Determination of Sulfonamides in Swine Plasma

Richard F. Bevill,* Kathleen M. Schemske, Herbert G. Luther, Elaine A. Dzierzak, Malinee Limpoka, and Deborah R. Felt

Membrane filtration, competitive drug displacement from proteins, thin-layer chromatography, and scanning densitometry have been combined to provide a rapid and sensitive method for the analysis of trace levels of sulfamethazine in animal plasma. A sensitivity of 0.05 μg/mL was achieved, and the concentrations of sulfamethazine in the plasma of 60 swine were shown to be closely correlated with coexisting concentrations in kidney tissues.

Sulfonamides are commonly used as promotants of animal growth and are frequently incorporated into rations fed to swine, cattle, sheep, and poultry (Lehmann, 1972). These compounds will produce residues in animal tissues which if consumed by humans may be detrimental to their health. In order to insure that meats contain "safe" concentrations of sulfonamides (≤0.1 μg/g), the Food and Drug Administration has established effective preslaughter drug withdrawal periods during which these drugs should not be fed to animals destined for human consumption. However, it has been demonstrated that willful or accidental misuse of sulfonamides during the withdrawal period resulted in excessive conentrations of these drugs in meats consumed by humans (Huber, 1971).

To control the misuse of sulfonamides the United States Department of Agriculture conducts a residue surveillance program based on the random collection and chemical analysis of animal tissues. Since tissue analysis is both time consuming and costly, a more rapid and economical method for residue screening has been sought.

Recently, the concentration of sulfonamides in animal plasma has been shown to provide an excellent index for predicting coexisting tissue concentrations of these drugs (Bourne et al., 1977). However, no assay methods have been reported which provide rapid and accurate quantitation of 0.1 μg/mL of sulfonamides in plasma. In order to evaluate plasma as a screening medium for sulfonamide residues in animal tissues, a method has been developed that utilizes thin-layer chromatography and spectrodensitometry for the identification and quantitative estimation of sulfonamides in animal plasma.

EXPERIMENTAL SECTION

Apparatus. A Schoeffel SD 3000 spectrodensitometer equipped with a Corning C-69 emission filter and a fluorescence reflectance attachment was used for the assay. The instrument was operated in the single-beam mode using an excitation wavelength of 310 nm. The CF-50 membrane cones used for filtration were obtained from the Amicon Corp., Lexington, Mass.

Reagents. All sulfonamides were of U.S.P. grade and were used without further purification. Phenylbutazone for Injection (U.S.P.) was obtained from Norden Laboratories, Inc., Lincoln, Nebr. The ethyl acetate and acetone were spectral grade from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. All aqueous solutions of sulfonamides were prepared in glass distilled water. Fluorescamine was obtained from Hoffmann-La Roche Inc., Nutley, N.J. Silica gel (type 60) was obtained from Brinkmann Instruments, Westbury, N.Y.

Thin-Layer Chromatography Plates. Water (68 mL) was added to 35 g of silica gel and the mixture was agitated vigorously for 30 s. A 0.25-mm layer of the slurry was spread on 20 × 20 cm glass plates with a Desaga/Brinkmann standard adjustable applicator obtained from Brinkmann Instruments, Westbury, N.Y. The plates were allowed to air-dry for 30 min and activated at 105 °C for 30 min. Activated plates were placed in a plate storage chamber containing calcium sulfate. Plates were prepared on the same day that they were to be used in assays.

Analytical Procedure. Five-milliliter portions of control, sulfamethazine fortified (0.2, 0.4, and 0.8 μg/mL) and sample plasma were pipetted into membrane cones. Twenty milligrams of phenylbutazone was added to the contents of each cone, and the cones were centrifuged at 2000*g* for 30 min. The clear centrifugates (2 mL) were transferred to screw-cap tubes, 2 μg of sulfaguanidine was added as an internal standard, and the solutions were extracted into 2.5 mL of ethyl acetate. Two milliliters of the ethyl acetate was transferred to graduated tubes and evaporated to 0.2 mL under a stream of nitrogen.

Ten microliters of each condensed extract was spotted on prescored (1 cm lanes) TLC plates. The plates were placed in sandwich chambers and developed with ethyl acetate. Solvent migration was limited to 10 cm from the sample origin. Following development the plates were allowed to air-dry for 30 min and dipped in a solution containing 10 mg of fluorescamine/100 mL of acetone. A vertically positioned stainless steel tank (24 × 24 × 1 cm) was used to facilitate the dipping procedure (Sigel, 1975). After drying for 5 min, the plate was scanned at an excitation wavelength of 310 nm. The total emission (above 520 nm) was recorded. Peak heights were manually determined.

Calculations. The quantitation of sulfamethazine was based on the method of internal standards. To determine the amount of sulfamethazine present in an unknown sample, a response factor was calculated from the average value of the ratio of peak heights of sample drug to standard drug:

$$\text{response factor } (f) = (H_s/H_{st})_d \times (C_{std}/C_s)$$

In this equation H_s/H_{std} is the ratio of peak heights of sample drug to standard drug and C_{std}/C_s is the concentration ratio of standard drug to sample drug. The amount of the sample drug in the unknown was calculated as follows:

$$\text{concentration} = (R \times C_{std}')/f$$

where R is the ratio of peak heights of the unknown

University of Illinois, College of Veterinary Medicine, Department of Veterinary Anatomy, Physiology and Pharmacology, Urbana, Illinois 61801.

0021-8561/78/1426-1201$01.00/0

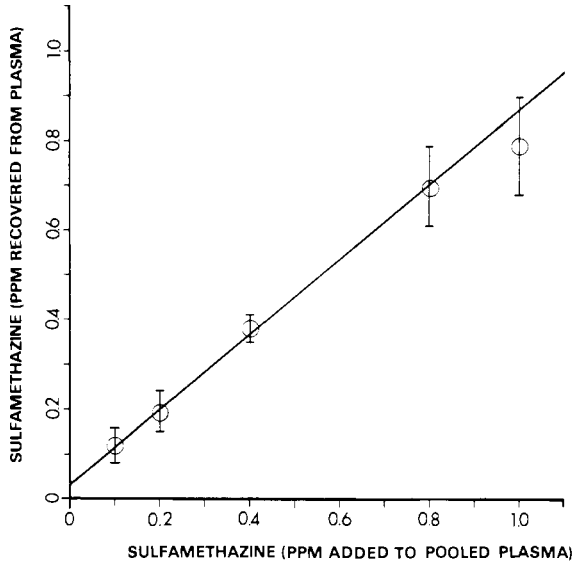

Figure 1. The recovery of sulfamethazine from swine plasma as determined by the measurement of a fluorescent derivative on thin layers of silica gel.

concentration of the sample drug to the known concentration of the standard drug (C_{std}').

RESULTS AND DISCUSSION

The direct extraction of plasma with organic solvents results in the formation of gelatinous precipitates and emulsions which interfere with the extraction process. In this study, the concentrations of proteins and other high molecular weight components of plasma which contribute to the formation of precipitates were reduced by filtering plasma through membrane cones. No precipitates or emulsions were formed when the plasma filtrates were extracted with ethyl acetate.

Sulfonamides are reversibly bound to plasma proteins (Anton, 1961). Numerous compounds, including phenylbutazone, competitively displace sulfonamides from protein binding sites and thus increase the amounts of free drug available for filtration. The influence of phenylbutazone on assay sensitivity was investigated by fortifying samples of sulfonamide-free swine plasma, obtained from four swine, with sulfamethazine to obtain concentrations of 0.1, 0.2, 0.4, 0.8, and 1.6 μg/mL. Each of the samples was assayed with and without the addition of phenylbutazone. A fourfold increase in the amount of drug measured was noted when phenylbutazone was added.

Sulfaguanidine was selected as an internal standard because of its limited use in swine. The drug is unsuitable for intramuscular or intravenous injection in animals, and following oral administration, the drug undergoes very limited absorption from the gastrointestinal tract. In addition to being an uncommonly used sulfonamide, the R_f value of sulfaguanidine was distinctly different from the other sulfonamides utilized in this study.

To determine the accuracy of the assay procedure, sulfamethazine was added to plasma obtained from six swine receiving a sulfonamide-free ration to obtain concentrations of 0.1, 0.2, 0.4, 0.8, and 1.0 μg/mL. Pooled plasma obtained from the same animals was fortified with identical concentrations of the drug. The response factor obtained when the fortified pooled plasma samples were assayed was used to calculate the concentration of drug in the fortified plasma of individual animals. As shown in Figure 1, there was good agreement between the results obtained when fortified pooled plasma was assayed. The lower limits of detection and measurement of sulfamethazine in swine plasma were 0.02 ppm and 0.05 ppm respectively.

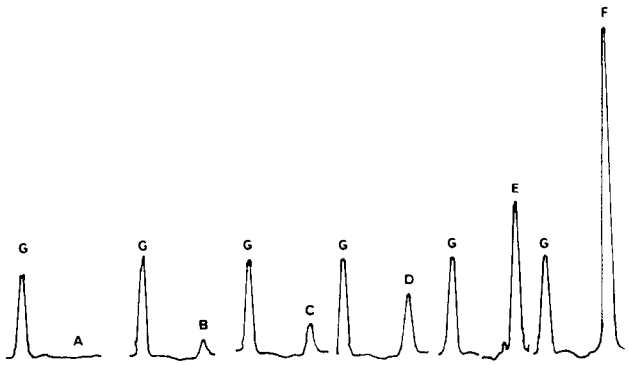

Figure 2. A typical recording obtained when thin-layer plates were spotted with 10 μL of control or fortified plasma extracts and developed and derivatized with fluorescamine prior to scan. Plasma was fortified with the following concentrations of sulfamethazine: (A) 0 μg/mL, (B) 0.1 μg/mL, (C) 0.2 μg/mL, (D) 0.4 μg/mL, (E) 0.8 μg/mL, (F) 1.6 μg/mL. Sulfaguanidine (G) concentration was 1.0 μg/mL.

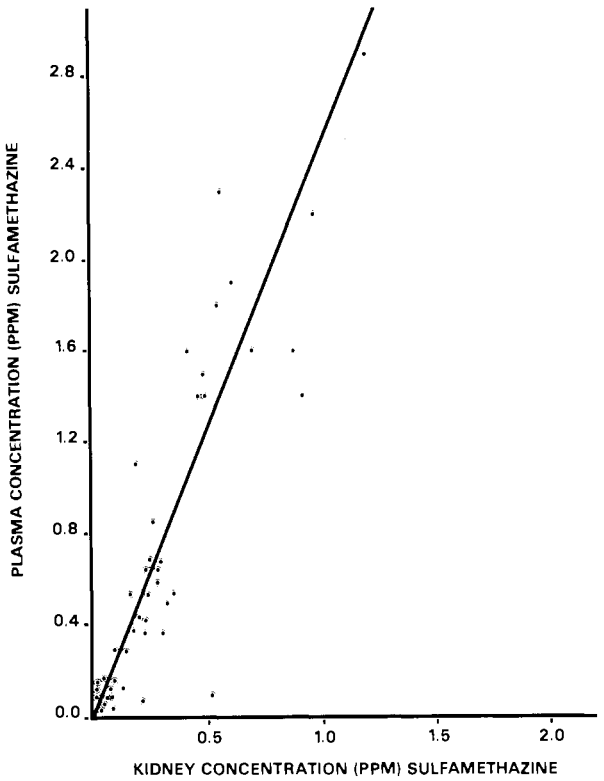

Figure 3. The concentration of sulfamethazine in the plasma and kidneys of swine being slaughtered for human consumption.

A typical recorded densitometer scan of a thin-layer plate to which control and fortified plasma extracts were applied, developed, and derivatized is presented in Figure 2. Control plasmas were consistently free of peaks other than those produced by the internal standard. The occurrence of sulfamethazine in unknowns was verified by comparing the *R*st values of unknowns with those obtained from fortified plasma samples.

Field Screening Study. Plasma and kidney tissues were collected from 243 swine slaughtered for human consumption at a midwestern abattoir. All samples were identified with respect to the animals of origin. After collection, the kidneys were cut into 2-cm cubes, placed in plastic bags, and stored in ice. Within 24 h following collection, the precut kidney tissues were frozen in liquid

nitrogen, blended to a tissue powder, and stored at −20 °C. Phenylbutazone was added to the plasma which was then filtered through membrane cones. Plasma filtrates were stored at −20 °C. The concentration of sulfamethazine in plasma was determined using the method described in this report. Kidney tissue from 60 animals whose plasma contained from 0 to 2.8 ppm sulfamethazine was analyzed for sulfonamide content (Bevill et al., 1977). The coexistent plasma and tissue concentrations determined for each animal are presented in Figure 3. A line with slope of 2.51 and intercept of 0.01 was obtained when the data were analyzed by least-square linear regression. The coefficient of correlation of the plasma–tissue points with the least-square regression line was 0.916.

The assay of sulfamethazine in swine plasma has been given primary attention in this report. However, the assay of other sulfonamides appears feasible since the TLC system described earlier in this paper provided a distinct separation of sulfathiazole, sulfamethazine, and sulfadimethoxine which had average and standard deviations of *R*st values of 2.6 ± 0.2, 4.9 ± 0.3, and 5.3 ± 0.3, respectively.

LITERATURE CITED

Anton, A. H., *J. Pharmacol. Exp. Ther.* **134**, 291 (1961).
Bevill, R. F., Sharma, R. M., Meachum, S. H., Wozniak, S. C., Bourne, D. W. A., Dittert, L. W., *Am. J. Vet. Res.* **38**(7), 973(1977).
Bourne, D. W. A., Bevill, R. F., Sharma, R. M., Gural, R. P., Dittert, L. W., *Am. J. Vet. Res.* **38**(7), 967 (1977).
Huber, W. G., *Adv. Vet. Sci. Comp. Med.* **15**, 101 (1971).
Lehmann, R. P., *J. Anim. Sci.* **35**, 1340 (1972).
Sigel, C. W., Wooley, J. L., Nichol, C. A., *J. Pharmacol. Sci.* **64**(6), 973 (1975).

Received for review July 25, 1977. Accepted June 15, 1978. This work supported in part by FDA Contract 223-74-7178 and the Illinois Agricultural Experiment Station.

Effects of Air, Ozone, and Nitrogen Dioxide Exposure on the Oxidation of Corn and Soybean Lipids

Roger I. Brooks and A. Saari Csallany*

This study was conducted to determine the oxidative effects induced by exposure of corn and soybean seeds to air, nitrogen dioxide (NO_2), and ozone (O_3). Whole, halves, and ground samples of soybean seeds and whole corn kernels were exposed to air, 15 ppm NO_2, or 1.5 ppm O_3 continuously for 100 h at room temperature. Lipid oxidation was measured by polyunsaturated fatty acid (PUFA) and tocopherol destruction and formation of fluorescent lipofuscin-like pigments. Exposure of whole soybean and corn seeds to air, 15 ppm NO_2, or 1.5 ppm O_3 was found to induce no PUFA and tocopherol destruction and no formation of lipofuscin-like pigments. Tocopherol and PUFA destruction and lipofuscin-like pigment formation were detected in samples of soybean seed halves exposed to 15 ppm NO_2 or 1.5 ppm O_3; however, only tocopherol destruction occurred in soybean halves exposed to air. Ground soybean samples exposed to air, 15 ppm NO_2, or 1.5 ppm O_3 incurred the greatest PUFA and tocopherol destruction and lipofuscin-like pigment formation.

Nitrogen dioxide and ozone are two of the most abundant oxidants found in polluted urban air (Stern, 1976). Each is capable of free radical formation, these free radicals being highly potent oxidizing agents in biological systems (Goldstein and Balchum, 1967; Dowell et al., 1971).

Studies on the effects of gaseous oxidants have focused upon their in vivo effects in animals and plants (Goldstein et al., 1969; Thomas et al., 1968; Tingey et al., 1973; Heagle, 1972) and their in vitro effects in animal and plant tissue homogenates (Fletcher and Tappel, 1973; Ting and Heath, 1968). Ozone and nitrogen dioxide have each been shown to cause serious physiological and biochemical damage to plants and animals (Menzel, 1976). Although cereal grains and oil seeds are commonly stored and air-dried in polluted urban environments for several months prior to processing, little is known about the deteriorative biochemical effects of exposure of these edible oil-bearing grains to gaseous oxidants. In addition to loss in nutritional value, oxidation of edible corn and soybean lipids also can result in adverse effects on the flavor, color, texture, and economic value of food products and animal feeds.

Corn contains approximately 3.5–5% oil and soybeans contain from 20–25% oil. Both oils are high in polyunsaturated fatty acid (PUFA) content. High oil contents and high levels of PUFA make these grains potentially susceptible to gaseous oxidant-induced oxidative damage, this being especially possible in commercial soybeans where an appreciable percentage of the seeds have cracked or broken seedcoats.

Tocopherols have been shown to exhibit antioxidant effects on the in vivo and in vitro oxidation of polyunsaturated lipids (Witting, 1975; Tappel, 1965). A delicate balance apparently exists between the amounts and distributions of PUFA and tocopherols in an oil-containing system. Nitrogen dioxide and ozone, being strong oxidizing agents, may upset this delicate balance and induce oxidation of PUFA and tocopherols. Therefore, a study was conducted to determine PUFA and tocopherol destruction induced by short-term, continuous exposure of corn and soybean seeds to concentrations of nitrogen dioxide and ozone approximately tenfold greater than those normally found in polluted urban air. In addition, lipid oxidation

Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108.

0021-8561/78/1426-1203$01.00/0