Effects of storage temperature and specimen handling on detection of sulfonamides in porcine tissues

by

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INTRODUCTION

Many different chemicals are used by the modern farmer in the production of agricultural commodities. Chemicals, utilized to increase productivity, include insecticides, herbicides, fertilizers, and a wide variety of feed additives. Many producers are apparently not well informed of the consequences that may result following misuse of these products. Product misuse has caused the death of livestock and/or the establishment of unacceptable levels of chemical residues in animal tissues.

The sulfonamides, especially sulfamethazine, have been used since the 1960s as feed additives in swine rations. Sulfamethazine, in combination with certain antibiotics, has been incorporated extensively in rations formulated for young swine. Unfortunately, approximately 12% of the tissues from hogs marketed for human consumption tested by the Food Safety and Quality Service (FSQS) of the United States Department of Agriculture (USDA) from 1974 to 1977 contained tissue concentrations greater than the tolerance limit of 0.10 μg/g established by the Food and Drug Administration (FDA) (Trabosh, 1978a). Some swine producers claimed that test procedures used by FSQS were not accurate at lower concentrations and that tissue mishandling could result in erroneously high test results.

This research was designed to determine if tissue mishandling could result in a change in the result obtained by the "official" FSQS procedure for quantitation of sulfamethazine levels in swine liver and muscle.

LITERATURE REVIEW

General Background

Sulfonamides were the first effective systemic antimicrobial compounds developed for medical usage. With their introduction, the era of modern antimicrobial chemotherapy began. These compounds quickly became the primary chemical defense against many infectious microorganisms. The use of sulfonamides for treatment of acute bacterial diseases declined following introduction of penicillin and other antibiotics. Sulfonamides are still used in specific situations where they are the most effective treatment and as feed additives for disease prevention and growth promotion.

The parent compound for the sulfonamides, sulfanilamide, p-aminobenzene-sulfonamide, was first synthesized by Gelmo (1908) for possible use in the dye industry. Significant medical usage of the sulfonamides was not reported until 1935, when Domagk (1935a,b,c) reported that Prontosil, 2:4 diaminoazobenzene-4-sulfonamide, protected mice from a highly pathogenic strain of hemolytic streptococcus and reduced the severity of staphylococcal infections in rabbits. The protective action of Prontosil in experimental streptococcal infections was soon confirmed by Levaditi and Vaisman (1935).

A group of French scientists, Trefouel et al. (1935) and Fourneau et al. (1936), hypothesized that Prontosil was broken down *in vivo* at the azo linkage to produce sulfanilamide, p-aminobenzene-sulfonamide. They reported that sulfanilamide was as effective as Prontosil in preventing

fatal streptococcal infections in experimental animals and also demonstrated that sulfanilamide was active not only in vivo but also in vitro. Prontosil was not active in vitro. Buttle et al. (1936) soon confirmed the work of Fourneau et al. (1936), reporting that p-aminobenzenesulfonamide paralleled Prontosil in therapeutic activity and was capable of curing experimental meningococcal infections in mice.

Sulfanilamide was soon used successfully by many investigators to treat several types of bacterial diseases in man. Heintzelman et al. (1937) reported favorable clinical results in ten cases of meningococcal meningitis treated with sulfanilamide.

Attempts to elucidate the mode of action of this compound were advanced by Stamp's (1939) discovery that streptococcal extracts neutralized the bacterial inhibitory action of sulfanilamide. Woods (1940) found that a yeast extract also contained a similar inhibitory substance and that these substances had the same physical and chemical properties as para-amino-benzoic acid (PABA). Woods (1940) postulated that sulfanilamide interfered in the utilization of PABA in some bacterial enzyme reactions.

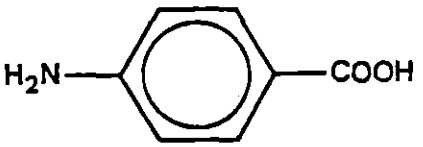
A system of nomenclature for sulfanilamide derivatives was developed by Crossley et al. (1938a,b,c). The parent compound, sulfanilamide, may be modified by substituting other groups for any of the hydrogens in the compound. The sulfanyl group ($-SO_2NH_2$) is the most important functional group and is assigned the number one position on the ring; derivatives of the amide nitrogen in this position are designated N^1 substituents. In the same manner, derivatives of the amino-nitrogen are designated as N^4

substituents (Bevill, 1982). Figure 1 shows the chemical structure of sulfanilamide.

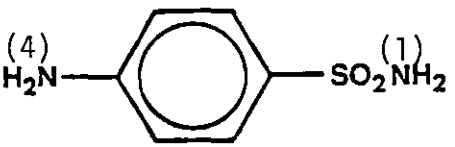
One of the sulfanilamide derivatives selected for commercial usage was a compound named sulfamethazine, (sulfadimidine), N^1-(4, 6-dimethyl-2-pyrimidinyl) sulfanilamide. This compound is a white to yellowish-white powder which by definition contains not less than 99.0% and not more than 100.5% sulfamethazine when calculated on the dry weight basis (The United States Pharmacopeia, 1970). The compound has a molecular weight of 278.33 and its empirical formula is $C_{12}N_{14}O_2N_4S$. It is almost odorless and has a slightly bitter taste. It is very slightly soluble in water and ether, slightly soluble in alcohol and soluble in acetone (Wood, 1955). Sulfamethazine may be prepared by reacting acetylsulfanilyl chloride with 2 amino-4, 6 dimethylpyrimidine in pyridin, followed by alkaline hydrolysis to the 2 (N^4-acetylsulfanilamido)-4, 6 dimethyl pyrimidine, the resulting sodium salt being neutralized with SO_2 (Wendholz, 1976). Sulfamethazine is amphoteric and forms salts in strongly acidic or basic solutions. Proton acceptance at N_4 accounts for its behavior as a base in acidic solutions. The sodium salt of sulfamethazine is more soluble in water than the parent compound and the solubility of the sodium salt increases as pH increases (Bevill and Huber, 1977).

Mechanism of Action

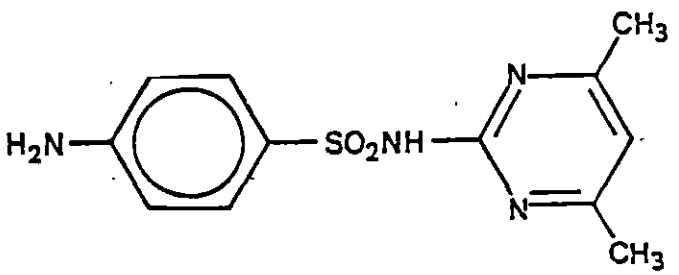
Woods (1940) postulated that sulfanilamide competed with para-aminobenzoic acid (PABA) for an enzymatic site in the formation of an essential bacterial metabolite since both compounds are similar in chemical struc-



Para-aminobenzoic Acid



Sulfanilamide



Sulfamethazine

Figure 1. Structural formulas of para-aminobenzoic acid, sulfanilamide, and sulfamethazine

ture. This theory was strengthened by the discovery that sulfonamides competitively blocked the enzymatic coupling of PABA and 2-amino-4-hydroxytetrahydropteridine, a step necessary in the formation of folic acid (Lehninger, 1975). Folic acid is converted into its coenzyme form, tetrahydro-folic acid (FH_4) by reduction. Tetrahydro-folic acid serves as an intermediate carrier of methyl, formyl, or hydroxymethyl groups in several enzymatic reactions in which one-carbon groups are transferred from one intermediate metabolite to another. Included are transfer reactions in connection with the intermediary metabolism of purines, pyrimidines, and amino acids necessary for biosynthesis of nucleotides, the direct precursors of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Lehninger, 1975). Sulfonamides, by inhibiting the formation of folic acid, exert a bacteriostatic effect on some bacteria. Sulfonamide-susceptible bacteria must produce folic acid from its components. Such bacteria are unable to utilize the preformed folic acid found in animal tissues.

Absorption, Metabolism, and Excretion

Sulfonamide absorption and movement from its site of administration is governed by passive diffusion. The rate of absorption is influenced by several factors including vascularity of the absorption site, extent of binding with material present at the administration site, and ionization state and lipophilicity of the drug (Bevill, 1982). Orally administered sulfamethazine is rapidly absorbed by healthy swine (Linkenheimer and Stolzenberg, 1965). The rate of absorption in diseased swine may be

altered. The rate of sulfamethazine absorption was significantly increased in milk-fed calves with diarrhea (Lapka et al., 1978). In contrast, water deprivation has been shown to slow the absorption of sulfaethoxypyridazine from the intestinal tract of cattle (Heath and Teske, 1973).

Distribution of sulfonamides from the blood into various tissues and other biological fluids is influenced by the ionization state of the sulfonamide, vascularity of the tissues, plasma protein binding to the sulfonamide and the presence of specific barriers to sulfonamide diffusion (Bevill, 1982). Passive diffusion governs the movement of the sulfonamide from blood to other fluids and tissues and the concentration attained in these tissues is a measure of the rate at which the drug equilibrates between blood plasma and these tissues. Eventually, a plasma-tissue equilibrium is established and this equilibrium occurs first in highly vascularized tissues. Once this equilibrium is established, a constant relationship exists between the concentration of the sulfonamide in plasma and that present in a specific tissue (Bevill, 1982). Tissue concentrations of sulfonamides are usually lower than plasma values. Studies in cattle, sheep, and swine have shown that the disappearance of a specific sulfonamide from plasma and tissues occurs at similar rates within a given species (Bourne et al., 1977; Bevill et al., 1977a,b). The half-life of sulfamethazine in swine plasma is 14-16 hours (Bevill and Huber, 1977).

Bevill et al. (1977a) reported that sulfamethazine metabolism fits a one compartment pharmacokinetic model in cattle. Sulfamethazine was eliminated as the parent compound and three metabolites: acetyl and

hydroxy derivatives and a polar metabolite. Similar excretion products were obtained in lamb studies with sulfamethazine (Bevill et al., 1977b; Bourne et al., 1977). The metabolism of sulfamethazine in lambs also fits a one compartment pharmacokinetic model (Dittert, 1977). Swine excrete sulfamethazine similarly but apparently are unable to hydroxylate sulfamethazine, as hydroxylated metabolites have not been found in swine urine following oral or intravenous administration of the compound (Bevill and Huber, 1977).

Usage and Residue Problems

Sulfonamides have been used extensively in the swine industry, and it has been estimated that 75% of the swine slaughtered in the United States received some sulfonamide during their relatively short lifetime (Cromwell, 1979). Sulfamethazine-antibiotic combinations in feed have been shown to be effective in improving growth rate and feed efficiency (Elliott et al., 1964; Melliere et al., 1968). This effect appears to be greatest in swine herds with chronic respiratory problems and young pigs appear to benefit the most. Sulfamethazine is approved for use as a feed additive in combination with certain antibiotics. These combinations include tylosin with sulfamethazine and a combination of three drugs: chlorotetracycline, sulfamethazine, and procaine penicillin. The level of sulfamethazine approved in these combinations is 100 grams per ton of complete feed (Leidahl, 1980).

The improper use of sulfonamides has resulted in a persistent problem of sulfonamide residues in swine tissues. The FSQS of the USDA began a

sulfonamide residue monitoring program in 1973. The initial violation rate for these compounds was over 10% and the rate continued to be over 10% through 1977. The number of swine tissue samples collected for analysis rose from 300 per year in 1974 to 9,410 in 1977. The violation rate dropped to 5.7% of 3,488 samples collected in 1981 (personal communication, L. R. Hensley, Residue Evaluation and Planning Division, FSIS, Washington, D.C., 1983). Residue levels or concentrations of sulfonamides over 0.1 μg/g or 0.1 part per million (ppm) in liver, kidney, or muscle are in violation (Federal Register, 1982). Tissues containing concentrations of a sulfonamide above 0.1 μg/g are considered adulterated and unfit for human consumption; however, marketing practices result in movement of the meat to the retail level before laboratory results are completed, except in herds with previous violations.

The reliability of the methods used by FSQS personnel to obtain these results has been questioned (Horwitz, 1981). The procedure utilized three different methods to identify and quantitate the sulfonamide levels in tissues. The samples were initially screened using a thin layer chromatographic (TLC) test. Positive samples were then analyzed using a gas-liquid chromatographic (GLC) procedure to identify the specific sulfonamide(s) present in the tissue sample. The amount of sulfonamide was then quantitatively determined by the Tishler method, option A (Horwitz, 1981).

The Tishler method (Tishler et al., 1968) is a colorimetric method which utilizes the Bratton-Marshall coupling reaction (Bratton and Marshall, 1939) and visible spectrophotometric techniques. The FSQS personnel believed that the combination of testing procedures reduced the

possibility of "false positives" or incorrect high results. The use of the colorimetric procedure for final quantitation was probably the weakest point in the entire testing regime. Although "clean-up" steps in the procedure remove some compounds that also couple with the Bratton-Marshall reagent, they do not remove all such compounds. The coupling reaction is nonspecific and primary aromatic amines other than sulfonamides may react in a similar manner. Low levels of two or more sulfonamides could be added together in this procedure. The Bratton-Marshall (B-M) procedure was originally developed to measure much higher levels: 0.5-1.0 mg/dl of sulfonamides in blood and urine (Bratton and Marshall, 1939). This procedure was later adapted by Tishler et al. (1968) for use on milk and tissues.

The advantages of the Tishler tissue procedure were speed of analysis, consistent recovery, and relatively inexpensive equipment necessary to perform the extraction and final readout.

Tishler et al. (1968) believed their procedure to be sensitive to at least 0.1 μg/g with a minimum recovery rate of 75%. Their results showed that sulfonamide-free control tissue could account for as much as 39±8 ng/g of the total measured in certain tissues. This amount was not considered by the authors to be significant, although in some cases, the control tissue would apparently account for one-half the total amount of "sulfonamide" measured. Similar or higher levels have been reported for sulfonamide-free control tissues as measured by the Tishler procedure (Righter et al., 1971; Lloyd et al., 1981). This added amount would be sufficient to cause a tissue sample containing a true sulfonamide level of

less than 0.1 µg/g, i.e., 0.06-0.09 µg/g, to measure greater than 0.1 µg/g and thus be considered to be in violation. In November 1979, Dr. Richard Ellis announced that the FSQS chemistry division was revising the method used to quantitate the amount of sulfonamides in swine tissues (USDA-APHIS, 1979). The chemistry division would replace the Tishler option C of the Bratton-Marshall test with the Tishler option A and would begin deducting a set background value of 0.05 µg/g from values obtained on liver and 0.02 µg/g from values obtained for muscle. They believed this correction would more accurately reflect the true sulfonamide residue in the tissue.

From June 1977 to January 1978, 13.3% of the tissues tested were found to have violative sulfonamide levels. Tissue concentrations of 0.11-0.20 µg/g were responsible for approximately 30% of the sulfonamide violations in liver tissues (Trabosh, 1978b). From January 1980 to December 1980, 5.6% of the swine tissues tested were found to have violative sulfonamide levels. Tissue concentrations of 0.11-0.20 µg/g were responsible for only 17.7% of the sulfonamide violations in liver tissues (Trabosh, 1981). This drop was apparently due, in part, to increased producer awareness of the problem and by the elimination of tissues which in the past would have been in violation, those which measured 0.10-0.14 µg/g, prior to subtracting the background amount of 0.05 ppm. The Tishler modification of the B-M procedure has been referred to as the "official" method for testing animal tissues for residues of sulfonamides. No collaborative study to validate this method could be found during an extensive search of the literature. Although various internal memoranda of the

Food and Drug Administration (FDA) and FSQS have stated that the Tishler method is the method of choice for examining animal tissues for sulfonamides, no statement could be found in any FDA regulations to support its alleged "official" status (Horwitz, 1981).

On February 1982, the Food Safety and Inspection Service (FSIS), previously FSQS, announced a policy change regarding acceptable methods for analyzing poultry and red meat tissues for sulfonamide drug residues (Anon., 1982). This announcement stated that on March 24, 1982, the FSIS field laboratories would discontinue the use of the Tishler A B-M method and would be implementing the sulfonamide thin-layer chromatography (STLC) method for determination of sulfonamides in poultry and red meat as the initial analysis of all tissue samples. If the results showed that these tissues contained 0.11-0.15 μg/g sulfonamides, the results would be confirmed by the gas chromatography/mass spectrometry (GC/MS) method before being reported as official (Anon, 1982).

Thin layer chromatography and GLC methods had been used previously by FSQS laboratory personnel. Unfortunately, recovery was low and deviation was high and these methods could not be used for quantitation until these problems were solved. A collaborative study to evaluate new GC and GC/MS methods determined that the GC/MS procedure provided the most reliable data and that the STLC method provided acceptable data if appropriate controls were used (Malanoski et al., 1981). No reports of a collaborative study on the STLC method could be found.

Methods of Analysis

Colorimetric

A wide variety of amines and phenols have been used to couple with diazotized sulfonamides. These include N-1-naphthyl-N'-dietylpropylene-diamine monohydrochloride, 1-(B-diethylaminoethylamino) naphthalene free from 1-naphthylamine, dimethyl-a-naphthylamine, ethyl-a-naphthylamine, 1-aceta-amido-8-naphthol-3,6-dilsulfonic acid, 1-amino-8-naphthol-3, 6-disulfonic acid, N-B-sulfatoethyl-m-toluidine,diphenylamine, a-naphthyl-amine, thymol, a-naphthol and B-naphthol (Snell and Snell, 1954). These compounds were replaced by a new coupling compound, N-(1-naphthyl) ethyl-enediamine dihydrochloride (NEDA), developed by Bratton and Marshall (1939). This compound offered several advantages over other compounds used and became the primary compound used for coupling with the sulfona-mides during analysis. The Bratton-Marshall method was later utilized by Tishler et al. (1968) in the development of a sensitive colorimetric method for determining sulfonamide concentrations in tissues and milk. The basic reactions following extraction from the tissues are that the sulfonamide is converted to a diazonium salt by the action of sodium nitrite. Ammonium sulfamate is used to neutralize excess sodium nitrite and the diazotized sulfonamide is coupled with NEDA. The amount of the resulting coupled complex is determined by measuring the absorbance of the sample and standard solutions against their respective blanks at 545 nm.

A simple qualitative screening test for primary aromatic amines in swine feeds using the Bratton-Marshall method has recently been developed

(Schwartz, 1982). This test is capable of detecting sulfamethazine levels of $\geq$0.15 ppm.

The majority of colorimetric methods has been based on these reactions with NEDA as the final coupling reagent.

New methods for sulfonamide analysis using chloramine-T in spectrophotometric and titrimetric analyses have been developed (Trieff et al., 1977); however, these methods have not been accepted by FSIS for tissue analysis.

Chromatographic methods of analysis

Thin-layer chromatographic (TLC), gas-liquid chromatography (GLC), and high performance liquid chromatography (HPLC) have been used for the determination of sulfonamide concentration in animal feed, tissues, and plasma.

Thin-layer chromatography methods have been used for the determination of sulfonamides in feeds (Cieri, 1976; Luchtefeld, 1976), plasma (Bevill et al., 1978) and tissues (Thomas et al., 1981; Phillips and Trafton, 1975). Cieri (1976) used an alcoholic solution of p-dimethylaminobenzaldehyde to detect sulfonamides on TLC plates after extraction from feeds. Identification of the specific sulfonamide was based on its RF value in comparison to standards. Quantitation was based on dilution and color intensity. Luchtefeld (1976) used N-(1-naphthyl) ethylene diamine (NEDA) to detect the sulfonamide on the plate and quantitation was based on dilution and color intensity. These methods are dependent on visual observation of the "spot" formed by the coupling reagent and the sulfonamide.

Thomas et al. (1981) developed a method that appears to be more sensitive. After extraction and development, the dry TLC plates are dipped in a fluorescamine solution. The fluorescamine-sulfonamide complex is then determined using scanning densitometry. The same detection method (Bevill et al., 1978) was used for plasma samples following various extraction procedures.

High performance liquid chromatographic methods have been used for the determination of sulfonamide concentrations in urine (Sharma et al., 1976), tissues (Johnson et al., 1975) and serum (Goehl et al., 1978). After extraction from the biological matrix, the sample is injected onto a specific column. Methanol is used to move the injected material along the column and the column eluants are read at 254 nm and/or 280 nm.

GLC methods have been used for the determination of sulfonamides in feed (personal communication, D. Sullivan, Vet A Mix, Shenandoah, Iowa, 1977) and swine tissues (Goodspeed et al., 1978; Manuel and Steller, 1981). The sulfonamide is extracted from the biological matrix, partitioned into an organic solvent, methylated with diazomethane and injected onto a specific column. Nitrogen or an argon-methane mixture of gases is used to move the injected material along the column and methylated compounds passing the detector are measured.

MATERIALS AND METHODS

Treatment Conditions

Time study

Six pigs were used for the time study. These animals were divided into two groups with three animals in each group. The pigs were fed one of two complete feeds for a minimum of ten days prior to slaughter. One group received sulfamethazine-free feed and the other group received feed containing 11.0 ppm sulfamethazine. The animals were housed individually in pens with concrete floors measuring approximately 3 x 3 meters. Pens were cleaned daily using shovels and water flushes. Water was suppled ad libitum via nipple watering devices. Feed was supplied ad libitum via metal self-feeders.

Freeze-thaw study

Three pigs were used for the freeze-thaw study. These animals were housed individually in pens with concrete floors measuring approximately 3 x 5 meters. Pens were cleaned daily using shovels and water flushes. Water was supplied ad libitum via open water pans. Feed was supplied ad libitum via metal self-feeders. These animals received control feed for a minimum of ten days prior to slaughter.

Feed preparation

A complete control feed was prepared in a tractor powered grinder-mixer. The control feed components consisted of shelled corn, soybean meal, and a commercially prepared vitamin-mineral premix. Analysis of the

feed components and the finished control feed by GLC failed to reveal the presence of any sulfonamide (less than .10 ppm).

A portion of the control feed was mixed with similar feed containing 109.8 ppm sulfamethazine to produce a finished product containing 11.0 ppm sulfamethazine.

Sample Collection and Preparation - Time Study

Time study

One animal was killed on each of three consecutive days. The animals were electrocuted and exsanguinated. After the skin and subcutaneous fat were removed, samples of liver and gluteal muscle were collected for processing. Maximum care was taken to insure the tissues collected were not contaminated by skin, fecal material, or urine.

The livers were cut into pieces approximately 1 cm^3. These pieces were mixed manually and randomly divided into two groups. One group was ground into a puree in a food blender. The resultant puree was poured into polyethylene bags, with approximately 50 grams of tissue in each bag. The other group of liver pieces was placed in polyethylene bags, with approximately 50 grams of tissue in each bag.

The muscle collected was trimmed free of visible fat, cut into pieces approximately 3 cm^3, chopped in a cast iron meat grinder, mixed manually, and placed in polyethylene bags with approximately 50 grams of tissue in each bag.

The polyethylene bags were identified as to tissue type and numbered. The bags were then selected randomly for the amount of time they would be

allowed to remain at 23°C. Samples selected as "0" hour samples were placed in a freezer at -23°C approximately 1 hour after the animals were killed. All other bags were spread out on an elevated tray in a covered water bath. Water bath temperature, 23°C, was monitored to assure that the temperature remained constant. Samples were then removed from the water bath at the following times: 6 hours, 12 hours, 24 hours, 48 hours, and 96 hours. These times were based on time expired from the time the animals were killed. Samples removed from the water bath were placed immediately in a freezer at -23°C.

Sample Collection and Preparation - Freeze-Thaw Study

Freeze-thaw study

Facilities at the Iowa State University Meat Laboratory were used to process these animals.

All animals were killed on the same day at approximately one-half hour intervals. Animals were stunned using a captive bolt device and exsanguinated. Animals were then lowered into a vat of boiling water and the hair was removed by automated machinery, followed by manual clean-up.

The body cavities were opened and the livers were removed. The livers were cut into pieces approximately 1 cm^3 and mixed manually. The pieces were placed in polyethylene bags with approximately 50 grams of liver per bag. The polyethylene bags were identified and numbered. The bags were then randomly selected for the number of freeze-thaw cycles to which they were subjected. All samples were placed in a freezer at -23°C. Samples were selected to undergo 0, 1, 2, or 3 freeze-thaw cycles. Sam-

ples selected to undergo zero freeze-thaw cycles were left in the freezer until used for analysis. The other samples were removed from the freezer after 24 hours and placed in a covered water bath at 23°C for 2 hours. They were then returned to the freezer until used for analysis. This process was repeated for the samples until they had been subjected to the predetermined number of freeze-thaw cycles. These samples were all returned to the freezer until they were analyzed.

Extraction Procedure for Liver and Muscle

The following procedure was used to extract sulfamethazine from the liver and muscle. Fifty grams of tissue were weighed from the original sample by shaving the frozen tissue into a sample cup. This tissue was then transferred to a glass blender jar. One hundred ml of a 50:50 acetone-chloroform (A:C) mixture were added and the mixture was blended at low speed for 1 minute. The fluid in the blender jar was filtered through Whatman #1 filter paper and glass wool into a 500 ml round bottom flask. An additional 100 ml of A:C mixture was then blended for 1 minute at low speed. The fluid in the blender jar was filtered through the same filter paper and glass wool into the same 500 ml round bottom flask. This process was repeated with a third 100 ml portion of A:C mixture. The filter paper and tissue in the filter paper were added to the contents of the blender jar and this mixture was blended for one minute at low speed. The fluid was then filtered through a new Whatman #1 filter paper into the same 500 ml round bottom flask. A final 100 ml portion of A"C mixture was added to the contents of the blender jar. The contents were blended for 1

minute. The fluid was then filtered into the same round bottom flask. Ten ml of 1N HCl were added to the contents of the round bottom flask and the flask was then placed on a rotary evaporator and the A:C mixture was evaporated from the flask using a combination of vacuum and heat. The remaining acid extract was transferred to a 250 ml separatory funnel. The 500 ml round bottom flask was rinsed using the following sequence of solvents: 25 ml hexanes (reagent grade), 25 ml hexanes, 3 ml acetone, 25 ml hexanes, 3 ml hexanes, 3 ml acetone, and 25 ml hexanes. Each rinse was added to the separatory funnel and the separatory funnel was shaken gently for 2 minutes and allowed to stand for 15 minutes. The lower (acid) layer was filtered through 9.0 cm glass fiber, Whatman grade GF/A filter paper into a 50 ml graduated cylinder. The 500 ml round bottom flask was rinsed with 5 ml 1N HCl and this portion was transferred to the separatory funnel. The separatory funnel was shaken gently for 1 minute and allowed to stand for 15 minutes. The lower layer was filtered through GF/A filter paper into the same 50 ml graduated cylinder. This process was repeated with another 5 ml of 1N HCl. Then, 1N HCl was added to the graduated cylinder to adjust the total volume to 30 ml. The sample was mixed and 15 ml of the sample were used for the colorimetric procedure. The remaining 15 ml were used for the GLC procedure.

The 15 ml portion of the sample used for the GLC procedure was transferred to a 60 ml polypropylene bottle. Nine and one-half grams of sodium citrate$\cdot 2H_2O$ were added and the bottle was shaken until the sodium citrate$\cdot 2H_2O$ was dissolved. The pH of the resultant mixture was adjusted to pH 5.5-5.6 with 4N NaOH. The sample was then poured into a 250 ml

separatory funnel. The bottle was rinsed with 25 ml methylene chloride ($MeCl_2$) which was added to the contents of the separatory funnel. The funnel was shaken gently for 1 minute and allowed to stand for 5 minutes for the phases to separate. The lower $MeCl_2$ phase was filtered through Whatman #40 paper into a pear-shaped 100 ml TS 24/40 flask. The $MeCl_2$ extraction was repeated with two separate 15 ml portions, and all extracts were combined in the same 100 ml pear-shaped flask. The $MeCl_2$ extracts were evaporated to dryness on a rotary evaporator. One-half ml of anhydrous methanol was added to the flask. The flask was swirled occasionally during the next 15 minutes. One-half ml diazomethane solution was added to the flask. The flask was swirled occasionally during the next 15 minutes. The contents of the flask were evaporated to dryness over N_2 at room temperature. A known volume of ethyl-acetate:isooctane (1:1) was added to the flask to dissolve the residue and give adequate peak height vs. minimum amount of background on the gas chromatograph.

Color Development

The 15 ml sample for color development was divided and 7.5 ml were placed in each of two test tubes. One ml of 0.1% sodium nitrite was added to each. The contents of the tubes were blended on a vortex mixer and allowed to stand for 3 minutes. One ml of 0.5% ammonium sulfamate was added to each tube. The contents of the tubes were combined on a vortex mixer and allowed to stand for 2 minutes. One-half ml N-(1-naphthyl) ethylenediamine dihydrochloride (NEDA) was added to one tube and 1/2 ml of water was added to the second tube. The contents of the tubes were

mixed on a vortex mixer and allowed to stand for 20 minutes. Sample absorbances were measured on a spectrophotometer set at a wave-length of 545 nm (Bausch & Lomb, Rochester, New York).

Calculation:

The amount of sulfamethazine in each sample was calculated using the following formulas.

$$A_S - A_N - A_W$$

$$A_{std} = A_{NS} - A_{WS}$$

$$\text{Sample ppm} = \frac{A_S}{A_{std}} \times \frac{\mu g \text{ sulfamethazine in standard volume}}{W} \times \frac{V_S}{V_{std}} \times \frac{100}{R}$$

where

A_S = net absorbance of unknown sample

A_N = absorbance of sample tube with NEDA

A_W = absorbance of sample with distilled water

A_{std} = absorbance of standard used that day

A_{NS} = absorbance of standard with NEDA

A_{WS} = absorbance of standard tube receiving distilled water

V_S = volume of acid extract of sample

V_{std} = volume of acid used to dissolve standard

W = weight of sample in grams

R = percent recovery for the samples extracted that day

To determine R, or percent recovery, the following procedure was used. Two or three samples were "spiked" with 5.0 µg sulfamethazine dissolved in methanol. One ml of methanol containing 5.0 µg of sulfametha-

zine was added to a tissue sample before the extraction procedure was begun. A duplicate sample was also extracted with no added sulfamethazine. The absorbances of both samples were measured after color development. The difference in absorbance between the samples was considered to be that contributed by the added "spike." This difference in absorbance was converted to µg sulfamethazine and divided by the µg sulfamethazine in the 1 ml "spike."

A standard curve was established over a wide range of sulfamethazine concentrations. This was accomplished by adding 0.0, 2.5, 5.0, 10.0, 15.0, 25.0, 125.0, and 250 µg sulfamethazine to individual 50 ml graduated cylinders. Each cylinder was brought to 30 ml with 2N HCl. Color was developed as explained above. Net absorbances A_{std} were then plotted (Y axis) on coordinate paper against concentration (X axis).

Gas Chromatographic Analysis of Sulfamethazine

The samples were analyzed for sulfamethazine on a Packard 600 gas liquid chromatograph (Packard Instrument Co., Downers Grove, Ill.) fitted with a ^{63}N: electron capture detector. The oven was equipped with a six feet long glass column, 1/4 in outside diameter and 1/8 in inside diameter, filled with 5% OV-7 gas chroma Q 100/120 mesh packing. The oven temperature was maintained at 270°C. The injection vaporizer was set at 280°C and the detector block was kept at 300°C. Nitrogen was used for the carrier gas. The flow rate was adjusted to 30 ml/minute. Injection solvents were nanograde purity. The amount of sulfamethazine in each sample was determined by calculations based on peak height and a calculation

factor (C.F.) determined for the standard concentration by dividing the standard ng injected by the peak height obtained from the injection. The mean of three or more injections was used to determine the C.F. The peak height per ml of sample was averaged for two or more injections. This value multiplied by the C.F. gave the concentration per µl of sample (C/µl). The C/µl x the dilution factor equaled the nanograms of drug in the sample (S_{NG}). The S_{NG} divided by the sample weight in grams x 1,000 equaled "uncorrected" ppm.

This value was corrected for recovery by multiplying the "uncorrected" value times 100 divided by percent recovery. Percent recovery was determined by the measured difference in a "spiked" sample vs. its "unspiked" duplicate.

Statistical Evaluation

Time study

F-values were determined from computed sums of squares and mean squares for the calculated sulfamethazine concentrations for each time period. Statistical significance was assigned to F-values according to Snedecor and Cochran (1967) using a split-plot design as shown in Cox (1971).

Freeze-thaw study

F-values were determined from computed sums of squares and mean squares for the calculated sulfamethazine concentrations for each freeze-thaw cycle. Statistical significance was assigned to F-values according to Ostle and Mensing (1975) using a randomized block design.

RESULTS

Time Study

Control tissues-liver

As measured by the Tishler color reaction, the uncorrected levels of "sulfamethazine" increased in both the blended and whole liver as the time the tissues were held at 23°C increased. The 48 hour and 96 hour values for blended liver were significantly higher ($P < .05$) than the values at shorter times. The 96 hour value for whole liver was significantly higher ($P < .05$) than values at shorter times. The uncorrected results for blended and whole liver as measured by the Tishler color reaction are shown in Table 1. A graphic illustration of these results is presented in Figure 2.

Table 1. Uncorrected time study Tishler control liver values

	Blended liver-ppm	Whole liver-ppm
0 hours	0.06±0.02	0.03±0.01
6 hours	0.10±0.05	0.04±0.01
12 hours	0.10±0.04	0.04±0.01
24 hours	0.11±0.04	0.06±0.02
48 hours	0.23±0.12*	0.10±0.04
96 hours	0.30±0.11*	0.38±0.15*

*$P < .05$.

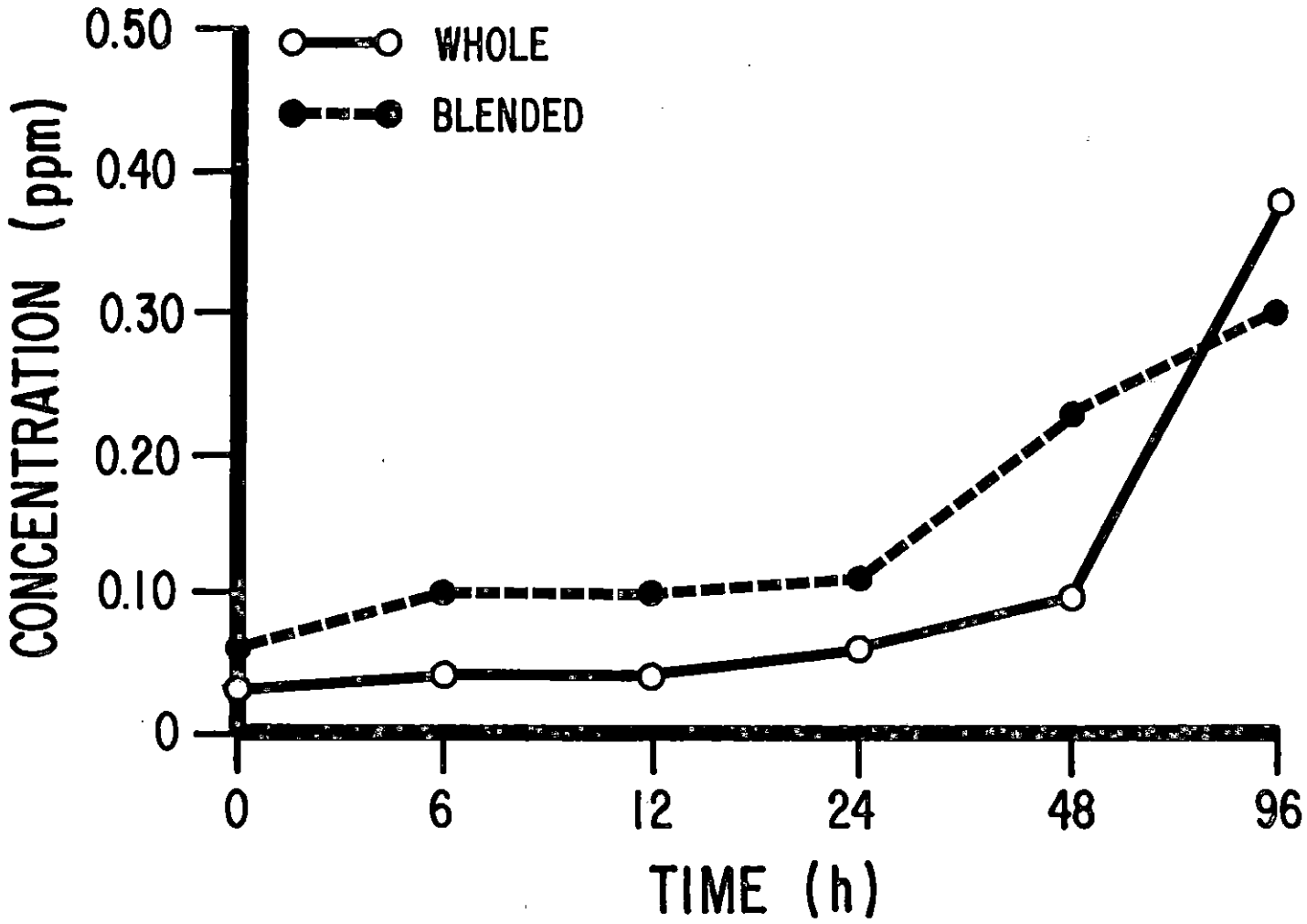


Figure 2. Uncorrected Tishler control liver values

The mean percent recovery values for the different time periods were variable but for both tissues were lowest at 96 hours. These values are shown in Table 2.

Table 2. Mean percent recovery-time study control liver values

	Blended liver-ppm	Whole liver-ppm
0 hours	64±28	78±19
6 hours	97±3	85±19
12 hours	91±14	59±19
24 hours	74±12	94±10
48 hours	74±15	75±44
96 hours	34±8	49±20

The corrected values for whole and blended control liver parallel the uncorrected values. The 48 hour and 96 hour values for blended liver were significantly higher ($P < .05$) than the values at shorter times. The corrected results for blended and whole liver as measured by the Tishler color reaction are shown in Table 3. A graphic illustration of these results is presented in Figure 3.

Gas-liquid chromatographic analysis of liver samples from pigs fed sulfonamide-free feed revealed that with one exception, the samples for all time periods were negative. One sample, a 0 hour sample was found to contain 0.01 ppm sulfamethazine. Three other 0 hour samples were negative. This finding suggests this sample may have been contaminated during the extraction procedures. Mean recovery for the negative samples was

Table 3. Corrected time study Tishler control liver values

	Blended liver-ppm	Whole liver-ppm
0 hours	0.07±0.01	0.04±0.01
6 hours	0.11±0.04	0.05±0.01
12 hours	0.10±0.03	0.06±0.01
24 hours	0.16±0.01	0.07±0.04
48 hours	0.35±0.04*	0.22±0.15
96 hours	0.50±0.18*	0.68±0.12

*$P < .05$.

87%. The 48 and 96 hour samples caused a problem with detector response and tended to greatly reduce sensitivity.

Control tissues-muscle

As measured by the Tishler color reaction, the uncorrected levels of "sulfamethazine" increased in the muscle from control animals as the time the tissues were held at 23°C increased. The 96 hour value was significantly higher ($P < .05$) than the values for shorter times. The uncorrected results for control muscle values are shown in Table 4. A graphic illustration of the results is presented in Figure 4.

The mean percent recovery values for the different time periods were variable but were lowest at 48 and 96 hours. These values are shown in Table 5.

The corrected values for control muscle parallel the uncorrected values. The 96 hour value was significantly higher ($P < .05$) than the values for shorter times. The corrected control muscle values are shown

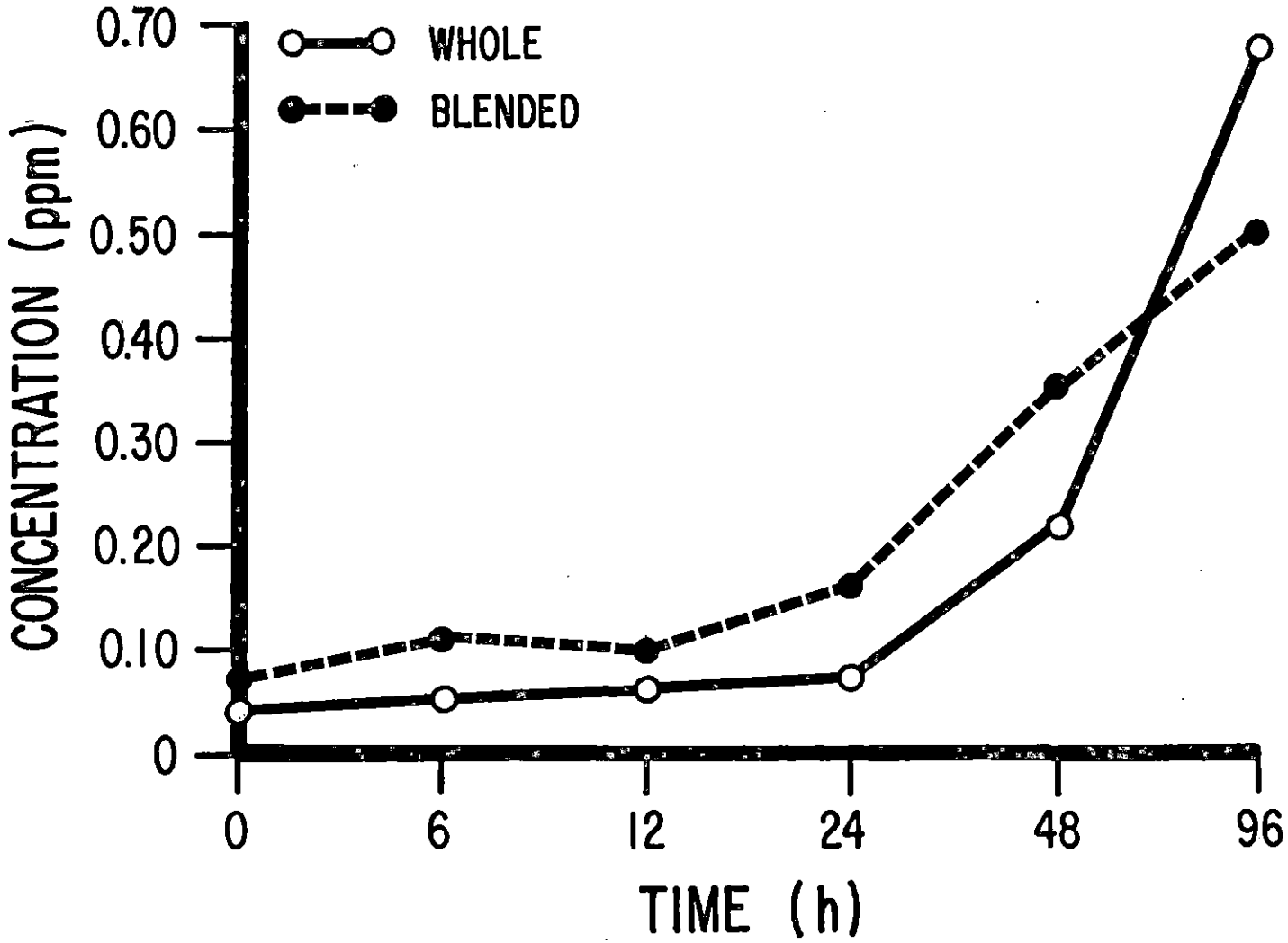


Figure 3. Corrected control liver values

Table 4. Uncorrected time study Tishler control muscle values

	Muscle-ppm
0 hours	0.02±0.01
6 hours	0.03±0.01
12 hours	0.03±0.01
24 hours	0.03±0.01
48 hours	0.03±0.01
96 hours	0.07±0.01*

*$P < .05$.

Table 5. Mean percent recovery on time study control muscle samples

	Muscle
0 hours	66±4
6 hours	55±26
12 hours	56±20
24 hours	53±38
48 hours	46±3
96 hours	39±9

in Table 6. A graphic illustration of these values is presented in Figure 5.

Positive tissues-liver

No significant differences were found in relation to treatment with time. As measured by the Tishler color reaction, the "sulfamethazine" level decreased in both the blended and whole liver through the first 48

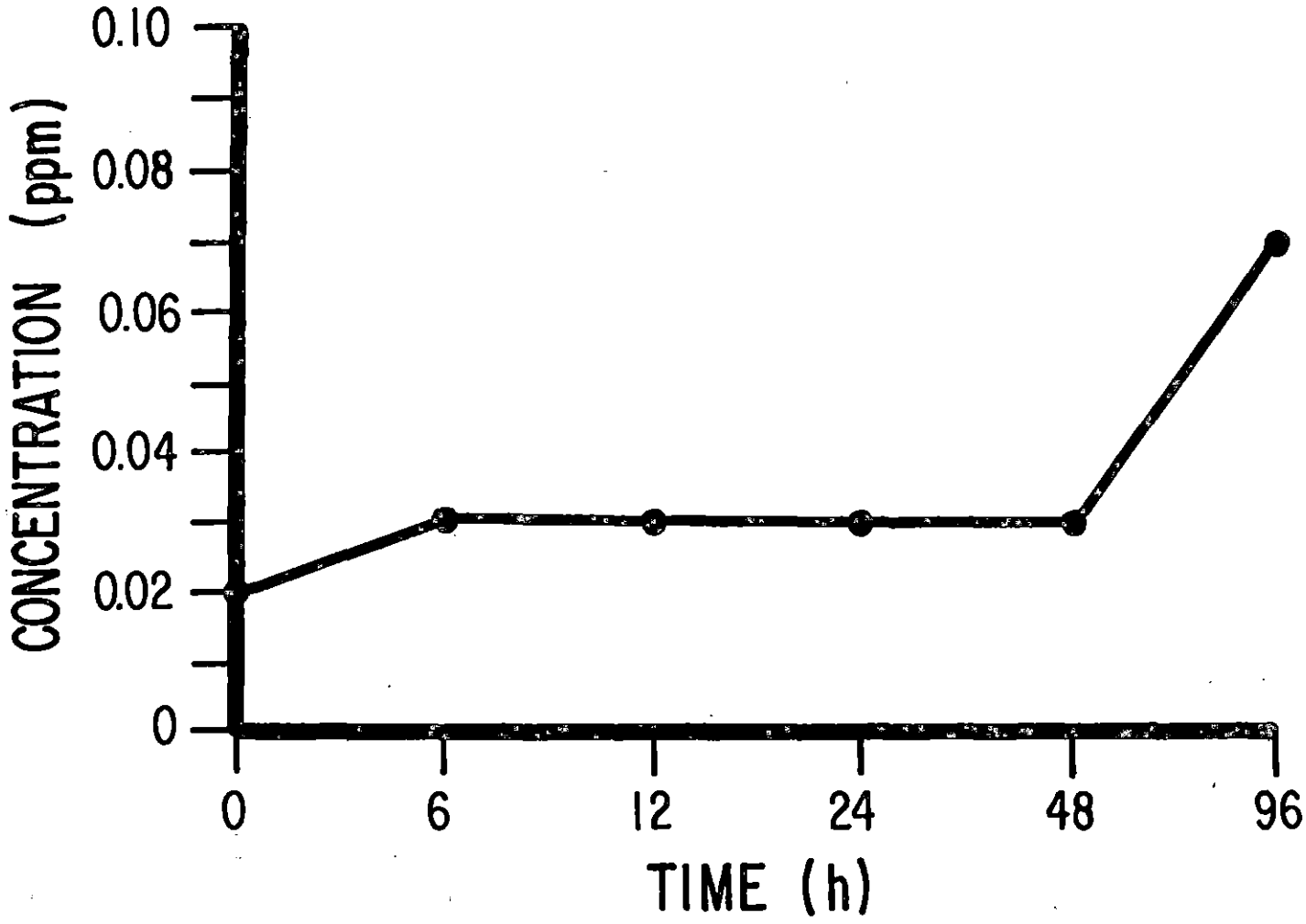


Figure 4. Uncorrected Tishler control muscle values

Table 6. Corrected time study Tishler control muscle values

	Muscle-ppm
0 hours	0.03±0.01
6 hours	0.06±0.03
12 hours	0.06±0.03
24 hours	0.06±0.03
48 hours	0.07±0.02
96 hours	0.18±0.03*

*P < .05.

hours and then increased between 48 and 96 hours. The change was not significant.

The uncorrected results for blended and whole liver values are shown in Table 7. A graphic illustration of these results is presented in Figure 6.

Table 7. Uncorrected time study Tishler positive liver values

	Blended liver-ppm	Whole liver-ppm
0 hours	0.26±0.07	0.28±0.07
6 hours	0.24±0.09	0.26±0.07
12 hours	0.20±0.06	0.23±0.06
24 hours	0.16±0.04	0.19±0.02
48 hours	0.10±0.10	0.16±0.04
96 hours	0.30±0.06	0.33±0.04

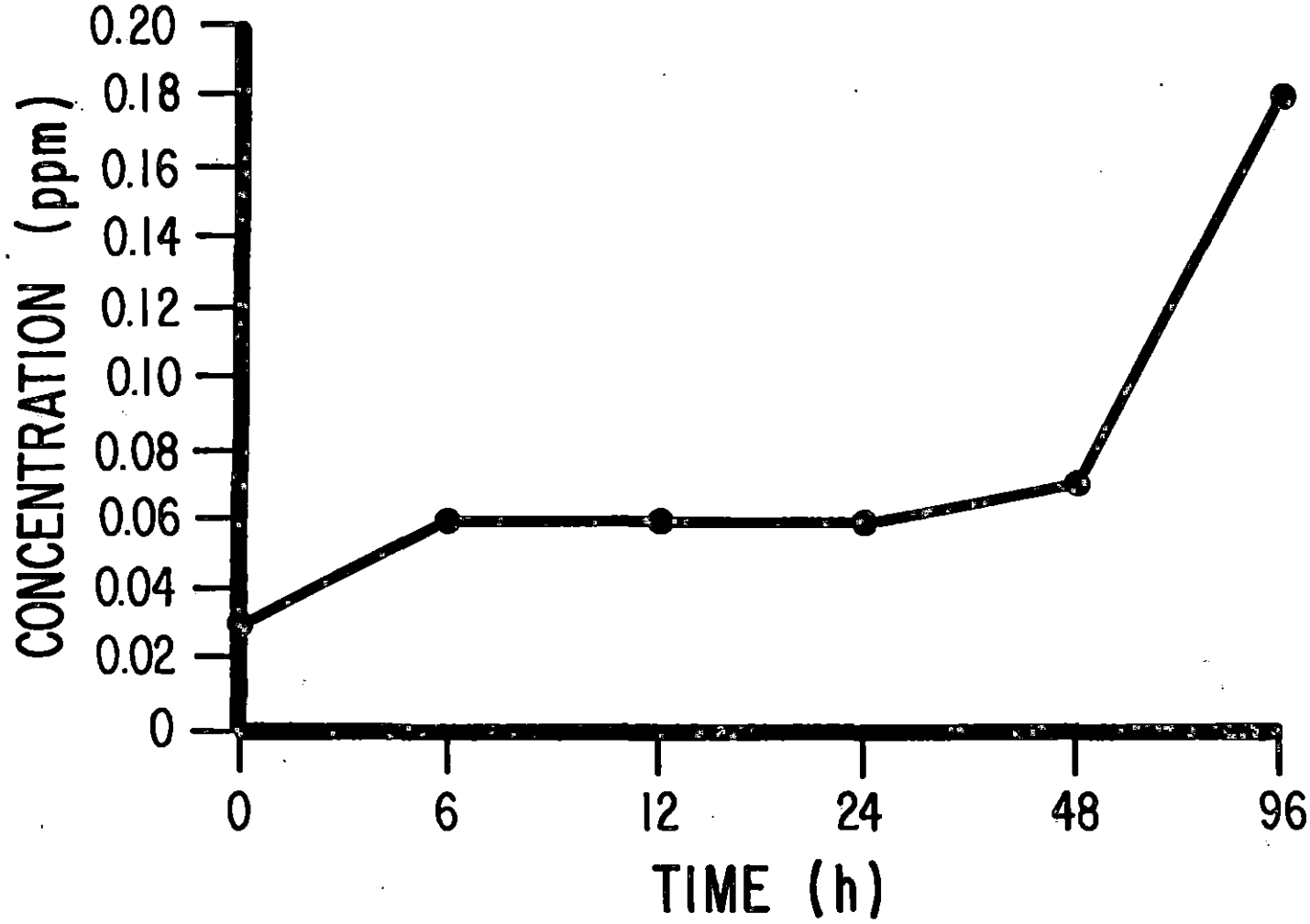


Figure 5. Corrected Tishler control muscle values

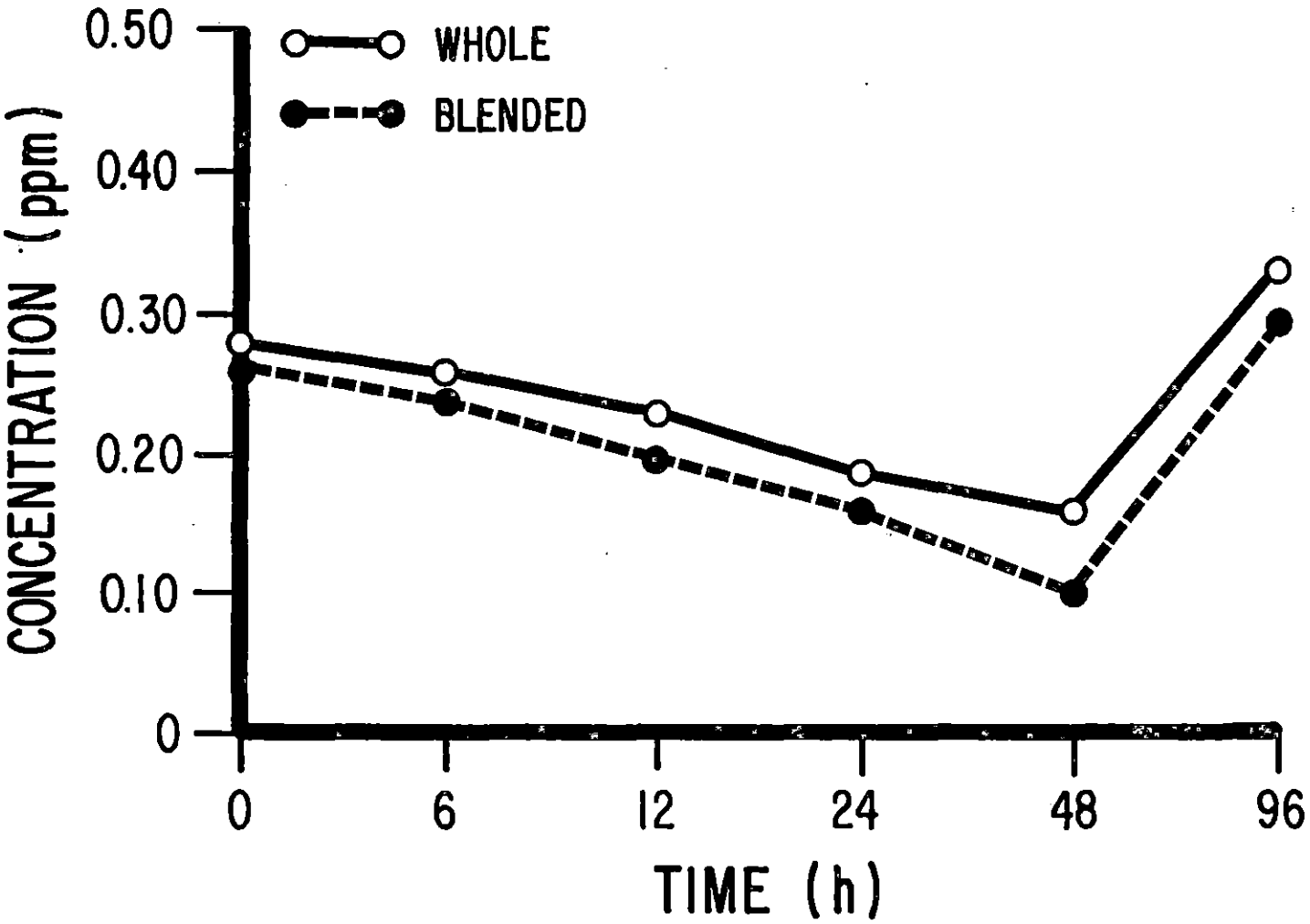


Figure 6. Uncorrected positive liver values

The mean percent recovery values for the different time periods were variable. These values are shown in Table 8.

Table 8. Mean percent recovery time study Tishler positive liver values

	Blended liver-ppm	Whole liver-ppm
0 hours	80±8	75 14
6 hours	64±5	72±16
12 hours	69±15	66±16
24 hours	59±3	77±18
48 hours	72±4	68±14
96 hours	62±11	65±4

The corrected results for blended and whole liver values are shown in Table 9. A graphic illustration of these results is shown in Figure 7.

Table 9. Corrected time study Tishler positive liver values

	Blended liver-ppm	Whole liver-ppm
0 hours	0.36±0.12	0.41±0.15
6 hours	0.35±0.16	0.41±0.17
12 hours	0.32±-0.12	0.39±0.16
24 hours	0.24±0.06	0.28±0.05
48 hours	0.15±0.02	0.24±0.05
96 hours	0.49±0.10	0.50±0.07

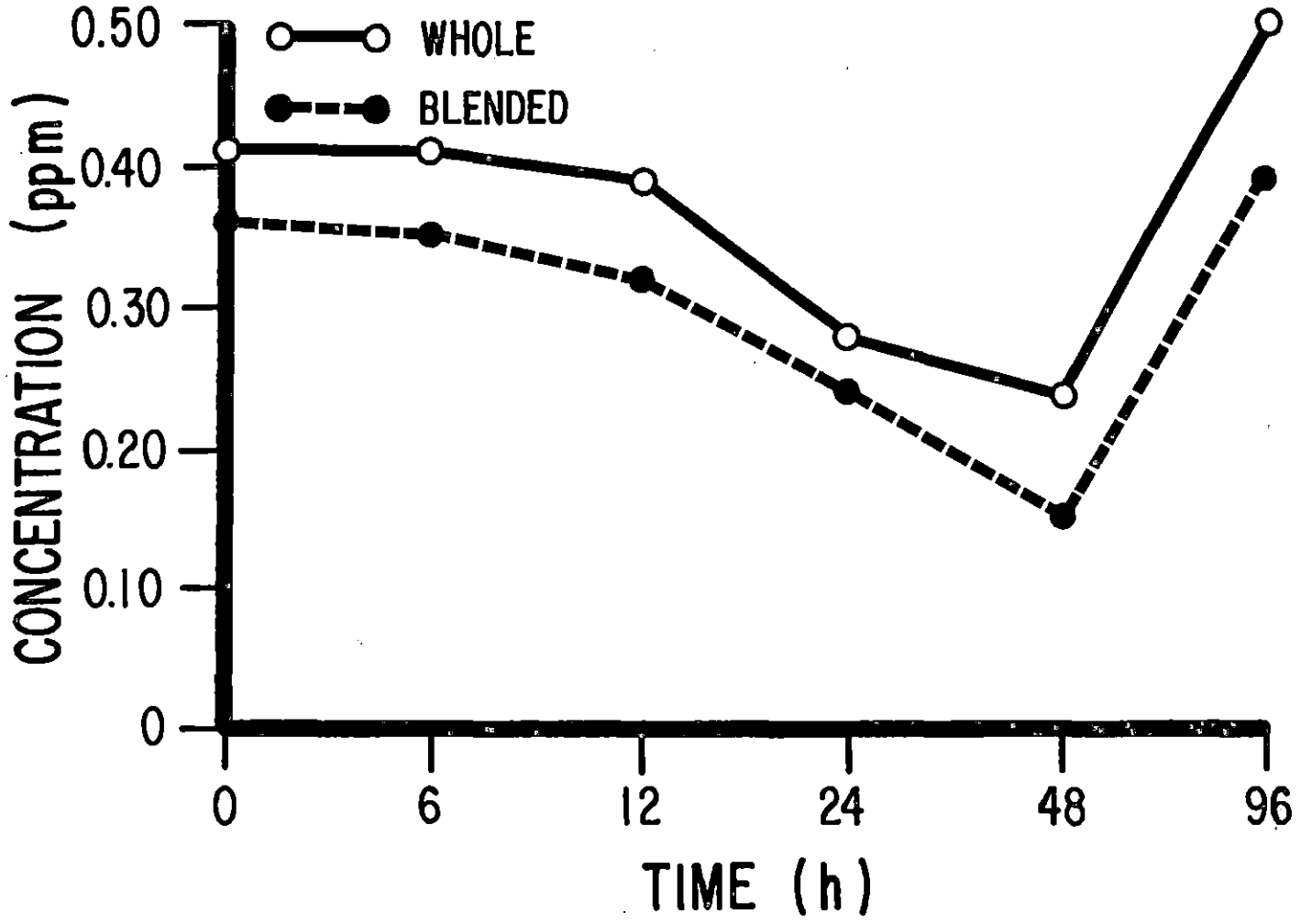


Figure 7. Corrected positive liver values

Gas-liquid chromatography analysis of liver samples from pigs fed feed containing 11.0 ppm sulfamethazine revealed no difference between blended and unblended samples. The combined results are shown in Table 10. The values in the table are corrected for recovery and mean recovery values are also included in this table.

The values for the 0, 6, 12, and 24 hour samples are not significantly different based on the GLC results. The values for the 48 and 96 hour samples were ignored because these samples fouled the detector preventing accurate analysis.

Table 10. GLC results on positive livers

Liver-ppm	Recovery %
0 hours 0.25	45
6 hours 0.20	50
12 hours 0.26	40
24 hours 0.24	38

Positive tissue-muscle

No significant differences were detectable by the Tishler method as the time increased. The values decreased up to 48 hours and then rose at 96 hours.

The uncorrected results for positive muscle values are shown in Table 11.

Table 11. Uncorrected time study Tishler positive muscle values

	Muscle-ppm
0 hours	0.08±0.03
6 hours	0.07±0.03
12 hours	0.06±0.01
24 hours	0.06±0.02
48 hours	0.06±0.03
96 hours	0.09±0.02

The mean percent recovery values for the different time periods were variable but were lowest at 96 hours. These values are shown in Table 12. The corrected results are shown in Table 13.

Table 12. Percent recovery positive muscle

	Muscle
0 hours	81±2
6 hours	84±4
12 hours	83±6
24 hours	71±2
48 hours	82±4
96 hours	60±5

Table 13. Corrected time study Tishler positive muscle values

	Muscle-ppm
0 hours	0.09±0.03
6 hours	0.08±0.03
12 hours	0.08±0.02
24 hours	0.08±0.02
48 hours	0.08±0.04
96 hours	0.13±0.03

Freeze-Thaw Study

Freeze thaw study - Tishler

As measured by the Tishler color reaction, the uncorrected and corrected levels of "sulfamethazine" increased as the tissues were subjected to the freeze-thaw treatment. The value after 4 freeze-thaw cycles (FTC) was significantly higher ($P < .05$) than the initial value. These results are shown in Table 14. A graphic illustration of these results are shown in Figure 8.

Table 14. Tishler liver values after freeze-thaw treatment

FTC	Uncorrected-ppm	Corrected-ppm
1	0.04	0.04
2	0.04	0.05
3	0.05	0.06
4	0.07*	0.09*

*$P < .05$.

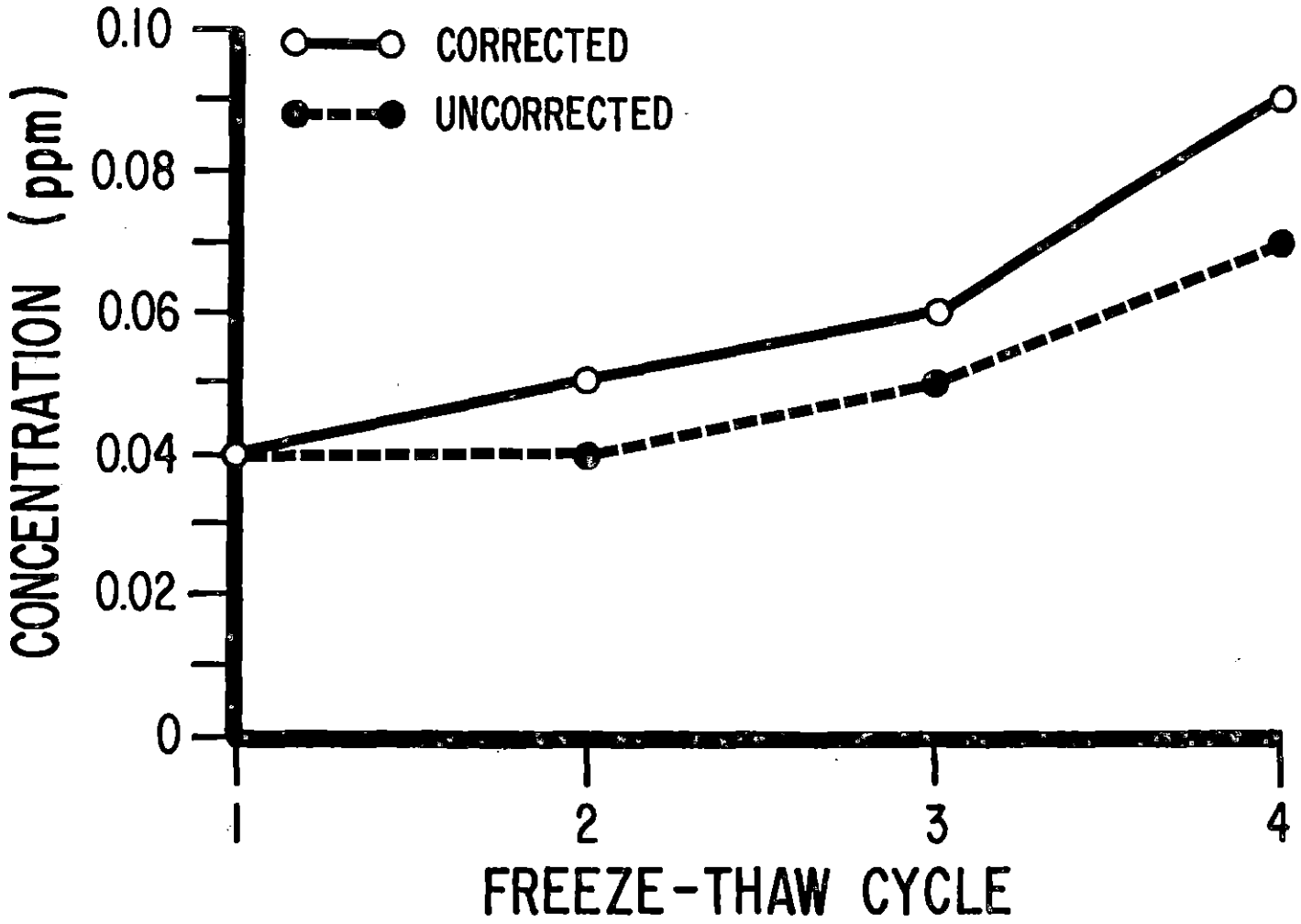


Figure 8. Tishler liver values after freeze-thaw treatment

Freeze-Thaw Study - GLC

All liver samples from these pigs which were fed control feed were negative for sulfamethazine when analyzed by GLC. The mean percent recovery for these samples was 56%. A great majority of these samples contained a substance which caused a significant response by the detector. The column retention time for this compound was approximately 1 minute longer than that for sulfamethazine. This peak tracing would begin to rise before the tracing for sulfamethazine began to drop. This compound was identified as nicotinamide by gas-liquid chromatography-mass spectrometry.

DISCUSSION

Time Study

Control tissues

The results of this study demonstrate that the "sulfonamide" concentration in liver and muscle, as measured by the Tishler method of analysis, can be affected if the tissue is stored at 23°C for some time. The effect of higher environmental temperatures is somewhat speculative but it is my opinion that the change would be accelerated at higher temperatures or relatively short storage time at higher temperatures could change the value considerably.

The difference in initial background concentrations of "sulfonamide" in the whole and blended liver samples from control animals may be due to rupture of cellular membranes by mechanical damage to the cells and/or the heat generated during the blending process. The difference in the levels suggests the effect of tissue treatment should be considered when doing collaborative studies between laboratories. The blended tissues would be more uniform samples but this treatment might influence the results of the study. The values in blended liver increased earlier than the whole liver values. This was probably caused by the same factors which created higher initial values in combination with an increase in available nutrients for putrifactive bacteria. Both values were at least 3 times their initial values at 48 hours and 5 times their initial values at 96 hours. Samples at these times were discolored and odoriferous.

The control muscle values also increased but not as quickly or to the extent of the liver values. Muscle would be a different type of media for bacterial growth and would probably contain fewer compounds readily converted to primary aromatic amines. The change in the muscle value was not significant through 48 hours but was at 96 hours.

Positive tissues

The "sulfonamide" level, as measured by the Tishler method of analysis, for both blended and whole liver dropped over the first 48 hours and then rose again at 96 hours. The decrease in measured "sulfonamide" levels in samples from pigs fed 11.0 ppm sulfamethazine feed may be due to a process produced by the putrefactive bacteria which are multiplying in the tissue. These bacteria may be partially inhibited by the residual sulfamethazine and its active metabolites in the tissues. As the sulfamethazine is incorporated into the bacterial wall in place of PABA, the sulfamethazine may become unavailable for extraction. At the same time, this action would prevent bacterial degradation of the tissue components into primary aromatic amines. Metabolism of sulfamethazine by tissue enzymes may also contribute to the change in values.

The measured amount of sulfamethazine in the muscle remained essentially the same through the first 48 hours and increased slightly by 96 hours. The initial concentration of sulfamethazine was lower in the muscle than the liver. The muscle degradation may have been slowed by this small amount of sulfamethazine which eventually was depleted.

Freeze-Thaw Study

The results demonstrate that the "sulfonamide level" of liver, as measured by the Tishler method of analysis, can be affected by repeated freezing and thawing. This finding suggests that samples should not be thawed for analysis and then refrozen for additional sulfonamide analyses. If a second analysis by this method is planned, the large sample should be divided while still frozen and only the portion of the sample to be analyzed allowed outside the freezer for a period of time sufficient to produce thawing. The portion to be analyzed should be processed promptly.

If tissue is not frozen immediately after collection and kept frozen until analysis begins, the Tishler results may be erroneously high or low. Samples should be handled in such a way to insure that they are taken from the animals as soon as possible and frozen within 30 minutes. Any sample arriving at the laboratory for analysis which is not frozen solid should be discarded. Samples which have partially thawed but still have some ice crystals in them may be undergoing some decomposition. Samples in the laboratory should be thawed quickly and the extraction procedures should begin as soon as possible. Samples should not be allowed to thaw out on a laboratory bench for an unspecified amount of time and samples which have thawed should not be frozen for later analysis.

The nicotinamide detected by GLC in these samples could be confused with and interpreted as a sulfonamide peak. This finding suggests that careful monitoring of retention times using various sulfonamide standards

is necessary when analyzing field specimens which could contain a variety of sulfonamide compounds.

SUMMARY

The literature on sulfonamide analysis and metabolism was reviewed. The Tisher method of analysis had been accepted as the "official" method of analysis by most laboratories. An extensive literature search failed to confirm this test as an "official" method.

Selected tissues from control swine consuming sulfonamide-free feed were subjected to the effects of storage at a temperature of 23°C for various times and of freeze-thaw treatments. Similar tissues from swine consuming feed containing 11.0 ppm sulfamethazine were subjected to the effect of storage temperature.

As measured by the Tishler color reaction, the "sulfonamide" concentration in the tissues from the control animals increased as the time the tissues were held at 23° C increased. An increase in "sulfonamide" concentration was also detected by the Tishler method in control tissues subjected to several freeze-thaw cycles.

Analysis of the same control tissues by a GLC method failed to detect a change in concentration in these tissues.

No significant differences in "sulfonamide" concentrations were detected by the Tishler or GLC methods in tissues from animals consuming feed containing 11.0 ppm sulfamethazine.

This study demonstrated that some types of tissue mishandling could alter the results obtained by the Tishler method of analysis.

REFERENCES

Anonymous. 1979. FSQS/FDA joint draft memorandum dated May 18, 1979. Food Chem. News 21(37):15-18.

Anonymous. 1982. Acceptable methods to determine sulfonamide residues in poultry and red met tissues. Federal Register 47(35):77±5.

Bevill, R. F. 1982. Sulfonamides. Pages 717-726 in N. H. Booth and L. E. McDonald, eds. Veterinary pharmacology and therapeutics. 5th ed. Iowa State University Press, Ames, Iowa.

Bevill, R. F., and W. G. Huber. 1977. Sulfonamides. Pages 894-911 in L. M. Jones, N. H. Booth, and L. E. McDonald, eds. Veterinary pharmacology and therapeutics. 4th ed. Iowa State University Press, Ames, Iowa.

Bevill, R. F., L. W. Dittert, and D. W. A. Bourne. 1977a. Disposition of sulfonamides in food-producing animals, IV: Pharmacokinetics of sulfamethazine in cattle following administration of an intravenous dose and three oral dosage forms. J. of Pharmaceutical Sci. 66(5): 619-623.

Bevill, R. F., R. M. Sharma, S. H. Meachum, S. C. Wozniak, D. W. A. Bourne, and L. W. Dittert. 1977b. Disposition of sulfonamides in food-producing animals: Concentrations of sulfamethazine and its metabolites in plasma, urine, and tissues of lambs following intravenous administration. Am. J. Vet. Res. 38(7):974-977.

Bevill, R. F., K. M. Schemske, H. G. Luther, E. A. Dzierzak, M. Limpoka, and D. R. Felt. 1978. Determination of sulfonamides in swine plasma. J. Agric. Food Chem. 26:1201-1203.

Bourne, D. W. A., R. F. Bevill, R. M. Sharma, R. P. Guaral, and L. W. Dittert. 1977. Disposition of sulfonamides in food-producing animals: Pharmacokinetics of sulfamethazine in lambs. Am. J. Vet. Res. 38(7):967-972.

Bourne, D. W. A., L. W. Dittert, G. D. Koritz, and R. F. Bevill. 1978. Disposition of sulfonamides in food-producing animals: VII. Disposition of sulfathiazole in tissue, urine, and plasma of swine following intravenous administration. J. Pharmacokinet. Biopharm. 6(2): 123-134.

Bratton, A. C., and E. K. Marshall, Jr. 1939. A new coupling component for sulfanilamide determination. J. Biol. Chem. 128:537-550.

Buttle, G. A. H., W. H. Gray, and D. Stephensen. 1936. Protection of mice against streptococcal and other infections by p-aminobenzene-sulphonamide and related substances. Lancet 1:1286-1290.

Cieri, U. R. 1976. Detection of sulfonamides in animal feeds. J. A. O. A. C. 59(1):56-59.

Cox, D. F. 1971. Methods for data analysis. Part II. Teaching notes. Dept. of Statistics, Iowa State University, 222 pp.

Cromwell, G. L. 1979. Sulfa residues. Animal Nutrition and Health 34(4):6,16.

Crossley, M. L., E. H. Northey, and M. E. Hultquist. 1938a. Sulfanilamide derivatives. I. Aminoarylsulfonamidoarylsulfonic acids and aminoarylsulfonamidoarylcarboxylic acids. J. Am. Chem. Soc. 60:2217-2222.

Crossley, M. L., E. H. Northey, and M. E. Hultquist. 1938b. Sulfanilamide derivatives. I. Disulfanilamides and related compounds. J. Am. Chem. Soc. 60:2222-2224.

Crossley, M. L., E. H. Northey, and M. E. Hultquist. 1938c. Sulfanilamide derivatives. III. Strepto-N-polylsulfanilyl-sulfanilamides and related compounds. J. Am. Chem. Soc. 60:2225-2227.

Dittert, L. W. 1977. Pharmacokinetic prediction of tissue residues. J. Toxicology and Environmental Health 2:735-756.

Domagk, G. 1935a. Chemotherapic der bakteriellen infektion. Anguv. Chem. 48:657-667.

Domagk, G. 1935b. Eine beitrag sur chemotherapie der bakteriellen infektionen. Deutshe Med. Wochenschr. 61:250-253.

Domagk, G. 1935c. Eine neue klasse von disinfektionsmittein. Deutsche Med. Wochenschr. 61:829-832.

Elliott, R. F., D. D. Johnson, and A. L. Shor. 1964. Effect of chlorotetracycline, sulfamethazine and procaine penicillin on the performance of starting pigs. J. Anim. Sci. 23:154-159.

Federal Register. 1982. Food and drugs 21 § 556670.

Fourneau, E., J. Trefouel, Mme. J. Trefouel, F. Nitti, and D. Bovet. 1936. Chimiotherapie des infections streptococciques par les derives du p-aminophenyl-sulfamide. Compt. Rend. Soc. de Biol. (Paris) 122:652-654.

Gelmo, P. 1908. Suphamides of p-aminobenzenesulphonic acid. J. Prakt. Chem. 77:369-382.

Goehl, T. J., L. K, Mathur, J. D. Strum, J. M. Jaffe, W. H. Pitlick, V. P. Shah, R. I. Paust, and J. L. Colaizzi. 1978. Simple high-pressure liquid chromatographic determination of trisulfapyrimides in human serum. J. Pharm. Sci. 67(3):404-406.

Goodspeed, D. P., R. M. Simpson, R. B. Ashworth, D. W. Shafer, and H. R. Cook. 1978. Sensitive and specific gas-liquid chromatographic-spectrophotometric screening procedures for trace levels of five sulfonamides in liver, kidney, and muscle tissue. J.A.O.A.C. 61: 1050-1053.

Heath, G. E., and R. H. Teske. 1973. Effect of water deprivation and forced exercise on blood concentrations of sulfaethoxypyridazine after its oral administration to calves. J.A.V.M.A. 163:749-752.

Heintzelman, J. H. L., P. B. Hadley, and R. R. Mellon. 1937. The use of p-aminobenzenesulphoamide in type III pneumococcus pneumonia. Am. J. Med. Sci. 193:759-763.

Holder, C. L., H. C. Thompson, and M. C. Bowman. 1981. Trace analysis of sulfamethazine in animal feed, human urine, and wastewater by electron capture chromatography. J. Chromatogr. Sci. 19:625-633.

Horwitz, W. 1981. Analytical methods for sulfonamides in foods and feeds I. Review of methodology. J.A.O.A.C. 64(1):104-130.

Johnson, K. L., D. T. Jeter, and R. C. Clairbourne. 1975. High-pressure liquid chromatographic determination of sulfamethazine residue in bovine tissue. J. Pharm. Sci. 64(10):1657-1660.

Lapka, R., Z. Urbanova, B. Kobylka, H. Raskova, J. Vanecek, and L. Polak. 1978. Pharmacokinetics of sulfadimidine in normal and diarrheic calves. Drug Metab. Dispos. 6:637-639.

Lehninger, A. L. 1975. Biochemistry. 2nd ed. Worth Publishers Inc., New York, N.Y.

Leidahl, R., editor. 1980. 1981 feed additive compendium. The Miller Publishing Company, Minneapolis, Minn.

Levaditi, C., and A. Vaisman. 1935. Action durative et preventive du chlorhydrate de 4'-sulfamido-2, 4-diamino-azobensene dans l'infection streptococcique experimentale. Compt. Rend. A. Cod. D. Sc. 200: 1694-1696.

Linkenheimer, W. H., and S. J. Stolzenberg. 1965. Pharmacologic characteristics of four sulfonamides in swine. Am. J. Vet. Res. 26(114): 1086-1094.

Lloyd, W. E., A. L. Jenny, D. F. Cox, and G. E. Rottinghaus. 1981. Relationship of sulfamethazine in swine diets and resultant tissue concentrations, using Tishler and gas liquid chromatographic methods. Am. J. Vet. Res. 42(2):339-343.

Luchtefeld, R. 1976. Use of thin-layer chromatography (TLC) in detecting sulfonamides in feeds. Off. Proc. Annu. Meet. Am. Assoc. Feed Microsc. 24:13-14.

Malanoski, A. J., C. J. Barnes, and T. Fazio. 1981. Comparison of three methods for determination of sulfamethazine in swine tissues: Collaborative study. J.A.O.A.C. 64(6):1386-1391.

Manuel, A. J., and W. A. Steller. 1981. Gas-liquid chromatographic determination of sulfamethazine in swine and cattle tissues. J.A.O.A.C. 64(4):794-799.

Melliere, A. L., W. P. Waitt, K. H. Baker, and J. A. Miyar. 1968. Tylosin-sulfamethazine for growing swine. J. Anim. Sci. 27:1777-1779.

Ostle, B., and R. W. Mensing. 1975. Statistics in research. 3rd ed. Iowa State University Press, Ames, Iowa. 596 pp.

Parks, O. W. 1982. Screening test for sulfamethazine and sulfathiazole in swine liver. J.A.O.A.C. 65(3):632-634.

Phillips, W. F., and J. E. Trafton. 1975. A screening method for sulfonamides extracted from animal tissues. J.A.O.A.C. 58(1):44-47.

Righter, H. F., J. M. Worthington, and H. D. Mercer. 1971. Tissue-residue depletion of sulfathiazone in swine. J. Anim. Sci. 33(4): 797-798.

Schwartz, D. P. 1982. Rapid screening test for sulfamethazine in swine feeds. J.A.O.A.C. 65(3):701-705.

Schwentker, F. F., S. Gelman, and P. H. Long. 1937. The treatment of meningococcic meningitis with sulfanilamide. J.A.M.A. 108:1407-1408.

Sharma, J. P., E. G. Perkins, and R. F. Bevill. 1976. High pressure liquid chromatographic separation, identification and determination of sulfa drugs and their metabolites in urine. J. Pharm. Sci. 65 (11):1606-1608.

Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods. 6th ed. Iowa State University Press, Ames, Iowa. 595 pp.

Snell, F. D., and C. T. Snell. 1954. Colorimetric methods of analysis. Vol. 4. 3rd ed. D. Van Nostrand Company, Inc., Princeton, N.J.

Stamp, T. C. 1939. Bacteriostatic action of sulfanilamide in vitro: Influence of fraction isolated from hemolytic streptococci. Lancet 2:10-17.

Suhre, F. B., R. M. Simpson, and J. W. Shafer. 1981. Qualitative quantitative determination of sulfamethazine in swine tissue by gas chromatographic/electron impact mass spectrometry using a stable isotope labeled internal standard. J. Agric. Food Chem. 29(4):727-729.

Thomas, M. H., K. E. Soroka, R. M. Simpson, and R. L. Epstein. 1981. Determination of sulfamethazine in swine tissues by quantitative thin-layer chromatography. J. Agric. Food Chem. 29(3):621-624.

Tishler, F., J. L. Sutter, J. N. Bathish, and H. E. Hagman. 1968. Improved method for determination of sulfonamides in milk and tissues. J. Agric. Food Chem. 16(1):50-53.

Trabosh, H. M. 1978a. Twelfth swine sulfonamide report. Food Safety and Quality Service, U.S. Department of Agriculture, Washington, D.C.

Trabosh, H. M. 1978b. Fourteenth swine sulfonamide report. Food Safety and Quality Service, U.S. Department of Agriculture, Washington, D.C.

Trabosh, H. M. 1981. Forty-fourth swine sulfonamide report. Food Safety and Quality Service, U.S. Department of Agriculture, Washington, D.C.

Trefouel, J., MMe. J. Trefouel, F. Nitti, and D. Bovet. 1935. Activite du p-aminophenylsulfamide sur les infections streptococciques experimentsales de la souris et du lapin. Compt. Rend. Soc. de Biol. (Paris) 120:756-758.

Trieff, N. M., V. M. S. Ramanujam, and G. C. Forti. 1977. Assay of p-aminobenzenesulfonamides with chloramine-t. Talanta 24:188-190.

U.S. Department of Agriculture-APHIS. 1979. Sulfa residues update. Nov. 9, 1979. USDA-APHIS, Veterinary Services, Washington, D.C.

United States Pharmacopeia. 1970. 18th revision. Mack Printing Company, Easton, Pa.

Windholz, M., editor. 1976. The Merck index. 9th ed. Merck and Co., Inc., Rahway, N.J.

Wood, H. C., editor. 1955. The dispensary of the United States of America. 25th ed. J. B. Lippincott Company, Philadelphia, Pa.

Woods, D. D. 1940. The relation of para-aminobenzoic acid to the mechanism of the action of sulfanilamide. Br. J. Exp. Pathol. 21: 74-90.

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