

DRUG RESIDUES IN ANIMAL TISSUES

Evidence for Transformation of Sulfamethazine to its N^4 -Glucopyranosyl Derivative in Swine Liver During Frozen Storage

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An analytical procedure for determining N^4 -glucopyranosylsulfamethazine (GPS) in swine livers is described. The sulfamethazine derivative is extracted from the tissues with water. GPS is isolated in sufficient purity for liquid chromatographic determination by a series of adsorption chromatographic procedures. Recovery of the conjugate from spiked swine livers was 82.2% with a coefficient of variation of 4.5%. Evidence is presented suggesting that sulfamethazine in swine livers is transformed to GPS during frozen storage. Two samples of swine liver in which incurred sulfamethazine residues were substantially depleted during frozen storage were analyzed for GPS. The conjugate accounted for 96.2% and 92.2% of the depleted sulfamethazine residues.

The Food Safety and Inspection Service (FSIS), U.S. Department of Agriculture, is responsible for screening edible animal tissues for violations of U.S. federal regulations concerning drug residues. Among the drugs under surveillance is sulfamethazine, which is widely used to promote growth and prevent disease in swine. Recent studies at FSIS (R. B. Ashworth, personal communication, 1982) revealed that a serious depletion of incurred sulfamethazine residues occurred in swine livers during frozen storage. Concern was expressed because swine tissues are normally held in frozen storage for varying lengths of time before analyses.

Others have observed the depletion of sulfamethazine residues during frozen storage. Cox and Krzeminski (1) reported that sulfamethazine residues decreased 13.9% in swine muscle and 12.6% in liver during 15 days storage at -20°C . These values were 4–6% higher than those observed by Saschenbrecker and Fish (2). The data of Murtha et al. (3) showed a 10% decrease in sulfamethazine levels in calf livers, following 40 days of storage at -20°C ; however, the authors attributed the difference in results to normal variations in the method.

Our laboratory was requested by FSIS to investigate the nature of the sulfamethazine depletion and was provided with samples in which incurred residues were substantially depleted during frozen storage. Our preliminary studies showed that substantial amounts of N^4 -glucopyranosylsulfamethazine (GPS) were present in the drug-depleted livers. This report presents our efforts to develop a quantitative method for GPS in swine liver and to relate the presence of this compound to the depletion of the parent drug during frozen storage.

METHOD

Reagents

(a) *Solvents*.—Methanol, isopropanol, acetonitrile, ethylacetate, and methylene chloride (Distilled-in-Glass®, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Acetone ("Baker Analyzed," J. T. Baker Chemical Co., Phillipsburg, NJ 08665.)

(b) *Sulfamethazine*.—American Cyanamid Co., Princeton, NJ 10540.

(c) *N^4 -Glucopyranosylsulfamethazine*.—See acknowledgments.

(d) *Glucose*.—National Bureau of Standards sample.

(e) *N -1-(Naphthyl)ethylenediamine (NEDA) dihydrochloride*.—Sigma Chemical Co., St. Louis, MO 63178.

(f) *Pipet tip*.—5 mL (Rainin Instrument Co., Woburn, MA 01801).

(g) *Large volume Pasteur pipet*.—4 mL (Fisher Scientific Co., King of Prussia, PA).

(h) *Resin*.—Duolite ES-863 (Diamond Shamrock Corp., Cleveland, OH 44114). Place 20 g resin in 60 mL sintered glass funnel attached to vacuum flask. Wash resin consecutively with five 50 mL portions of methanol, acetone, and water. Store resin at 4°C in water. Prewashed resin is sufficient for 12 columns packed in the following manner: Cut 3.5 cm off tip of large volume Pasteur pipet and insert small plug (0.5 cm) of fine glass wool. Add slurry of resin to obtain column 5.5 cm high. Add 0.5 cm plug of glass wool to top of resin. Wash resin with additional 10 mL water before use. (Note: Do not let column run dry.)

(i) *Neutral alumina*.—Brockman Activity I, 80–200 mesh (Fisher Scientific Co.). Insert 5 mm glass bead into 5 mL pipet tip. Layer glass bead with 0.5 cm sea sand followed by 2.2 cm bed of neutral alumina. Add 0.25 cm layer of sea sand. Wash column with three 2 mL portions of 90% methanol before use.

Apparatus

(a) *Shaker*.—IKA-Vibrax VXR Shaker (Tekmar Co., Cincinnati, OH 45222).

(b) *Centrifuges*.—Sorvall Superspeed Centrifuge-Type SS-1 Rotor (Ivan Sorvall, Inc., Norwalk, CT). International Clinical Centrifuge-Rotor No. 273 (International Equipment Co., Needham Heights, MA 02194).

(c) *Vortex stirrer*.—Super Mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(d) *Liquid chromatography (LC)*.—Altex Model 100A pump (Altex Scientific Inc., Berkeley, CA 94710) connected to Schoeffel Model SF770 Spectroflow variable wavelength detector operated at 266 nm. Altex Model 210 sampling valve with 50 μL loop. Column: 25 cm \times 4.6 mm id LC-18 (Supelco Inc., Bellefonte, PA 16823). Mobile phase: water-acetonitrile-isopropanol (94.5 + 5.0 + 0.5). Samples elute isocratically at flow rate of 1 mL/min.

Analytical Procedure

To 2.5 g frozen, ground liver in 50 mL polypropylene screw-cap centrifuge tube, add 13.25 mL water. Shake vigorously 20 min. Centrifuge 5 min at 3000 rpm. Remove aqueous suspension with disposable Pasteur pipet and filter through plug of glass wool packed in large volume disposable pipet, collecting 7.5 mL filtrate. Pass filtrate through column of Duolite ES-863. Wash column with 20 mL water. Elute column with 6 mL methanol, applying slight air pressure, when required, to maintain flow. Let methanol percolate through all but 1.5 cm resin (ca 1.25–1.50 mL methanol added to column) before collecting effluent. After collection, dilute effluent to 10 mL with methanol and pass sample through column of neutral alumina. Wash column with 5 mL methanol. Remove excess

methanol from column with air pressure and continue pressure until column dries as evidenced by disappearance of condensed moisture on outside of column (ca 15 min). Elute column by gravity flow with 7 mL water. Dilute effluent to 10 mL with water and pass through second column containing Duolite ES-863. Wash column with 10 mL water and elute with 6 mL methanol as described above. Evaporate effluent to dryness in 9 mL screw-cap vial under stream of nitrogen at 50°C. Add 0.4 mL LC mobile phase to residue. Vortex-mix for 30 s and centrifuge 1 min at 2500 rpm. Immediately inject 50 μ L sample into LC column. (Note: All phases of procedure are performed in absence of direct light.)

Recovery Studies

Drug-free, frozen, ground liver samples were spiked, by injection, with GPS at concentrations of 0.95, 0.63, 0.32, and 0.16 ppm—equivalent to 0.6, 0.4, 0.2, and 0.1 ppm sulfamethazine, respectively. Unspiked liver samples served as controls. Recoveries were determined by comparison of LC peak heights with a standard curve prepared from varying concentrations of GPS (0.025–0.16 μ g). Retention time was 11.5 min.

Isolation of GPS

Twelve 2.5 g samples of a frozen swine liver, substantially depleted of its incurred sulfamethazine residue following frozen storage, were carried through the analytical procedure described, omitting the LC analysis step. Instead, effluents from the final ES-863 columns were combined and evaporated to dryness. The residue was dissolved in 0.6 mL methanol and 0.2 mL was spotted across the inner 3 plates of a 10 \times 12.5 cm 4-scored TLC Uniplate (five 10 \times 2.5 cm plates) having a 250 μ m layer of silica gel G (Analtech, Newark, DE). The outer plates were spotted with single spots of the extract. The plate had been previously washed with methanol and dried at 100°C for 1 h. The plates were developed in a No. 4 American Medical Museum jar with ethyl acetate–methanol–water (4 + 1 + 0.1) and dried in a forced air oven for 10 min at 50°C. The outer plates were removed and subjected to hydrolysis, and the spots were visualized with a modified Bratton-Marshall (B-M) spray reagent (4) as described in the TLC studies section. The area of the remaining TLC plate corresponding to the R_f of the unknown was scraped from the plate. The adsorbent was packed in a large volume Pasteur pipet plugged with glass wool and eluted with methanol–methylene chloride (3 + 1). The eluants from 3 plates were combined and evaporated to dryness.

Thin Layer Chromatography (TLC) Studies

(a) *Development.*—2.5 \times 10 cm glass plate with 250 μ m layer of silica gel G. Developing chamber: 11.2 \times 4.7 cm large mouth screw-cap bottle. Developing solvent: ethyl acetate–methanol–water (3.5 + 1.5 + 0.1). Develop plates 0.5 cm and dry in forced air oven for 5 min at 50°C. Redevelop plates to height of 1.0 cm, then 6.0 cm, with oven-drying between and after attaining final height. R_f values: sulfamethazine 0.9; GPS 0.6; glucose 0.5.

(b) *Acid hydrolysis.*—Expose TLC plates to HCl fumes for 5 min in 11.2 \times 4.7 cm screw-cap bottle, followed by 3-min exposure to ammonia vapors in a second bottle. Remove excess ammonia by heating plates 5 min in 50°C forced air oven. Use 10 mL beaker in screw-cap bottle as receptacle for 5 mL concentrated HCl or ammonium hydroxide.

(c) *Visualization techniques.*—(1) *Modified B-M spray reagent.*—Expose plates 3–5 s to nitrous acid vapors generated by the addition of sodium nitrite to 8% aqueous phos-

phoric acid. Allow 20 s for excess nitrous acid to dissipate. Spray with 0.4% NEDA in methanol to produce pink spot for sulfamethazine. (2) *Bounias reagent* (5).—Spray plate with 6.5 mM solution of NEDA in methanol containing 3% sulfuric acid. Heat plates 5–10 min at 100°C to develop colors for GPS and glucose which ranged from pink to grey-violet, depending on the length of heating. (3) *Combination reagent.*—Expose plates to diazotization reaction. Allow 20 s for excess nitrous acid vapors to dissipate. Spray with Bounias reagent. Sulfamethazine produces pink color immediately, while glucose and GPS develop color after heating at 100°C.

Chemical Ionization–Mass Spectrometry (CI-MS)

Spectra were obtained at 250 eV with a Varian Model MAT 311A mass spectrometer interfaced to the Incos data system. Ammonia was the ionizing gas at a source pressure of 5×10^{-4} torr. Samples were introduced by vaporization from a direct insertion probe.

Results and Discussion

Preliminary TLC studies on crude extracts of sulfamethazine-depleted frozen swine liver, obtained by extraction with polar solvents, revealed the presence of a tailing B-M positive compound following excessive exposure to nitrous acid vapors, prior to spraying with the NEDA coupling agent. This result was not observed when nonpolar solvents were used or when the TLC plate was exposed to the conventional diazotization reaction. These observations suggested that a readily hydrolyzed polar sulfamethazine derivative of significant concentration was present in the drug-depleted samples. Subsequent studies showing that the unknown behaved similarly to GPS led to the development of the analytical procedure for isolation and determination of the conjugate.

Tentative identification of the isolated unknown as GPS was concluded based on a series of chromatographic results. The unknown had the same retention time (11.5 min) as the authentic compound in the LC system. TLC of the unknown revealed the same R_f value as that of the authentic compound and both reacted similarly to the visualization reagents. Development of the TLC plates, followed by acid hydrolysis, resulted in a positive B-M reaction following diazotization and visualization with NEDA; however, the unhydrolyzed compound required prolonged exposure to nitrous acid vapors for a positive B-M reaction. Similar results were obtained for the unknown isolated by LC, although partial hydrolysis occurred during evaporation of the mobile phase. Subjecting the unknown and authentic compound to acid hydrolysis prior to development resulted in B-M positive spots with R_f values identical to sulfamethazine. The results of these studies indicated that the unknown was a derivative of sulfamethazine with substitution in the N^4 -position.

Presumptive evidence that the unknown contained a sugar moiety at the N^4 -position was attained by TLC and visualization with the NEDA–methanol–sulfuric acid spray of Bounias (5), with or without diazotization, as follows: (a) spraying with the Bounias reagent following TLC development gave positive spots for the unknown and authentic compound at R_f 0.6; (b) spraying with Bounias reagent following hydrolysis and development resulted in a positive reaction for both the authentic and unknown compound at the R_f of glucose (R_f 0.5), without a positive spot at the R_f of GPS; (c) diazotization prior to spraying with the Bounias reagent, following hydrolysis and plate development, resulted in positive spots at the R_f values of glucose and sulfamethazine for both the unknown and authentic compound. The results of the TLC studies are summarized in Table 1.

Table 1. Summary of TLC studies and visualization reactions at *N*⁴-glucopyranosylsulfamethazine (GPS) isolated from sulfamethazine-depleted stored liver

Treatment	Color reaction at <i>R_f</i> of authentic compound		
	Glucose	GPS	Sulfamethazine
TLC-B-M reaction	-	-	-
TLC-hydrolysis, B-M reaction	-	+	-
Hydrolysis-TLC, B-M reaction	-	-	+
TLC-Bounias reagent	-	+	-
Hydrolysis-TLC, Bounias reagent	+	-	-
Hydrolysis-TLC, diazotization-Bounias reagent	+	-	+

The identity of the isolated compound was confirmed by CI-MS. The most abundant ion of the authentic and isolated compound was *m/z* 124 (protonated dimethylaminopyrimidine) (6). A molecular ion was not observed in either the authentic or isolated compound. Other characteristic fragments of the authentic and isolated compound and their relative abundances, respectively, are *m/z* 279-protonated sulfamethazine (5.2–7.2%); *m/z* 180 (3.7–1.2%); and *m/z* 162 (2.0–0.8%).

Figures 1a and 1b compare the LC chromatograms of extract of control tissue with that of liver fortified with 0.32 ppm GPS

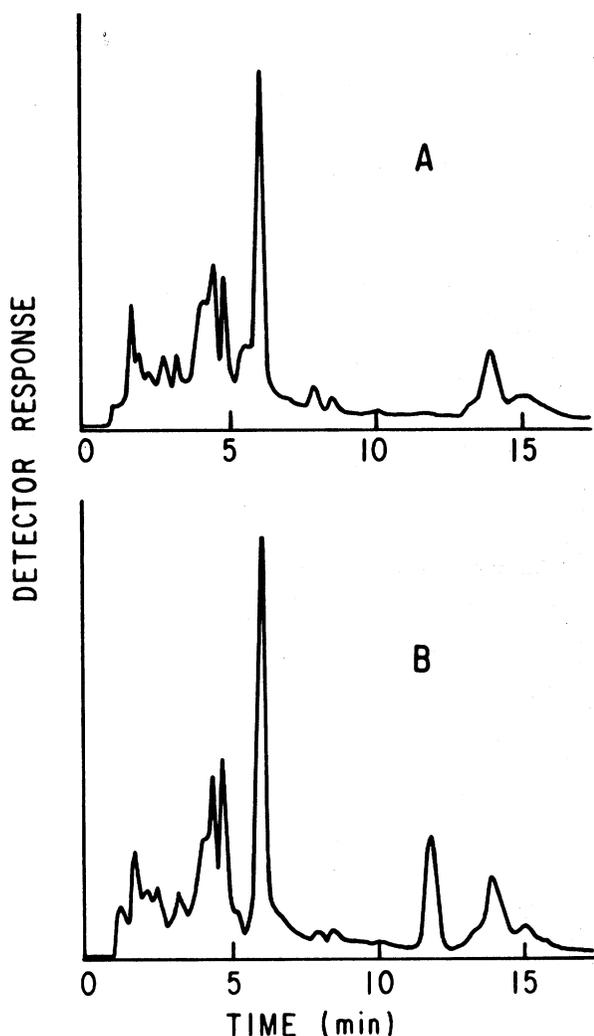


Figure 1. LC chromatograms of A, liver extract of control tissue; B, extract of liver spiked with 0.32 ppm *N*⁴-glucopyranosylsulfamethazine. Detector response at 266 nm and 0.02 AUFS.

(0.2 ppm sulfamethazine equivalent) before analysis. No interfering peaks at the retention time of GPS were observed in control tissues, even at 0.01 AUFS. Detector response to GPS concentrations of 0.025 to 0.16 μ g was linear.

The percent recoveries of GPS from 12 swine liver samples spiked with concentrations ranging from 0.16 to 0.95 ppm (0.1–0.6 ppm sulfamethazine equivalent) as determined by the LC procedure are presented in Table 2. The mean recovery was 82.2%; coefficient of variation was 4.5%. Recoveries were based on the analysis of 1/2 of the aqueous phase (liver contains 71.6% water (7)) of the initial extract.

To determine if GPS forms spontaneously during the isolation procedure, swine livers were spiked with 0.6 ppm sulfamethazine and carried through the procedure. As evidenced by LC chromatograms, no conjugate was present in the liver extracts. Sulfamethazine itself elutes from the neutral alumina column with 90% methanol and is therefore not present in the methanol effluent of the second ES-863 column. Giera et al. (6) observed the formation of the conjugate during evaporation of methanol extracts of swine livers.

Several aspects of the analytical procedure are worth noting. (a) The centrifuged aqueous extract contains substantial amounts of suspended material. The suspended material is removed from the initial ES-863 column by the aqueous rinse and does not interfere with subsequent steps of the procedure. (b) Applying pressure to the alumina column during aqueous elution of adsorbed materials should be avoided, to decrease the amount of slow-moving material on the LC column. Nevertheless, the LC columns must be washed with methanol–water (75 + 25) following 4 LC determinations to remove these relatively nonpolar materials and prevent their interfering with subsequent determinations. (c) It is important that methanol is evaporated from the alumina column before elution with water to prevent leaching of GPS from the second ES-863 column, which serves to remove dissolved alumina in the aqueous effluents. (d) All steps of the procedure are performed in rapid sequence because a slow hydrolysis of the glucoside occurs in aqueous solutions. (e) The procedure is not conducive to routine analyses of swine livers. An analyst performing 2 analyses concurrently can complete 4–6 samples in 8 h.

To relate the presence of GPS to the depletion of the parent drug during frozen storage, 2 samples of swine liver which initially had incurred sulfamethazine residues of 0.57 (Sample A) and 0.69 ppm (Sample B) were analyzed for the parent drug and the conjugate after 2 years of storage at –20°C. LC and TLC analyses for sulfamethazine were performed on extracts obtained by the screening procedure previously described (4). The stored samples contained 0.04 ppm and 0.05 ppm sulfamethazine, respectively. Previous analyses of

Table 2. Recovery of *N*⁴-glucopyranosylsulfamethazine added to swine liver

Added, ppm ^a	Recovery, % ^b
0.16	86.0
0.16	80.6
0.16	80.2
0.32	82.7
0.32	86.7
0.32	77.7
0.63	79.3
0.63	84.6
0.63	80.0
0.95	87.9
0.95	78.9
0.95	81.2

^aEquivalent to 0.1, 0.2, 0.4, and 0.6 ppm sulfamethazine, respectively.

^bBased on analysis of 1/2 the aqueous extract.

these samples after 1 year (Sample A) and 18 months of storage (Sample B) showed sulfamethazine contents of 0.4 and 0.27 ppm (R. B. Ashworth, 1982, personal communication), respectively, suggesting a gradual depletion of the parent drug during frozen storage. The results, performed in quadruplicate, of the analyses following the 2-year storage period showed Sample A and B contained 0.81 ± 0.03 ppm and 0.93 ± 0.03 ppm GPS—equivalent to 0.51 and 0.59 ppm sulfamethazine, respectively. Hence, the N^4 -glucoside can account for 96.2% and 92.2% of the depleted sulfamethazine, respectively, in the stored livers.

The results of these studies, although providing presumptive evidence that sulfamethazine undergoes unknown reactions during frozen storage to form GPS, do not preclude the possibility that a portion or all of the conjugate was present in the zero-time samples. Giera et al. (6) reported that the length of frozen storage seemed to increase the conjugate level in tissue extracts. Unequivocal evidence, obtained by analyzing swine livers for the parent drug and the conjugate initially and periodically during frozen storage, must be obtained to reach a definite conclusion. If such studies prove the presumptive evidence is correct, previous studies (8, 9) identifying GPS as a major in vivo metabolite of sulfamethazine metabolism will need to be re-evaluated. Nevertheless, an analytical procedure that determines both sulfamethazine and its N^4 -glucopyranosyl derivative would be desirable to determine the combined residues in swine livers.

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