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POSTMORTEM STABILITY OF DRUGS IN BLOOD AND TISSUES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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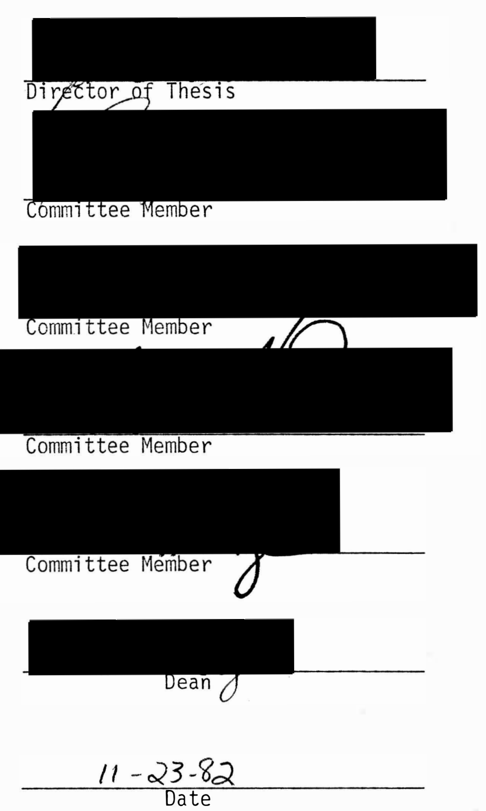

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ABSTRACT

Title of Thesis: POSTMORTEM STABILITY OF DRUGS IN BLOOD AND TISSUES

Barry Steven Levine, B.S.

Medical College of Virginia-Virginia Commonwealth University, 1982

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Dr. James C. Valentour

The delay that occurs between specimen acquisition and analysis in clinical and forensic toxicology requires the establishment that no changes in drug concentration have occurred during this timed interval. In this research, several groups of common drugs were examined for stability in serum, blood and tissues at room temperature and at 4°C. Specifically, benzodiazepines, barbiturates, lidocaine, procainamide and nortryptyline were studied. Decreases were found in the following drugs: chlordiazepoxide, norchlordiazepoxide, demoxepam, and nitrazepam. No changes were found in the other drugs. More detailed work was performed on chlordiazepoxide (CDP). Two breakdown products, demoxepam and nordiazepam (ND) were identified and quantitated. A series of experiments at various pH's in the presence and absence of fluoride/oxalate ($F^-/C_2O_4^=$) were undertaken to examine chemical and microorganism effects on CDP breakdown in blood and buffer. At pH5, the rate of CDP breakdown was the same in blood and buffer and no nordiazepam was formed in either medium. This was determined by comparing the slopes of ln(CDP) vs. time for each condition; the slopes were about -.06 for each. All slopes are in $(days)^{-1}$. At pH

6.5 and pH 8, in the absence of $F^-/C_2O_4^=$, CDP was less stable in blood than buffer (slopes of -.13 and -.27 versus -.036 and -.039) and ND was formed in the blood. At these pH's, $F^-/C_2O_4^=$ stabilized CDP in blood such that it was more stable than buffer (slopes of -.0047 and -.0066 versus -.038 and -.036).

INTRODUCTION

I. OBJECTIVE AND SIGNIFICANCE

The basic hypothesis of this research is that drugs vary in their stability in blood and tissues after death and various factors can contribute to this variability. This problem can be studied under two conditions: 1) *in situ* - when the blood and tissues are in the body; or 2) *in vitro* - after specimens have been removed from the body. It was decided that the second condition would be studied in this research; not only could more data about a larger group of drugs be obtained, but the practical difficulties in retaining human intact cadavers could be avoided. Once it was decided to study *in vitro* stability of drugs, the next step was to design protocols to study the problem in blood and tissues. This frequently would involve the development of methods to enable the quantification of the drugs in decomposed specimens. Drugs encountered in forensic and clinical toxicology laboratories would be examined initially. Then other drugs would be studied such that relationships between drug stability and functional groups could be made. Chemical and microorganism contributions to drug instability would be examined in those cases where an initial screening indicated drug instability.

Information generated in this research has applications in both the forensic and clinical toxicology laboratories. Ideally, all medical examiner and coroner cases should be autopsied within hours after death

and when the situations dictate, the related toxicological analyses should be performed as rapidly as possible after receipt of the specimens. However, in the real world, this rarely occurs. A more likely scenario is that an autopsy is performed within 48 hours of a death and the toxicological analyses may occur several days to several weeks or even months after sample acquisition. In light of this, certain precautions are undertaken to ensure the integrity of the specimens. For example, tissue specimens are stored in freezers at -20°C between sample receipt and analysis. For blood, besides its storage at 4°C, chemicals such as sodium fluoride and potassium oxalate are added to inhibit microorganism growth and to prevent blood coagulation. Furthermore, there are those occasional forensic cases requiring toxicologic analysis involving bodies found weeks or months after death. Even clinical samples may be sent to other laboratories, often times by mail under non-refrigerated conditions. The point is that for whatever reason, toxicologic tests are frequently performed some time after specimen acquisition. Since the results of these tests in forensic cases can become involved in criminal or civil litigation, it is important that the results be an accurate indication of events at the time of death. In other words, it is important to know if changes in toxicant concentration between death and analysis have occurred.

Even though a relatively small number of toxicants have been analyzed for postmortem stability, there is sufficient evidence to suggest that changes in concentration can occur over relatively short periods of time. Therefore, it is important that the toxicologist be cognizant of these changes. By initially studying classes of compounds, the greatest amount of information can be obtained in the shortest

amount of time. Of course, there are structural differences within a given class of compounds and these differences can provide the basis for further research when differences in stability within a given class are found.

II. BACKGROUND

Many factors can affect the postmortem blood and tissue concentrations of drugs. In their book, Gonzales _et al_. (1) discussed many of these factors. They included: 1) the entrance of microorganisms into the blood from the gastrointestinal tract; 2) external conditions such as temperature, humidity, or the surrounding medium - air, water, soil, etc.; 3) the presence of other poisons; and, 4) cellular disintegration leading to the release of degradative enzymes. Parker _et al_. (2) enumerated other influences on the postmortem quantification of drugs, including postmortem diffusion of the drug, presence or absence of gastric contents, and postmortem body position.

To this point, some work has been done on the effect of these conditions on postmortem concentrations of drugs and other toxicologically important chemicals. The following is a review of the work done to date on blood and tissues.

A. _Anticonvulsant drugs and barbiturates_.

Attempts to quantify the effects of putrefaction on tissue concentrations of barbiturates date back to 1942 (3). However, due to limitations in methodology, the first meaningful quantitative results were not produced until 1957 by Algeri (4). 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 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 (5) 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-humidity, temperature, etc.

Postmortem effects on sodium secobarbital were studied by Parker _et al_. in 1971 (2). Groups of rats were administered lethal

doses of the drug; 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 hours respectively following death. A UV spectrophotometric method (6) was used for analysis. The concentrations of secobarbital in postmortem rat livers increased greatly during the first 24 hours and increased to a slightly higher level after 48 hours. A slight decrease in secobarbital concentration was observed at 72 hours in comparison to the 48 hour 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 9 days, analysis of the liver indicated significant concentrations of the drug. This indicated a significant postmortem diffusion from the stomach. This postmortem diffusion was found to be independent of the position of the body.

Sunshine and Hackett (7) 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*. (8) observed a 20% decrease in plasma phenytoin concentrations when stored at 4°C for eight weeks. However, Schäfer (9) reported no decrease in the concentrations of phenytoin, phenobarbital, or primidone when stored in serum at 4°C for about 12 weeks. Although Pippenger *et al*. (10) found significant bacterial growth in pooled serum samples sent by mail, no significant changes in phenytoin, phenobarbital, primidone, and ethosuximide concentrations were found. Wilensky (11) found no changes in the concentrations of mephenytoin, phenobarbital, primidone, and phenytoin after 6 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; (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 appears to be conflicting data concerning the stability of anticonvulsant drugs and barbiturates in blood and in tissues. One possible explanation for these conflicts is that much of the data were collected using UV spectrophotometry for quantitation. Not only is this less sensitive than chromatographic methods, it is also

subject to more frequent interferences from decomposition products than are chromatographic methods.

B. Carbon monoxide (CO)

The first quantitative work on postmortem effects on carbon monoxide concentration was published in 1940 by Gettler and Freimuth (12). Previous qualitative work by Weimann (13) demonstrated that in two fatalities caused by CO poisoning, exhumations of the bodies over a month later revealed that CO was still found in large amounts in the blood. Gettler and Freimuth exposed human cadavers to CO in a sealed container. After examining heart blood using a modified Van Slyke procedure (14), they found that CO does not reach the heart even after the body is exposed to CO for 42 hours. This implies that there is no postmortem absorption of CO. Their next experiments involved the analysis of blood samples with normal CO content to determine whether the putrefaction process produced any CO. Specifically, CO content of an aliquot of blood standing at room temperature was compared to another aliquot of the blood kept in a refrigerator. Even after 12 weeks, no significant amount of CO was produced during putrefaction. This study was expanded to include abnormal levels of CO. In eight of the ten cases reported, with varying degrees of time after exposure and varying amounts of CO, a loss in CO content and CO capacity was found. In the other two cases, there was no change in CO content or CO capacity.

Dominguez et al. (15) used dogs to study the effect of post-mortem changes on carboxyhemoglobin (CO-Hb) levels. After exposure to 1% CO in air for 25-45 seconds, the animals were submerged in offshore waters at 29.5°C in marine plywood containers. After four days, liver,

kidney, lungs, heart and any sanguineous fluid present in the thoracic cavity were obtained. These specimens were treated in one of two ways: 1) specimens were kept frozen until analysis; or 2) specimens were frozen until at least 24 hours prior to analysis when it was allowed to thaw at a temperature of about 25°C. Gas chromatography was used for analysis. When rigid preservation measures were employed, no increase in CO levels were observed; this was true whether normal or slightly higher than normal CO levels were present. However, significantly elevated CO levels were found in the samples which thawed in advance of the analysis. This *in vitro* formation of carbon monoxide had also been reported by Sjöstrand (16) who hypothesized that one molecule of CO is released when each of the porphyrin rings in the hemoglobin molecule splits. It was concluded by Dominguez *et al*. that the amount of carboxyhemoglobin is not significantly altered when 1) the specimens are frozen until examined and 2) the blood extracted from tissue is quickly analyzed for carboxyhemoglobin.

Markiewicz (17) also stressed the importance of the conditions under which the sample containing carboxyhemoglobin is stored. Blood samples, obtained 24 hours after death and containing various amounts of carboxyhemoglobin were allowed to stand at room temperature for varying lengths of time. A modified Wollf procedure (18) was used for quantitation. Increasing concentrations of carboxyhemoglobin were observed from one to six days; after this increase, decreased levels were observed. Increased carboxyhemoglobin also appeared in blood stored in a refrigerator, but this took longer to occur in comparison to the blood samples at room temperature. Moreover, covering the tube containing the blood with paraffin decreased the magnitude of these effects. Another set of

experiments involved heating the blood to 65°C. An even greater increase in carboxyhemoglobin was found. Studies were also undertaken to examine the conditions which would favor hemoglobin degradation and therefore, increase CO generation. Conditions conducive to increased hemoglobin breakdown included low pH, hemolysis, decreased partial pressure of O_2, decreased catalase activity and the appearance of peroxides.

Recently, Winek and Prex (19) used spectrophotometric and gas chromatographic procedures to study the changes in CO-Hb concentrations in blood after storage for 30 and 150 days. For the most part, greater losses in CO-Hb were observed when gas chromatography using thermal conductivity detection was employed for analysis. The spectrophometric procedure worked well when the blood was fresh, but pigments formed during the decomposition process altered the carboxyhemoglobin and deoxyhemoglobin spectral scans. These interferences are not a problem when gas chromatography is used. The overall loss of CO-Hb might be due to: oxygen competing with CO at the iron heme binding site of hemoglobin; oxidation of carboxyhemoglobin to methemoglobin and a partial loss of CO binding capacity.

It appears that if blood containing CO is permitted to remain at room temperature for long periods of time, changes in CO-Hb concentration are seen. However, if the specimens are stored at reduced temperatures, then no significant changes in CO-Hb concentration occur. Moreover there is no absorption of CO after death.

C. Cardiac glycosides

In 1973, Iisalo and Nuutila (20) used a radioimmunoassay procedure to analyze serum digoxin levels preceding and following deaths in

three fatalities involving an overdose of digoxin. In one case, a greater than three fold increase was observed in the concentration of digoxin in the postmortem serum sample when compared to a premorten sample. One of the other two cases also showed this effect; however, it was less pronounced. An "accumulated absorption" was given by the authors as the cause for this postmortem increase in digoxin concentration. No further explanation of the term "accumulated" absorption was given by the authors.

Selesky et al. (21) studied fifteen fatalities in which there was a history of digoxin use. In the only case in which there was an antemortem and postmortem blood sample, no difference in the digoxin concentration between the two samples was observed. This finding was in agreement with Holt and Bernstead (22) who stated that elevated post-mortem concentrations of digoxin probably reflected elevated levels before death. Both groups used radioimmunoassay for digoxin analysis. Moreover, because they observed a consistently higher level in heart blood than in blood from the femoral vein, they recommended that blood from the femoral vein be used for any retrospective analysis.

To reconcile these discrepancies, Vorpahl and Coe (23) conducted a thorough study to correlate antemortem and postmortem digoxin concentrations. Using samples from 26 autopsies, they found that the average postmortem serum digoxin concentrations was significantly higher in samples obtained from the heart, subclavian vein, and femoral vein than the antemortem levels. Radioimmunoassay was used for quantitation. Furthermore, all postmortem heart serum levels were greater than ante-mortem heart serum levels when the postmortem interval exceeded one hour. Assuming that 2 ng/ml is a toxic serum digoxin concentration, 48%

of the cases would have been diagnosed as victims of digoxin toxicity if the antemortem value was used; this percentage would increase to 89% if the postmortem cardiac serum value was used. It was hypothesized that after death, the equilibrium of drug between blood and tissue changes such that the drug leaves the tissue and enters the nearby surrounding blood. Analysis of vitreous humor showed that in contrast to serum digoxin levels, vitreous humor digoxin levels usually decreased in the postmortem period in comparison to the "antemortem" period. However, when an elevated postmortem vitreous humor digoxin level was found, it was indicative of antemortem toxic serum levels. They concluded that a combination of femoral venous serum and vitreous humor are most indicative of antemortem digoxin toxicity.

Aderjan et al. (24) corroborated much of this work, obtaining blood samples from patients before death and within thirty minutes following death. At autopsy, blood samples from the left ventricle and femoral vein were secured. All of these patients died from causes other than digitalis overdose. Using a specialized radioimmunoassay procedure, they found that in none of these cases were there significant changes in digitalis concentration between the blood obtained prior to death and that obtained thirty minutes after death. However, when these specimens were compared to the heart blood samples obtained at autopsy, six of the eight heart blood specimens were significantly higher than the antemortem blood levels; in the other two cases, essentially no changes were noticed. On the other hand, the femoral vein digoxin concentrations were only slightly increased at autopsy in comparison to the antemortem blood concentrations. As with Vorpahl and Coe, the explanation of Aderjan et al. for their observations was that a new

equilibrium between the blood and tissues was established after death. Among those tissue studied, those mainly involved in this redistribution were the heart and the kidneys.

The data collected on cardiac glycosides clearly indicates that changes in blood concentrations do occur after death and that the site of acquisition is very important to the interpretation of results. The cause of these changes involve a postmortem redistribution of cardiac glycosides between the heart and nearby blood such that there is a decreased cardiac glycoside concentration in the heart and a greater concentration in the surrounding blood. Since medical examiners usually obtain their blood specimens from the heart, it would be recommended that in those cases where digitalis toxicity is suspected, a blood sample from the femoral vein and vitreous humor also be obtained.

D. Cocaine

Since cocaine has an ester linkage and since there are various esterases in the blood, it would be reasonable to expect that the concentration of cocaine in blood would change in freshly removed postmortem blood. This was suggested by Jatlow and Bailey (25) who using gas chromatography-nitrogen detection found that after 15 minutes and 30 minutes at room temperature, plasma cocaine concentrations decreased by 6.5% and 12.5% respectively. Moreover, the rate of loss of cocaine was approximately doubled at 37°C. Twenty four hours after blood storage at either -15°C or on solid CO_2, varying decreases in blood cocaine concentration to a maximum of 30% were observed. However, when the extract residues were stored in a desiccator at -15°C, no decreases were observed for at least 72 hours. These obervations were made during the development of a sensitive analytical method for the

detection of cocaine in plasma. Divorichik (26) reported that the rate of hydrolysis of cocaine was slowed if blood samples were immediately iced upon collection. Furthermore, by quick-freezing the samples in liquid nitrogen while shaking the tubes, the sample can be stored at -15°C for at least six weeks without any change in cocaine concentration.

Taylor _et al_. (27) incubated serum containing ^{14}C and ^{3}H labeled cocaine at 37°C and determined the metabolites formed using gas chromatography. After one hour, 20% of the cocaine was converted to benzoylecgonine, ecgonine, and ecgonine methyl ester; the ecgonine methyl ester was present in the highest concentration. After four hours of incubation, two-thirds of the cocaine was hydrolyzed; this time, benzoylecgonine was the major breakdown product.

The above findings were disputed by Stewart _et al_. (28) who found no significant cocaine degradation when plasma samples fortified with labeled cocaine were stored for four days at -15°C. They hypothesized that the findings of Jatlow and Bailey could be explained by the alkaline pH (9.8) in which the cocaine was extracted. Their experiments showed that cocaine was rapidly converted to benzoyl ecgonine at that pH. Thin-layer chromatography was employed to separate metabolites. They determined that the conversion of cocaine to ecgonine methyl ester in human plasma occurs enzymatically through the action of plasma cholinesterase. However, there is no detectable enzymatic conversion of cocaine to benzoylecgonine in human plasma. These data would imply that the ecgonine methyl ester should be the only significant metabolite produced from cocaine by human plasma.

The most detailed analysis of the *in vitro* hydrolysis of cocaine in blood was reported by Baselt (29). Using a gas chromatographic method, he found that at a cocaine concentration of .1 mg/L in refrigerated and nonfluoridated blood no detectable amount of cocaine was observed by day 8. However, if the blood was refrigerated and fluoridated with .5% sodium fluoride, 40-60% of the cocaine could be detected three weeks after the addition of cocaine to the blood. A similar pattern was observed when a cocaine concentration of 1 mg/L was used. In refrigerated, fluoridated samples, 80% of the original amount of cocaine was still present. Moreover, the hydrolysis of cocaine was found to be temperature dependent; a greater rate of disappearance of drug was observed in fluoridated blood stored at room temperature as opposed to fluoridated blood stored in a refrigerator.

Because plasma cholinesterase remains active in blood *in vitro*, proper specimen preservation is required to ensure sample integrity. Freezing samples at -15°C immediately upon receipt has been shown to delay cocaine breakdown. Sodium fluoride, an esterase inhibitor as well as a microorganism inhibitor, can also delay cocaine breakdown.

E. Cyanide

Decreases in postmortem concentrations of cyanide (CN^-) in blood and tissues were first reported by Curry (30) in 1963. In one fatality resulting from inhalation of gaseous hydrocyanic acid, a blood sample taken at death showed a cyanide concentration of .35 mg/100 ml while a blood sample taken the next day at autopsy revealed a cyanide concentration of .05-.1 mg/100 ml. A microdiffusion separation technique was coupled with a colorimetric procedure for analysis. There

have been several mechanisms proposed for this postmortem disappearance of cyanide. The most obvious is the conversion of cyanide to the naturally occurring metabolite thiocyanate ion (CNS^-). Ballantyne _et al_. (31) added small amounts of potassium cyanide to human and rabbit serum and after one hour, only between 31 and 44% of this added amount was detected as CN^-. An increase in CNS^- was found, but not enough to completely account for this drop of CN^- levels. However, when these experiments were repeated with whole blood a much slower decrease in CN^- levels were observed. This suggested that CN^- had a high affinity for red blood cells; this was demonstrated by other workers (32, 33, 34). Moreover, they reported that 62-74% of cyanide was still detected in whole blood after 24 hours, apparently in agreement with the observation of Barr (32) that cyanide is 70% bound to red cells. As with serum, only a percentage of the cyanide not accounted for appeared as thiocyanate. Proposed mechanisms for decreases in cyanide concentration included a hydrolysis reaction to form ammonium formate (35) and conversion to aminomalonotrile (36, 37, 38, 39).

Further work by Ballantyne and coworkers (39) illustrated the differences between removal of blood immediately after death and stored versus removal of blood at various times after death. They demonstrated that significant amounts of cyanide were detected at three weeks when the blood was removed at death and stored at 10-15°C. When the blood remained in the body until analysis, the amount of cyanide detected in blood decreased during the first week after death such that only about 30% of the amount of CN^- present at death was detected as CN^-. By the end of the second week, less than 3% of the CN^- originally present was detected; by the end of 21 days, CN^- was barely detected. They

combined a nitrogen aeration method for separation with spectrophotometry for CN^- quantitation. They hypothesized that these differences were probably due to autolysis and putrefaction taking place in the intact body, thus providing many possible routes of transformation for cyanide. These researchers suggested that spleen is the tissue of choice for the analysis of cyanide when there has been some time between death and the acquisition of specimens.

Naroyanaswani *et al*. (40) added CN^- to the stomach and followed its disappearance with time at room temperature. Quantitating by a UV spectrophotometric procedure, they found that there could be a 60% decrease in CN^- concentration within 2 hours after addition to the stomach. The pH of the stomach was 6.5. After 20 days, approximately 80% of the added CN^- was not detected. When the pH of the stomach was 8.0, only 10% of CN^- disappeared after 20 days. The authors recommended the storage of visceral tissues in alkaline medium to prevent the loss of CN^- from postmortem samples.

Besides these observations of decreased concentrations of cyanide with time, increases in cyanide concentration have also been observed. Bernt *et al*. (41) found that blood and gastric contents not containing cyanide yielded significant amounts of cyanide when analyzed two to four weeks after storage at 4°C. However these findings were not duplicated when the blood and gastric contents were incubated for the same length of time at 37°C. Gettler and Baine (42) used colorimetry and found insignificant postmortem production of cyanide at room temperature. Curry *et al*. (43) did exhaustive experiments using visible spectrophotometric methods to clarify the postmortem synthesis of cyanide. Among their results were the following: 1) cyanide production

can occur in blood even when no bacteria were present in the blood; 2) specimens which were sources of postmortem cyanide production included brain, liver, kidney, uterus, gastric contents, and intestinal contents; 3) fluoride inhibited the production of cyanide in blood; 4) blood enzymes and *Pseudomonas pyocyaneus* were the probable causes of cyanide production; and 5) the blood enzymes worked better in the production of cyanide at 37°C while the bacteria produced more cyanide at 4°C than 37°C.

To summarize, the majority of the data suggests that decreases in cyanide concentration occur in postmortem blood and tissues. Some of the cyanide could be converted to thiocyanate or lost as hydrocyanic acid (HCN). An important fact to consider is the pH of the specimen; in an acidic medium, more of the cyanide would be lost as HCN.

F. *Ethanol*

It is not surprising that much of the work that has been done on postmortem changes in drug concentrations has been done on ethanol. A review of postmortem changes in ethanol concentration was written by Parker (44) in 1974 while Corry (45) devoted part of her review on sources of antemortem and postmortem ethanol to discuss the postmortem production of ethanol.

Evidence for postmortem production of ethanol dates back to 1935 when Nicloux (46) found that mice which had no ethanol before death produced as much as 100 mg/100 g of ethanol after death. The actual amount of ethanol produced depended on the temperature and condition of storage of the bodies. Nicloux (47) also published the earliest work done with human corpses; in three cases in which there was no evidence to suggest that the people involved consumed alcohol prior to death.

Concentrations of ethanol in tissues ranging from 40-132 mg/100 g were found. The method of analysis used by Nicloux involved triple steam distillation with various clean-up steps to remove other volatile materials.

After Nicloux, several other researchers have studied this effect using animals. In 1971, Narikawa and Kotoku (48) used gas chromatographic methods to obtain the following results: 1) a maximum of 100 mg/100 ml ethanol was produced in the intraperitoneal fluid of mice incubated at 30°C in air for 4-5 days; 2) greater postmortem ethanol concentrations were found when glucose was injected into mice before death; 3) using rabbit corpses, they found that after 7 days at 15°C, ethanol concentrations were higher in pleural fluid and cardiac blood than in femoral vein blood and vitreous humor. Davis _et al_. (49) determined that even under sterile conditions, ethanol was detected in brain, liver, and lung of mice after 5 days. However, no ethanol was found when germ-free mice were used, implying that postmortem ethanol production was due to microorganisms. Iribe _et al_. (50) confirmed the work of these two groups and found that rats burned to death showed less ethanol production than did control rats.

Redetzki _et al_. (51) used enzymatic methods of analysis to demonstrate that smaller amounts of ethanol were produced postmortem in humans in the extremities than in the deeper sites. This was verified by Wolthers (52) who said that smaller amounts of newly produced ethanol in the periphery were probably related to the amount of available oxygen and to slower cooling of tissues in the extremities. Pleuckhahn (53) gave a range of ethanol concentrations in human heart blood from eight putrefied bodies of 33-127 mg/100 ml; in half of these cases, it was

known that the individual did not consume ethanol before death. Moreover, the concentrations of ethanol in femoral blood was lower than in blood from a deep tissue, the heart. Gonzales et al. (1) observed an increased ratio of liver ethanol concentration to brain ethanol concentration in putrefied bodies that were submerged as opposed to bodies putrefying in air. Gormsen (54) reported that in an autopsy of a person not consuming ethanol prior to death, a blood concentration of 20 mg/100 ml and gastric concentration of 230 mg/100 g were found 20 hours after death. Various fungi and yeast were observed to be present also. In a subsequent paper (55), Gormsen stated that unless alcohol is found in urine as well as in blood and tissues, any alcohol concentration detected is meaningless in determining whether alcohol was present prior to death. O'Toole et al. (56) ascertained that under sterile conditions, human body tissues remained free of bacteria and fungi for at least twenty hours after death. Moreover, they suggested that postmortem ethanol production is caused by perimortem infections or other contaminations rather than microorganisms existing in the body before death.

Clark and Jones (57) studied postmortem ethanol production in 26 human cadavers which were refrigerated within 4 hours after death. In no blood was an ethanol concentration greater than 100 mg/l found at autopsy (3-27 hours after refrigeration); this was true whether the blood gave a positive or negative blood culture.

Besides the work done on intact animal and human bodies, many researchers have studied postmortem production of ethanol in isolated tissues. Freimuth et al. (58) allowed isolated human blood, brain, and liver not previously containing ethanol to sit at 20-26°C for thirteen

days. The lowest amount of ethanol was produced in the blood; the greatest amount of ethanol was produced in the brain. Moreover, refrigerated samples produced less ethanol. This latter finding was confirmed by Gonzales *et al*. (1). More of these types of data have been subsequently reported (59-62).

Plueckhahn and Ballard (63) attempted to isolate some of the microorganisms which were involved in the postmortem production of ethanol. Among those organisms which were able to ferment glucose and other reducing substances were *E*. *coli*, *Streptococcus fecalis*, *Proteus sp.*., and *Candida sp*. However, only aerobic organisms were isolated in this study. Blackmore (64) isolated those organisms that produced ethanol from the bodies of crew members from a crashed airplane. Moreover after isolation, these organisms were added to media containing carbohydrates and amino acids to determine the ability of each organism to produce ethanol.

Blood, unlike other tissues, can have chemicals added to it to delay or prevent postmortem changes. Several studies have been undertaken to study the ability of various chemicals to prevent changes in alcohol concentration. In 1965, Glendening and Waugh (65) used sodium fluoride and potassium oxalate as preservatives and found no significant postmortem production of alcohol in three months. Test tubes sealed with black-rubber stoppers were used to store the blood. Bradford (66), using sodium nitrate and mercuric chloride as a preservative, found no change in alcohol concentration over six months. However, if sodium fluoride was used instead of mercuric chloride, both increases and decreases in blood alcohol concentration were observed. Pleuckhahn (53) stated that in blood samples obtained from an autopsy, no less than 5 mg

of sodium fluoride per milliliter of blood should be added for proper preservation. Furthermore, samples must be stored below 6°C if the analysis is not done within twenty-four hours following collection. Russel (67) observed decreases in blood alcohol concentration with .75% w/V potassium oxalate, both in the presence and absence of .25% w/V sodium fluoride. Blood samples were stored at 4-6°C for up to five weeks. However, the samples not containing fluoride showed greater decreases in alcohol concentration. Hayden et al. (68) found upon reanalysis of 200 blood samples stored at 4°C for 30 to 50 days, no significant changes in blood alcohol concentration occurred. Fluoride and oxalate were used as the preservative. Some samples analyzed after refrigerated storage for one year showed no significant changes in alcohol concentration.

Brown et al. (69) studied the stability of ethanol in stored blood using three parameters-temperature, fluoride concentration, and time. They interpreted their observed losses of ethanol in terms of three mechanisms: 1) temperature-dependent oxidation of ethanol to acetaldehyde; 2) microorganism destruction of ethanol in the absence of fluoride, and which could be prevented with .5% w/V sodium fluoride; and 3) diffusion from polypropylene cups used for storage of blood in Great Britain.

Postmortem diffusion of alcohol from tissues to surrounding blood has been studied by several researchers. In 1949, Bowden and McCallum (70) reported diffusion from the stomach to heart blood, but there were some problems with their data. In 1956, Gifford and Turkel (71) introduced 86 proof whiskey into the stomach of cadavers between 1 and 55 hours following death. The bodies were then autopsied between 10

and 24 hours. later. A blood sample was taken from the femoral vein and from various parts of the heart before and after the introduction of alcohol. Significant diffusion of alcohol from the stomach to the heart was observed; no change in alcohol concentration was observed in the femoral vein. In a subsequent study (72), blood samples were obtained from the femoral vein and from the heart in 75 autopsies. Of the 51 cases which showed the presence of alcohol in the blood, 35 of these had cardiac blood with a higher alcohol content than femoral blood, presumably due to postmortem diffusion of alcohol from the stomach. From their findings, they suggested that in postmortem cases, blood from the femoral vein should be used to obtain a blood alcohol concentration at the time of death. Pluekhahn (53) did similar experiments with over 200 cadavers and collected blood from various sites throughout the body. Postmortem diffusion of alcohol was found to occur from the stomach into the pericardial fluid and the pleural fluid within several hours after death. However, alcohol concentrations in blood from the intact chambers of the heart remained the same for up to 48 hours following death.

In conclusion, there is a large amount of data illustrating the postmortem production of ethanol by a wide range of microorganisms in blood and in tissues. This microorganism activity can be inhibited by the addition of one of several chemicals to the blood. When analysis of blood for ethanol was performed in the absence of these chemicals, it is important to consider ethanol concentrations in urine, vitreous humor, or tissues to ensure proper interpretation of the blood ethanol concentration.

G. Methaqualone

Kaeferstein (73) studied the effects of putrefaction on tissue concentrations of methaqualone using gas chromatography, Methaqualone concentrations decreased by 28% in blood, kidney, and brain after 26 months of laboratory storage following removal from a fresh cadaver. Refrigerated storage of the tissues retarded these changes. A brain homogenate stored at 30°C for 9 weeks showed no change in drug concentration.

H. Methylphenidate

In vitro hydrolysis of methylphenidate to α-phenyl-α-piperidine acetic acid in urine was studied by Schubert (74). Using thin layer and gas chromatography he found that when the urine was stored at 21°C, only 32% of the methylphenidate added to the urine remained intact while 74% of the methylphenidate was unchanged when the urine was stored at 4°C.

I. Narcotics

It had been known for a long time that *in vitro* deacetylation of heroin to monoacetylmorphine occurs very rapidly in blood (75-77). Nakamura *et al.* (78) studied *in vitro* hydrolysis of heroin in human blood to determine the rate of degradation and production of human metabolites. Fresh blood and serum obtained from a normal male subject, and postmortem, heparinized whole blood were used in the experiments. To these phosphate buffered (pH 7.4) liquids were added known amounts of heroin hydrochloride and incubated at 37.5°C. At various time intervals, aliquots of the serum or blood were removed and analyzed for heroin and its metabolites by gas chromatography. Hydrolysis of heroin occurred twice as fast in whole blood than in serum; the half-life in whole blood was 9 minutes while it was 22 minutes in serum. Moreover, in serum, no cleavage of 6-monoacetylmorphine was observed while in whole blood, the hydrolysis continued, resulting in the formation of morphine. Nonetheless, in both serum and whole blood, the concentration of 6-monoacetylmorphine was relatively large, suggesting that postmortem blood samples should be examined for monoacetylmorphine in heroin overdoses.

Garrett and Gürkan (79) used dog plasma to obtain further information on the hydrolysis of heroin. In the absence of a preservative, the apparent half-life of heroin was 8 minutes. The addition of 10 mg of sodium fluoride increased the half-life to 40 minutes; tetraethyl pyrophosphate also increased the half-life of heroin. When heroin was added to plasma kept at room temperature for twenty-four hours, no significant loss of heroin was observed thus indicating that the enzyme system involved is rapidly inactivated at room temperature.

Garrett and Gürkan confirmed the findings of Nakamura et al. in that the major product observed in the plasma was 6-monoacetylmorphine.

The enzyme system responsible for the hydrolysis of heroin was elucidated by Smith and Cole (80). It had been previously determined (76,77) that cholinesterase probably was not involved in the degradation of heroin. They concluded that an arylesterase, one of a group of esterases which hydrolyze phenolic esters only, was responsible for the hydrolysis of heroin in human plasma. Furthermore, since only 30-35% of heroin hydrolysis occurred in the plasma, there were esterases present on the outer surface of the red blood cells to account for the remaining ability to hydrolyze heroin.

Fransioli et al. (81) studied the detectability of methadone and propoxyphene in frozen and formalin-fixed tissues at various intervals following autopsy. Using EMIT[R] and gas chromatography for quantitation, they examined brain, kidney, liver, lung, and spleen. In general, decreases in drug concentration over a two year span were observed in all tissues. However in those tissues in which drug was originally found, it was still detected after this time period. Since this study was retrospective in nature, the original tissue concentrations were not obtained.

Bednarczyk et al. (82) noted a greater than 2.5 fold increase in the blood concentration of propoxyphene and a four fold increase in norpropoxyphene when comparing premortem blood concentrations and postmortem blood concentrations. Even though the patient was on a respirator for over two days after ingestion before death occurred, the authors ruled out the possibility of continued drug absorption following hospitalization. Instead, they suggested that during the "induced state of

simulated life", organ necrosis caused the release of drug originally bound to tissues. Furthermore, emphasis was placed on the toxicologic interpretation of postmortem samples when the victim was on a respirator for a long period of time.

J. Pancuronium

Wingard et al. (83) studied the effect of storage on a serum solution of .5 mg/l pancuronium. Employing a fluorometric procedure, an initial analysis of the serum solution was made. Samples from this solution were removed, frozen, and analyzed at various time intervals following freezing. They observed that fluorescence readings were significantly lower within a twenty-four hour period. The authors did not know whether this change in fluorescence was due to drug instability or to some other factor. Poklis and Melanson (84) studied the effects of storage on pancuronium concentrations in postmortem blood, serum, and urine. Samples were stored in a refrigerator and aliquots were removed and analyzed over a 10 day period. While urine showed slight decrease over a week period, a sharp decrease in the serum concentration of pancuronium was observed. Decrease in the postmortem blood concentration of pancuronium was dramatic, but inconsistent. The fact that decreases were observed in serum and blood, but not urine, suggested to the authors that irreversible binding of the drug to blood components had occurred. Nonetheless, they concluded that use of the blood for identification of the drug is useful. They recommended the analysis of pancuronium be done as quickly as possible upon receipt of the specimen.

K. Pesticides

Kyofuji (85) examined the postmortem stability of various groups of pesticides in many biological fluids and tissues. Basically,

he found that organic phosphate pesticides such as parathion, diazinon and malathion, showed rapid decreases in tissue concentration to the point where only 7-16% of the orginal concentration remained after 7 days. Moreover, endrin and ruberon concentrations decreased by 65-70% over a week period. Conversely, other organic chlorine pesticides, such as hexachlorocyclohexane and pentachlorophenol, were more stable over the same length of time. Tsunenari et al. (86) studied the postmortem stability of paraquat in rat tissues. He found a decrease within paraquat concentration in the stomach and cecum while a postmortem rise in paraquat concentration was observed in the liver. In all of the above experiments, the tissues remained in the cadaver until analysis. The authors hypothesized that decreases in paraquat concentration in the GI tract were due to its chemical degradation by bacteria produced or activated during the putrefaction process. Alternatively, postmortem diffusion from the stomach to the liver through the portal vein could explain both the decreases observed in the stomach and increases observed in the liver. Increases in liver paraquat concentration could also be due to release of chemical from tissue binding sites.

L. Phencyclidine (PCP)

Clardy and Ragle (87) examined the stability of PCP stored in blood containing sodium fluoride as a preservative and potassium oxalate as an anticoagulant. After an initial quantitation by gas chromatography-mass spectrometry, the bloods were stored for a time in the refrigerator and then at room temperature. No changes in PCP concentrations were found in the blood samples after 18 months.

M. Theophylline

Jonkman *et al*. (88) studied the stability of theophylline in serum, plasma, and saliva for 1 week at 6°C and 25°C and for 6 months at -20°C. In all cases, they found less than 7% decrease in theophylline concentration in these fluids. Their assay included extraction of blood and analysis by liquid chromatography. From these results, they concluded that storage or transportation of theophylline containing samples for a few days cannot significantly alter the reported value.

N. Tricyclic antidepressants

Bandt (89) presented a paper concerning the reliability of postmortem serum tricyclic antidepressant concentrations at various times after death. Antemortem concentrations were compared to postmortem concentrations using a gas chromatography-electron impact mass spectrometry method for analysis (90). Of the nine cases discussed, eight were found to have highly significant increases in the serum drug concentration as the postmortem interval increased. There was some evidence to suggest that this same effect occurred in vitreous humor, but the rate of change of concentration of tricyclic antidepressant was more gradual. As a mechanism for these changes, the author proposed that following death, the decreasing pH of the body changed the equilibrium between bound and free drug in tissues such that less drug was bound to tissues. This free drug worked its way into the extracellular fluid by passive diffusion. It was concluded that when determining the relationship between tricyclic antidepressants and death, it was necessary to obtain a blood sample as close to death as possible; when this is not feasible, factors such as history, liver

concentrations, gastric concentrations, and the ratio of parent drug to its active metabolite in the liver must also be considered.

O. Summary

The above discussion illustrates that although a relatively small number of drugs have been analyzed for stability in blood and in tissues, there is sufficient evidence to suggest that changes in drug concentration as a function of time do occur. It is believed that this research will increase or improve the current knowledge on the subject in several ways. Firstly, this research will establish protocols where these questions can be examined in an organized manner. Moreover, drugs will be examined which have not been studied previously, such as benzodiazepines and lidocaine. Furthermore, it will examine barbiturates, a group of drugs in which there is a conflict in the previously reported data. One of the strengths of the work proposed is that the drug analyses are performed using chromatographic methods; one problem with much of the previously published data is that the different analyses used spectrophotometric procedures for quantitation; these procedures lack the sensitivity and specificity of chromatographic procedures.

EXPERIMENTAL

I. MATERIALS

A. Standards

1. Benzodiazepines. Diazepam, nordiazepam, flurazepam, N-1-desalkylflurazepam, chlordiazepoxide, norchlordizepoxide, nitrazepam and demoxepam were donated by Hoffman-LaRoche Co., Nutley, N. J. 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.

2. Oxybarbiturates. Free acids or sodium salts of the following barbiturates were obtained from the Medical College of Virginia Hospitals Toxicology Laboratory: amobarbital, butabarbital, pentobarbital, phenobarbital, secobarbital. Methanolic standards of concentration 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.

3. Thiopental. Five grams of sodium thiopental was dissolved in HCl and extracted with methylene chloride. The methylene chloride layer was separated and evaporated to dryness. The purity of the solid was determined by dissolving a known amount of solid in a known volume of .5N NaOH and then, obtaining a UV scan. The absorbance at 305 nm and the $A^{1\%}_{1cm}$ (930) were used to calculate the purity. A 200

mg/l methanolic standard was then prepared. The internal standard was 1 mg/ml phenolphthalein in methanol.

4. Lidocaine (USP-NF) and mepivicaine (USP-NF). 200 mg/l aqueous solutions.

5. Nortryptyline (USP-NF). 200 mg/l aqueous solution of the free base.

6. Procainamide (Squibb injector vial). 93 mg/l methanolic solution of the free base.

7. Disopyramide (G.D. Searle and Company). 200 mg/l aqueous solution of the free base.

B. Reagents.

1. Buffers

a. Borate solution (pH 9.2). 14 g sodium borate (Fisher S-249) in 200 ml distilled water.

b. Phosphate buffer (pH 11). 1.0M sodium phosphate monobasic (pH 4.3) added to 1.0M sodium phosphate tribasic to bring the final pH to 11.

c. Phosphate buffer (pH 5.5). Equal amounts of .5M KH_2PO_4 and .5M Na_2HPO_4 (17.0 g of KH_2PO_4 diluted to 250 ml with distilled water and 17.7 g of Na_2HPO_4 diluted to 250 ml distilled water). The solutions are mixed to bring the final pH to 5.5.

2. Solvents

a. Methylene chloride, toluene, hexane, isoamyl alcohol, methanol (Fisher HPLC or pesticide grade).

b. Extraction solvents: I. toluene: hexane: isoamyl alcohol (78:20:2). II. methylene chloride.

3. Other reagents

a. Sodium fluoride (NaF): (Baker) potassium oxalate ($K_2C_2O_4$): (Mallinkrodt). Each tube containing $F^-/C_2O_4^=$ had 10 mg NaF and 3.75 mg $K_2C_2O_4$ per ml blood.

b. HCl: (Heico); NaOH: (Baker)

c. TMPAH. .1M trimethylphenylammonium hydroxide in methanol. (Eastman Kodak Co.). For methylation of barbiturates 1 part TMPAH: 2 parts methanol was used

C. Specimens

Blood was obtained from cadavers autopsied at the Office of the Chief Medical Examiner's office 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 the drugs of interest was collected, combined, and stored at -20°C until used.

Tissues were obtained from autopsied cases from the Office of the Chief Medical Examiner's for the Commonwealth of Virginia. Drug identity was confirmed by GC/MS. Between receipt and analysis, the tissues were stored in waxed cardboard containers at -20°C.

II. EXPERIMENTAL PROCEDURE

A. Blood

An aliquot of a methanolic standard of the drug to be analyzed was added to a glass container and evaporated to dryness at 65°C. The residue was reconstituted with blood obtained from cadavers within 48 hours after death and stirred magnetically for 1-1.5 hours. After an initial quantitation the blood was then divided into 2 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 the "spiked" drug.

B. Serum

When approximately 30-40 ml of serum containing the drug to be analyzed was pooled, an initial drug quantitation was performed. The serum was divided into 2 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 quantitated.

C. Tissues

The tissues were cut into small pieces and divided up 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 1 month time intervals, a 1 part tissue to 2 part water homogenate

was prepared using a portion stored at each temperature. Aliquots of these homogenates were then analyzed.

D. Blood for special chlordiazepoxide/demoxepam study

Blood was obtained from cadavers within 24 hours after death and frozen at -20°C until needed. The pH of the blood was adjusted by the addition of glacial acetic acid or concentrated ammonium hydroxide. Aqueous phosphate buffer (0.1M) was adjusted to the same pH with 4N NaOH. An aliquot of a methanolic standard of chlordiazepoxide or demoxepam was added to a glass container and evaporated to dryness at 65°C. The residue was reconstituted with either blood or buffer. After an initial quantitation, the blood and buffer were each divided up into 4 parts: 1) 25°C without fluoride/oxalate; 2) 4°C without fluoride/oxalate; 3) 25°C with fluoride/oxalate; 4) 4°C with fluoride/oxalate. At various intervals, aliquots from each part was analyzed.

E. Methodologies

The methodology employed for the quantitation of benzodiazepines came directly from the paper by Peat and Kopjak (19). Moreover, the analysis of disopyramide, nortryptyline, and lidocaine is based on this procedure, with a change in internal standard being the only difference. The gas chromatographic procedure for oxybarbiturates was presented by Blanke and Saady (92); the liquid chromatographic procedure for oxybarbiturates and thiopental uses the same extraction conditions. Procainamide is quantitated using the method of Rocco, et al. (93).

III. INSTRUMENTATION

The specific instrument conditions for each drug analysis are given in Table 1.

A. Gas chromatographs (GC)

HP5880 with nitrogen:phosphorus detector. A 2 m x 2 mm I.D. glass column packed with 3% OV ^{R} 101 on 80/100 Supelcoport was used. The carrier gas was helium at a flow rate of 30 mL/min. The injection port temperature 225°C and the detector temperature was 300°C.

Hewlett-Packard 5730 with ^{63}Ni electron capture detector; 1.3 m x 2 mm I.D. glass column packed with 3% OV-17 ^{R} or 3% OV-7 ^{R} on 80/100 Gas Chrom Q was used. The carrier gas was methane: argon (10:90) at a flow rate 30 ml/min. The oven temperature was 250°C. and the detector temperature was 300°C.

B. Gas Chromatograph/Mass Spectrometer

(GC/MS) Hewlett-Packard 5985A; A 1m x 2 mm I.D. glass column packed with 3% OV-101 on 80/100 Supelcoport was used. The carrier gas was helium at a flow rate of 33 ml/min. The initial oven temperature was 220°C. with a 10°C. per minute rise to 280°C.

C. Liquid chromatograph

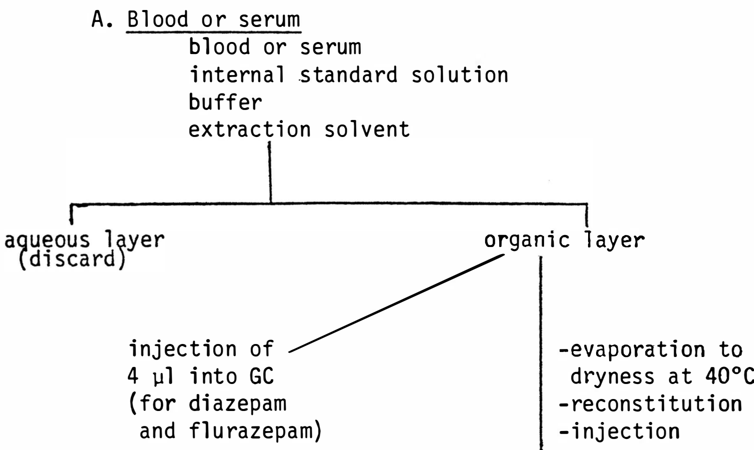
(LC) Hewlett-Packard 1080 and an Altex Model 332 Gradient liquid chromatograph attached to a Gilson Holochrome variable wavelength detector and a HP 3390A integrator were used for liquid chromatographic procedures.

TABLE 1. Specific Instrument Conditions for the Different Drug Analyses

Drug	Ref. for Method	Methodology	Instrument	Column	Temp. Program	Mobile Phase	Wavelength of Detection (nm)
Diazepam Flurazepam	91	GC	HP5730	3% OV^{R} 7 on 80/100 GasChromQ	250°C isothermal	10% methane in argon	---
Chlordiazepoxide Demoxepam Nitrazepam Norchlordiazepoxide Nordiazepam	91	LC	Altex Model 332	Altex Ultrasphere ODS 5μ;4.6 mm ID x 25 cm	ambient temperature	70% methanol 30% water flow 1 ml/mn	240
Oxybarbiturates	92	LC	HP1080	Chromanetics 10μ;4.6 mm IDX 25 cm	ambient temperature	65% methanol 35% water flow 1 ml/mn	220
		GC	HP5880	3% OV^{R} 101 on 80/100 Supelco- port	140°C for 1 min ↑15°C/min→220°C ↑20°C/min→280°C	helium	---
Thiopental	92	LC	Altex Model 332 25 cm	Altex ODS 10μ;4.6 mm IDx	ambient temperature flow 2 ml/min	60% methanol 40% water	290
Lidocaine Nortryptyline	91	GC	HP5880 port	3% OV^{R} 101 on 80/100 Supelco- ↑20°C/min→280°C	195°C for 1 min ↑15°C/min→220°C	helium	---
Procainamide	93	LC	Altex Model 332	Altex ODS 10μ;4.6 mm IDx 25 cm	ambient temperature	40% methanol 59% water .4% triethylamine pH adjustment to 4.8 with acetic acid	278
Disopyramide	91	GC	HP5880	3% OV^{R} 101 on 80/100 Supelco- port	220°C for 1 min 20°C/min→280°C		

IV. EXTRACTION

A. Blood or serum

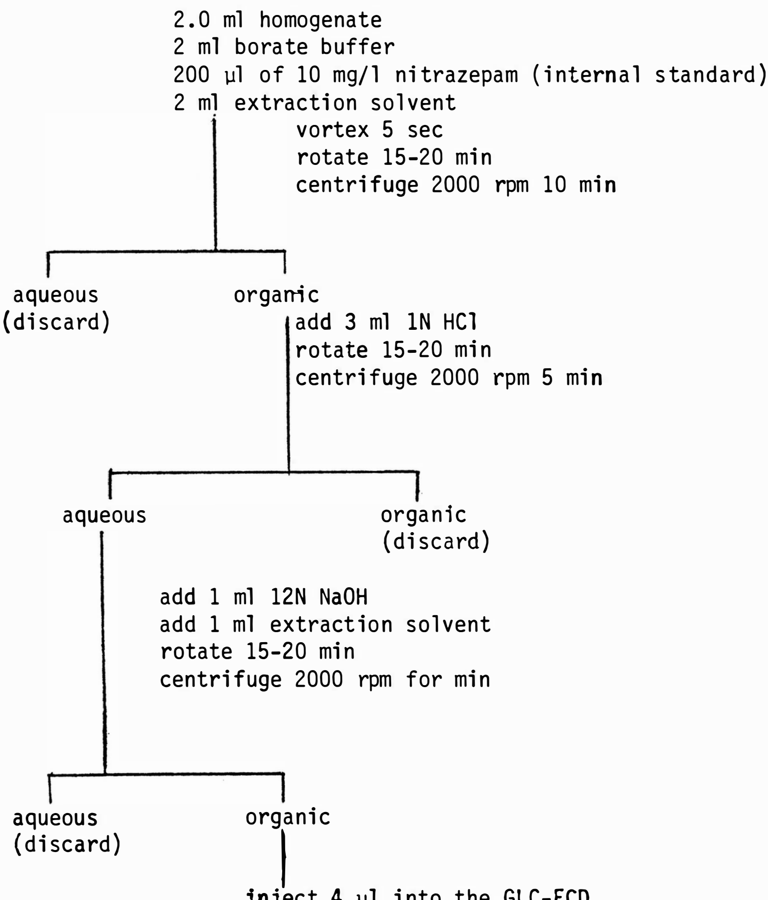


The specifics for each drug extraction are given in Table 2.

TABLE 2. Specific Extraction Conditions

Drug	Internal Standard	Buffer	Extraction Solvent	Reconstitution Chemical	Reconstitution Volume(ml)	Volume(μl) Injected
Diazepam	1μg flurazepam	borate	I	-----	-----	4
Flurazepam	1μg flurazepam	borate	I	_____	_____	4
Chlordiazepoxide Norchlordiazepoxide Nordiazepam	5 μg diazepam	borate	I	methanol	0.3	20
Nitrazepam	5 μg nordiazepam	borate	I	methanol	0.3	20
Oxybarbiturates (GC)	20 μg butabarbital	phosphate (pH 5.5)	II	.033M TMPAH	0.1	1
Oxybarbiturates (LC)	20 μg barbital	phosphate (pH 5.5)	II	methanol	0.3	20
Thiopental	200 μg phenolphthalein	phosphate (pH 5.5)	II	methanol	0.3	20
Lidocaine	10 μg mepivicaine	borate	I	methanol	0.1	1
Disopyramide	10 μg mepivicaine	phosphate (pH 11)	I	methanol	0.1	1
Nortryptyline	10 μg lidocaine	phosphate (Ph 11)	I	methanol	0.1	1
Procainamide	10 μg N-propionyl procainamide	phosphate (pH 11)	II	methanol	0.3	20

B. Tissues - benzodiazepines



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 ratio or height ratio of analyte peak to internal standard peak in comparison to ratios from fortified tissue standards.

C. Tissues - oxybarbiturates

2.0 ml homogenate
200 µl of 200 mg/l butabarbital (internal standard)
2 ml phosphate buffer - pH 5.5
15 ml toluene: hexane: isoamyl alcohol (78:20:2)

Rotate mechanically for 10 min
centrifuge

aqueous layer (discard) | organic layer

3 ml of saturated borate buffer (pH 9.5)

rotate mechanically for 10 min
centrifuge

aqueous layer | organic layer (discard)

1 ml 1N HCl

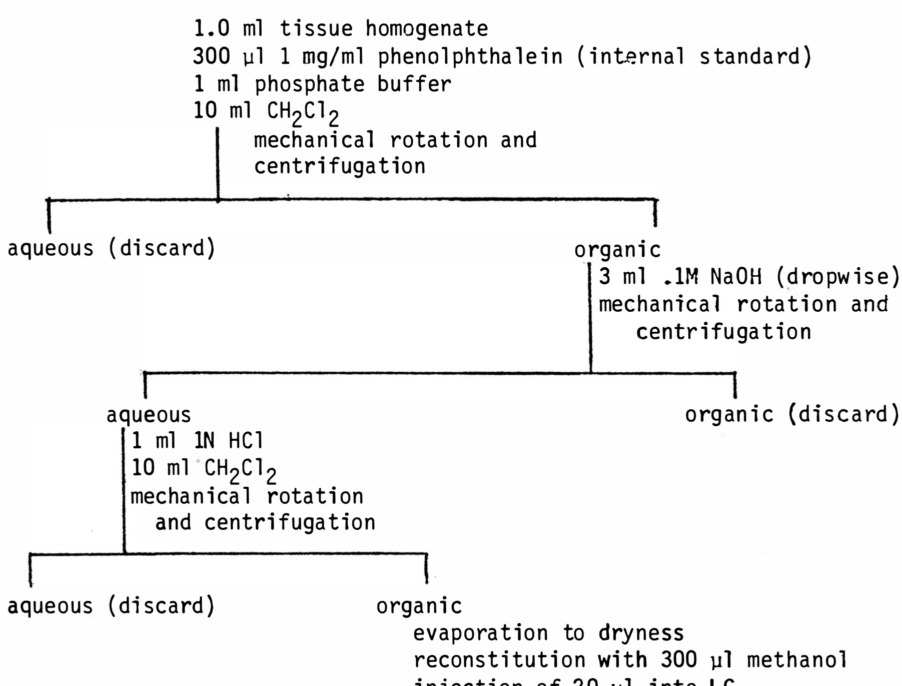
10 ml methylene chloride

rotate mechanically for 10 min
centrifuge

aqueous layer (discard) | organic layer

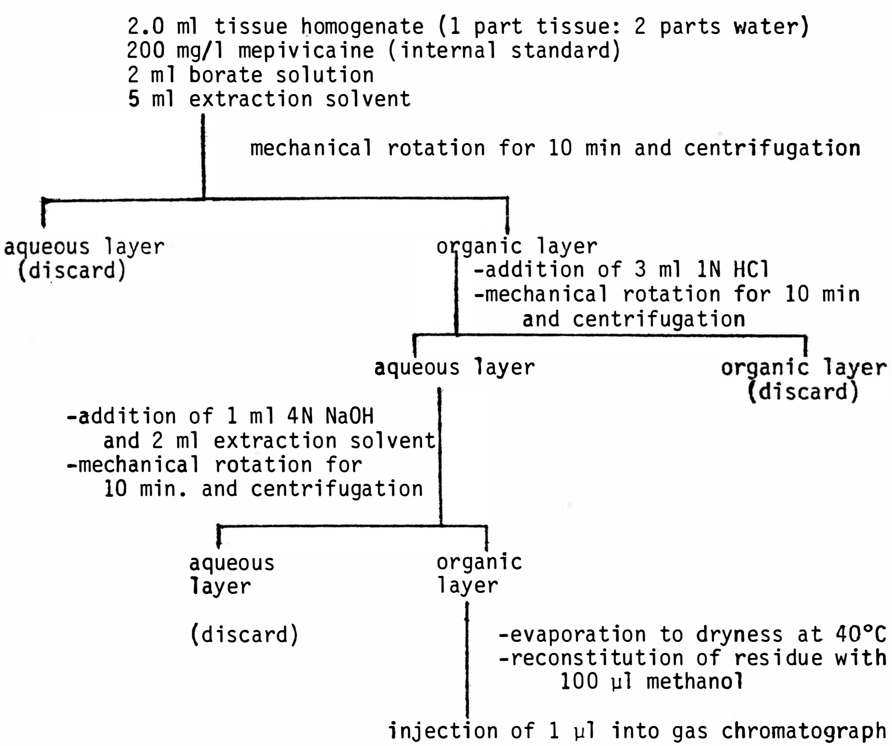
evaporated to dryness at 40°C
reconstitute residue with 100 µl of .033M TMPAH in methanol
injection of 1 µl into gas chromatograph

D. Tissues - thiopental



evaporation to dryness
reconstitution with 300 μl methanol
injection of 20 μl into LC

E. Tissues - lidocaine



V. RECOVERY STUDY FOR BARBITURATE LC METHOD

Two groups of blood standards were prepared; one group had 50 μl of the internal standard added to it while the other group had no internal standard. Buffer and 5 ml of methylene chloride were added to both groups of blood standards. After mechanically rotating for 5 minutes, the methylene chloride from the group containing the internal standard was filtered into conical centrifuge tubes. In the group without the internal standard, 2.5 ml of the methylene chloride was filtered into conical centrifuge tubes. Then, 25 μl of the internal standard solution was added to these tubes. Both groups were evaporated to dryness and reconstituted with 200 μl of mobile phase. 20 μl was injected. The percent recovery was calculated by dividing the area ratio (barbiturate to internal standard) of the samples to which the internal standard was added before extraction by the area ratio of the samples to which the internal standard was added after the extraction and multiplying by 100.

VI. FRACTION COLLECTION

Fractions collected (for benzodiazepines) 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.

VII. STATISTICAL MANIPULATIONS

Linear regressions for blood, serum, and buffer data were performed and 95% confidence intervals for the slope (95% CI) were obtained (94). See Appendix 1 for greater detail. Tissue data was compiled as follows: For each tissue, the initial quantitation was assigned a value of 100%. Successive quantitations were made and the "percent (%) of original present" was calculated. Data from each drug in each tissue were then compiled and an average "% of original present" was obtained for each time period.

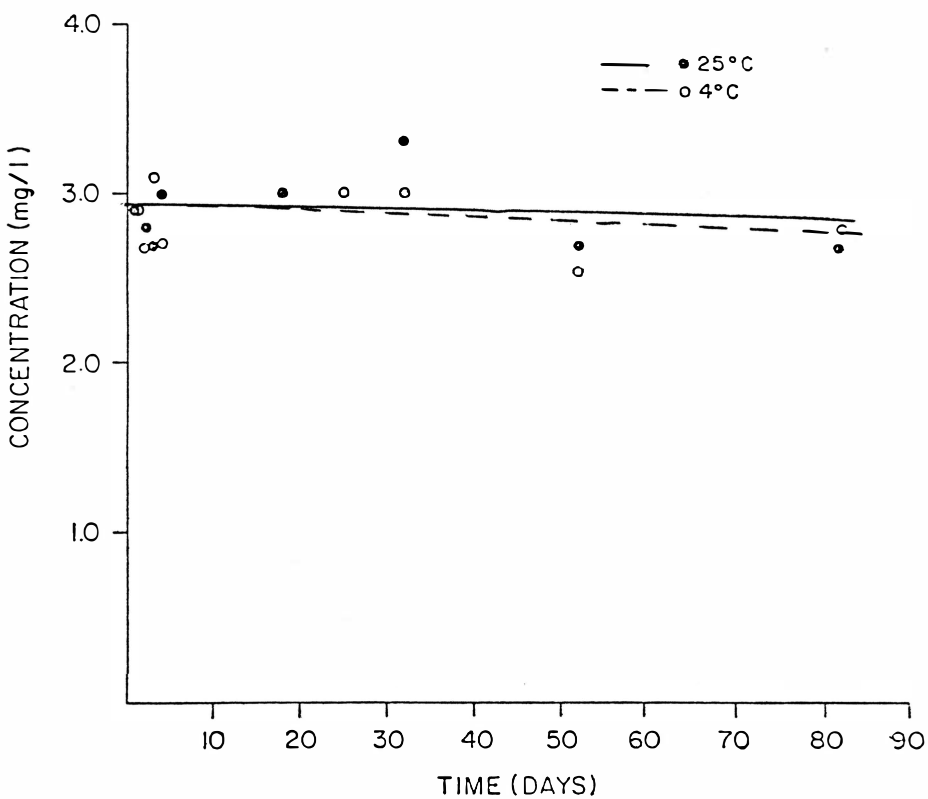
RESULTS

I. BENZODIAZEPINES

A. Blood

1. Diazepam and nordiazepam. The results from the storage of blood containing diazepam at 25°C and 4°C are shown in Figure 1. It indicates that diazepam is very stable in blood when stored at room temperature or at refrigerated temperature over a 90 day period, since no statistically significant changes in the slope were observed under either condition. Moreover, even after 5 months, no changes in concentration at either temperature were detected. However, conflicting data were produced when similar experiments were performed with nordiazepam. An initial study demonstrated a decrease in nordiazepam concentrations at 25°C and no significant change at 4°C (Table 3). The data obtained at 25°C were rearranged by plotting the natural logarithm of concentration versus time and then performing the linear regression. Since the higher correlation coefficient was obtained when ln(ND) was plotted vs. time as opposed to a plot of (ND) vs. time, the decomposition of nordiazepam followed pseudo first order kinetics. However, when these experiments were repeated, no significant changes were observed in either blood or buffer over a 2 month period (Table 4). To date, no explanation has been found to explain these conflicting data.

FIGURE 1. Storage of diazepam in blood at 25°C and 4°C.



25°C - slope=-.0012; intercept=2.9; 95% CI=(-.11, .009)
4°C - slope=-.0026; intercept=2.9; 95% CI=(-.010, .005)

TABLE 3

Storage of blood containing nordiazepam I

TIME (days)	25°C		4°C	
	(ND)	ln(ND)	ND	ln(ND)
0	3.4	1.22	3.4	1.22
3	2.0	.69	2.8	1.03
5	2.5	.92	3.1	1.13
12	1.6	.47	2.5	.92
19	0.8	-.22	2.5	.92
33				
49	0.5	-.69		
75	0.4	-.92	2.6	.96
Slope	-.032	-.027	-.0068	-.0023
Intercept	2.3	.84	2.9	1.06
Corr. Coef.	-.81	-.92	-.51	-.50
95% CI for Slope			(-.020.006)	

(ND) = nordiazepam concentration (mg/l)

TABLE 4

Storage of Blood Containing Nordiazepam-II

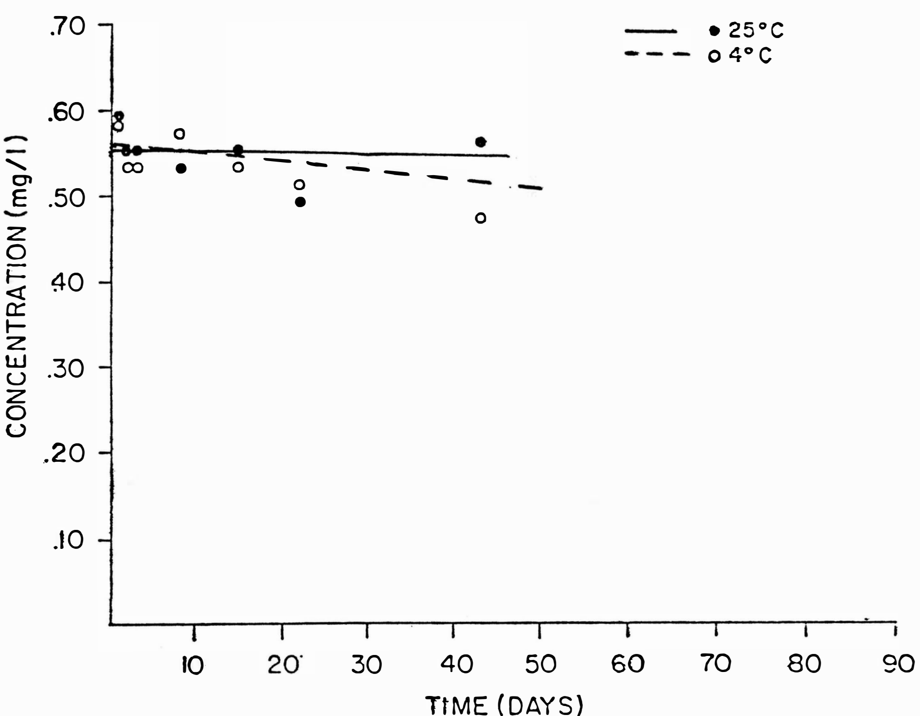
TIME (days)	Concentration (mg/l) 25°C	4°C
0	5.1	5.1
3	4.3	5.0
7	5.1	4.3
10	5.0	4.8
14	4.9	4.6
28	4.4	4.4
42	4.7	4.7
49	4.4	4.5
63	4.9	4.7
Slope	-.0037	-.0038
Intercept	4.8	4.8
95% CI for Slope	(-.025,.017)	(-.022,014)

2. Flurazepam and desalkylflurazepam. Figures 2 and 3 illustrate the storage effects of flurazepam and N-1-desalkylflurazepam at 4°C and 25°C. No significant changes in the concentration of either parent drug or metabolite were found.

3. Chlordiazepoxide (CDP) and norchlordiazepoxide (NCDP). The decay curves for these compounds in blood are given in Figures 4 and 5. It is clear from the graph that when both the parent drug and metabolite are stored at room temperature, their presence is not detected within 18 days after their addition to the blood.

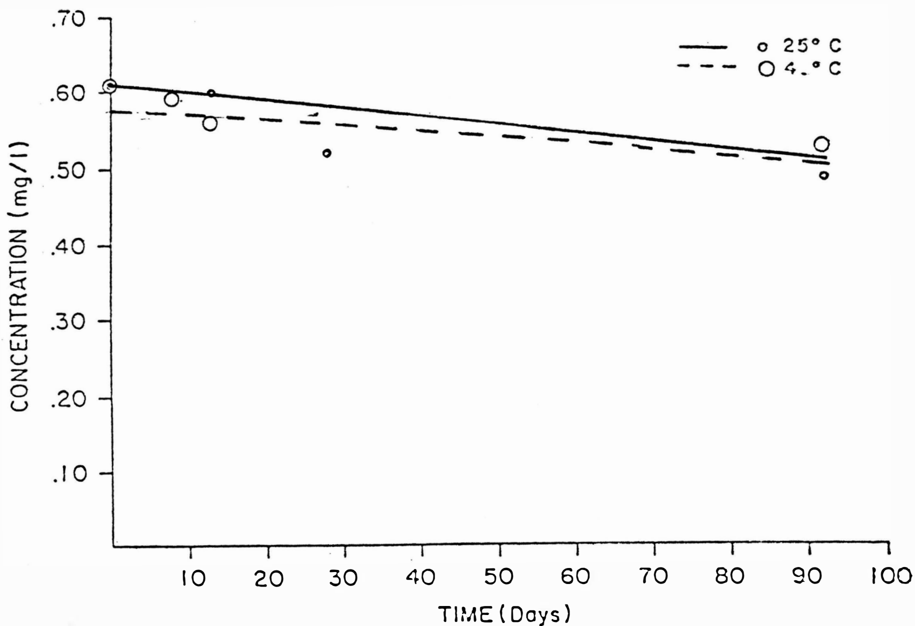
To obtain further information on the degradation of chlordiazepoxide (CDP) and norchlordiazepoxide (NCDP) experiments were undertaken to identify and possibly quantitate the breakdown products from these drugs. By analyzing different LC fractions as discussed in the Experimental section (p. 43), two breakdown products were tentatively identified by retention times or GC/MS-demoxepam (DEM) and nordiazepam (ND). The structures of these as well as the other benzodiazepines examined are given in Table 5. Demoxepam was identified by GC and LC retention time; no mass spectrum was obtained. Thermal instability of demoxepam prevented it from eluting intact from the GC column to the mass spectrometer. 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. Representative data from these experiments are given in Figures 6 and 7. Since demoxepam appeared earlier (2 days) than nordiazepam (4 days), it was evident that demoxepam was the first breakdown product.

FIGURE 2. Storage of flurazepam in blood at 25°C and 4°C.



25°C - slope=-5.0×10^{-4}; intercept=.55; 95% CI=(-.0058, .0048)
4°C - slope=-0020; intercept=.56; 95% CI=(-.0057, .0017)

FIGURE 3. Storage of N-1-desalkylflurazepam in blood at 25°C and 4°C.



25°C - slope=-.0028; intercept=.61; 95% CI=(-.0071, .0015)
4°C - slope=-.0020; intercept=.58; 95% CI=(-.0058, .0018)

FIGURE 4. Storage of chlordiazepoxide in blood at 25°C and 4°C.

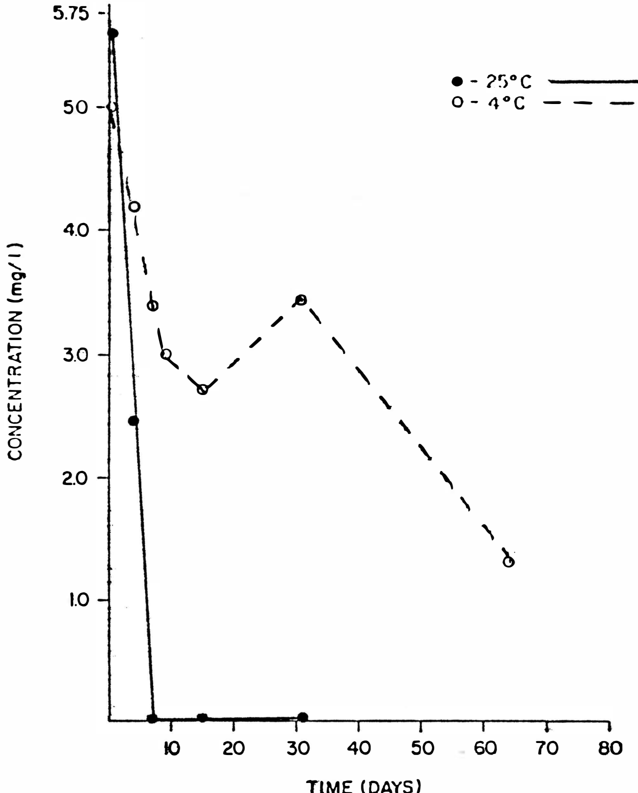


FIGURE 5. Storage of norchlordiazepoxide in blood at 25°C and 4°C.

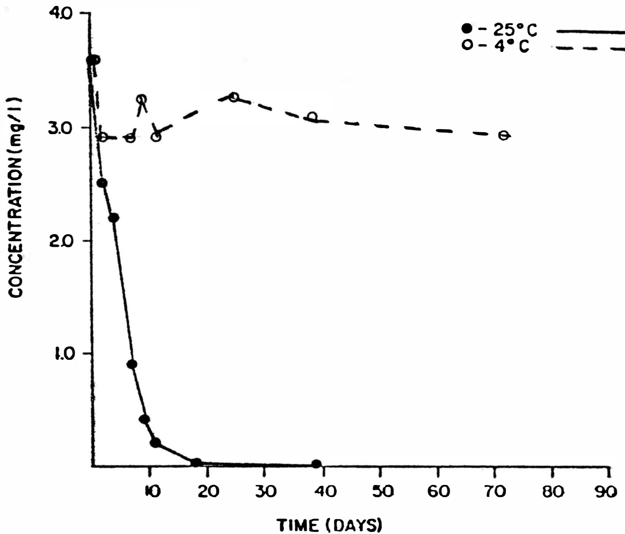


TABLE 5. Structures of Benzodiazepines

$R_1 = CH_3-$ Diazepam
$R_1 = H-$ Nordiazepam

$R_2 = NHCH_3-$ Chlordiazepoxide
$R_2 = NH_2-$ Norchlordiazepoxide

Demoxepam

$R_3 = CH_2CH_2N(C_2H_5)_2-$Flurazepam
$= H-$N-1-desalkyl Flurazepam

Nitrazepam

FIGURE 6. Demoxepam concentrations in norchlordiazepoxide blood solutions stored at 25°C.

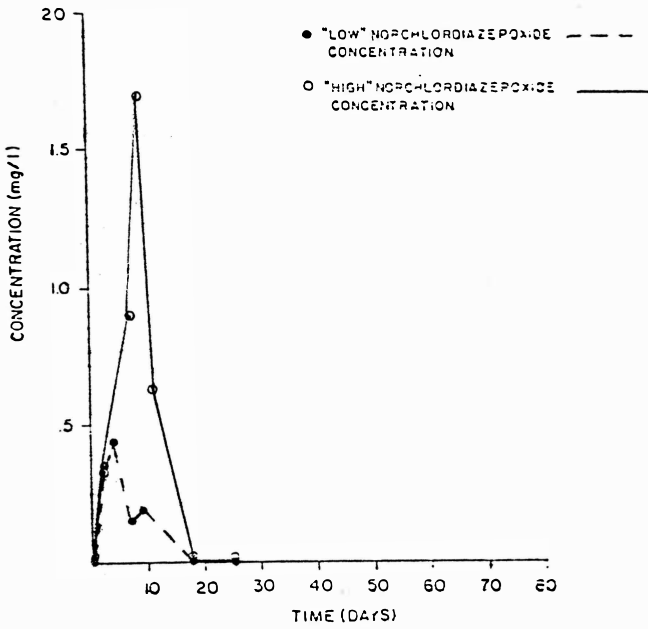
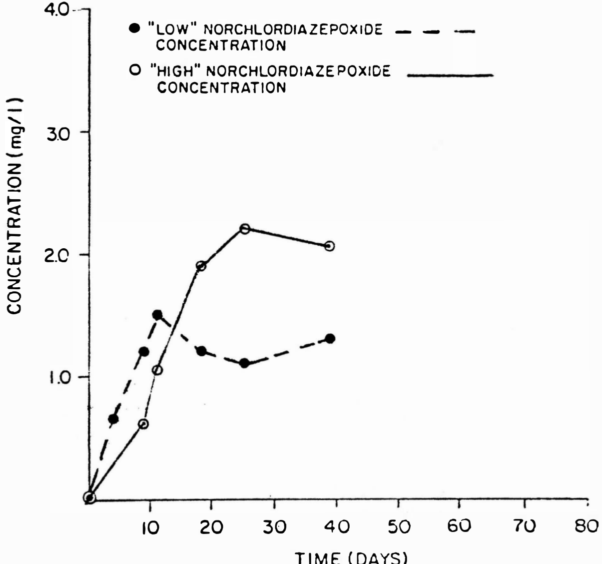


FIGURE 7. Nordiazepam concentrations in norchlordiazepoxide blood solutions stored at 25°C.



It was decided to study the mechanism of chlordiazepoxide breakdown in greater detail. Specifically, it was assumed that at least 2 different processes can be occurring in the blood: 1) chemical degradation of chlordiazepoxide in a pH-dependent aqueous medium and 2) microorganism induced degradation. To study these effects, a series of tubes were set up as outlined in the experimental section (Special chlordiazepoxide study). Presumably, the presence of fluoride, a microorganism inhibitor, would prevent any changes induced by microorganisms and aqueous buffer could be used as an indicator of changes occurring in any aqueous medium. Three pH's were studied: 5, 6.5, and 8 and each experiment was carried on over a 3-4 week period. The data from these experiments are given in Tables 6-11. Qualitatively, when chlordiazepoxide was stored in blood at room temperature at pH 8 and pH 6.5 in the absence of $F^-/C_2O_4^=$, none of the CDP added (5 mg/l) was detected after 15 days. Approximately 25-40% of this was converted to ND through the intermediate DEM. This was illustrated by the fact that the concentration of DEM, not originally present, increased to a maximum of 1.6 mg/l by day 6 and then decreased to <0.1 mg/l by day 15. This would suggest that virtually all of the CDP that was converted to DEM was then converted to ND. Not all of DEM was converted to ND because a small, but detectable amount of the 2-amino-5-chlorobenzophenone (see Discussion) was observed. No attempt was made to quantitate the benzophenone because no pure standard was available. Tentative identification of the benzophenone was made based on the LC and GC-ECD retention times in comparison to a qualitative benzophenone standard. The presence of $F^-/C_2O_4^=$ in blood at pH's 8 and 6.5 significantly changed the observations. Approximately 80-90% of the

TABLE 6

Special CDP Blood/Buffer Study: pH 8, no F/C_2O_4

TIME (days)	25°C BLOOD (CDP)	25°C BLOOD ln(CDP)	25°C BUFFER (CDP)	25°C BUFFER ln(CDP)	4°C BLOOD (CDP)	4°C BLOOD ln(CDP)	4°C BUFFER (CDP)	4°C BUFFER ln(CDP)
0	5.7	1.74	5.0	1.61	5.7	1.74	5.0	1.61
2	5.4	1.69	4.8	1.57	5.1	1.63	4.9	1.59
4	4.9	1.59	4.2	1.44	4.9	1.59	5.1	1.63
8	2.4	.88	4.0	1.39	5.3	1.67	4.2	1.44
10	0.9	-.11	4.1	1.41	4.8	1.57	4.5	1.50
14	0.2	-1.61	2.7	.99	4.9	1.59	4.4	1.48
16	N.D.	----	2.8	1.03	4.9	1.59		
18	N.D.	----	2.4	.88	4.6	1.53	4.5	1.50
22	N.D.	----	2.3	.83	4.4	1.48	3.8	1.34
25	N.D.	----	2.0	.69	4.6	1.53	4.0	1.39
Slope	-.44	-.27	-.13	-.039	-.036	-.0072	-.043	-.0096
Intercept	5.7	2.11	4.9	1.64	5.4	1.68	5.0	1.61
Corr. Coef.	-.97	-.93	-.97	-.98	-.82	-.82	-.86	-.86
95% CI for Slope					(-.057,-.015)		(-.065,-.021)	

(CDP) = CDP concentration (mg/l)
N.D. = none detected

TABLE 7

Special CDP Blood/Buffer Study: pH 8, F/C_2O_4

TIME (days)	25°C BLOOD (CDP)	25°C BLOOD ln(CDP)	25°C BUFFER (CDP)	25°C BUFFER ln(CDP)	4°C BLOOD (CDP)	4°C BLOOD ln(CDP)	4°C BUFFER (CDP)	4°C BUFFER ln(CDP)
0	5.7	1.74	5.0	1.61	5.7	1.74	5.0	1.61
2	5.0	1.61	4.5	1.50	5.1	1.63	4.8	1.57
4			4.6	1.53	5.3	1.67	5.3	1.67
8	5.0	1.61	4.0	1.39	5.3	1.67		
10	4.9	1.59	3.2	1.16	4.9	1.59	4.3	1.46
14	5.0	1.61	2.8	1.03	4.9	1.59	4.3	1.46
16			2.6	.96	4.6	1.53	4.2	1.44
18			2.8	1.03			4.2	1.44
22	4.6	1.53	2.3	.83	4.4	1.48	4.6	1.53
25	4.6	1.53	2.2	.79	4.6	1.53	4.1	1.41
Slope	-.033	-.0066	-.12	-.036	-.042	-.0084	-.036	-.0077
Intercept	5.3	1.68	4.8	1.60	5.5	1.70	5.0	1.60
Corr. Coef.	-.84	-.84	-.96	-.98	-.89	-.90	-.76	-.77
95% CI for Slope					(-.058,-.026)		(-.062,-.010)	

(CDP) = CDP concentration

TABLE 8

Special CDP Blood/Buffer study: pH 6.5, no F/C_2O_4

	25°C				4°C			
	BLOOD		BUFFER		BLOOD		BUFFER	
TIME (days)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)
0	4.9	1.59	5.0	1.61	4.9	1.59	5.0	1.61
2	4.7	1.55	4.4	1.48	4.8	1.57	4.6	1.53
4	4.2	1.44	3.8	1.34	4.6	1.53	4.2	1.44
8	1.8	.59	3.1	1.13	4.1	1.41	4.3	1.46
10	1.6	.47	2.8	1.03	4.4	1.48	3.8	1.34
14	1.4	.34	2.3	.83			3.5	1.25
18	N.D.	----	2.3	.83	3.9	1.36	3.5	1.25
22	N.D.	----	2.2	.79	4.1	1.41		
24	N.D.	----	2.0	.69	4.0	1.39	4.0	1.39
28	N.D.	----	1.9	.64	4.2	1.44	3.9	1.36
Slope	-.028	-.13	-.10	-.036	-.028	$-.63\times10^{-4}$	-.034	-.011
Intercept	4.9	1.67	4.3	1.49	4.7	1.55	4.5	1.52
Corr. Coef.	-.97	-.96	-.92	-.96	-.80	-.78	-.67	-.73
95% CI for Slope					(-.047,-.009)		(-.062,-.006)	

(CDP) = CDP concentration (mg/l)
N.D. = None detected

TABLE 9

Special CDP Blood/Buffer Study: pH 6.5, F/C_2O_4

	25°C				4°C			
	BLOOD		BUFFER		BLOOD		BUFFER	
TIME (days)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)
0	6.0	1.79	5.0	1.61	6.0	1.79	5.0	1.61
2	5.3	1.67	4.3	1.46	5.5	1.70	4.7	1.55
4	5.2	1.65	3.6	1.28	5.0	1.61	4.0	1.39
8	5.4	1.69	3.2	1.16	5.4	1.69	4.2	1.44
10	5.3	1.67	3.1	1.13	5.3	1.67	3.9	1.36
14	5.5	1.70	2.4	.88	5.0	1.61	3.7	1.31
18	5.4	1.69	2.3	.83	5.5	1.70	4.3	1.46
22	5.0	1.61	2.2	.79	5.1	1.63	4.0	1.39
24	5.2	1.65	1.8	.59	5.4	1.69	3.7	1.31
28			1.6	.47			3.8	1.34
Slope	-.018	-.0047	-.11	-.038	-.014	-.0023	-.031	$-8.2x10^{-4}$
Intercept	5.6	1.71	4.4	1.50	5.5	1.70	4.5	1.52
Corr. Coef.	-.56	-.68	-.95	-.97	-.38	-.36	-.70	-.70
95% CI for Slope					(-.026,-.002)		(-.057,-.005)	

(CDP) = CDP concentration (mg/l)

TABLE 10

Special CDP Blood/Buffer Study: pH 5, no F/C_2O_4

TIME (days)	25°C				4°C			
	BLOOD		BUFFER		BLOOD		BUFFER	
	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)
0	5.2	1.65	5.4	1.69	5.2	1.65	5.4	1.69
1	4.7	1.55	4.9	1.59	5.1	1.63	5.3	1.67
2	4.8	1.57	4.8	1.57	5.1	1.63	5.2	1.65
5	3.8	1.34	4.0	1.39	4.9	1.59	4.7	1.55
7	3.3	1.19	3.6	1.28	4.1	1.41	4.4	1.48
9	2.4	.88	2.6	.96	3.7	1.31	4.2	1.44
12	2.4	.88	2.5	.92	3.7	1.31	4.5	1.50
14	1.8	.59	1.9	.64				
16	1.6	.47	1.8	.59			4.4	1.48
19	2.1	.74			4.0	1.39	4.5	1.50
21	1.2	.18	1.5	.41			4.7	1.55
23			1.4	.34			4.8	1.57
Slope	-.18	-.064	-.18	-.062	-.083	-.033	-.028	-.0053
Intercept	4.8	1.62	4.9	1.65	5.0	1.67	5.0	1.61
Corr. Coef.	-.95	-.96	-.96	-.99	-.81	-.95	-.56	-.53
95% CI for Slope					(-.11,-.054)		(-.055,-.001)	

(CDP) = CDP concentration (mg/l)

TABLE 11

Special CDP Blood/Buffer Study: pH 5, F/C_2O_4

	25°C				4°C			
	BLOOD		BUFFER		BLOOD		BUFFER	
TIME (days)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)	(CDP)	ln(CDP)
0	5.2	1.65	5.4	1.69	5.2	1.65	5.4	1.69
1	4.9	1.59	5.0	1.61	5.1	1.63	4.8	1.57
2	4.7	1.55	4.9	1.59	5.1	1.63	5.0	1.61
5	4.6	1.53	3.9	1.36	5.1	1.63	5.2	1.65
7	3.3	1.19	3.1	1.13	4.5	1.50		
9	2.9	1.06	3.0	1.10	4.6	1.53	4.9	1.59
12	2.6	.96			4.1	1.41	4.5	1.50
14	2.5	.92	2.9	1.06	4.6	1.53	4.8	1.57
16							4.6	1.53
19			2.3	.83	4.2	1.44	4.5	1.50
21	1.8	.59	1.6	.47			4.6	1.53
23	1.5	.41	1.2	.18	4.0	1.39		
Slope	-.16	-.053	-.16	-.056	-.053	-.011	-.031	-.0064
Intercept	4.9	1.65	5.0	1.67	5.1	1.64	5.1	1.64
Corr. Coef.	-.96	-.99	-.96	-.97	-.90	-.89	-.79	-.78
95% CI for Slope					(-.094,-.010)		(-.056,-.006)	

(CDP) = CDP concentration (mg/l)

CDP added was detected at the end of the 3 week period. Small amounts of DEM and no ND were detected.

The storage of CDP in buffer at pH 8 and 6.5 at 25°C differed from either condition observed in blood at corresponding pHs. CDP broke down to DEM at both pHs and the total CDP and DEM, on the average, was about 90% of the total CDP added to the buffer. $F^-/C_2O_4^=$ had no effect on CDP stability in buffer at either pH.

The results from storing the blood and buffer at pH 5 at 25°C contrasted to their counterparts at the other pHs. For example no ND was detected in either of the blood samples. Larger concentrations of DEM were observed in blood samples with and without $F^-/C_2O_4^=$ (approximately 3 mg/l). Moreover, the total of CDP and DEM concentrations, on the average, approximated 80-90% of the CDP originally added to the blood. Furthermore, the presence of $F^-/C_2O_4^=$ had little or no effect on the breakdown of CDP.

Tables 6-11 also give data on the effects of these storage conditions on CDP stability in blood at 4°C. It is clear that under all conditions, CDP is more stable at 4°C than at 25°C. However, in all cases, there were statistically significant decreases in CDP concentration over the 3-4 week period, since 0 did not fall within the 95% confidence interval for the slopes.

The data in Tables 6-11 at 25°C were rearranged by plotting ln (CDP concentration) versus time and obtaining the slopes, intercepts and correlation coefficients of these lines by performing linear regressions. In most cases ln(CDP) vs. t plots gave equal or slightly better correlations than (CDP) vs. t plots. The slopes are compiled in Table 12. From this table, many points are evident: 1) in

TABLE 12

Slopes of ln(CDP) vs t in Blood and Buffer at Different pHs at 25°C*

	pH 8	pH 6.5	pH 5
Blood	-.27	-.13	-.064
Blood+$F^-/C_2O_4^=$	-.0066	-.0047	-.053
Buffer	-.039	-.036	-.062
Buffer+$F^-/C_2O_4^=$	-.036	-.038	-.056

* - units are in $(days)^{-1}$

blood without $F^-/C_2O_4^=$, CDP is most stable at pH 5 and least stable at pH 8; 2) in blood with $F^-/C_2O_4^=$, CDP is most stable at pH 6.5 and pH 8; 3) the presence of $F^-/C_2O_4^=$ has no effect on the stability of CDP in buffer; 4) in buffer, CDP is most stable at pH 6.5 and pH 8; 5) the presence of $F^-/C_2O_4^=$ in blood has its greatest stabilizing effect at pH 6.5 and pH 8, making it more stable than in the corresponding buffer solutions; 6) the presence of $F^-/C_2O_4^=$ in blood at pH 5 has no effect and moreover, the stability of CDP in blood approximates the stability of CDP in buffer at pH 5.

To learn more about nordiazepam formation, it was decided to perform similar types experiments as above except that demoxepam was used as the starting material. Specifically, it was hoped that the pH dependence of DEM stability and ND formation could be ascertained in greater detail. The following is a summary of the results obtained: 1) few changes (<10%) were found in all conditions at 4°C; 2) 20-30% decrease in demoxepam in buffer was seen at all pH's at 25°C but none of the DEM was converted to nordiazepam; the blood data at 25°C are summarized in Table 13.

Since the data suggest that the conversion of demoxepam to nordiazepam occurred by microbial action, it became necessary to determine the type of microorganisms being produced. To accomplish this, blood stored at 25°C and 4°C in the absence and presence of F/C_2O_4 were analyzed for anaerobic bacteria, aerobic bacteria, and yeast. Blood at pH's 5 and 6.5 were analyzed because the nordiazepam formation was seen at pH 6.5 and not at pH 5. The results are given in Table 14. At both pH's, there appears to be little qualitative difference in the types of organisms present under each condition. However, since no

TABLE 13. Summary of Results From Special Demoxepam Study - Blood at 25°C

pH	$F^-/C_2O_4^=$ PRESENT or ABSENT	RESULTS
5	absent	- 20% decrease in DEM concentration - No ND formation
5	present	- 20% decrease in DEM concentration - No ND formation
6.5	absent	- 50% decrease in DEM concentration - [A]about 50% of original DEM converted to ND
6.5	present	- 10-15% decrease in DEM concentration - No ND formed

[A]The sensitivity of the analytical procedure for DEM was 10% and therefore, not sensitive enough to state that all DEM that disappeared was converted to ND.

TABLE 14. Results of Microorganism Study

pH	Storage Condition of Blood	Bacteria Detected		Yeast Detected
		Anaerobic	Aerobic	
6.5	25°C - F/C_2O_4	none	enteric Streptococci *Pseudomonas aeruginosa* *E. coli*	none
6.5	4°C - F/C_2O_4	none	enteric Streptococci *Pseudomonas aeruginosa* *E. coli*	none
6.5	25°C + F/C_2O_4	none	enteric Streptococci Pseudomonas *E. coli*	*Sacchanomyces* species
6.5	4°C + F/C_2O_4	none	enteric Streptococci Pseudomonas	none
5.0	25°C - F/C_2O_4	none	enteric Streptococci	none
5.0	4°C - F/C_2O_4	none	enteric Streptococci *Pseudomonas maltophilia*	none
5.0	25°C + F/C_2O_4	none	none	none
5.0	4°C + F/C_2O_4	none	enteric Streptococci	none

quantitative determinations were made, it is possible that an increased number of microorganisms can account for nordiazepam formation in one of the samples and not in the others.

4. Nitrazepam. Table 15 illustrates the stability of nitrazepam in blood and buffer (pH 7.5). It is evidence that nitrazepam is unstable in blood, dropping from an initial concentration of 5 mg/l at day 0 to none detected by day 24. Linear regressions of (NIT) vs. t and ln(NIT) vs. t suggest a pseudo-first order decay process, but the difference in r values is quite small. Nitrazepam appears to be quite stable when stored at 4°C in blood and buffer over a 2 month period as indicated by the 95% CI for the slopes. About 30% of nitrazepam disappears in buffer at 25°C. and linear regressions of (NIT) vs. t and ln(NIT) vs. t do not indicate whether this process is 0-order or pseudo-first order.

B. Tissues

The tissue data for benzodiazepines are given in Figures 8-10. Several points are suggested from these data: 1) There was no significant change in the concentration of diazepam in both liver and brain for 3 months following its storage at either room temperature or at refrigerated temperature (Fig. 8). 2) When livers containing nordiazepam are stored at 25°C, there is approximately a 50% decrease in nordiazepam concentration by day 25 (Figure 9). At least 80% of flurazepam originally present is detectable when the livers were stored at 25°C or 4°C (Figure 10).

N-1-desalkylflurazepam was not quantitated along with flurazepam because a change in the extraction procedure was necessary.

TABLE 15

Stability of Nitrazepam in Blood and Buffer

TIME (days)	25°C BLOOD (CDP)	25°C BLOOD ln(CDP)	25°C BUFFER (CDP)	25°C BUFFER ln(CDP)	4°C BLOOD (CDP)	4°C BLOOD ln(CDP)	4°C BUFFER (CDP)	4°C BUFFER ln(CDP)
0	4.5	1.50	4.1	1.41	4.5	1.50	4.1	1.41
4	4.0	1.39	4.4	1.48	4.6	1.53	4.5	1.50
8	2.0	.69	4.3	1.46	4.4	1.48	4.3	1.46
11	1.4	.34	3.9	1.36	5.0	1.61	4.1	1.41
15	1.0	0	4.0	1.39	4.4	1.48	4.1	1.41
18	0.5	-.69	3.0	1.10	4.0	1.39	4.1	1.41
22	0.7	-.36	3.1	1.13			4.0	1.39
24	N.D.	----	2.8	1.03	4.6	1.53	3.7	1.31
31	N.D.	----	3.2	1.16	4.5	1.50		
36	N.D.	----	3.2	1.16	4.6	1.53	4.3	1.46
43	N.D.	----	2.9	1.06	4.6	1.53	4.3	1.46
Slope	-.18	-.10	-.036	-.010	7.1×10^{-4}	3.0×10^{-4}	7.2×10^{-4}	1.8×10^{-4}
Intercept	4.1	1.56	4.2	1.45	4.5	1.50	4.1	1.43
Corr. Coef. for Slope	-.94	-.96	-.81	-.81	(-.023,.021)		(-.0082,.0096)	

FIGURE 8. Effect of temperature on the storage of tissues containing diazepam.

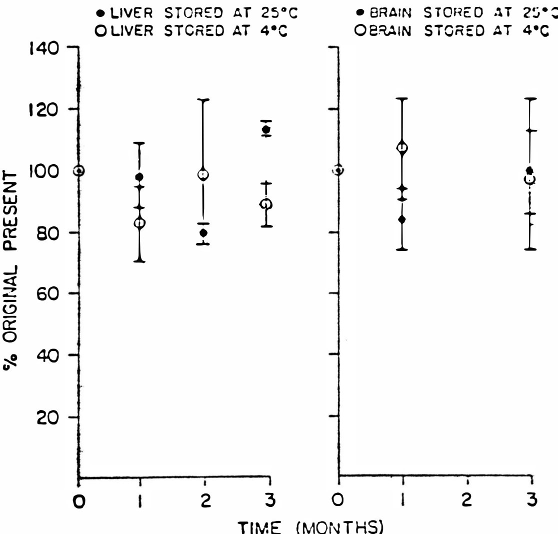


FIGURE 9. Effect of temperature on the storage of tissues containing nordiazepam.

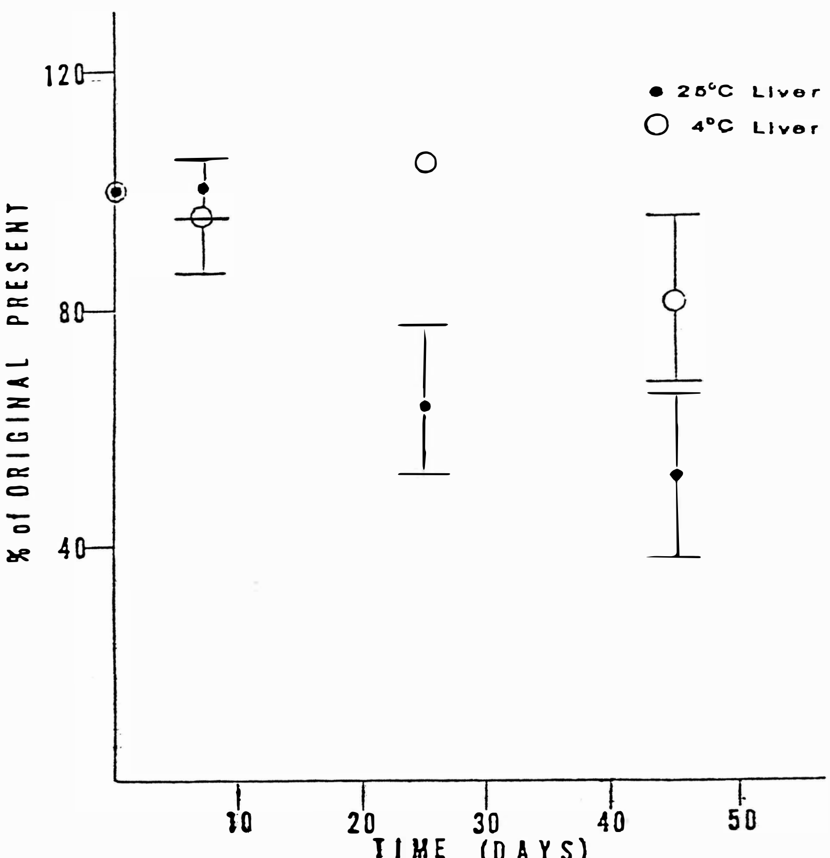
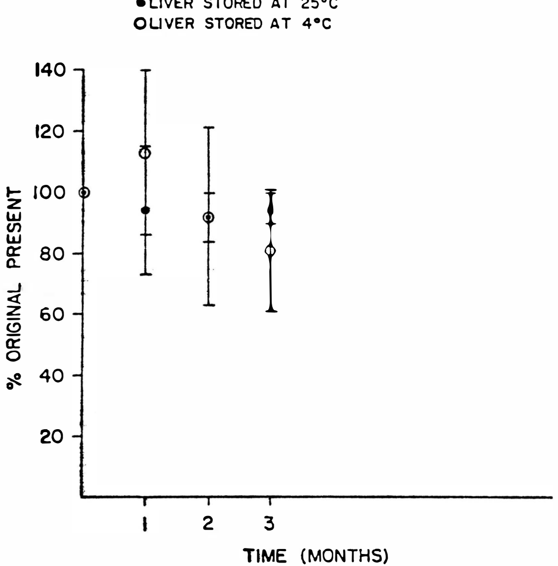


FIGURE 10. Effect of temperature on the storage of tissues containing flurazepam



This was due to a peak interferring with the flurazepam peak. Gas chromatographic retention times suggested that this peak was hydroxyethyl flurazepam, a metabolite of flurazepam. In order to remove this interference, a less-polar solvent, hexane, was employed in the initial extraction step. However, this change caused the more polar metabolites of flurazepam such as N-1-desalkylflurazepam to remain largely in the aqueous layer, thus preventing their quantitation. Moreover, due to the small quantities of flurazepam detected in the brain (<0.1 mg/l), no attempts were made to submit them to these experiments.

II. BARBITURATES

A. Oxybarbiturates

1. LC Method Development

a. Linearity and sensitivity

The linearity of plots of peak area ratios of the barbiturate peak to the internal standard peaks was established by adding known amounts of the barbiturate to blood not containing barbiturate and then submitting the blood solutions to the extraction procedure. The standard curves obtained are illustrated in Figures 11 and 12. The curves were determined to be linear (corr >.99) for amobarbital, butabarbital, pentobarbital, and secobarbital from 0.2 mg/l to 60 mg/l. Since the concentrations of phenobarbital encountered in clinical and forensic labs are somewhat higher than those of the other barbiturates, higher concentrations of phenobarbital were tested. Linearity (corr >.99) was established from 0.5 mg/l to 120 mg/l for phenobarbital. Since the lowest therapeutic concentration of pentobarbital and secobarbital is .5 mg/l, this method has sufficient sensitivity for the detection of therapeutic concentrations of the barbiturates.

FIGURE 11

The ordinate plots the area ratio of barbiturate to barbital, the internal standard. Linear regression of each line yielded the following:

butabarbital : slope = .029; intercept = .0063; r>.99

pentobarbital : slope = .031; intercept = -.048; r>.99

amobarbital : slope = .037; intercept = -.02; r>.99

secobarbital : slope = .038; intercept = .005; r>.99

FIGURE 11. Standard curve for blood barbiturates.

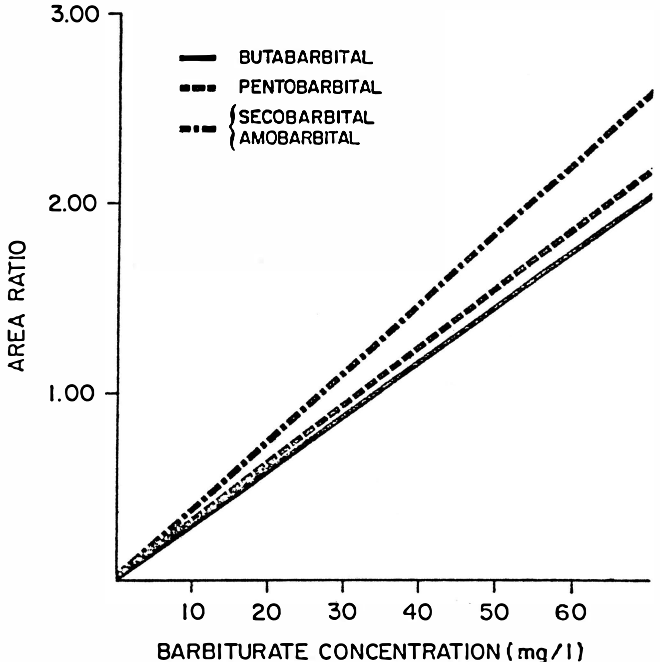
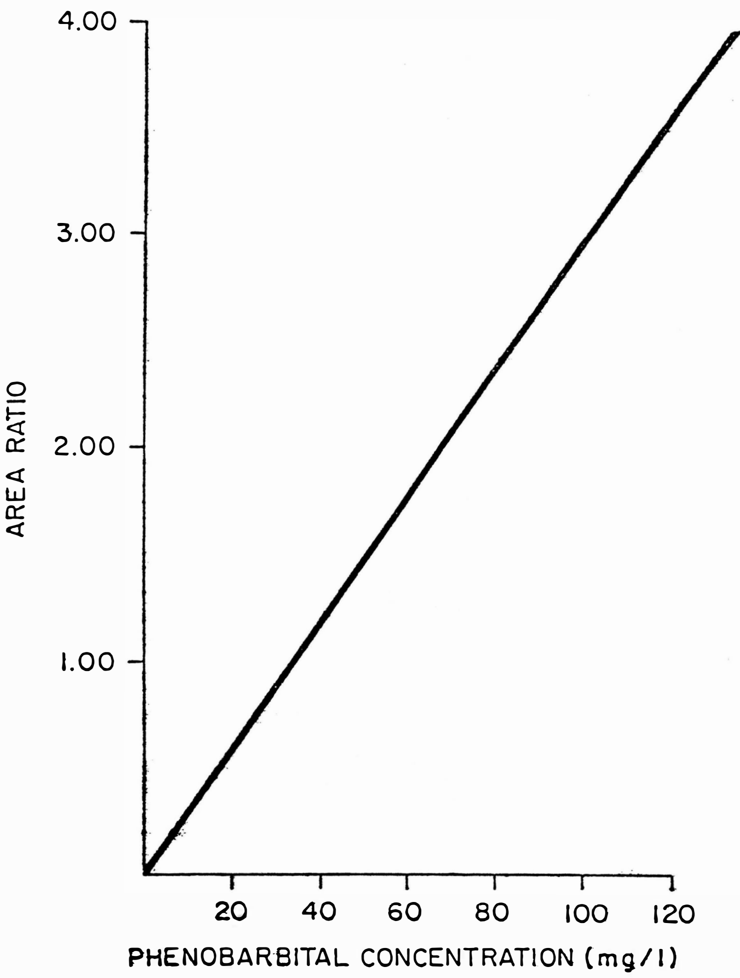


FIGURE 12

The ordinate plots the area ratio of phenobarbital to barbital, the internal standard. Linear regression of the line yielded the following:

slope = .043; intercept = .20; $r > .99$

FIGURE 12. Standard curve for blood phenobarbital.



b. Precision

The reproducibility of the method was determined by preparing 1 blood sample, containing 20 mg/l of the barbiturates and dividing into 5 portions. These portions were extracted and chromatographed. The coefficient of variation for the area ratio of barbiturates to internal standard is given in Table 16.

c. Specificity

From the pH of the extraction, it would be expected that weak acid and neutral drugs would enter the organic layer. Therefore a series of these compounds were injected to determine their retention times. These retention times are given in Table 17. Several points become evident from the table. For example, pentobarbital co-chromatographs with amobarbital. This means that either one of these drugs can be quantitated as long as the other is not present. Moreover, an alternate method would be required for qualitative identification. Fortunately, these drugs are rarely co-administered. A more severe limitation is that primidone and phenobarbital are poorly resolved. Phenobarbital is a metabolite of primidone and both compounds would appear in a sample from a patient receiving primidone. Butalbital and butabarbital, two drugs which are difficult to separate using gas chromatography, differ in their retention times by about 0.2 minutes. This would not only allow quantitation, but also can permit qualitative identification.

TABLE 16

Coefficient of Variation for the Barbiturate Determination at 20 mg/l in Blood

Barbiturate	Coefficient of Variation (%)
amobarbital	8.1
butabarbital	5.0
pentobarbital	7.3
phenobarbital	2.4
secobarbital	7.9

TABLE 17

Retention Times of some Co-extracted Drugs using HPLC barbiturate procedure

Drug	Retention Time (± .05 min.)
amobarbital	4.8
aprobarbital	3.1
barbital	2.2
butabarbital	3.6
butalbital	3.8
carbamazepine	6.0
cyclobarbital	3.7
glutethimide	4.5
hexobarbital	4.3
meprobamate	not observed
mesantoin	3.8
methaqualone	8.0
pentobarbital	4.8
phenobarbital	2.8
phenytoin	3.9
primidone	2.9
secobarbital	5.4
thiopental	5.8

d. Recovery

Recovery studies for barbiturates were performed as outlined in the Experimental Section (p. 43). The average of these results for each is given in Table 18.

e. Patient Samples

To determine further the validity of this method, serum samples containing phenobarbital were obtained from the Medical College of Virginia Hospitals Toxicology Laboratory. These samples are assayed for phenobarbital using the gas chromatographic method of Blanke and Saady (92). These specimens were also run through this LC method and plots of the concentrations using the 2 methods are given in Figure 13. Before these samples were assayed by the liquid chromatographic method, a standard curve for serum phenobarbital levels was generated. Good agreement between the two methods was found (r=.99) for phenobarbital samples.

2. Stability in blood and tissues

Data of concentration versus time for the barbiturates in blood and serum are given in Tables 19-24. Two sets of data were obtained, one set for "therapeutic" concentrations and one set for "toxic" concentrations. Since 0 is within the 95% confidence interval (CI) for the slope, it can be stated that there is no statistically significant change in the barbiturate concentrations over the 2-3 month period at 25°C and 4°C. (See Appendix for further discussion)

TABLE 18

Extraction Efficiency of Barbiturates by Methylene Chloride

Barbiturate	Average % Recovery
amobarbital	72 ± 1
butabarbital	81 ± 11
pentobarbital	73 ± 12
phenobarbital	82 ± 14
secobarbital	79 ± 12

FIGURE 13

The x-axis gives the concentration of serum samples containing phenobarbital as obtained by the GC method of Blanke and Saady (92). The y-axis gives the concentrations of the same specimens as obtained by this LC method. Linear regression yielded the following:

slope = 1.00; intercept = .069; r =.99; n = 18

FIGURE 13. Scatter plot for serum phenobarbital concentrations using a GC method (92) and this LC method.

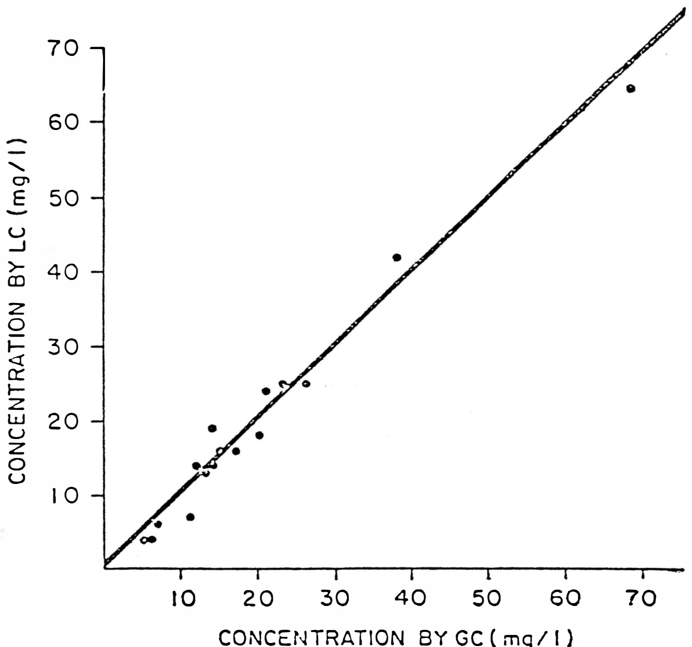


TABLE 19

Storage of Blood Containing Amobarbital

at 25°C and 4°C

Time(days)	"Therapeutic" Concentration(mg/l)		"Toxic" Concentration(mg/l)	
	25°C	4°C	25°C	4°C
0	2.2	2.2	21.8	21.8
7	2.6	2.3	21.7	23.4
14	2.4	2.7	19.4	23.3
42	2.0	1.8	17.2	19.1
49	2.2	2.0	18.2	22.8
58	2.4	2.0	17.0	19.4
81	2.5	2.1	20.3	20.1
Slope	1.6×10^{-4}	-.0052	-.0036	-.039
Intercept	2.3	2.3	20.7	22.8
95% CI for slope	(-.007,.007)	(-.01,.001)	(-.086,.078)	(-.11,.028)

TABLE 20

Storage of Blood Containing Butabarbital

at 25°C and 4°C

Time(days)	"Therapeutic" Concentration(mg/l)		"Toxic" Concentration(mg/l)	
	25°C	4°C	25°C	4°C
0	6.2	6.2	20.6	20.6
7	4.5	4.5	16.8	18.1
14				18.7
35	5.3	4.5	19.6	18.9
42	5.0	4.7	18.3	19.4
49	4.7	5.5	17.3	18.6
60	5.1	4.8	18.6	18.8
82	4.8	5.8	18.6	18.4
Slope	-.0084	.0010	-.0077	-.013
Intercept	5.2	5.1	18.8	19.5
95% CI for Slope	(-.020,.004)	(-.029,.031)	(-.056,.031)	(-.043,017)

TABLE 21

Storage of Blood Containing Pentobarbital

at 25°C and 4°C

Time(days)	"Therapeutic" Concentration(mg/l)		"Toxic" Concentration(mg/l)	
	25°C	4°C	25°C	4°C
0	1.7	1.7	19.3	19.3
7		1.7		21.4
14			19.2	20.4
35	1.4	1.2	19.7	20.3
42	1.1	1.2	19.7	20.3
49	2.3	2.1	20.8	18.8
60	1.8	1.5	20.5	19.9
83	1.9	1.8	19.7	19.4
Slope	.0042	.0023	.0091	-.0048
Intercept	1.5	1.6	19.5	19.7
95% CI for Slope	(-.013,.021)	(-.018,.014)	(-.016,.034)	(-.011,.0012)

TABLE 22

Storage of Blood Containing Phenobarbital

at 25°C and 4°C

Time(days)	"Therapeutic" Concentration(mg/l)		"Toxic" Concentration(mg/l)	
	25°C	4°C	25°C	4°C
0	20.7	20.7	60.5	60.5
7	20.6	21.0		57.3
14	19.4	21.0	56.6	58.6
21	18.5	21.3	53.0	56.2
28		18.0		
35	21.7	21.2	61.7	58.0
42	22.7	22.4	59.4	52.2
81	20.5	20.8	55.8	61.3
Slope	.013	.0035	-.020	.008
Intercept	20.2	21.1	58.4	57.5
95% CI for Slope	(-.62,.66)	(-.17,.18)	(-.17,.21)	(-.13,.15)

TABLE 23

Storage of Blood Containing Secobarbital

at 25°C and 4°C

Time(days)	"Therapeutic" Concentration(mg/l) 25°C	4°C	"Toxic" Concentration(mg/l) 25°C	4°C
0	2.3	2.3	18.1	18.1
7	1.9	2.7	22.5	22.2
14	2.1	2.5	18.2	22.3
21	2.9	2.9	19.6	17.8
28	2.6	2.8		
35	2.1	2.4	18.3	19.4
42	3.0	2.9	22.0	20.8
72		2.3	19.5	19.3
Slope	0.15	.008	.026	.0016
Intercept	2.1	2.4	19.3	20.1
95% CI for Slope	(-.002,.032)	(-.004,.020)	(-.029,.081)	(-.056,.060)

TABLE 24

Storage of Serum Containing
Phenobarbital at 25°C and 4°C

Time(days)	Phenobarbital Concentration(mg/l) 25°C	4°C
0	20	20
7	21	20
14	20	20
21	19	19
27	19	19
37	20	20
42	20	20
56	19	20
70	19	19
Slope	-.018	.006
Intercept	20	20
95% CI for slope	(-.041,.005)	(-.026,.014)

The first 2 data points for each barbiturate were obtained using the liquid chromatographic method discussed above. However, due to the presence of interference substances, a gas chromatographic method (92) had to be used to finish the experiments. This is discussed in greater detail in the Discussion (p. 120).

Data from livers containing barbiturates are given in Table 25. The figures were obtained by combining data from individual livers 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 2 month period at 4°C and 25°C.

B. Thiopental

1. LC method development

a. Sample Chromatograms, linearity, and sensitivity

Figure 14 illustrates sample chromatograms of a blood extract containing thiopental and internal standard. A standard curve plotting area ratio of thiopental to phenolphthalein versus thiopental concentration was prepared by extracting blood after the addition of known amounts of thiopental (Figure 15). The plot was linear from 1 mg/l to 60 mg/l thiopental (corr >.99). Since plasma concentrations greater than 7 mg/l are associated with anesthesia (94), this procedure can be used to quantitate thiopental therapeutically as well as in overdose situations.

TABLE 25. Barbiturate Tissue Study

Time (months)	% OF ORIGINAL PRESENT ($\bar{x} \pm s$)							
	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	83±5	91±23	78±13
2	77±5	76±9	100	100	90±6	86±10	97±16	91±12

FIGURE 14

The vertical axis is time (min) and the horizontal axis is detector response.

14 a) liquid chromatogram of a blood extract containing thiopental and internal standard.

14 b) liquid chromatogram obtained after extracting a blank blood sample.

FIGURE 14. Sample chromatogram for thiopental.

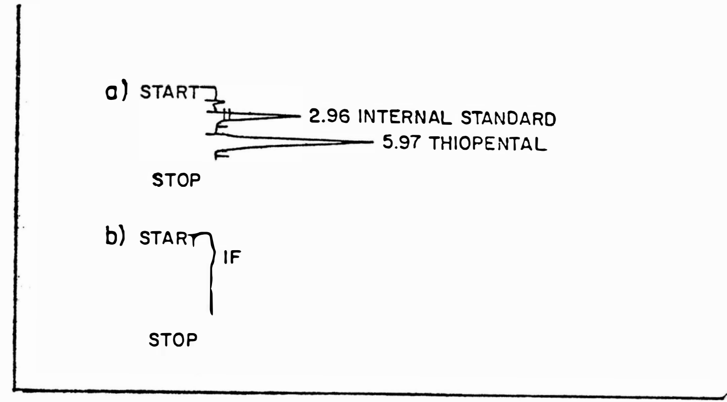
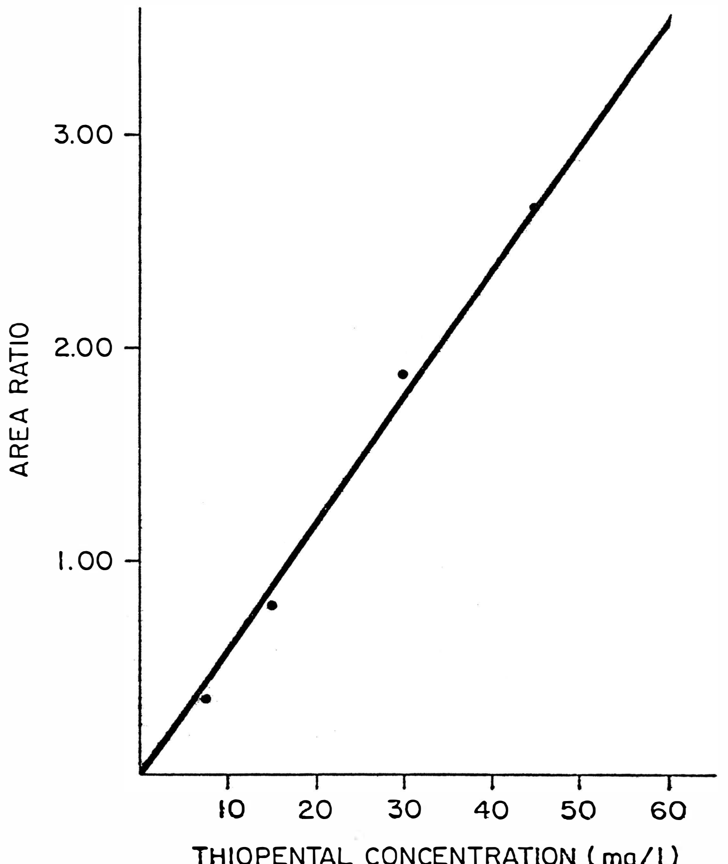


FIGURE 15

The ordinate plots area ratio of thiopental to internal standard. Linear regression yielded the following:

slope = -.062; intercept = -.09; $r > .99$

FIGURE 15. Standard curve for blood thiopental.



b. Precision

Within day precision was determined by preparing 6 samples, each containing 30 mg/l thiopental. These samples were then extracted and chromatographed. The mean and stardard deviation of the 6 area ratios were used to calculate the coefficient of variation (3.9%).

Day to day precision was calculated by preparing a 30 mg/l standard whenever thiopental samples were analyzed (approximately once a week over a 2 months period). Day to day precision was 8.2%.

c. Specificity

Weakly acidic and neutral drugs would co-extract with thiopental and internal standard. Therefore, methanolic standards of these drugs were injected into the liquid chromatograph to determine whether they interfere with this assay. Table 26 lists the drugs examined and found not to interfere. Moreover, only carbamazepine gave a peak when present in therapeutic concentrations in the blood.

2. Thiopental stability in blood and aqueous buffer

Table 27 illustrates the stability of thiopental in blood and in phosphate buffer at pH 7.0. Since 0 is within the 95% confidence interval for the slopes of these curves, no significant changes in thiopental concentration occurred under any of the conditions tested. Pentobarbital, a metabolite of thiopental and the equivalent oxybarbiturate to thiopental, was quantitated at the beginning and end of the 2 month period; none was detected in any of the samples.

TABLE 26

Co-extracted Drugs Found not to Interfere with Thiopental Assay

amobarbital
butabarbital
butalbital
carbamazepine
glutethimide
meprobamate
mesantoin
methaqualone
methyprylon
pentobarbital
phenobarbital
phenytoin
primidone
secobarbital

TABLE 27

Storage of Thiopental in Blood and Buffer at 25°C and 4°C

TIME(days)	CONCENTRATION (mg/l)			
	BLOOD		BUFFER	
	25°C	4°C	25°C	4°C
0	25	25	16	16
7	22	25	15	14
14	25	27	13	13
26	22	25	14	18
40	21	24	17	17
63	27	29	13	14
Slope	.024	.042	-.018	-.007
Intercept	23	26	15	15
95% CI for Slope	(-.076,.12)	(-.015,.12)	(-.090,.054)	(-.096,.082)

III. LIDOCAINE

A. GC method development

1. Sample chromatograms, linearity and sensitivity

Sample chromatograms for the lidocaine assay are given in Figure 16. The linearity of lidocaine concentration vs peak area ratio of lidocaine to internal standard was established by adding known amounts of lidocaine to serum, blood, or tissue homogenate, not containing lidocaine and then extracting after addition of internal standard. The curves were observed to be linear (r >.99) from 0.1 mg/l to 13 mg/l lidocaine. An example is illustrated in Figure 17. Since the therapeutic concentration of lidocaine in serum is 1-5 mg/l, this procedure provides a linear curve into the toxic range of lidocaine.

2. Precision

The "within day" precision of the method was determined by extracting 10 samples, each containing 4.6 mg/l lidocaine. The coefficient of variation was 2.9%. Moreover, replicate injections of the same sample over a two hour period showed less than a 2% change in area ratio of lidocaine to internal standard, indicating stability of the reconstituted residue.

The "day to day" precision of the method was calculated from 4.6 mg/l serum lidocaine quality control sample freshly prepared and run with each batch of lidocaine over a 3 months period. The coefficient of variation was 3.8%. During this period lidocaine analyses were performed by 8 technicians.

FIGURE 16

The vertical axis is time (min) and the horizontal axis is detector response.

16 A): gas chromatogram obtained after extracting a blank serum sample

16 B): gas chromatogram obtained after extracting a serum sample containing lidocaine and internal standard

16 C): gas chromatogram obtained after extracting an aliquot of a linear homogenate spiked with lidocaine and internal standard

FIGURE 16. Sample chromatogram for lidocaine.

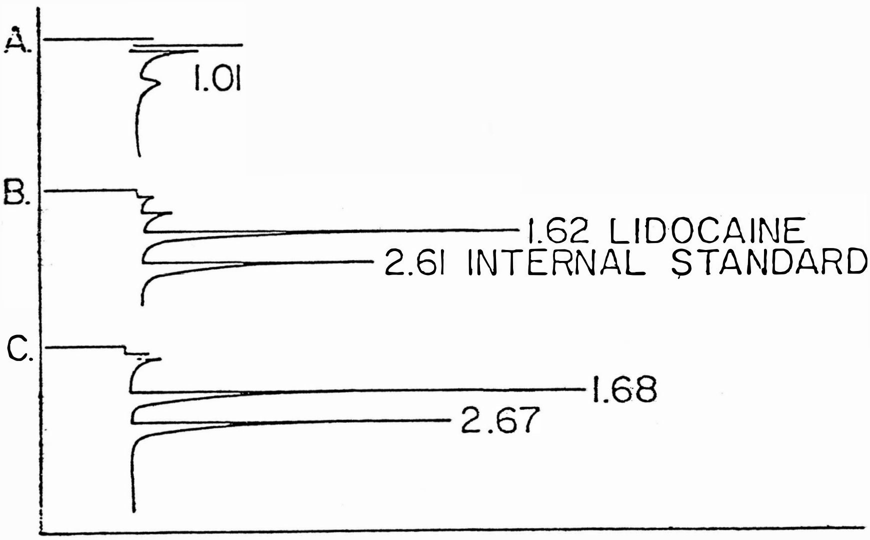
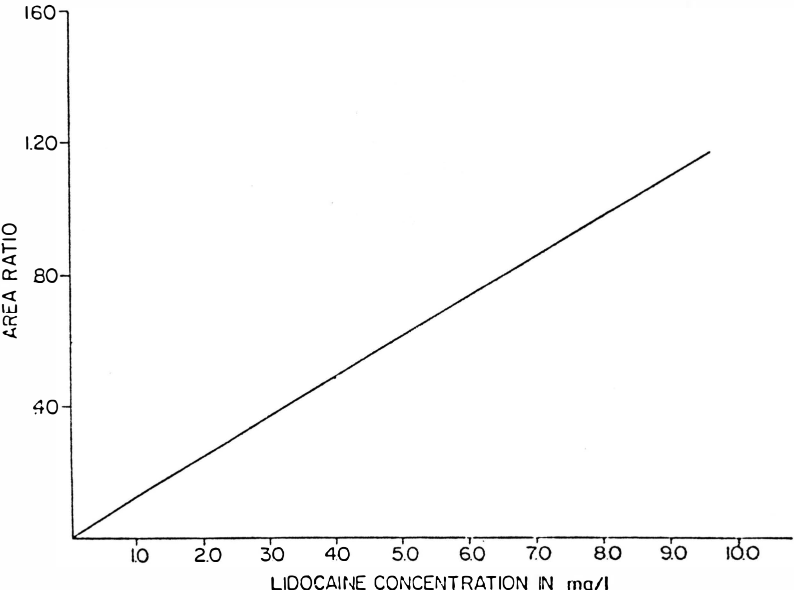


FIGURE 17

The ordinate plots area ratio of lidocaine to mepivicaine, the internal standard. Linear regression yielded the following:

slope = .14; intercept = .007; $r>.99$

FIGURE 17. Standard curve for serum lidocaine.



3. Specificity

A list of basic and neutral drugs which were extracted and found not to interfere with the quantification of lidocaine is given in Table 28. In other words, baseline separation between each of these drugs, lidocaine, and internal standard, was obtained.

Monoethylglycylxylidide (MEGX), an inactive metabolite of lidocaine, was also subjected to the extraction procedure. Its retention time was 1.3 minutes. In all patient samples quantitated for lidocaine, no interferences by MEGX were observed.

B. Lidocaine stability in blood, serum and liver

The data obtained from the pooled serum and pooled blood study are given in Tables 29-30. After 70 days, approximately 80% of the lidocaine initially present was still detected in serum; there appeared to be little temperature effect; statistical evaluation indicated that these were insignificant changes. No significant loss in lidocaine was seen in blood at either temperature as indicated by the 95% confidence interval for the slope of the concentration versus time plots.

The results from the 4 livers studied are given in Table 31. After the two month period, approximately 70% of lidocaine originally present was still detected when the livers were stored at room temperature. Livers kept in the refrigerator retained 87% of the lidocaine originally present over the same length of time.

IV. NORTRYPTYLINE, DISOPYRAMIDE, AND PROCAINAMIDE

TABLE 28

Drugs Found Not to Interfere with the Lidocaine Assay when Present in Therapeutic Concentration

Amitryptyline	Methadone
Chlordiaxepoxide	Methaqualone
Codeine	Nordiazepam
Desipramine	Nortryptyline
Diazepam	Procainamide/NAPA
Disopyramide	Propoxyphene
Doxepin	Propranolol
Flurazepam	Quinidine
Imipramine	
Meprobamate	

TABLE 29

Stability of Lidocaine in Pooled Serum

Time(days)	Concentration(mg/l) 25°C	4°C
0	6.3	6.3
7	5.8	6.0
14	5.6	6.2
21	5.5	5.9
27	5.2	5.2
37	5.3	5.3
56	5.4	5.2
70	5.3	5.6
Slope	-.011	-.013
Intercept	5.9	6.1
95% CI for Slope	(-.06,.04)	(-.084,.058)

TABLE 30

Stability of Lidocaine in Pooled Blood

Time(days)	Concentration(mg/l) 25°C	4°C
0	2.3	2.3
7	2.6	2.3
14	2.2	2.2
21	2.2	2.4
27	2.0	2.2
37	2.5	2.0
43	2.0	2.2
70	2.3	2.2
Slope	-.0019	-.0023
Intercept	2.3	2.3
95% CI for Slope	(-.010,.0063)	(-.0063,.0020)

TABLE 31

Lidocaine Liver Study

Time(months)	% Original Present ($\bar{x}$±s) 25°C	4°C
0	100	100
1	75±4	90±12
2	70±35	87±16

The concentration versus time data at 25°C and 4°C for these drugs are given in Tables 32-34. The data suggests that no significant changes in drug concentration were found at either temperature under these storage conditions. This is evident from the fact that 0 is contained within the 95% confidence interval for the slope for all 3 drugs at 25°C and 4°C.

A point brought up during this research was the effect that *in vitro* ethanol formation might have on drug quantitations. Therefore, it was decided to analyze blood specimens at 25°C and at 4°C at the beginning and end of the experiment for ethanol. A gas chromatographic method was used for ethanol quantitation (96). No ethanol was found in any of the specimens over the 2 month period.

TABLE 32

Storage of Nortryptyline in Blood

Time(days)	Concentration(mg/l) 25°C	4°C
0	5.1	5.1
17	5.2	4.3
14	4.4	4.4
28		5.3
44	4.5	
47	3.8	3.8
56	3.8	4.6
Slope	-.022	-.010
Intercept	5.1	4.9
95% CI for Slope	(-.14,.10)	(-.024,004)

TABLE 33

Storage of Disopyramide in Blood

Time(days)	Concentration(mg/l) 25°C	4°C
0	5.6	5.6
15	5.2	5.2
22	5.4	
29	6.0	4.9
43	6.0	5.1
56	5.1	6.0
Slope	-.0012	.0045
Intercept	5.2	5.2
95% CI for Slope	(-.030,.028)	(-.031,.021)

TABLE 34

Storage of Procainamide in Blood

Time(days)	Concentration(mg/l) 25°C	4°C
0	5.8	5.8
7	5.1	5.5
14	4.9	5.3
21	5.0	5.4
30	5.3	5.1
56	4.6	4.7
Slope	-.0015	-.0018
Intercept	5.4	5.7
95% CI for Slope	(-.023,.019)	(-.0033,.0003)

DISCUSSION

I. BENZODIAZEPINES

The methods used in the performance of the experiments involving benzodiazepines were based on the paper by Peat and Kopjak (91) which employed 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 liquid chromatography. However, the production of an endogenous interference product unrelated to the drug made the quantitation of demoxepam difficult and frequently impossible.

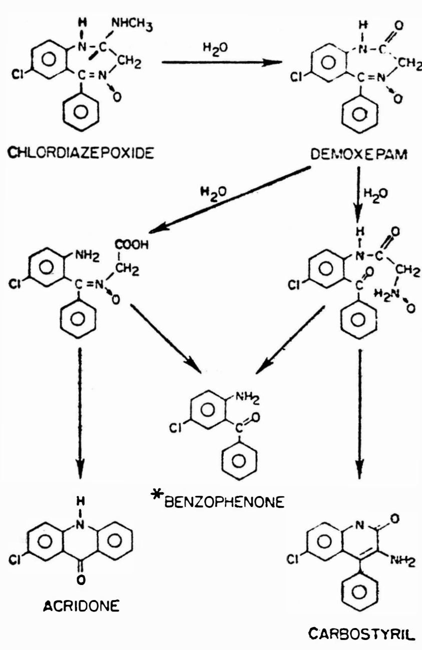
The quantitation of benzodiazepines in tissues involved a back extraction into acid followed by extraction from the aqueous layer after alkalinization. These clean-up steps were necessitated by the presence of neutral substances, especially lipids, in brain and liver.

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 interpretation 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 initially effects of dehydration were negligible.

The results indicate that diazepam stored in blood for up to 5 months was stable, even at room temperature. Flurazepam and its N-1-desalkyl metabolite also showed similar stability. 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 1-2 weeks. Even at refrigerated temperatures, a decrease was observed initially before a leveling off occurred. The data with norchlordiazepoxide 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 CDP breakdown in water is given in Figure 18 (97). In these experiments, the formation of demoxepam was observed. However, one compound produced during the aging process from chlordiazepoxide and norchlordiazepoxide

FIGURE 18. Chemical degradation of chlordiazepoxide in water. (95)



which was not reported to be formed in water, is nordiazepam, which involves the loss of the nitrone oxygen.

The experiments in which CDP was added to blood and aqueous buffer at different pHs in the presence and absence of F/C_2O_4 revealed a large amount of information about the mechanism of CDP breakdown at room temperature. In order to make more quantitative comparisons between blood and buffer at the various pHs, some simple pharmacokinetic calculations were made. Specifically, it has been reported that CDP decomposes in aqueous solutions by a first order process (98). That means that the rate of CDP breakdown is directly proportional to the CDP concentration (96). Mathematically,

$$\frac{-dC}{dt} = kC$$

where C = CDP concentration

$\frac{-dC}{dt}$ = rate of CDP breakdown

k = proportionality constant

Rearranging the above equation,

$$\frac{dC}{C} = -kdt$$

Integrating the left side of the equation from the initial CDP concentration Co to some CDP concentration C and integrating the right side from t=0 to t=t, one obtains

$$\ln C - \ln Co = -kt$$

Again rearranging,

$$\ln C = -kt + \ln Co$$

This is an equation of a line in the form y=mx+b; where

b = intercept

m = slope

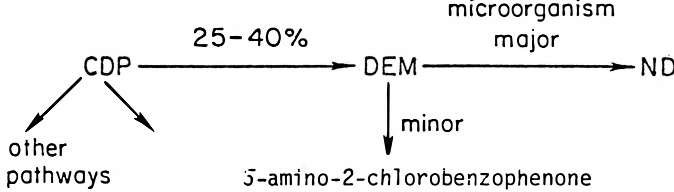
These mathematical manipulations imply that a plot of ln C vs t yields a straight line with slope -k and intercept ln Co. Therefore, samples with the same rate of decomposition should have the same slope. In this way, quantitative comparisons between the different blood and aqueous specimens can be made. Data from Tables 6-11 at 25°C were compiled in such a way. The data suggest that breakdown of CDP is affected by pH and microorganism (Figure 19).

To summarize, it appears that at pH 8 and pH 6.5, blood is not simply an aqueous solution. In one condition (blood without F/C_2O_4), a compound (ND), is formed which does not appear in aqueous buffer; in the other condition (blood with F/C_2O_4), CDP appears to be more stable than in aqueous buffer (Fig. 19a). However, at pH 5, blood does act like a simple aqueous solution in that the rate of decomposition is approximately the same in blood and buffer, both in the presence and absence of F/C_2O_4 (Fig. 19b). This would suggest that at pH 6.5 and 8.0, there is some component of the blood, which, when not subject to microorganism attack, can protect CDP breakdown in blood. This component might then be destroyed at pH 5 which then causes the blood to behave as an aqueous solution.

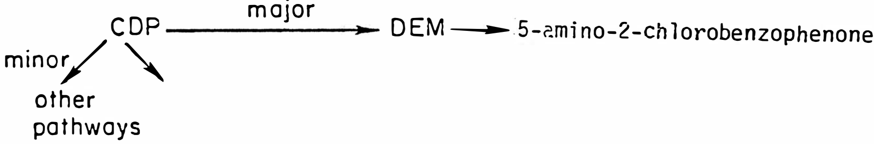
These types of calculations with CDP breakdown have previously been performed in various aqueous media by Maulding _et al_. (99). Specifically, they made Arrhenuis plots for the conversion of CDP to DEM in pH 7 phosphate buffer with ionic strengh 0.5M. By extrapolating their plot of log k vs. =1/T to 298 K, a k value of .0072 $(\text{days})^{-1}$ was obtained. In the experiments performed in this research, a k value for ln(CDP) vs. t plot of phosphate buffer pH 6.5 and ionic strength 0.6M was .036 $(\text{day})^{-1}$ (Table 12). Converting the data to log k, a k value of

FIGURE 19. Proposed mechanism for chlordiazepoxide breakdown.

a. blood, pH 6.5, 8



b. buffer, pH 5, 6.5, 8; blood pH 5



.015 $(day)^{-1}$ obtained. Therefore there is approximately a 2-fold difference between the 2 sets of data. Considering the differences in starting concentration (.90 mg/ml vs. 5 mg/l) and methodology (thin-layer chromatography vs. liquid chromatography), the data reported here is in good agreement with the previously published data.

The decay pattern observed with chlordiazepoxide and norchlordiazepoxide at room and refrigerated temperatures has been previously observed with clonazepam, another benzodiazepine. Knop *et al*. (100) reported that a plasma clonazepam concentration of 25 μg/l disappeared within 6 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.

Because of this reported decrease in clonazepam concentration at 25°C, it was decided to examine another benzodiazepine containing a nitro group, nitrazepam. Like clonazepam, nitrazepam was found to disappear at room temperature, from 5 mg/l initially, to none detected by day 25.

The data from the tissues corroborates many of the results from the blood data. Diazepam, for example, was found to be stable in tissues as well as in blood. Flurazepam also appeared to be moderately stable. One problem that arose during the research was the acquisition of tissues containing chlordiazepoxide. Throughout a 2 year period, 2 livers containing CDP were obtained and prepared as outlined in the Experimental Section (p.33). However, the initial analysis showed no CDP present in the liver even though CDP was detected in blood in both cases. Moreover nordiazepam was detected both livers. This was determined by LC and GC retention times. Even though nordiazepam is a

metabolite of CDP, it is unlikely that it would be detected in the liver without the detection of other metabolites, norchlordiazepoxide and demoxepam. Since nordiazepam is a breakdown product of CDP, it would appear that CDP had broken down before the commencement of the experiment. The livers were stored in a freezer at -20°C prior to the experiment, but it could not be determined how long the livers were at body temperature, room temperature or 4°C before placement in the freezer. In general for medical examiner cases in Virginia, autopsies are performed within 48 hours after death and tissue specimens are placed in the freezer within hours after autopsy. Nevertheless, it can be said with relative certainty that CDP is very unstable in liver, even though no quantitative data were obtained.

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. (101) 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 within. Similar findings with chlordiazepoxide and diazepam were also observed by van der Kleign (102) in mice. Moreover, the mice livers also demonstrated an unequal pattern of distribution after intravenous administration of diazepam and chlordiazepoxide. This pattern of unequal distribution has been observed with imipramine (103) and barbiturates (104). 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 100-150 g), and because of the multiple data points required, only 10 g portions could be used for each quantitation.

Many implications arise from these data both for clinical and forensic 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 6 month period when stored in amber bottles in the freezer. Under refrigerated conditions in regular bottles, decomposition was more extensive. Norchlordiazepoxide was found to be even more unstable. Quantitation of chlordiazepoxide and its metabolites should be performed as rapidly as possible. When samples are sent to other laboratories, it is preferable that they be shipped frozen or in refrigerated containers. In forensic cases, when analyses are not performed as quickly as in clinical situations, it is recommended that the samples contain a chemical preservative and be stored in the freezer until the time of analysis. Moreover, the interpretation of chlordiazepoxide and metabolite concentrations in post-mortem 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 which 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 (Figure 20), they are also breakdown products of chlordiazepoxide in blood.

II. BARBITURATES

A. Oxybarbiturates. There are several advantages to the LC method developed for barbiturate quantitation. Its sensitivity allows the detection of sub-therapeutic concentrations of the most frequently prescribed barbiturates. No derivatization to obtain good peak efficiency was required. Furthermore, an isocratic mobile phase using methanol and an unbuffered aqueous solution was employed. This mobile phase increases the lifetime of the analytical column, requires a lower pump pressure and avoids the intensive clean-up during the shut-down procedure. A brief flushing of the column with methanol at the end of the day is sufficient. The turn-around time for this procedure is less than one hour; therefore, it is quite useful in emergency toxicology situations.

Besides the many advantages to this method, there are also some limitations to it. The wavelength of detection is 210 nm, thus requiring a variable wavelength detector. Amobarbital and pentobarbital are not separable using this method. Therefore, an alternate method to determine the identity of the barbiturate is required. The biggest shortcoming of this method is the poor resolution between primidone and

FIGURE 20. Metabolism of chlordiazepoxide.

CHLORDIAZEPOXIDE

NORCHLORDIAZEPOXIDE

NORDIAZEPAM

DEMOXEPAM

OXAZEPAM

conjugation as glucuronide
and excretion

phenobarbital. Because of this, this system cannot be used for samples from patients receiving primidone. Also, the ability to collect fractions coming off the detector allows simpler confirmation by mass spectrometry. Confirmation is necessary in forensic cases in which these drugs may be a factor in the cause of death.

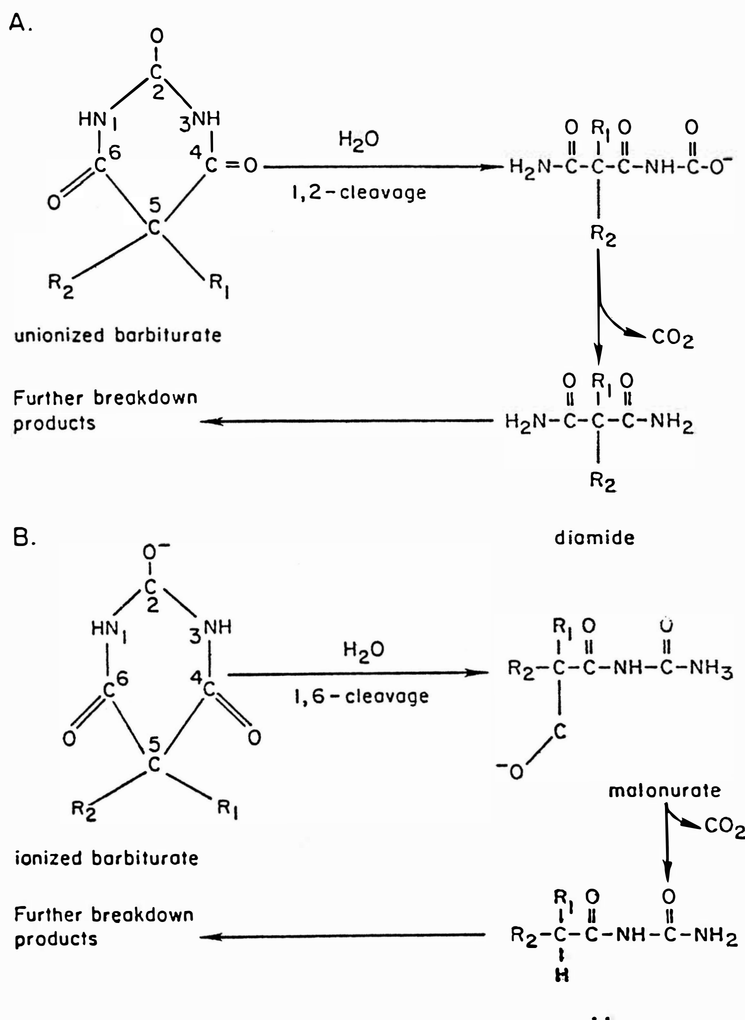
The analysis of barbiturates in decomposed blood and tissues is somewhat more difficult than the analysis of more routine toxicologic specimens. In this research, a liquid chromatographic method was specifically developed to quantitate barbiturates with the hope that the advantages of liquid chromatography in barbiturate analysis could be utilized. For example, no derivatization is required and more polar metabolites, if produced, could be more easily quantitated using liquid chromatography. However, as the research developed, liquid chromatography became unsuitable for quantitation because of the presence of interfering substances produced during the decomposition process. When this problem first developed, it could be solved by adjusting the pH of the water in the mobile phase, but eventually, the interference peaks became so large that even this did not work. A clean-up step where the initial organic extract was back extracted into base and then back extracted after acidification also permitted quantitation, but this proved to be quite time comsuming. Finally, it became necessary to return to gas chromatography with nitrogen phosphorus detection for quantitation. Even this did not solve all of the problems with quantitation of barbiturates in liver. Initially a three step extraction using methylene chloride as the extraction solvent was used. However, in decomposed tissue, this caused the co-extraction of interference products which rendered quantitation impossible. This

problem was aleviated for the most part by using a slightly more polar solvent, toluene:hexane:isoamyl alcohol (78:20:2) for the initial extraction.

It appears that amobarbital, butabarbital, pentobarbital, phenobarbital, and secobarbital are quite stable when stored in blood both at room temperature and at 4°C over a 2 to 3 month period. Since barbiturate instability in aqueous alkaline medium is well known (104), 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 utility 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 suggests that the majority of blood samples are slightly acidic or neutral, which means that the blood pH favors chemical stability of barbiturates.

Some work has been done on identifying the breakdown products of barbiturates in aqueous solutions. Goyan et al. (106) first proposed 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-positions 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 Figure 21.

FIGURE 21. Degradation pathways for barbiturates (106).



This mechanism was verified by Tishler *et al*. (107) who were able to follow spectrophotometrically the degradation of phenobarbital and the formation of phenyl-ethylacetylurea, the ureide formed from the breakdown of phenobarbital. Using the absorbance at 245 nm and 260 nm and some algebraic manipulations, they were able to quantitate the ureide in the presence of phenobarbital and the diamide. While studying the kinetics of hydrolysis of barbituric acid derivatives, Garrett *et al*. (105) discovered that in alkaline solution, conversion of barbital to diethylmalonuric acid is reversible; that is, once the diethylmalonuric acid is formed, either decarboxylation to the acetylurea derivative or recyclization to barbital can occur. Thin-layer chromatography was used to separate and identify some of the degradation products. The reversibility of barbital degradation was confirmed using mass spectrometry by Gardner and Goyan (108). However, there was no evidence to suggest the reversibility of this reaction with other barbiturates. For example, Fretwurst (109) reported a 50% recovery of degraded barbital as diethylmalonuric acid but no phenylethylmalonuric acid was found in the degradation of phenobarbital. It would appear that the decarboxylation of phenylethylmalonuric acid is much faster than the decarboxylation of diethylmalonuric acid, rendering the initial breakdown step of phenobarbital essentially irreversible by equilibrium forces.

Besides chemical stability, a factor in blood which is a greater problem than in water is the presence of microorganisms. Since no chemical preservatives were added to the blood, microorganism growth could continue relatively umimpeded at room temperature and this was verified by some of the physical characteristics of the blood. The data

suggest that microorganism enzymes did not alter the structure of the barbiturate ring. These data are in agreement with some of the data on barbiturate stability in blood and are in conflict with others. For example, Coutselinis and Kearis (5) found decreases in pentobarbital concentration in dog blood when stored at room temperature over a two month period. However, one potential problem with these data is that quantitation of pentobarbital was by a UV spectrophotometric method, which would be more susceptible to interferences from decomposition products than would the gas chromatographic method used in the latter part of these experiments. Schäfer (9) reported no change in serum phenobarbital concentration when stored at 4°C for about 12 weeks. Wilensky (11) found no changes in serum phenobarbital concentrations after 6 months of storage in glass containers without exposure to light at room temperature.

The liver data corroborated the findings in blood, that is, no significant changes were observed over a two month period. This conflicts with previously reported data which reports both increases and decreases in barbiturate concentration (2, 6, 7). As with the conflicts observed in blood and discussed in the preceding paragraph, it is believed that differences in storage conditions and in methodology can account for these differences.

B. Thiopental. Among the advantages that liquid chromatography has over gas chromatography is its ability to handle thermally unstable compounds, ie., compounds which decompose at the high temperatures of the gas chromatographic injection port, oven and detector. Thiopental is one such compound. A UV scan of thiopental

suggested that a detection wavelength of 290 nm would provide the greatest sensitivity for quantitating thiopental.

The biggest problem in the development of this assay was the search for an internal standard. Specifically, a weakly acidic compound with sufficient UV absorbance at 290 nm was needed. This latter requirement excluded most of the common barbiturates. Ideally, an internal standard should be structurally similar to the analyte. Thiamylal, thioamobarbital was tested, but insufficient resolution from thiopental was obtained. The only chemical that could be found which met all of the criteria was phenolphthalein; its phenolic groups enabled its extraction at pH 5.5. Moreover, it had sufficient UV absorbance at 290 nm.

Pentobarbital, a metabolite of thiopental might also require quantitation when thiopental is present. Since pentobarbital is a breakdown product of thiopental when analyzing by gas chromatography, the simultaneous quantitation of thiopental and pentobarbital by GC is virtually impossible. Unfortunately, pentobarbital has little UV absorbance at 290 nm and thus, cannot be quantitated with thiopental at that wavelength. However, since pentobarbital and thiopental are clearly separated, it is possible to quantitate pentobarbital at 210 nm. Phenolphthalein also has a UV absorbance at 210 nm and can be used as an internal standard for pentobarbital quantitation as well. The fact that all 3 compounds have sufficient absorbance at 210 nm would suggest the use of this wavelength for simultaneous quantitation of thiopental and pentobarbital. The problem with this is the decreased UV absorbance of thiopental at 210 nm in comparison to 290 nm. Moreover, detection at

210 nm is more difficult because of the greater presence of interference substances from the blood or from the reagents.

The method described for the quantitation of thiopental offers many advantages. Its sensitivity permits quantitation in sub-therapeutic concentrations as well as in larger concentrations (95). No interferences from co-extracted drugs or endogenous products were detected. Moreover it permitted the quantitation of thiopental in decomposed blood.

It was somewhat surprising to find that thiopental was stable over a 2 month period both in blood and in buffer. Narbutt-Mering and Weglowskou (110) identified the decomposition products of thiopental in pharmaceutical preparations. Specifically, they artifically aged the preparations by heating at 110°C for 3 to 24 hours and identified the breakdown products by paper chromatography. The scheme which they derived is given in Figure 22. Since no changes in thiopental concentration was found in either medium studied, no attempts were made to identify any of these products in the specimens tested in this study.

III. LIDOCAINE

Although numerous gas chromatographic procedures for lidocaine using nitrogen detection have been published (111-117), several problems with these methods were apparent. For example, some require such time consuming steps as multiple extractions (114,115) or derivatization (110). Previous attempts at lidocaine quantification resulted in instability of the standard. The method developed illustrated the stability of the standard curve and speed of analysis without

FIGURE 22. Decomposition of thiopental in pharmaceutical preparations

thiopental

H_2O

ethyl-methylbutyl-
thiobarbituric acid

CO_2

ethyl-methylbutyl-
acetyl urea

2-methylbutyl butanoic acid

+

$NH_2-C(=S)-NH_2$

Thiourea

sacrificing the needed specificity, sensitivity, and precision. No cardioactive drugs which might be co-administered with lidocaine were found to interfere with lidocaine analyses. Furthermore, its sensitivity enables the quantitation of sub-therapeutic concentrations of lidocaine. Its precision is also comparable to other published gas chromatographic methods for lidocaine.

Lidocaine has been implicated in numerous accidental and homicidal deaths (118). Because of this, its chemical stability in body fluids and tissues takes on great significance. Today, little work has been published on the stability of lidocaine in biological matrices. However, ethylglycylxylidide and glycylxylidide, two metabolites of lidocaine, are known to be unstable in plasma and urine. Adjepon-Yamoah and Prescott (119) found significant losses of each when stored for one month in neutral or alkaline solution at 4°C to -20°C.

It appears that from the data presented here, lidocaine is relatively stable in serum, blood, and liver when stored at either room temperature or at 4°C. In all specimens analyzed, at least 70% of the amount originally present was recovered after 2 months. This stability in biological samples is not too surprising since lidocaine is quite stable when stored in aqueous solution. Extremes in pH are required before lidocaine is hydrolyzed to 2,6-dimethylaniline and N,N-diethylaminoacetic acid (120).

IV. DISOPYRAMIDE, NORTRYPTYLINE, PROCAINAMIDE

To gain information about the stability of other N-containing structures, not included in the study of benzodiazepines, barbiturates, and lidocaine, the following drugs were also examined in blood: disopyramide, nortryptyline, and procainamide. Nortryptyline contains a secondary amine, procainamide contains a primary amine, and disopyramide has an amide linkage with a primary amine. It was found that each drug was stable in blood over a two month period, both at 25°C and 4°C. This would suggest that drugs or chemicals containing these functional groups are stable under the experimental design followed.

The stability of disopyramide in blood was also observed in aqueous solution (121). However, little data in the literature were found concerning the aqueous stability of procainamide and nortryptyline. Therefore, concurrent with the blood experiment, solutions of procainamide and nortryptyline in phosphate buffer (pH 7.5) were prepared and analyzed with the blood samples. No significant changes in concentration in either drug were found over the two month period.

CONCLUSION

This research clearly indicated that drugs can vary in their *in vitro* stability in blood and in tissues and that numerous factors can alter stability. One approach to analyzing all of the data presented here in its entirety is to review the structures of the drugs involved, placing specific emphasis on the nitrogen containing functional groups possessed by these chemicals. Table 35 lists the drugs, placing specific emphasis on the nitrogen-containing functional groups and their stability or instability in blood. From Table 35, a number of conclusions can be drawn. Firstly, the basic structure of the barbiturate ring is stable in blood, since no changes in oxybarbiturates or thiopental were observed. The stability of the benzodiazepine ring requires a more detailed analysis. To review, chlordiazepoxide (CDP), norchlordiazepoxide (NCDP), and demoxepam (DEM) decomposed in blood at 25°C. CDP and NCDP broke down to DEM which was then converted to nordiazepam; these changes do not directly involve the benzodiazepine ring. The conversion of DEM to ND involves the loss of the nitrone oxygen. Therefore, it appears from the data that nitrones are unstable in blood under the storage conditions studied. However, one other benzodiazepine was found to be unstable in blood at 25°C, nitrazepam. Since no breakdown products of nitrazepam were identified and quantitated, it cannot be said with certainty whether the nitro group was being changed or the benzodiazepine ring structure was being broken

TABLE 35

Nitrogen-Containing Functional Groups
of Drugs Examined

Skeletal Structure	Drug	Stable/Unstable in Blood at 25°C
R O | ‖ N—C / \\ Ø CH_2 \\ / C=N | Ar	diazepam	stable
	flurazepam	stable
	nordiazepam	?
	nitrazepam	unstable
	N-1-desalkylflurazepam	stable
N-R \\ N=C / \\ Ø CH_2 \\ / C=N | ↓ Ar O	chlordiazepoxide	unstable
	norchlordiazepoxide	unstable
H O | ‖ N—C / \\ Ø CH_2 \\ / C=N | ↓ Ar O	demoxepam	unstable
Ar - NO_2	nitrazepam	unstable

TABLE 35 (continued)

Nitrogen-Containing Functional Groups of Drugs Examined

Skeletal Structure	Drug	Stable/Unstable in Blood at 25°C
Barbiturate ring: H–N, C=O, C=O, N–H, C=O, $C(R_1)(R_2)$	barbiturates	stable
$R - C(=O) - NH_2$	disopyramide	stable
$R - C(=O) - NHR_1$	lidocaine	stable
	procainamide	stable
$R - NH_2$	procainamide	stable
$R - NH - R_1$	nortryptyline	stable
$N(R)(R_1)(R_2)$	disopyramide	stable
	flurazepam	stable
	lidocaine	stable

NOTES: R, R_1 and R_2 are alkyl groups
Ar is an aryl group

ϕ = (benzene ring)

down. In other words, it is possible that the nitro group is contributing to the instability of the ring, making it more susceptible to breakdown. Of all the drugs studied, nitrazepam most closely resembles nordiazepam structurally, the only difference being the nitro group on nitrazepam. Since conflicting data exists on nordiazepam, no further statement about the site of nitrazepam degradation can be made.

Other N-containing functional groups appeared to be stable according to the data presented: amides, anilides, amines, and imines.

In those chemicals unstable at 25°C, their decomposition can be traced to both chemical and microorganism effects. For example, the breakdown of chlordiazepoxide to demoxepam occurred in blood and aqueous buffer, suggesting that this is an effect seen in all aqueous media; however, the conversion of demoxepam to nordiazepam is not seen in water and is greatly delayed by fluoride, a microorganism inhibitor. The decomposition of nitrazepam at 25°C was not studied in great detail. Nevertheless, nitrazepam breakdown was seen in both aqueous buffer and blood, suggesting that it was, at least in part, a breakdown involving aqueous media.

The loss of the nitrone oxygen as seen in the breakdown of chlordiazepoxide, norchlordiazepoxide, and demoxepam has been reported previously (122) and can occur in several ways. For example, nitrones can react with electrophiles (E) in aqueous or alcoholic media to form the deoxygenated product via the following mechanism;

$$=\overset{+}{N}(-O^{-})- \; + \; E \longrightarrow =\overset{+}{N}(-O\text{-}E)- \longrightarrow =N- \; + \; E=O$$

The deoxygenation may also occur upon mild heating in various solvents or upon heating more vigorously either alone or in the presence of various solid catalysts. Therefore, nitrones are unstable in other solutions besides blood.

The work presented has applications in the clinical and forensic toxicology laboratories. Biological specimens containing chlordiazepoxide or nitrazepam should not be exposed to 25°C for long periods of time to protect the integrity of the specimens. However, these findings can be applied in a more general sense. For example, any biological specimen containing chemicals with nitrones must be preserved properly to ensure proper and meaningful analysis. Specifically, blood and tissues should be frozen immediately upon receipt and kept there until the time for analysis. Although it is unclear to date about the stability of the nitro functional group, it too should be stored under these rigorous storage conditions until more data are obtained.

In conclusion, it should be stated that knowledge concerning the stability of all chemicals requiring analysis by clinical and forensic toxicologists is important for proper interpretation of results. This research has focused on drugs with nitrogen-containing functional groups, but this group, albeit a large group, is by no means the only group of interest to the toxicologist. Much more work of this type is required in the future, since there are still large groups of chemicals which have not been studied thoroughly.

REFEREMCES

1. Gonzales, T. A., Vance, M., Helpern, M., and Umberger, C. J., Legal Medicine, Pathology, and Toxicology, New York, Appleton, Century, Crofts, Inc., 1954.

2. Parker, J. M., Winek, C. L., and Shanor, S. P., "Post-mortem changes in tissue levels of sodium secobarbital", Clin. Tox., 4(2): 265-272, (1971).

3. Barbieri - Palmieri, C., "Sulla possibilitadi determinare quantitativamente i barbiturici nei cadaveri in vari tempi dopo la morte", Zacchia, 7:203-222, (1942).

4. Algeri, E. J., "The determination of barbiturate after putrefaction", J. For. Sci., 2(4):443-455, (1957).

5. Coutselinis, A., and Kiaris, H., "The influence of putrefaction on the determination of barbiturates in blood", Med. Sci. Law, 10: 47-49, (1970).

6. Goldbaum, L., "Determination of barbiturates - ultraviolet spectrophotometric method with differentiation of several barbiturates", Anal. Chem. 24:1604-1607, (1952).

7. Sunshine, I. and Hackett, E., "Chemical findings in cases of fatal barbiturate intoxications", J. For. Sci., 2(2):149-158, (1957).

8. van der Kleijn, E., Guelen, P. J. M., van Wijk, C., and Baars, I., "Clinical pharmacokinetics in monitoring chronic medication with anti-epileptic drugs, "in Clinical Pharmacology of Anti-Epileptic Drugs, H. Schneider, et al., Eds. Springer-Verlag, New York, 1975, pp. 11-33.

9. Schäfer, H. R., General discussion in Clinical Pharmacology of Anti-Epileptic Drugs, H. Schneider, et al. Eds. Springer-Verlag, New York, 1975, pp. 307-309.

10. Pippenger, C. E., Penry, J. K., White, B. G., Daly, D. D. and Buddington, R., "Interlaboratory variability in determination of plasma antiepileptic drug concentrations", Arch. Neur. 33:351-355, (1976).

11. Wilensky, A. J., "Stability of some antiepileptic drugs in plasma", Clin. Chem. 24(4):722-723, (1978).

12. Gettler, A. O. and Freimuth, H. C., "The carbon monoxide content of the blood under various conditions", Am. J. Clin. Path. 10:603-616, (1940).

13. Weimann, W., "Carbon Monoxide in exhumed bodies", Deut. Zeit. Ges. Ger. Med., 17:48, (1931).

14. Sendroy, J. and Liu, S. H. "Gasometric determinations of oxygen and carbon monoxide in blood", J. Biol. Chem., 89:113, (1930).

15. Dominguez, A. M., Halstead, J. R., and Domanski, T. J., "The effect of postmortem changes on carbonxyhemoglobin results", J. For. Sci., 9(3):330, (1964).

16. Sjöstrand, T., "The formation of carbon monoxide by in vitro decomposition of hemoglobin in bile pigments", Acta physiologica scand., 26:328, (1952).

17. Markiewicz, J., "Investigations of endogenous carboxyhemoglobin", J. For. Med., 14:16, (1967).

18. Wollf, E., "Une méthode très simple pour la determination de petites quantités d'oxyde carbone dans le sang," Ann. Med. leg. 27:221, (1947).

19. Winek, C. L. and Prex, D. M., "A comparative study of analytical methods to determine postmortem changes in carbon monoxide concentration", Forensic Sci. Int. 18:181-187, (1981).

20. Iisalo, E. and Nuutila, M., "Myocardial digoxin concentrations in fatal intoxications", The Lancet, 3:257, (1973).

21. Selesky, M., Spiehler, V., Cravey, R. H., and Elliot, H. W., "Digoxin concentrations in fatal cases", J. For. Sci. 22(2):409-417, (1977).

22. Holt, D. and Bernstead, J., "Postmortem assay of digoxin by radioimmunoassay", J. Clin. Path., 28:483-486, (1975).

23. Vorpahl, T. E., and Coe, J. J., "Correlation of antemortem and postmortem digoxin levels", J. For. Sci. 23(2):329, (1978).

24. Aderjan, R., Buhr, H. and Schmidt, G., "Investigation of cardiac glycoside levels in human post mortem blood and tissues *determined* by a special radioimmunoassay procedure", Arch. Tox. 43(2):107-114, (1979).

25. Jatlow, P. I. and Bailey, D. N., "Gas-chromatographic analysis for cocaine in human plasma with use of a nitrogen detector", Clin. Chem 21(13):1918-1921, (1975).

26. Divorichik, B., Miller, S. and Graham, W. "Gas chromatographic determination of cocaine in whole blood and plasma using a nitrogen sensitive flame ionization detector", J. Chrom. 135(1):141-148, (1977).

27. Taylor, D., Estevez, V. S., Englert, L. F. and Ho, B. T., "Hydrolysis of carbon-labeled cocaine in human serum", Res. Comm. Chem. Path. Pharm 14(2):249-257, (1976).

28. Stewart, D. J., Inaba, T., Tang, B. K. and Kalow, W. "Hydrolysis of cocaine in human plasma by cholinesterase", Life Sci. 20:1557-1563, (1973).

29. Baselt, R. "On the stability of cocaine in biological fluids", Presented at the 30th annual AAFS meeting, St. Louis, February 23, 1978.

30. Curry, A. S., "Cyanide poisoning", Acta Pharmacol. Toxicol. 20:291-294, (1963).

31. Ballatyne, B., Bright, J., Williams, P. "An experimental assessment of decreases in measurable cyanide levels in biological fluids", J. For. Sci. Soc. 13:111-117, (1973).

32. Barr, S., "The microdetermination of cyanide: Its application to the analysis of whole blood:", Analyst, 91:268-272, (1966).

33. Sunshine, I. and Finkle, B., "The necessity for tissue studies in fatal cyanide poisoning", Intern. Arch. Gewerbepath. Gewerbehyg. 20:558-561, (1964).

34. Von Zeynek, R. V., Z. Physiol. Chem. 33:426, (1901).

35. Ioanid, N. et Bors G., "Les empoisonnements par les glucosides cyanogénétiques ou à ses sels, "Ann. méd. lég. 40:43-48, (1960).

36. Guantelli, M. A., Meth. Forensic Sci., 3:222, (1964).

37. Ioanid, N. and Bors, G., "Contribution chimicojudiciare à l'études des empoisonnements par l'acide cyanhydrique", Ann. méd. lég. 41:275-279, (1961).

38. Oke, O. L., World Rev. Nutrition and Dietetics 11:170, (1969).

39. Ballantyne, B., Bright, J. E., and Williams, P., "The postmortem rate of transformation of cyanide", Forensic Sci. 3:71-76, (1974).

40. Narayanaswani, K., Jand, S., Kotangale, R. S., Nath, P. and Bisaria, V. S., "Changes in cyanide as a function of storage time in autopsy material by UV spectrometry", Indian J. Med. Res., 73:291-295, (1981).

41. Bernt, A. C., Kerdeu, C., Prokop, O., "Zur frage der Veruertbarkeit von cyanidbefunden im leichenmaterial", Dtsch. Z. ges. gerichtl. Med. 51:522-533, (1961).

42. Gettler, A. O. and Baine, J. O., "The toxicology of cyanide", Amer. J. Med. Sci. 195:182-198, (1938).

43. Curry, A. S., Price, D. E., and Rutter, E. R., "The production of cyanide in post mortem material", Acta Pharmacol. et Toxicol. 25:339-344, (1967).

44. Parker, J., "Postmortem drug level changes", Toxicology Annual, C. L. Winek, Ed., Dekker, New York, pp. 151-165, (1974).

45. Corry, J. E. L., "A Review - Possible sources of ethanol ante- and post-mortem: its relationship to the biochemistry and microbiology of decomposition", J. Appl. Bac. 44:1-56, (1978).

46. Nicloux, M., "Sor de l'alcool dans le cadavre d'un mammifere alcoolisé. Neoformation d'alcool chez les animaux témoins", comptes Rendus des Séances de la Societé de Biologie, Paris, 120:1306-1309, (1935).

47. Nicloux, M., "Neoformation d'alcool ethylique dans le cadavre humain en voie de putrefaction", Compte Rendu des Séances de la Societé de Biologie, Paris, 121:975-978, (1936).

48. Narikawa, R., and Kotoku, S., "Medico-legal evaluation of the ethanol levels in cadavric blood and urine", Yonago Acta Medica 15:61-69, (1971).

49. Davis, G. L., Leffert, R. L. and Rantanen, N. W., "Putrefactive ethanol sources in postmortem tissues of conventional and germ free mice", Arch. Path. 94:71-74, (1972).

50. Iribe, K., Nakai, A., Ueno, M. and Mukal, S. "Relations between production of alcohol from body fluid and LDH activity in decomposition in corpes", Reports National Research Institute of Political Science Tokyo 27:8-11, (1974).

51. Redetzki, H. Johannsmeier, K. and Dotzauer, G. "Faulnis and Athylalkohol", Deutsche Zeitschrift fur diegesamte gerichtliche Medizin 41:424-434, (1952).

52. Wolthers, H., "Undersøgelser over post-mortel", Ålkoldannelse academic dissertation, København, Christtreus Bogtrykkeri.

53. Pleukhahn, V. D., "The significance of blood alcohol levels at autopsy", Med. J. Aust. 2:118-124, (1967).

54. Gormsen, "Yeasts and the production of alcohol post-mortem", J. For. Med. 1:170-171, (1954).

55. Gormsen, "Alcohol production in the dead body - Further investigations", J. For. Med. 1:314-315, (1954).

56. O'Toole, W. F., Saxena, H. M. K., Golden, A. and Ritts, R. E., "Studies of postmortem microbiology using sterile autopsy technique", Arch. Path. 80:540-547, (1965).

57. Clark, M. A. and Jones, J. W., "Studies on putrefactive ethanol production, I: Lack of spontaneous ethanol production in intact human bodies", J. For. Sci. 27(2):366-371, (1982).

58. Freimuth, H. C., Volatile, M. T. and Fisher, R. S., "The results of studies on the determination of ethyl alcohol in tissues", J. Criminal Law, Criminology and Police Science, 42:529-533, (1951).

59. Schwerd, W. and Garhammer, C. L., "Uber der Nachwers von niederen primaren Alcoholen der aliphatischen Reihe und deren Bildung in faulendem Blut", Deutsche Zeitschrift fur die Gesamte Gerichtliche Medizin 42:75-90, (1953).

60. Schwerd, W., "Die Beurteilund von Alkoholbefunden im Leichenblut", Deutsche Zeitshrift fur die Gesamte Gerichtliche Medizin 43:221-231, (1954).

61. Powlus W. and Janitzki, V., "Untersuchungen am Leichenblut nach Widmark and nach der ADH-Methode", Deitsche Zeitschrift fur die Gesamte Gerichtliche Medizin 48:403-410, (1959).

62. Christopoulous, G., Kirch, E. R. and Gearien, J. E., "Determination of ethanol in fresh and putrefied post-mortem tissues", J. Chrom. 87:455-472, (1973).

63. Plueckhahn, V. D. and Ballard B., "Factors influencing the significance of alcohol concentrations in autopsy blood samples", Med. J. Austrailia 1:939-947, (1968).

64. Blackmore, D. J., "The bacterial production of ethyl alcohol", J. For. Sci. Soc. 8:73-78, (1968).

65. Glendening, B. L. and Wangh, T. C., "The stability of ordinary blood alcohol samples held various periods of time under different conditions", J. For. Sci. 10:192-200, (1965).

66. Bradford, L. W., "Preservations of blood samples containing alcohol", J. For. Sci. 11:214-216, (1966).

67. Russel, A. B., "Post-mortem alcohol survey in some Australian states", Med. J. Aust. 2:948-951, (1971).

68. Hayden, P. M., Layder, M. T. and Hickey, M. D., "The stability of alcohol content in samples of blood and urine", Irish J. Med. Sci. 146:6-11, (1977).

69. Brown, G. A., Neylan, D., Reynolds, W. J. and Swalldon, K. W., "Stability of ethanol in stored blood. I", Anal. Chim. Acta. 66:271-283, (1973).

70. Bowden, K. M. and McCallum, N. E. W., "Blood alcohol content: Some aspects of its post-mortem uses", Med. J. Australia, 2:76-81, (1949).

71. Gifford, H. and Turkel, H. W., "Diffusion of alcohol through the stomach wall after death", J. Amer. Med. Ass. 161:866-868, (1956).

72. Turkel, H. W. and Gifford, H., "Erroneous blood alcohol findings at autopsy", J. Amer. Med. Ass., 164:1077-1079, (1957).

73. Kaeferstein, H., "Detectability of methaqualone in putrefaction", Beitr. Gerichtl. Med. 38:217-221, (1980).

74. Schubert, B., "Detection and identification of methylphenidate in human urine and blood samples", Acta. Chem. Scand. 24:433-438, (1970).

75. Eddy, N. and Howes, H., "Studies of morphine, codeine, and their derivatives VIII. Monoacetyl, diacetylmorphine and their hydrogenated derivatives", J. Pharmacol. 53:430-439, (1935).

76. Wright, C., "The enzymatic deacetylation of heroin and related morphine derivatives by blood serum", J. Pharmaco. 71:164-177, (1941).

77. Wright, C., "The deacetylation of heroin and related compounds by mammalian tissues", J. Pharmacol. 75:328-337, (1942).

78. Nakamura, G., Thornton, T. and Noguchi, T., "Kinetics of heroin deacetylation in aqueous alkaline solution and in human serum and whole blood", J. Chrom. 110:81-89, (1975).

79. Garrett, E. and Gurkan, T., "Pharmacokinetics of morphine and its surrogates II: methods of separation of stabilized heroin and its metabolites from hydrolyzing biological fluids and applications to protein binding and red blood cell partition studies", J. Pharm. Sci. 68(1):26-32, (1979).

80. Smith, D. and Cole, W., "Identification of an arylesterase as the enzyme hydrolyzing diacetylmorphine (heroin) in human plasma", Biochem. Pharm. 25(1):367-370, (1976).

81. Fransioli, M. G., Szabo, E. T. and Sunshine, I., "Detection of methadone and propoxyphene in stored tissue", J. Anal. Tox. 4(1):46-48, (1980).

82. Bednarczyk, L. R., Wetli, C. V. and Balkon, J., "Case report: respiratory toxicology", J. For. Sci. 26(2):373-380, (1981).

83. Wingard, L. B., Abouleish, E., West, D. C. and Goehl, T. J., "Modified fluorometric quantitation of pancuronium bromide and metabolites in human maternal and umbilical serums", J. Pharm. Sci. 68(70):914-916, (1979).

84. Poklis, A. and Melanson, E. G., "A suicide by pancuronium bromide injection: evaluation of the fluorometric determination of pancuronium in postmortem blood, serum, and urine", J. Anal. Tox. 4(6):275-280, (1980).

85. Kyofuji, T., "Studies on the extraction of poisonous substances from cadaveric materials of synthetic organo-pesticides poisoning and effects of postmortem changes on their extraction", J. Kumamoto Med. Sco. 44:767-817, (1970).

86. Tsunenari, S., Yonemitsu, K., Uchimura, Y. and Kanda, M., "The influence of putrefactive changes on the determination of paraquat in autopsy materials", For. Sci. Int. 17:51-56, (1981).

87. Clardy, D. O. and Ragle, "Stability of phencyclidine in stored blood", Clin. Tox. 18(3):929-934, (1981).

88. Jonkman, J. H. G., Franke, J. P., Shoenmaker, R. and deZeeuw, R. A., "Stability of theophylline in serum, plasma, and saliva stored at different temperatures", Clin. Chem. 27(12):2071-2072, (1981).

89. Bandt, C. M., "Postmortem changes in the serum levels of tricyclic antidepressants", Presented at the 32nd annual AAFS meeting, New Orleans, February, (1980).

90. Hemphill, G. and Breutzman, D., "The determination of therapeutic concentrations of amitryptyline and nortryptyline in serum using electron impact mass fragmetography", Clin. Chem., 24(6), 991, (1978).

91. Peat, M. A. and Kopjak, L., "The screening and quantitation of diazepam, flurazepam, chlordiazepoxide and their metabolites in blood and plasma by electron-capture gas chromatography and high pressure liquid chromatography", J. For. Sci. 24(1):46-54, (1979).

92. Blanke, R. V. and Saady, J. J., "Rapid simultaneous determination of six anticonvulsant drugs by GLC", 26th S. E. Regional Meeting, Am. Chem. Soc., (1974).

93. Rocco, R. M., Abbott, D. C., Glese, R. W., and Karger, B. L., "Analysis for procainamide and N-acetylprocainamide in plasma or serum by high-performance liquid chromatography", Clin. Chem. 23(4):705-708, (1977).

94. Bhattacharyya, G. K. and Johnson, R. A., Statistical Concepts and Methods, New York, John Wiley and Sons, 1977, pp 334-359.

95. Baselt, R., "Analytical Procedures for Therapeutic Drug Monitoring and Emergency Toxicology". Biomed. Pub., Davis California, p. 278, (1980).

96. Curry, A. S., Walker, G. W. and Simpson, "Determination of ethanol in blood by gas chromatography", Analyst 91:742, (1966).

97. Carstensen, J. T., Su, K. S. E., Maddrell, P., Johnson, J. B. and Newmark, H. N., "Thermodynamic and kinetic aspects of parenteral benzodiazepines", Bulletin of the parenteral drug association 25:193-202, (1971).

98. Tinoco, Jr., I., Sauer, K. and Wang, J. C., Physical Chemistry - Principles and Applications in Biological Sciences, Prentice-Hall, Inc., Englewood Cliffs, N. J., 1978, pp. 260-266.

99. Maulding, H. V., Nazareno, J. P., Pearson, J. E. and Michaelis, "Practical kinetics III: benzodiazepine hydrolysis", J. Pharm. Sci., 64(2):278-284, (1975).

100. Knop, H. J., van der, Kleign, E. and Edmunds, L. C., "The determination of clonazepam in plasma by gas-liquid chromatography", Pharmaceutisch Weekblad, 110:297-309, (1975).

101. Placidi, G. F., Tognoni, G., Pacifici, G. M., Cassano, G. B. and Morselli, P. L., "Regional distribution of diazepam and its metabolites in the brain of cat after chronic treatment", Psychopharmacology 48:133-137, (1976).

102. van der Kleign, E., "Kinetics of distribution and metabolism of diazepam and chlordiazepoxide in mice", Arch. Int. Pharmacodyn, 178(1):193-215, (1969).

103. Cassano, G. B. and Hansson, E. "Autoradiographic distribution studies in mice with C^{14} imipramine", Int. J. Neuropsych. 2:269, (1966).

104. Cassano, G. B., Ghetti, B., Gliozzi, E. and Hansson, E., "Autoradiographic distribution study of 'short acting' and 'long acting' barbiturates: ^{35}S-thiopentone and ^{14}C-phenobarbitone", Brit. J. Anesth. 39:11, (1967).

105. Garrett, E. R., Boharski, J. T. and Yakatan, G. J., "Kinetics of hydrolysis of barbituric acid derivatives", J. Pharm. Sci. 60(2):1145-1154, (1971).

106. Goyan, J. E., Shaikh, Z. and Austian, J., J. Pharm. Sci. 49:627, (1960).

107. Tishler, F., Sinsheimer, J. E. and Goyan, J. E., "Mechanism of phenobarbital degradation", J. Pharm. Sci. 51:3), 214-216, (1962).

108. Gardner, L. A. and Goyan, T. E., "Mechanism of phenobarbital degradation", J. Pharm. Sci., 62(6):1026-1027, (1973).

109. Fretwurst, F., Arzneim-Forsch, 8:44, (1948).

110. Narbutt-Mering, A. B. and Weglowska, W., "Identification of decomposition products of the thiopental type drugs", Acta Poloniae Pharmaceutica, 22(1):15-22, (1965).

111. Adjepon-Yamoah, K. K. and Prescott, L. F., "Gas-liquid chromatographic estimation of lidocaine, ethylglycylxylidine, glycylxylidide and 4-hydroxyxylidine in plasma and urine. J. Pharm. Pharmac. 26:889-893, (1974).

112. Irgens, T. R., Henderson, W. M. and Shelver, W. H., "GLC analysis of lidocaine in blood using an alkaline fmale-ionization detector. J. Pharm. Sci. 65(4):608-610, (1976).

113. Hucker, H. B. and Stauffer, S. C., "GLC analysis of lidocaine in plasma using a novel nitrogen-sensitive detector". J. Pharm. Sci. 65(6):926-927, (1976).

114. Rosseel, M. T. and Bogaert, M. G., "Determination of lidocaine and its desethylated metabolites in plasma by capillary column gas-liquid chromatography". J. Chrom. 154:99-102, (1978).

115. Park, G. B., Erdtmansky, P. E., Brown, R. R., Kullberg, M. P. and Edelson, J., "Analysis of mepivacaine, bupivacaine and etidocaine, lidocaine, and tetracaine". J. Pharm. Sci. 69(5):603-605, (1980).

116. Gal, J., Freedman, M. D., Kumar, E. and Freed, C. R., "A rapid and simple microassay for lidocaine in human blood plasma using gas-liquid chromatography with nitrogen detection". Therapeutic Drug Monitoring 3:177-180, (1981).

117. Hawkins, J. D., Bridges, R. R. an Jennison, T. A., "A single-step assay for lidocaine and its major metabolite, monoethylglycinexylidide, in plasma by gas-liquid chromatography and nitrogen phosphorus detection". Therapeutic Drug Monitoring 4:103-106, (1982).

118. Baselt, R. C., "Disposition of toxic drugs and chemicals in man: Volume 2 - peripherally-acting drugs and common toxic chemicals", Biomed. Pub., Canton, Connecticut, pp. 57-61, (1978).

119. Adjepon-Yamoah, K. K. and Prescott, L. F., "Gas-liquid chromatographic estimation of lignocaine, ethylglycylxylidide, olycylxylidide and 4-hydroxyxylidine in plasma and urine, J. Pharm. Pharm. 26:889-893, (1974).

120. Personal communication, Analytical Division, Wyeth Drug Company.

121. Ranney, R. E., Dean, R. R., Karim, A. and Radzialowski, F., "Disopyramide phosphate: Pharmacokinetics and pharmacologic relationships of a new antiarrhythmic agent", in Archives Internationales de pharmacodynamie et de therapie, May 1971, p. 92.

122. Katrizky, A. R. and Lagouski, J. M., "Chemistry of the heterocyclic n-oxides", London, Academic Press, p. 143-231, (1971).

123. Personal communication, Dr. Vernon Chinchilli, Assistant Professor, Biostatistics, Medical College of Virginia.

APPENDIX

The statistics used to compare the slopes of the concentration vs. time figures and tables are summarized below (94, 123):

$y = \alpha + \beta X$

α = y-intercept

β = slope

The method of least squares (LSQ) is used to obtain the best straight line through the data points:

$y = \alpha + \beta X$

α = y-intercept obtained from LSQ fit

β = slope obtained from LSQ fit

Once α and β are obtained, these values are used for hypothesis testing:

H_0 : $\beta = 0$, that is, there is no change in concentration as a function of time.

H_1 : $\beta \neq 0$, that is, there is a change in concentration as a function of time.

The data from each figure and table can be used to obtain a 95% confidence interval (CI) for β. Since this is a 2-sided test, the 95% CI can be used for hypothesis testing. If 0 falls within this confidence interval, then H_0 cannot be rejected at the 95% confidence interval and there is no statistically significant difference between 0 and β. This would then indicate that there are no changes in drug concentration as a function of time.

VITA