue and fluids from non-overdose cases. J Anal Toxicol 2003;27:145–8.

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