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Postmortem Stability of Barbiturates in Blood and Tissues

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ABSTRACT: The stability of five commonly prescribed barbiturates and thiopental in blood and liver at room temperature and at 4°C was studied. Gas chromatography was used for oxybarbiturate analysis while liquid chromatography was used to quantitate thiopental. In blood and liver, greater than 75% of the drugs were detected at the end of the two- to three-month period. These changes were not considered significant; therefore, barbiturates appear to be stable in blood and liver under the conditions of these experiments.

KEYWORDS: toxicology, barbiturates, chromatographic analysis, blood, tissues (biology)

Barbiturates are frequently prescribed sedative, hypnotic, or antiepileptic medication. Because of their widespread use, the potential for accidental or intentional abuse of barbiturates is great. Therefore, there are numerous requests for the clinical or forensic toxicologist to quantitate barbiturates in blood and tissues. Since days or weeks may separate sample acquisition and quantitation, it is necessary to ensure that barbiturates are sufficiently stable over this period of time. Attempts to study this problem date back to 1942 [*1*]. However, because of limitations in methodology, the first meaningful quantitative results were not produced until 1957 by Algeri [*2*]. Ultraviolet (UV) spectrophotometric methods were combined with paper chromatography to analyze a liver obtained from a pentobarbital death. After analysis, immediately upon autopsy, the liver was placed in a museum jar with a loose-fitting cover at 27°C and parts of the liver were removed at random intervals for up to 90 days and analyzed for pentobarbital. After 90 days, slight increases in pentobarbital concentration were observed. As a control a normal liver not containing barbiturates was also allowed to decompose under the same conditions; no barbiturates or interfering substances were produced. Algeri suggested that pentobarbital degradation did not occur because reducing

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substances produced in an acid medium during the putrefaction process would inhibit "oxidation" of the barbiturate. The fact that an increase was observed was attributed to decreased protein binding, interference of material from bordering zones, and decreased water content with a subsequent loss of weight of decomposing material.

The findings of Algeri were in apparent conflict with work of Coutselinis and Kiaris [*3*] who studied the effect of putrefaction on the concentration of pentobarbital in dog blood. Blood samples stored at room temperature for two months were compared to blood samples refrigerated at 4°C for the same length of time. Pentobarbital was quantitated using a UV spectrophotometric method. The concentration of pentobarbital in the room temperature samples decreased with increasing time; the refrigerated samples showed little change. Two explanations were given for the decrease in pentobarbital concentration: (1) the "oxidation" of the barbiturates despite the presence of the reducing substances produced during the putrefaction process and (2) the other factors of putrefaction such as humidity, temperature, and so forth.

Postmortem effects on sodium secobarbital were studied by Parker et al in 1971 [*4*]. Groups of rats were administered lethal doses of the drug via a stomach tube; one group was analyzed for liver secobarbital levels immediately after death, thus acting as a control. Three other groups were similarly analyzed at 24, 48, and 72 h, respectively, following death. A UV spectrophotometric method [*5*] was used for analysis. The concentrations of secobarbital in postmortem rat livers increased greatly during the first 24 h and increased to a slightly higher level after 48 h. A slight decrease in secobarbital concentration was observed at 72 h in comparison to the 48-h concentration. In a second series of experiments, a group of rats were asphyxiated and upon death, were given a dose of sodium secobarbital via a stomach tube. After nine days, analysis of the liver indicated significant concentrations of the drug. This was interpreted as a significant postmortem diffusion from the stomach. This postmortem diffusion was found to be independent of the position of the body.

Sunshine and Hackett [*6*] used UV spectrophotometric methods to compare the concentration of barbiturates in formalin-fixed tissues to the concentration of barbiturates originally found after autopsy. It was concluded that if "significant" amount of barbiturate were present in the original sample, then it will be identifiable in formalin-fixed tissues up to three years after formalin fixation. However, a quantitative comparison between original and formalin-fixed tissues demonstrated tissue dependent differences. For example, fixation removed the drug from the liver more quickly than from other tissues. Conversely, the spleen had a significantly higher barbiturate concentration than the other tissues after formalin fixation.

The introduction of therapeutic drug monitoring of antiepileptic drugs within the past decade has made the establishment of drug stability an important consideration in the clinical as well as the forensic toxicology laboratory. Specific points of concern include the storage of serum controls and the shipment of patient samples through the mail. Moreover, there is some controversy about the length of time serum controls can be stored without a significant decrease in drug concentration. van der Kleijn et al [*7*] observed a 20% decrease in plasma phenytoin concentrations when stored at 4°C for eight weeks. However, Schäfer [*8*] reported no decrease in the concentrations of phenytoin, phenobarbital, or primidone when stored in serum at 4°C for about twelve weeks. Although Pippenger et al [*9*] found significant bacterial growth in pooled serum samples sent by mail, no significant changes in phenytoin, phenobarbital, primidone, and ethosuximide concentrations were found. Wilenksy [*10*] found no changes in the concentrations of mephenytoin, phenobarbital, primidone, and phenytoin after six months of storage in glass containers without exposure to light at room temperature. Several explanations were offered by Wilensky for these conflicting data: (1) the presence of interfering peaks in the gas chromatographic methods produced during the decomposition process, (2) bacterial growth, and (3) the pH of the serum. Besides the fact that barbiturates are unstable in base, different breakdown products are found if the barbiturates are stored in aqueous acid or aqueous base.

In summary, there appear to be conflicting data concerning the stability of anticonvulsant drugs and barbiturates in blood and in tissues. Therefore, it was decided to study this problem with oxybarbiturates and thiopental in blood and in liver.

Experimental Procedure

Standards

Oxybarbiturates—Sodium salts or free acid forms of the following barbiturates were obtained: amobarbital (Lilly), butabarbital (extracted and purified from tablets), pentobarbital (USP-NF), phenobarbital (Winthrop Labs), and secobarbital (extracted and purified from tablets). Methanolic standards with a free acid concentration of 200 mg/L were prepared. Amobarbital was used as the internal standard for butabarbital; butabarbital was used as the internal standard for the other oxybarbiturates.

Thiopental—Five grams of sodium thiopental (injectable) were dissolved in dilute hydrochloric acid and extracted with dichloromethane. The organic layer was separated and evaporated to dryness. The purity of the standard was assessed by comparing its extinction coefficient at 305 nm with that reported in the literature [*11*]. A 200-mg/L standard in methanol was then prepared.

Phenolphthalein (Baker)—A solution of 1 mg/mL in methanol was used as an internal standard for thiopental quantitation.

Reagents

Phosphate Buffer (pH 5.5)—0.5*M* potassium phosphate (KH_2PO_4) (Baker) and 0.5*M* sodium phosphate (Na_2HPO_4) (Baker) were prepared and the solutions were mixed to bring the final pH to 5.5.

Dichloromethane, Toluene, Hexane, Isoamyl Alcohol, and Methanol—(Fisher pesticide or high pressure liquid chromatographic (HPLC) grade).

TMPAH—0.1*M* trimethylphenylammonium hydroxide, (TMPAH) in methanol (Eastman Kodak Co.). For methylation of oxybarbiturates, a 0.033*M* solution was used.

Specimens

Blood was obtained from cadavers autopsied at the Office of the Chief Medical Examiner for the Commonwealth of Virginia. It was stored in a freezer at −20°C until used. Before addition of drugs, the blood was analyzed to ensure that no interferences with the added drugs occurred.

Serum was obtained from the Medical College of Virginia Hospital Toxicology laboratory. The serum from patients receiving phenobarbital was collected, combined, and stored at −20°C until used.

Tissues were obtained from autopsied cases from the Office of the Chief Medical Examiner for the Commonwealth of Virginia. Drug identity was confirmed by gas chromatography/mass spectroscopy (GC/MS). Between receipt and analysis, the tissues were stored in waxed cardboard containers at −20°C.

Blood

An aliquot of a methanolic standard of the barbiturate analyzed was added to a glass container and evaporated to dryness at 65°C. The residue was reconstituted with cadaver blood and stirred magnetically for 1 to 1.5 h. After an initial quantitation the blood was then divided into two portions: one portion was stored in a stoppered Erlenmeyer flask at room temperature and the other was stored in a stoppered Erlenmeyer flask at 4°C. At various

times over a three-month period, aliquots of the blood were analyzed for the added barbiturate.

Serum

When approximately 30 to 40 mL of serum containing the drug to be analyzed was pooled, an initial drug quantitation was performed. The serum was divided into two portions: one was stored in a capped test tube at 4°C and the other was stored in a capped tube at 25°C. Aliquots were removed periodically and analyzed.

Tissues

The tissues were cut into small pieces and divided into 10-g portions. One portion was analyzed initially and the other portions were divided into two groups. Two portions were stored in separate capped glass tubes at room temperature while the other portions were stored in separate, capped glass test tubes in the refrigerator. At one-month time intervals, a one-part tissue to two-parts water homogenate was prepared using a portion stored at each temperature. Aliquots of these homogenates were then analyzed.

Instrumentation

Gas Chromatograph—A Hewlett-Packard 5880 gas chromatograph with a nitrogen sensitive detector was used. The column (2-m by 2-mm inner diameter) was packed with 3% OV-101, 100-120 mesh (Supelco). The carrier gas was helium at a flow of 30 mL/min. The injector temperature was 200°C and the detector temperature was 300°C. The oven temperature program was held at 150°C for 1 min, followed by a 15°C/min rise to 280°C.

Liquid Chromatograph—To quantitate thiopental, an Altex model 332 gradient liquid chromatograph attached to a Gilson Holochrome variable wavelength detector and a Hewlett-Packard 3390 integrator was employed. An RP-8 (25-cm by 4.6-mm inner diameter) 10-μm column was used. The mobile phase was methanol:water (60:40) at a flow of 2 mL/min. The wavelength of detection was 290 nm.

Extraction

Blood or serum—

- 0.5-mL blood or serum
- 0.5-mL phosphate buffer
- 20-μg internal standard for oxybarbiturates or 200-μg phenolphthalein for thiopental
- 5-mL dichloromethane
- mechanical rotation and centrifugation

aqueous layer (discard)

organic layer

oxybarbiturates

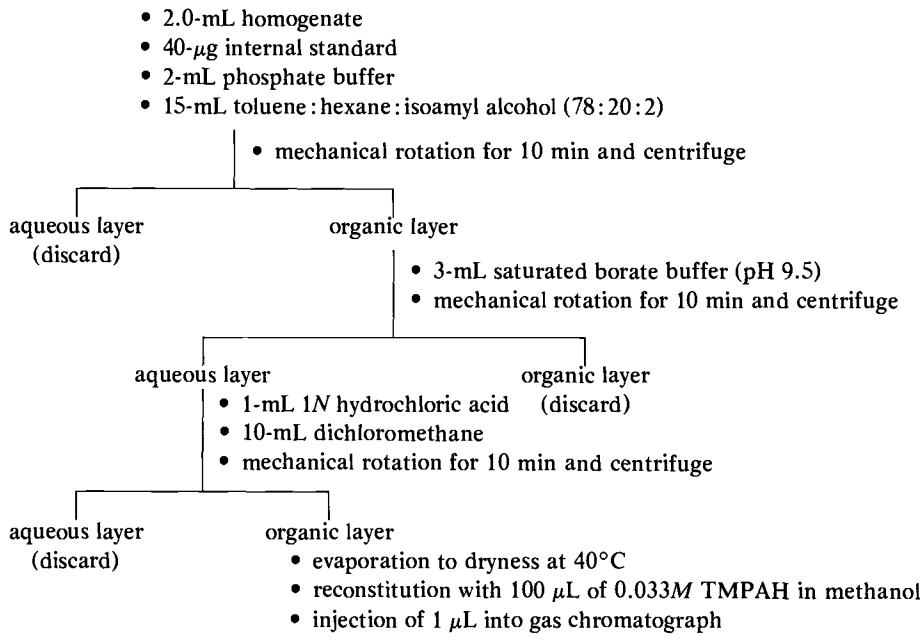
- reconstitution with 100 μL of 0.033*M* TMPAH
- injection of 1 μL into gas chromatograph

thiopental

- reconstitution with 300-μL methanol
- injection of 20 μL into liquid chromatograph

These methods are based on those previously reported [12,13].

Tissue



Statistical Manipulations

Deviations in concentration between the initial analysis and successive analyses were calculated for each drug. These deviations were used to calculate an "average" deviation for each drug over the three-month period. From this, an average percent change (deviation/initial quantitation) was determined [*14*].

Tissue data were compiled as follows: for each drug, the initial concentration was assigned a value of 100%. Successive analyses were made and the "% of original present" was calculated. Data from each drug in each tissue were compiled and an average "% of original present" was obtained for each time period.

Results

Results from the barbiturate blood study are given in Table 1. For the oxybarbiturates, two sets of data were obtained, one set for "therapeutic" concentrations and one set for "toxic" concentrations. From the data, greater than 75% of the barbiturates originally present were detected at the end of the three-month period. Moreover, it appears that this stability of oxybarbiturates occurs in blood over a wide concentration range.

Results from livers containing barbiturates are given in Table 2. They were obtained by combining data from liver as outlined in the Experimental Section. For all of the barbiturates studied, greater than 75% of the barbiturates originally present were detected at the end of the two-month period at 4 and 25°C.

Discussion

The analysis of barbiturates in decomposed tissues was found to be more difficult than the analysis of more routine toxicologic specimens. Ordinarily, an extraction with any organic solvent such as methylene chloride followed by a base cleanup is sufficient to remove any

TABLE 1—*Barbiturate blood data.*

Barbiturate	Original Concentration, mg/L	Average Percent Change	
		25°C	4°C
Amobarbital	2.2	± 9	±11
	21.8	−13	± 8
Butabarbital	6.2	−21	−19
	20.6	−12	− 9
Pentobarbital	1.7	±18	±18
	19.3	± 4	± 5
Phenobarbital	20.7	± 5	± 3
	60.5	± 6	± 6
Secobarbital	2.3	±17	±13
	18.1	±11	±11
Thiopental	25.0	±10	± 6
Phenobarbital (serum)	20.0	± 3	± 3

TABLE 2—*Barbiturate tissue study.*

Time, Months	Percent of Barbiturates Originally Present ($\bar{x}$ ± Standard Deviation)							
	Amobarbital		Pentobarbital		Phenobarbital		Secobarbital	
	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C
0	100	100	100	100	100	100	100	100
1	91 ± 8	91 ± 8	100	100	88 ± 5	88 ± 5	91 ± 23	78 ± 13
2	77 ± 5	76 ± 9	100	100	90 ± 6	86 ± 10	97 ± 16	91 ± 12

interfering substances which might be present. However, in decomposed tissue this multistep extraction still resulted in the coextraction of interference products which rendered quantitation impossible. This problem was alleviated for the most part by using a slightly more polar solvent, toluene:hexane:isoamyl alcohol (78:20:2), for the initial extraction.

It appeared that amobarbital, butabarbital, pentobarbital, phenobarbital, secobarbital, and thiopental are quite stable when stored in blood and liver at 4 and 25°C over a two- to three-month period. Since barbiturate instability in aqueous alkaline medium is well known [*15*], it was important that the pH of the blood be monitored throughout the time course of this experiment. It was found that the pH of the blood at the beginning of the experiment was 6.4 and remained under 7.0 throughout. This would explain the observed chemical stability of the barbiturates in the blood. To increase the use of these data, a series of blood samples obtained from the Office of the Chief Medical Examiner of Virginia was examined for pH. The pH values ranged from 5.0 to 7.0, with the majority of the specimens having a pH of 6.0 to 6.5. This suggested that the majority of the blood samples were slightly acidic or neutral, which meant that the blood pH favored chemical stability for barbiturates.

Although no breakdown of barbiturates in blood was observed, breakdown products of barbiturates in aqueous solutions have been identified. Goyan et al [*16*] first proposed the mechanisms for the hydrolysis of barbiturates; these mechanisms depended on whether the barbiturates were in ionized or unionized forms. Unionized barbiturates were hydrolyzed at the 1,2-position while ionized barbiturates were hydrolyzed at the 1,6-position. Depending on the site of attack, different breakdown products could be formed. These schemes are demonstrated in Fig. 1. Others have verified this mechanism for both oxybarbiturates [*17,18*] and thiopental [*19*].

Besides chemical stability, a factor that is a greater problem in blood than in water is the

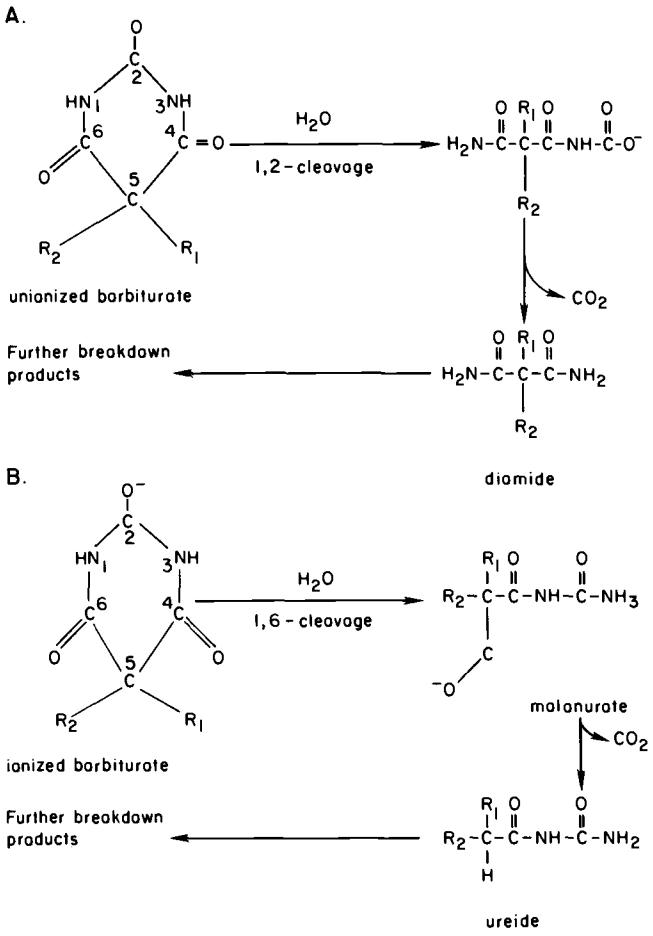


FIG. 1—*Barbiturate degradation in aqueous solutions:* (a) *unionized barbiturate and* (b) *ionized barbiturate.*

presence of microorganisms. Since no chemical preservatives were added to the blood, microorganism growth could continue relatively unimpeded at room temperature and this was verified by some of the physical characteristics of the specimens. The data suggest that microorganism enzymes did not alter the structure of the barbiturate ring. Much of the previously published data agree with this. Moreover, some of the conflicting data can be explained either by differences in experimental design or by differences in method. For example, Algeri's observed increase in pentobarbital concentration in liver with time could be explained rather easily by dehydration of the tissue while in the museum jar. In the experiments reported here, the tissues used for each quantitation were weighed at the beginning of the experiment to enable more meaningful comparisons between the data points.

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