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Postmortem Stability of Benzodiazepines in Blood and Tissues

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ABSTRACT: The stability of benzodiazepines in blood and tissues was examined in this study. Specifically, diazepam, chlordiazepoxide, flurazepam, and their desalkyl metabolites were studied over several months. Diazepam, flurazepam, and *N*-1-desalkylflurazepam were stable when stored in blood at room temperature while chlordiazepoxide, norchlordiazepoxide, and nordiazepam were found to be unstable under similar storage conditions. Data from tissues containing these chemicals corroborated the results from blood.

KEYWORDS: pathology and biology, benzodiazepines, postmortem examinations

Over the past 15 years, benzodiazepines have been prescribed for an increasing number of physiological and psychological illnesses. The prototype of this class of drugs, chlordiazepoxide (Librium®) was first introduced in the United States in 1963 as antianxiety medication. Although still in use, chlordiazepoxide has largely been replaced by diazepam (Valium®). Moreover, flurazepam (Dalmane®), is a frequently prescribed hypnotic drug. The structures of these drugs and their desalkylated metabolites are given in Fig. 1. The pharmacokinetics of these drugs are well known and this subject is reviewed by Bellantuono et al [*1*]. Because of their widespread use, it is not surprising that benzodiazepines have been implicated in fatal and nonfatal drug overdoses. However, it should be noted that few if any cases have been documented in which benzodiazepines have been the sole drug present in an overdose situation. Nevertheless, their appearance in association with alcohol and other drugs cause their quantitation to be quite significant in the interpretation of forensic and clinical toxicologic results.

Since days or even weeks may pass between specimen acquisition and drug quantitation, the establishment of drug stability in blood is necessary for proper interpretation of results. Little if any data have been reported on this subject. This research examines various aspects of this

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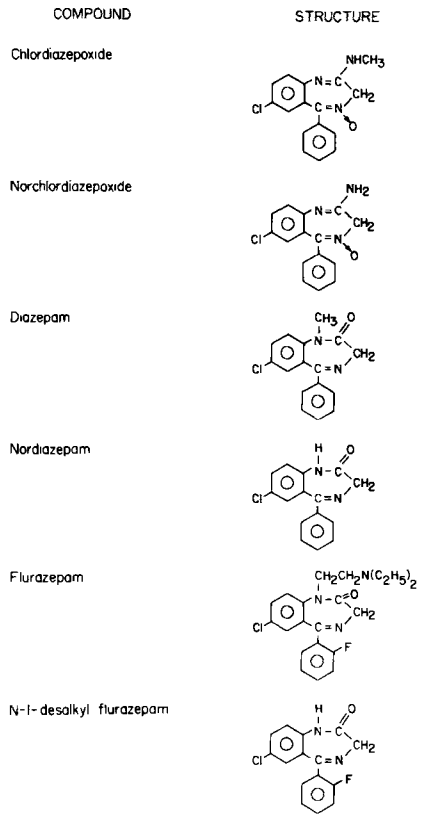


FIG. 1—*Structure of some commonly prescribed benzodiazepines and their desalkylated metabolites.*

problem. After a careful review of the methods for the quantitation of benzodiazepines it was decided that the method of Peat and Kopjak [*2*], which uses electron capture gas chromatography (GC-ECD) and liquid chromatography (LC) would be the most advantageous for this work. Moreover, the ability to collect the eluent from the LC was used during these experiments.

Experimental Procedure

Experimental Design

Blood—An aliquot of a methanolic standard of the benzodiazepine to be analyzed was added to a glass container and evaporated to dryness at 65°C. No breakdown of these chemicals occurred during this process. The residue was reconstituted with blood obtained from cadavers within 24 h after death. Before addition of the blood, it was found not to contain benzodiazepines. After an initial quantitation the blood was then divided into two portions: one portion was stored in a corked Erlenmeyer flask at room temperature and the other was stored in a corked Erlenmeyer flask at 4°C. At various times, aliquots of the blood were quantitated for benzodiazepines.

Tissues—Upon receipt, tissues from cases in which a drug screen suggested the presence of benzodiazepines were stored in waxed cardboard containers in a freezer at −20°C. Before the first analysis, approximately 100 g of tissue were cut into small pieces. These pieces were divided into ten portions of about 10 g each and placed in 25-mL screw cap test tubes (see Discussion). The tubes were corked and divided into three groups: four tubes were stored in a refrigerator at

4°C, four tubes were stored in a hood at about 25°C, and the remaining test tubes were used for the initial quantitation. At approximately one month intervals, one portion stored at 4°C and one portion stored at 25°C were homogenized and analyzed for benzodiazepines.

Materials—Diazepam, nordiazepam, flurazepam, *N*-1-desalkylflurazepam, chlordiazepoxide, norchlordiazepoxide, and demoxepam were donated by Hoffman-LaRoche Co., Nutley, NJ. Standards with the equivalent of 10 mg/L of free base in methanol were prepared. Diazepam was used as the internal standard except when diazepam was studied in which case flurazepam was used as the internal standard.

Reagents—The borate buffer (pH 9.2) was 14 g of sodium borate (Fisher S-249) in 200 mL of distilled water.

The extraction solvent was toluene:hexane:isoamyl alcohol (78:20:2). All solvents were Fisher high pressure liquid chromatography (HPLC) or pesticide grade.

The methanol was Fisher HPLC grade.

Instrumentation—The gas chromatograph (GLC-ECD) was a Hewlett-Packard 5730 with ^{63}Ni electron capture detector with a 1.3-m by 2-mm glass column packed with 3% OV-17 or 3% OV-7 on 80-100 Gas Chrom Q. The carrier gas was methane:argon (10:90) at a flow rate of 30 mL/min. The oven temperature was 250°C-isothermal, and the detector temperature was 300°C.

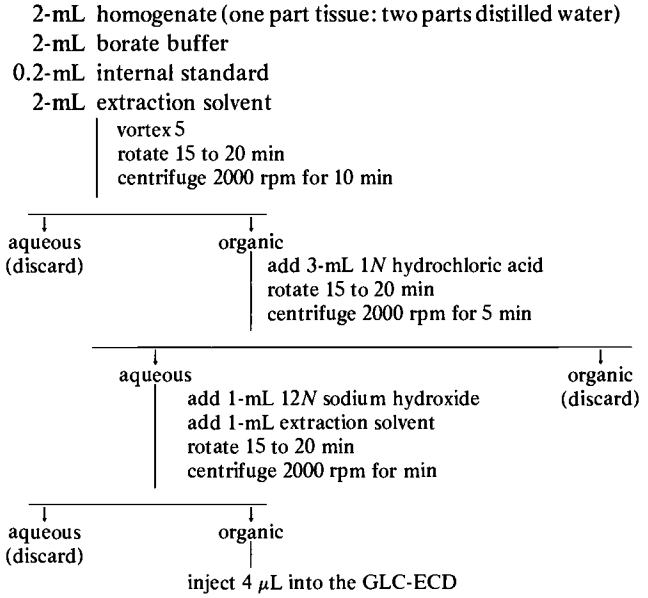
The gas chromatograph/mass spectrometer (GC/MS) was a Hewlett-Packard 5985A with a 1-m by 2-mm glass column packed with 3% OV-101 on 80-100 Supelcoport. The carrier gas was helium at a flow rate of 33 mL/min. The oven temperature was 220°C initially, with a 10°C/min rise to 280°C.

The liquid chromatograph (LC) was a Hewlett-Packard 1080 with a RP-8 column at 35°C, a mobile phase of methanol:water (65:35) at a flow rate of 2 mL/min, and a detector at 240 nm.

Extraction and Quantitation

Blood—As mentioned previously, the quantitation of benzodiazepines was performed using the method of Peat and Kopjak [2]. Diazepam and flurazepam were quantitated by GC-ECD; nordiazepam, chlordiazepoxide, and norchlordiazepoxide were quantitated by LC.

Tissues



The only deviation from this procedure was for tissues containing flurazepam, where the initial extraction was with 2-mL *n*-hexane (see Discussion). Quantitation was based on the area or height ratio of analyte peak to internal standard peak in comparison to ratios from fortified tissue standards.

Fraction Collection—Fractions collected from the liquid chromatographic column were made basic with borate buffer and extracted with the extraction solvent. The organic extracts were then subjected to the following: (1) injection of an aliquot of the extract into the GLC-ECD and (2) evaporation of the extraction solvent followed by reconstitution with methanol and injection into the GC/MS.

Results

Blood

Diazepam was found to be very stable when stored at room temperature or at a refrigerated temperature; no changes in concentrations were observed over a five-month period. However, as shown in Fig. 2, nordiazepam was less stable. Decreases in nordiazepam concentration were observed at both temperatures, with the greater and more rapid decreases occurring in the blood stored at room temperature. Flurazepam and *N*-1-desalkylflurazepam demonstrated slight decreases in concentration (<20%) over time at both temperatures. The decay curves for chlordiazepoxide and norchlordiazepoxide in blood are given in Fig. 3 and 4. It can be seen that when both the parent drug and metabolite were stored at room temperature, their presence was not detected after 18 days from the time of addition to the blood. When these blood solutions were stored in the refrigerator, an initial decrease in drug concentration was observed, followed by a leveling off of the concentration.

Blood received by forensic toxicologists frequently contains sodium fluoride and potassium oxalate as a preservative and anticoagulant, respectively. Therefore, a series of experiments were undertaken to examine the effects of F^- and $C_2O_4^=$ on blood containing chlordiazepoxide stored at room temperature. The presence of these chemical preservatives had little effect on norchlordiazepoxide preservation as the drug disappeared within 20 days at both low (2 mg/L) and high (5 mg/L) concentrations (Fig. 5). However, F^- and $C_2O_4^=$ did partially pro-

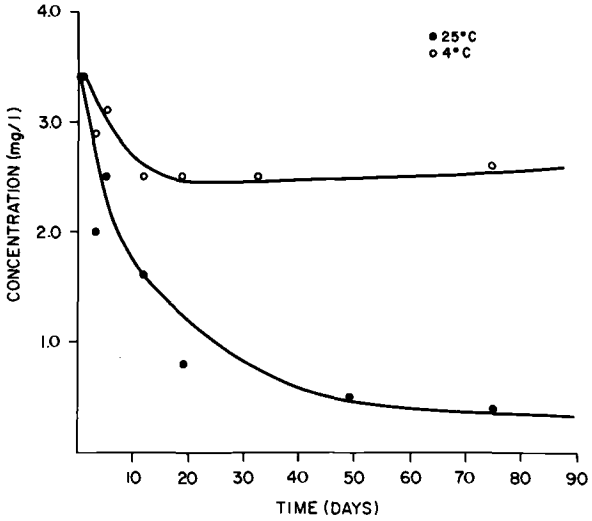


FIG. 2—*Effect of storage of blood containing nordiazepam at 25 and 4°C.*

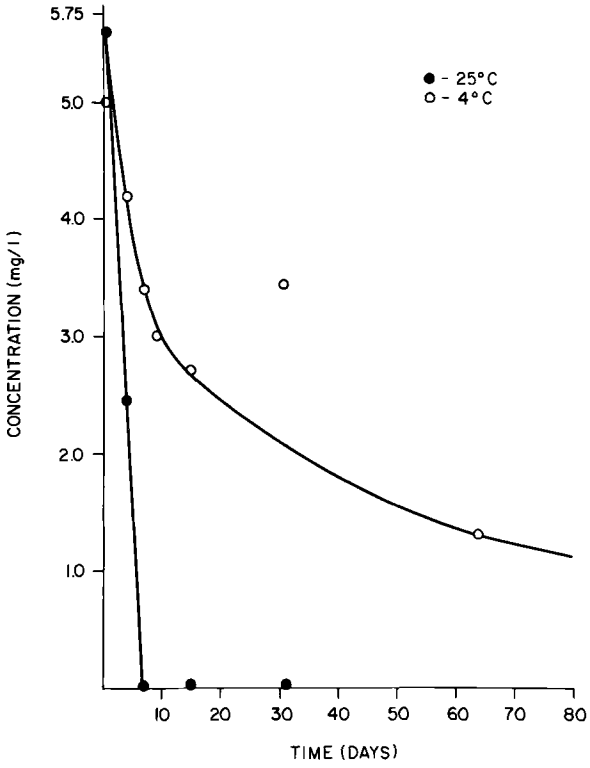


FIG. 3—*Effect of storage of blood containing chlordiazepoxide at 25 and 4°C.*

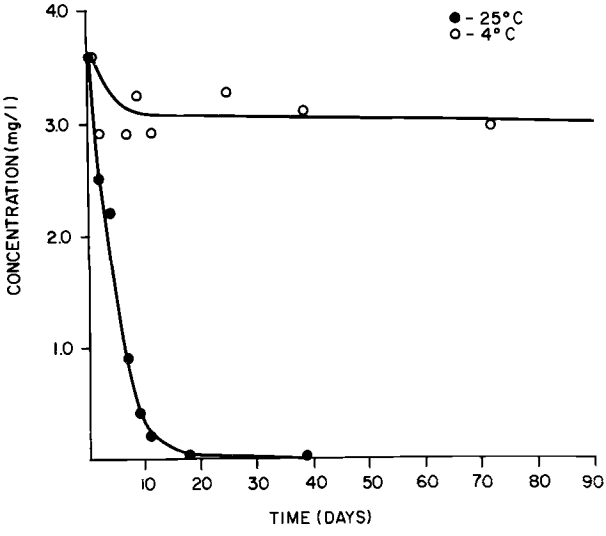


FIG. 4—*Effect of storage of blood containing norchlordiazepoxide at 25 and 4°C.*

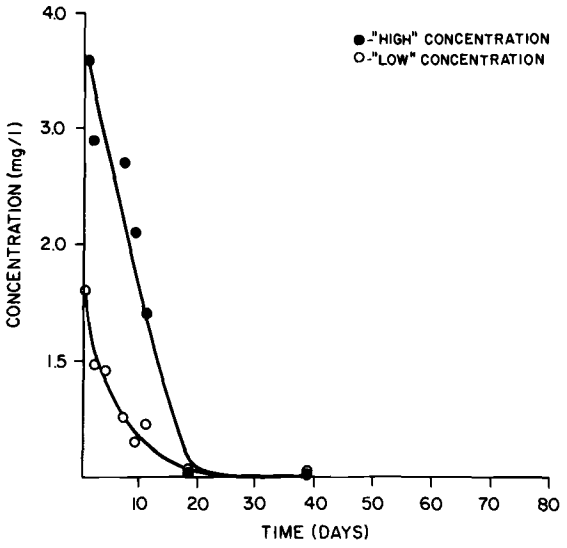


FIG. 5—*Storage of norchlordiazepoxide at 25°C with F^- and $C_2O_4^=$*

tect chlordiazepoxide from degradation in comparison to that observed at 25°C in the absence of F^- and $C_2O_4^=$ (Fig. 6).

To obtain further information on the degradation of chlordiazepoxide and norchlordiazepoxide, experiments were undertaken to identify and possibly quantitate the breakdown products from these drugs. By analyzing different LC fractions as discussed in the Experimental Section, two breakdown products were tentatively identified: demoxepam and nordiazepam. Demoxepam was identified by GC and LC retention time; no mass spectrum was obtained because of thermal instability of demoxepam. Nordiazepam was identified by GC and LC retention times and by GC/MS. Blood standards of these drugs were prepared and, when possible, these compounds were also quantitated when present. Since demoxepam appeared earlier (two days) than nordiazepam (four days), it was evident that demoxepam was the first breakdown product of norchlordiazepoxide (Fig. 7). Because of the presence of an interfering peak from the blood itself, demoxepam was not quantitated in the chlordiazepoxide blood study, but its presence was confirmed by GLC-ECD. Specifically, the LC fraction corresponding to the retention of demoxepam was collected, re-extracted and injected onto the GC. Any conversion of demoxepam to nordiazepam was delayed by the addition of F^- and $C_2O_4^=$ (Fig. 8). Nordiazepam was not seen in norchlordiazepoxide blood solutions with F^- and $C_2O_4^=$ until 18 days after the original addition of norchlordiazepoxide (data not shown). In comparison, nordiazepam appeared four days after the addition of norchlordiazepoxide to blood not containing F^- and $C_2O_4^=$ (Fig. 9).

Tissues

The identity of the benzodiazepines was suggested by their retention times on the gas chromatograph and confirmed by gas GC/MS. The data collected from these tissue studies were obtained as follows. For each tissue, the initial quantitation was assigned a value of 100%. Successive quantitations were made and the "% of original present" was calculated. Data for each drug in each tissue were then compiled and an average % of original present was obtained for each time period. When analyzing these data, it is important to keep in mind that the co-

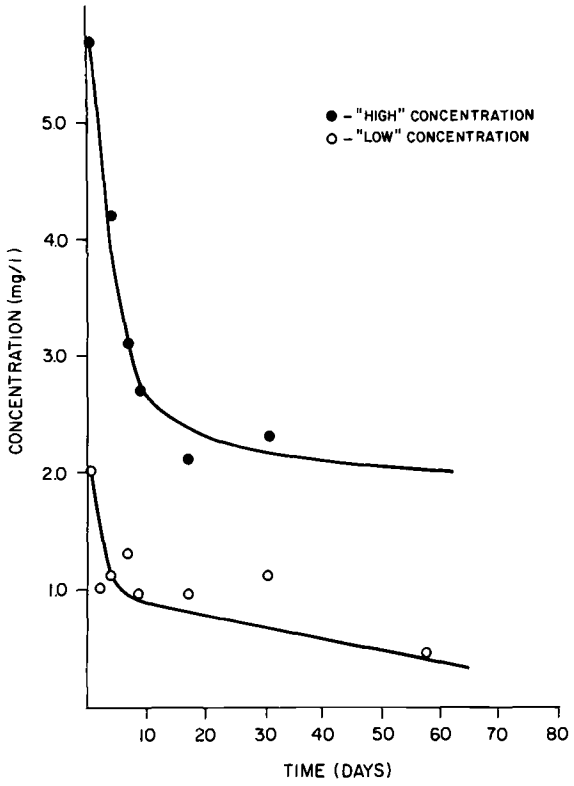


FIG. 6—*Storage of chlordiazepoxide at 25°C with F^- and $C_2O_4^=$*

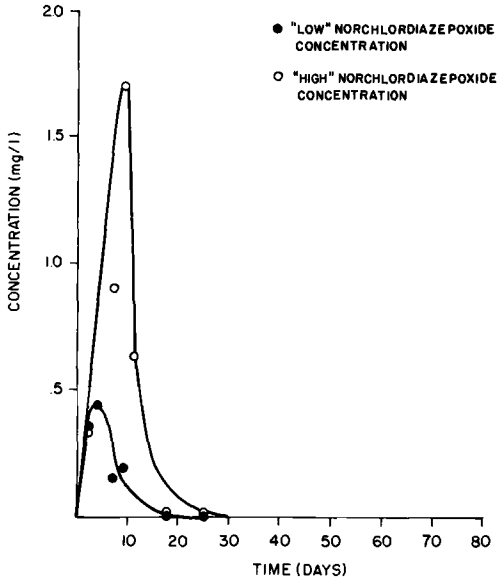


FIG. 7—*Demoxepam concentrations in norchlordiazepoxide blood solutions stored at 25°C.*

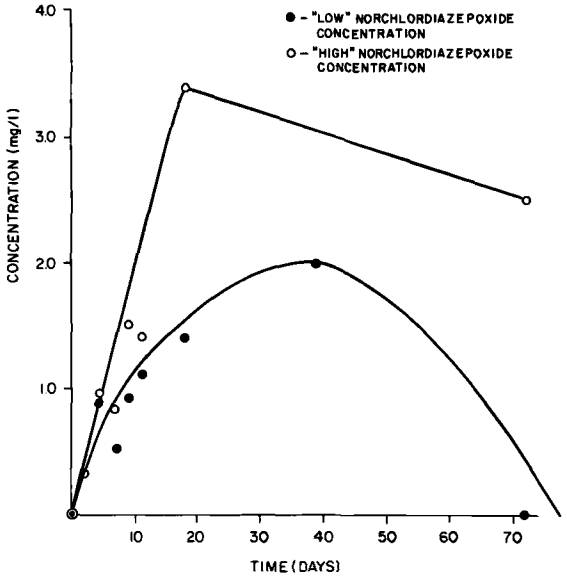


FIG. 8—*Demoxepam concentrations in norchlordiazepoxide blood solutions stored at 25°C with* F^- *and* $C_2O_4^=$.

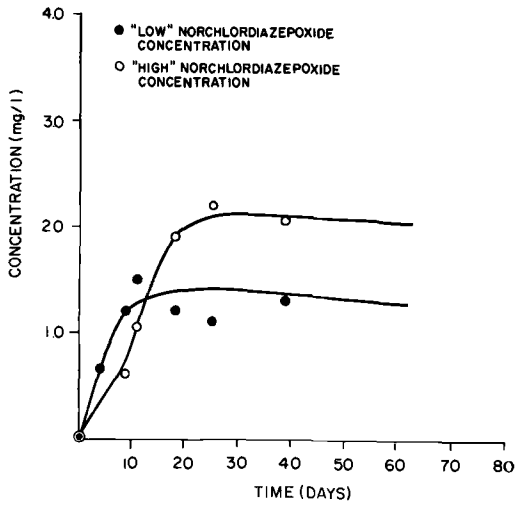


FIG. 9—*Nordiazepam concentrations in norchlordiazepoxide blood solutions stored at 25°C.*

efficient of variation of triplicate samples within a homogenate was 15 to 20%. Several factors are suggested from these data:

1. There was no significant change in the concentration of diazepam in both liver and brain for three months following its storage at either room temperature or at refrigerated temperature. This is in agreement with the data obtained from the blood experiments (Fig. 10).

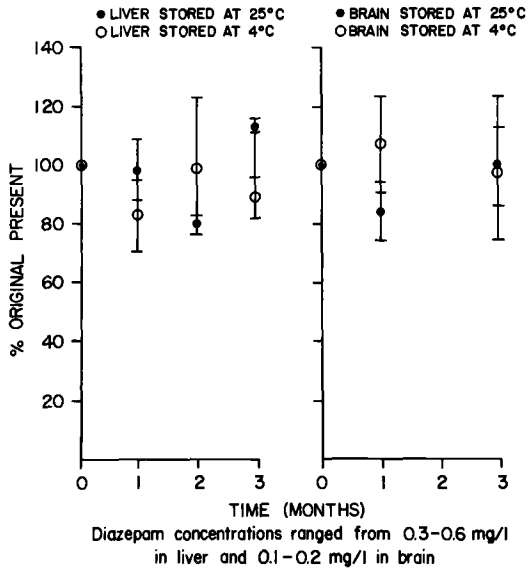


FIG. 10—*Effect of temperature on the storage of tissues containing diazepam.*

2. The data from the one reported case containing nordiazepam suggested a decrease at both temperatures after two months of storage at each temperature, with the greatest decrease occurring in the liver stored at room temperature (Fig. 11).

3. Slight changes in the concentration of flurazepam in the examined livers were found over a three-month period (Fig. 12); this is similar to the results obtained from the blood experiments.

N-1-desalkylflurazepam was not quantitated along with flurazepam because a change in the extraction procedure was necessary. This was a result of a metabolite of flurazepam interfering with the flurazepam peak. To remove this interference, a less polar solvent, hexane, was used in the initial extraction step. However, this change caused the more polar metabolites of flurazepam to remain largely in the aqueous layer, thus preventing their quantitation. Moreover, because of small quantities of flurazepam detected in the brain, no attempts were made to submit them to these experiments.

Discussion

The methods used in the performance of these experiments were based on the paper by Peat and Kopjak [*2*] which used a combination of electron capture gas chromatography (GC-ECD) and liquid chromatography (LC) to analyze for benzodiazepines. These methods afforded several advantages including rapid analysis time, sensitivity, and good recovery (>85%). Furthermore, because of the specificity of the electron capture detector, interferences by putrefactive-base production were not a significant problem in the analyses in which gas chromatography was used. Unfortunately, chlordiazepoxide and norchlordiazepoxide could not be quantitated by GC-ECD because of their thermal instability at the high oven temperature. Moreover, the polarity of nordiazepam caused such poor peak efficiency that reproducibility became a significant problem. These problems were alleviated by using LC. However, the production of an endogenous interference product unrelated to the drug made the quantitation of demoxepam difficult and frequently impossible, especially in the blood used in the chlordiazepoxide studies.

The quantitation of benzodiazepines in tissues involved a back extraction into acid followed by

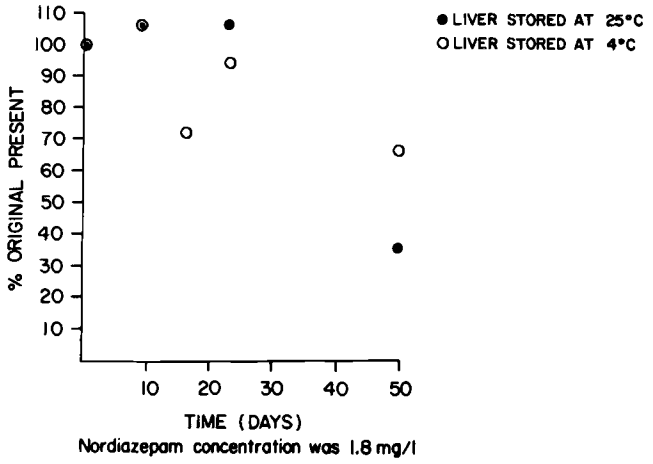


FIG. 11—*Effect of temperature on the storage of a tissue containing nordiazepam.*

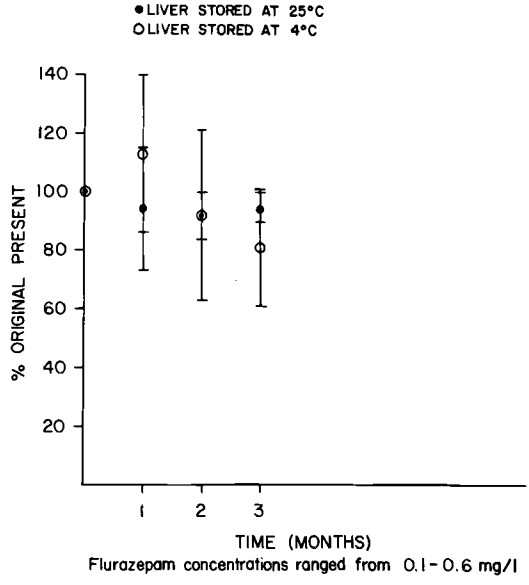


FIG. 12—*Effect of temperature on the storage of tissues containing flurazepam.*

extraction from the aqueous layer after alkalinization. These cleanup steps were necessitated by the presence of neutral substances, especially lipids in brain and liver.

The method of tissue storage deserves some discussion. Specifically, it was decided to cut the tissue into small pieces and place weighed portions in stoppered glass test tubes. There were several reasons for storage in this manner as opposed to keeping the tissue intact or as an aqueous homogenate. Changes in drug concentrations of tissue homogenates may not accurately represent changes in intact tissue. For example, viscosity or ionic strength may influence a drug's stability. Conversely, keeping the tissue entirely intact may present problems for interpre-

tation if there was a lack of homogeneity within the tissue. Storage in the form of small pieces was felt to be the best compromise. Also by weighing the portions initial effects of dehydration were negligible.

The results indicate that diazepam stored in blood for up to five months was stable, even at room temperature. Even though the data with flurazepam indicate slight decrease with time at 4 and 25°C, it nonetheless appears to be moderately stable under these storage conditions. On the other hand, chlordiazepoxide is unstable especially at room temperature. In a solution originally at 5-mg/L chlordiazepoxide, the drug was not detectable within one to two weeks. Even at refrigerated temperatures, a decrease was observed initially before a leveling off occurred. The norchlordiazepoxide data were qualitatively similar, although the time until complete disappearance of the compound was slightly longer.

This instability of chlordiazepoxide observed in blood has previously been reported in aqueous solutions. The mechanism of chlordiazepoxide breakdown in water is given in Fig. 13 [3]. In the experiments reported here, the formation of demoxepam was observed. Furthermore, a standard of the benzophenone was obtained and a methanolic solution was injected into the liquid chromatograph to obtain its retention time. At no time during the analyses of chlordiazepoxide and norchlordiazepoxide was a peak with a retention time corresponding to the benzophenone observed (< 0.3 mg/L).

One compound produced during the aging process from chlordiazepoxide and norchlordiazepoxide that was not reported to be formed in water, is nordiazepam. This involves the loss of the nitrone oxygen. A two-step mechanism for the degradation of chlordiazepoxide and norchlordiazepoxide in blood is suggested (Fig. 14). The initial step may be a temperature dependent, chemical breakdown of the original compound in an aqueous medium to demoxepam. The demoxepam may then be converted to nordiazepam, possibly by a reductase enzyme produced by microorganisms in the putrefractive process. Further evidence for this proposed

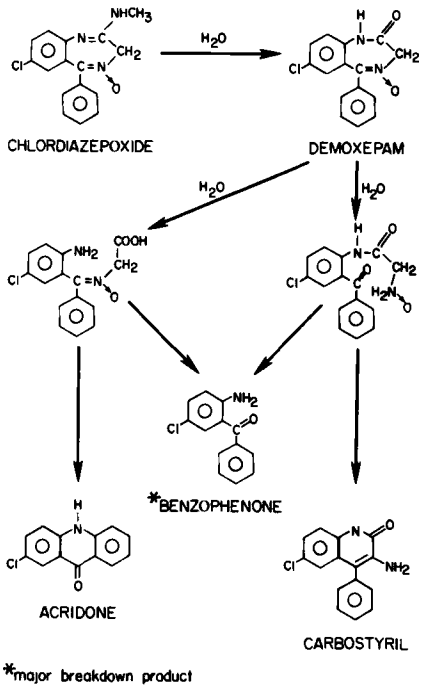


FIG. 13—*Chemical degradation of chlordiazepoxide in water.*

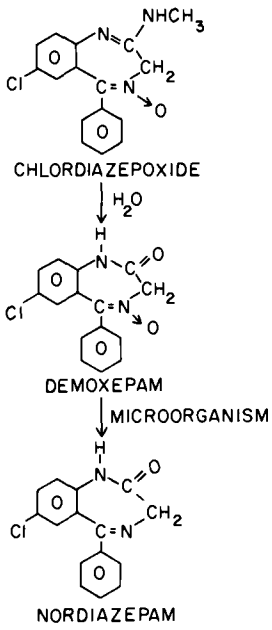


FIG. 14—*Proposed mechanism for breakdown of chlordiazepoxide in blood.*

mechanism is suggested by the norchlordiazepoxide data. In the blood solution not containing F^- and $C_2O_4^=$, demoxepam was rapidly converted to nordiazepam; in the presence of F^- and $C_2O_4^=$, the conversion of demoxepam to nordiazepam was delayed, causing the observed increase in demoxepam concentration. The fact that the presence of F^- and $C_2O_4^=$ slowed the conversion of chlordiazepoxide to demoxepam suggests that the conversion to demoxepam might include both chemical and microorganism-induced steps.

The decay pattern observed with chlordiazepoxide and norchlordiazepoxide at room and refrigerated temperatures has been previously observed with clonazepam, another benzodiazepine. Knop et al reported that a plasma clonazepam concentration of 25 μg/L disappeared within six days when stored at 20°C. Approximately 85% of clonazepam originally present remained after 20 days of storage at 1°C; about 95% remained after the same length of time when stored at −20°C [*4*].

The data from the tissues corroborate many of the results from the blood data. Diazepam, for example, was found to be stable in tissues as well as blood. Nordiazepam appeared to decrease with time when stored at room temperature, both in blood and in tissues. However, one major shortcoming in these experiments was the lack of tissues containing chlordiazepoxide.

It was found that the coefficient of variation within a homogenate was 15%. This can explain some of the large variations seen in Figs. 10 and 12. Several explanations can be offered for the relatively large coefficient of variation within a homogenate: (1) lack of homogeneity of the homogenate, (2) contamination of the tissue with surrounding blood, or (3) difference in drug distribution within a tissue. Intratissue differences in drug distribution are known for certain chemicals. Similar distribution studies have been done with benzodiazepines. Placidi et al [*5*] found severalfold differences in the concentration of diazepam and its three major metabolites in the different lobes of the brain and in the white and gray matter. Similar findings with chlordiazepoxide and diazepam were also observed by Van der Kleign [*6*] in mice. Moreover, the mice livers also demonstrated an unequal pattern of distribution after intravenous administration of diazepam and chlordiazepoxide. This has been observed with imipra-

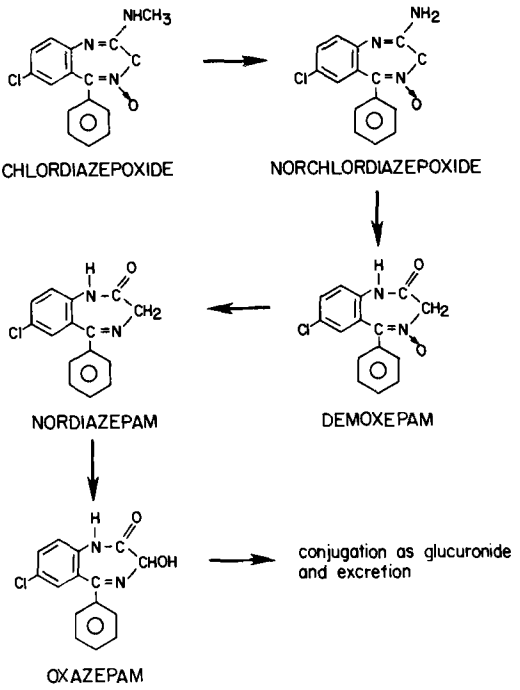


FIG 15—*Metabolism of chlordiazepoxide.*

mine [7] and barbiturates [8]. These animal studies indicate the importance of using a large mass of tissue when obtaining an "average" drug concentration in a tissue. Because of the relatively small amount of tissue available for the studies reported here (about 100 to 150 g), and because of the multiple data points required, only 10-g portions could be used for each quantitation.

Many implications arise from the results reported in this paper, both for clinical and forensic science laboratories. Acquisition and analysis of specimens should always be done promptly. However in the case of chlordiazepoxide greater care is required than with other drug-containing specimens. Chlordiazepoxide plasma or whole blood controls should be stored in the freezer until they are required. Chlordiazepoxide standards should be prepared in alcohol, stored in amber bottles, and periodically monitored for the presence of demoxepam. For example, in this laboratory, it was found that approximately 5% of a methanolic standard of chlordiazepoxide was converted to demoxepam over a six-month period. Norchlordiazepoxide was found to be even more unstable. Quantitation of chlordiazepoxide and its metabolites should be performed as rapidly as possible. When specimens are sent to other laboratories, it is preferable that they be shipped frozen or in refrigerated containers. In forensic science cases, where analyses are not performed as quickly as in clinical situations, it is recommended that the specimens contain a chemical preservative and be stored in the freezer until the time of analyses. Moreover, the interpretation of chlordiazepoxide and metabolite concentrations in postmortem cases becomes much more difficult when there is a time lag of greater than several days between death and specimen acquisition. The data show that an initial chlordiazepoxide concentration of 5 mg/L, a concentration considered toxic, can rapidly decrease within days to a concentration that could be considered therapeutic if the body remains at room temperature. Furthermore, if the body remains at room temperature for too long, then nordiazepam would be detected. Specific care must also be exhibited in interpreting concentrations of demoxepam

and nordiazepam; not only are they metabolites of chlordiazepoxide (Fig. 15), they are also breakdown products of chlordiazepoxide in blood.

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