Enzyme Immunoassay for Screening of Sulfamethazine in Swine

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A competitive solid-phase enzyme immunoassay was developed to measure residues of sulfamethazine in swine plasma. In the assay, sulfamethazine residue in plasma was allowed to compete with the enzyme conjugate for binding to a limited amount of anti-sulfamethazine antibodies immobilized on microtiter wells through a precoated layer of protein A. Standard curves were constructed over 0.01–1 μg/mL of sulfamethazine. Plasma was used in the assay without any sample preparation or extraction step. Of 17 swine plasma samples obtained from a slaughterhouse, 3 samples were positive for sulfamethazine by enzyme immunoassay (0.3–0.4 μg/mL) and had a comparable value by a TLC method. Recoveries of sulfamethazine in spiked plasma samples between 0.016 and 0.5 μg/mL ranged from 97.6 to 106.3% (mean recovery 103%). Precision of the assay as determined by interwell and inter- and intraassay variabilities was below 10%. Among 36 sulfonamide analogues tested for specificity, only sulfamerazine showed significant cross-reaction in the assay. The assay is rapid, sensitive, and convenient to perform. Eighty plasma samples can be analyzed for sulfamethazine in less than 2 h. The test is readily adaptable to high-volume testing of swine plasma in slaughterhouses.

Sulfonamide antibiotics are commonly incorporated into swine feed as promotants of growth and for control of certain diseases in animals (Van Houweling and Kingma, 1969; Lehmann, 1972). These antibiotics are retained in tissues of animals eating medicated diet. Consumption of meat from animals containing these antibiotic residues may result in development of hypersensitivity to these drugs and preferential selection of bacterial mutants that are resistant to these antibiotics also used in the treatment of human diseases (Mercer, 1975). Normally, tissue residues in animals are controlled by withdrawing the antibiotics from feed weeks before their slaughter when concentrations of the sulfonamides are presumed to reach below their safety level. This procedure often results in a number of animals reaching slaughterhouses with a substantially excessive amount of unmetabolized drugs still present in their tissues.

The Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) has recently started a Residue Avoidance Program (RAP) designed for checking animals before they are slaughtered (RAP, 1982; MPI, 1985) to ensure a residue-safe meat reaching the public. Although different sulfa–antibiotic combinations have been approved for use in swine feed, sulfamethazine has been identified as the major problem in approximately 95% of all sulfonamide tissue violations (Ashworth, et al., 1985) in the United States. The Food and Drug Administration (FDA) requires a 15-day withdrawal period for feed containing sulfamethazine (*Fed. Regist.*, 1977) and has established its tolerance limit in tissues for human consumption as 0.1 ppm (Code of Federal Regulations, 1983).

Current methods for analysis of sulfamethazine in animal tissues and feeds include colorimetry (Bratton et al., 1939), gas–liquid chromatography (GLC) (Manuel and Steller, 1981), high-pressure liquid chromatography (HPLC) (Cox and Krzeminski, 1982), and thin-layer chromatography (TLC) (Williams, 1984; Beville et al., 1978). These methods require extensive sample extraction and cleanup and, therefore, are not readily applicable to routine screening of the drug in a large number of samples.

Enzyme immunoassays have recently been reported as alternative methods for detection of sulfamethazine (Fleeker and Lovett, 1985; Fleeker, 1986). However, these methods require extraction of the antibiotic from the sample and have longer assay protocol, rendering them impractical for routine analysis in hog slaughterhouses.

We report here the development of a competitive enzyme immunoassay (EIA) for routine screening of sulfamethazine in swine plasma that does not require any extraction and is completed in less than 2 h.

MATERIALS AND METHODS

Materials. Sulfamethazine, sodium nitrite, thimerosal, bovine thyroglobulin, Sephadex G-50, horseradish peroxidase (HRP) (type VI), sodium metaperiodate, sodium borohydride, casein, 2,2′-azino(3-ethylbenzothiazoline)-sulfonic acid (ABTS), sodium dodecyl sulfate (SDS), ammonium sulfate, 30% hydrogen peroxide, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylformamide (DMF), succinic anhydride, *p*-nitrophenyl chloroformate, 2,2′-oxybis(ethylamine) dihydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Tween-20 and recombinant protein A (r-protein A) were bought from J. T. Baker Chemical Co. (Phillipsburg, NJ) and Behring Diagnostics (La Jolla, CA), respectively. All other inorganic and organic chemicals were of reagent grade or better. Disposable syringe filter (0.45 μm) was purchased from Glass Works (Corning, NY). Nunc microtiter plates (high binding) were from Denmark.

New Zealand female white rabbits were used for antibody production. Blood samples from trichina-positive and trichina-negative swine were procured from laboratory animals raised under controlled conditions. Field samples of plasma were obtained from a commercial hog slaughterhouse.

Preparation of Sulfamethazine–Thyroglobulin (Immunogen) Conjugate. A 57-mg portion of sulfamethazine was diazotized and conjugated to 100 mg of bovine thyroglobulin according to the published procedure (Fleeker and Lovett, 1985). The conjugate was dialyzed four times against 50 mM ammonium bicarbonate solution and purified on a column of Sephadex G-50. Fractions containing protein were pooled, lyophilized, and stored at −20 °C.

Production of Anti-Sulfamethazine Antibody. Portions of 1 mg each of the sulfamethazine–thyroglobulin (immunogen) conjugate in 0.5 mL of saline mixed with 1 mL of complete Freund's adjuvant was injected into each of the four New Zealand female white rabbits. After 5 weeks, each rabbit was boosted by injecting 0.5 mg of the

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conjugate in 0.25 mL of saline emulsified with 0.5 mL of incomplete Freund's adjuvant. The animals were bled periodically through the ear vein, and the serum was separated by centrifugation at 15000*g* for 20 min. Anti-sulfamethazine antibodies were purified from the serum by precipitation with ammonium sulfate (Garvey et al., 1977). Titer of the antibodies was determined by a direct conjugate binding assay described later in this section. Rabbits were boosted every 2 weeks until a satisfactory titer was obtained.

Preparation of Sulfamethazine–HRP Enzyme Conjugate. The sulfamethazine–enzyme conjugate was prepared according to published procedures (Singh, 1978). The conjugate was dialyzed three times against 10 mM phosphate-buffered saline (PBS), pH 7.2. Aqueous 10% sodium thimerosal was added to the conjugate solution to a final concentration of 0.01%. The solution was then filtered through 0.45-μm filter disk and stored at −20 °C in 50% glycerol.

Preparation of Plasma Samples. Swine blood was collected in the presence of EDTA (1–2 mg of EDTA/mL of blood) and spinned at 1500 rpm in a table top centrifuge to separate out the plasma. The plasma was stored frozen at −20 °C or in liquid form at 4 °C in the presence of 0.01% thimerosal for an extended period of time.

Preparation of Sulfamethazine Standards in Plasma. A 1 mg/mL master stock solution of sulfamethazine was prepared in DMF. The master stock solution was diluted with 20 mM PBS, pH 7.2, to give a stock solution of 100 μg/mL of the drug. The stock solution was further diluted with plasma to make a working stock of 1 μg/mL sulfamethazine. Sulfamethazine standards were prepared by making serial dilutions of 1 μg/mL working stock solution. Each standard was divided into 0.5-mL aliquots in vials and lyophilized. Lyophilized standards were stored at −20 °C.

Immobilization of Antibody on Microtiter Wells. Each well of the microtiter strip was filled with 200 μL of solution of r-protein A (2 μg/mL in 10 mM PBS, pH 7.2). These strips were sealed and incubated at 4 °C for 5 h. The r-protein A solution was aspirated, and the wells were washed five times with saline–Tween (0.9% NaCl containing 0.05% Tween-20). Each well was then incubated with 300 μL of solution of 0.1% casein in 20 mM PBS, pH 7.2, at 37 °C for 30 min and washed as described above. Each well was then filled with 200 μL of antibody solution (12.5 μg/mL in 50 mM carbonate–bicarbonate buffer, pH 9.6) and incubated at 4 °C overnight (15–20 h). The solution was aspirated and the wells were air-dried at room temperature for 2–3 h. The antibody-immobilized microtiter wells thus prepared were stored at 4 °C.

Competitive Direct Enzyme Immunoassay (EIA). The enzyme conjugate (sulfamethazine–HRP) was diluted 1:1000 with 20 mM PBS, pH 7.2, containing 0.01% casein (w/v) and mixed with standards or samples in 10:1 ratio. Plasma samples in 50-μL volume usually obtained from 200–300 μL of whole blood was mixed with 500 μL of dilute enzyme conjugate for analysis in duplicates. The mixture was transferred to wells (200 μL/well) and incubated at 37 °C for 1 h. The wells were washed five times with 20 mM PBS, pH 7.2, containing 0.05% Tween-20 (v/v). Each well was then filled with 100 μL of ABTS substrate solution (Ram et al., 1986) and incubated at 37 °C for 20 min for color (bluish-green) development. The color development was stopped with 100 μL of 1% SDS solution (w/v). Absorbance of the color was monitored at 405 nm in an EIA plate reader. Percent binding was calculated from the absorbance obtained in the absence (B_0) and presence (B) of sulfamethazine in standards/samples as follows: percent binding = $(B/B_0) \times 100$. A standard curve was prepared by plotting log [sulfamethazine] vs percent binding or logit percent binding, which is defined as ln $(\%[(B/B_0)/100] - \%B/B_0)$. The content of sulfamethazine in an unknown sample was determined from the standard curve (Figure 2).

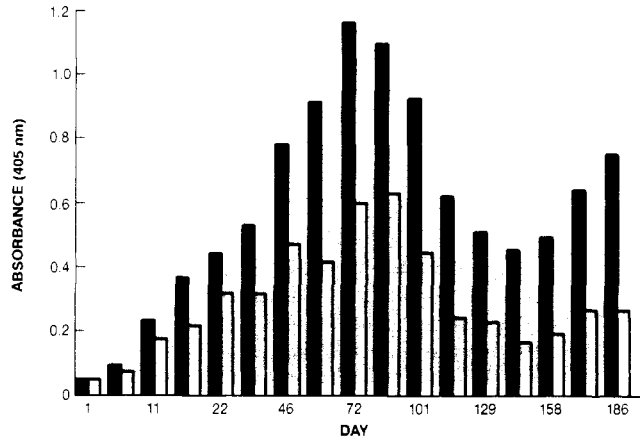

Figure 1. Evaluation of sensitivity of EIA test with the antibody purified from each bleed at 0 μg/mL (■) and 0.1 μg/mL (□) sulfamethazine.

The EIA performed in the absence of sulfamethazine was termed as binding assay.

Determination of Sulfamethazine by TLC. The test was performed in accordance with the USDA handbook on sulfa-on-site test (SOS). After standards and samples were spotted, the plate was developed with methanal to a height of approximately 1 cm, dried, and then redeveloped with ethyl acetate to a height of 6–7 cm. Sulfamethazine in plasma samples was determined by comparing their fluorescence intensities with those of the standards.

RESULTS

Antibody Production and Evaluation. A titer of antibodies purified from serum of four rabbits bled at various intervals were determined by conjugate binding assay. Each rabbit gave a slightly different response to antibody formation during the immunization period. Generally, a successive increase in titer of the antibody was observed in each case, peaking off around 95 days for two rabbits and 71 days for the other two rabbits during the course of initial and three booster injections. Titers of the antibodies generally fluctuated for one rabbit during subsequent booster injections.

In order to verify whether the color development during conjugate binding assay was due to a specific reaction involving anti-sulfamethazine antibodies, a competitive EIA was performed at 0 and 0.1 μg/mL sulfamethazine. A representative result is shown in Figure 1. Sulfamethazine competed effectively with sulfamethazine–HRP conjugate for antibody binding. The inhibition in binding as indicated by decrease in absorbance at 0.1 μg/mL relative to 0 μg/mL successively increased in spite of the decrease in titer of the antibodies (Figure 1). This indicated that sensitivity of the assay was independent of titer of the antibody.

EIA Standard Curve. Since the primary objective of the study was to determine sulfamethazine in swine plasma, standards in plasma (0.01–1 μg/mL in sulfamethazine) were used to generate standard curves (Figure 2). A linear dose–response curve was obtained by plotting log–logit of percent binding against sulfamethazine concentration (Figure 2 inset).

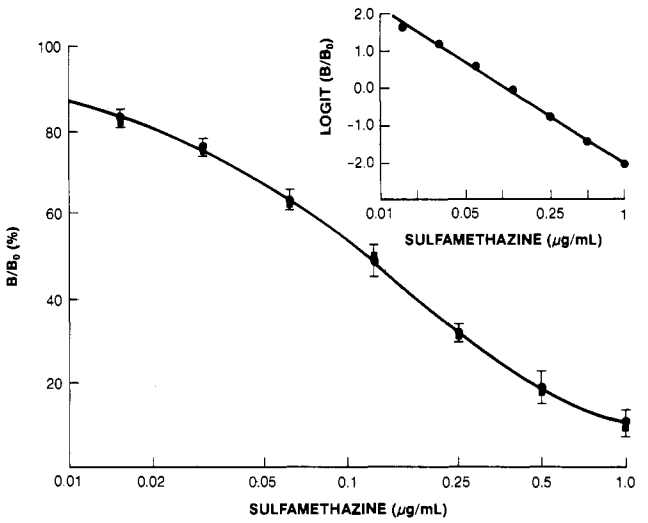

Figure 2. EIA standard curves for sulfamethazine prepared using standards in plasma before (■) and after (●) lyophilization. Each point represents an average of four determinations. Error bars are only shown for lyophilized plasma standards to maintain clarity of the figure.

Table I. Recovery of Sulfamethazine Spiked in Plasma at Various Concentrations by EIA

sulfamethazine, μg/mL		
added	recovered	% recovery
0.016	0.018	112.5
0.031	0.032	103.2
0.063	0.067	106.3
0.125	0.122	97.6
0.250	0.251	100.4
0.500	0.500	100.0

Sulfonamides including sulfamethazine are reversibly bound to plasma proteins (Anton, 1961). The use of standards prepared in plasma thus should balance out any interference arising due to interaction of sulfamethazine with the sample plasma proteins. Standard curves obtained by using plasma standards from three different pigs maintained on sulfamethazine-free diet were identical (curves not shown), indicating little or no effect by any variation in plasma from different animals. Plasma standards can be stored lyophilized at −20 °C to avoid any deterioration. The dose–response curves using plasma standards before and after lyophilization were essentially identical, indicating no loss in the drug concentrations during lyophilization (Figure 2).

Recovery of Sulfamethazine. The accuracy of the method was checked by recovery experiments. Known amounts of sulfamethazine were added in swine plasma in varying concentration and assayed in quadruplicate (Table I). Recovery of sulfamethazine ranged from 97.6% to 112.5% with a mean value of 103.3%.

Sensitivity of the Assay. A 50% inhibition in binding was obtained around 0.1 μg/mL of sulfamethazine, which falls in the midregion of the EIA standard curve. The lower limit of detection of sulfamethazine was 0.1 μg/mL (Figure 2).

Field Study. Fifteen field and two laboratory samples of swine plasma were analyzed by EIA and results compared with the SOS test (Table II). Both methods showed three positives, with EIA values ranging from 0.35 to 0.48 μg/mL while the corresponding values by the SOS method were approximately 0.4 μg/mL.

Trichinosis is a common problem in swine affecting both producers and consumers (Murrell, 1985). In order to test whether plasma from swine suffering from trichinosis interfered in the sulfamethazine EIA, a known trichina-positive sample was obtained and compared with a trichina-negative sample (Table II). Both samples were negative for sulfamethazine, indicating no interference due to trichinosis in pigs.

Table II. Determination of Sulfamethazine in Field Plasma Samples by EIA and SOS Test

	sulfamethazine, μg/mL	
sample no.	EIA	SOD
1	0.35	≈0.4
2	0.42	≈0.4
3	0.48	≈0.4
4[a]	0.0	ND[c]
5[b]	0.0	ND
6–17	0.0	ND

[a] Negative for *Trichinella spiralis*. [b] Positive for *Trichinella spiralis*. [c] None detected.

Table III. Interwell Variability of Absorbance in EIA of Sulfamethazine

sulfamethazine,[a] μg/mL	absorbance	% CV
0	1.978 ± 0.097	4.9
0.1	0.975 ± 0.063	6.5
0.5	0.395 ± 0.036	9.1

[a] Each sample was assayed in replicates of 32 in a single run.

Table IV. Interassay Variability of EIA Standard Curve for Sulfamethazine

sulfamethazine std,[a] μg/mL	% binding		
	mean	SD	% CV
0.016	83.1	1.8	2.2
0.031	76.0	1.8	2.4
0.063	64.2	2.2	3.4
0.125	48.4	2.5	5.2
0.25	31.4	1.6	5.1
0.5	18.3	1.5	8.2
1.0	10.5	1.2	11.4

[a] Each standard was assayed in quadruplicate on 14 different days.

Table V. Interassay Variability of Sulfamethazine in Two Known Samples Determined from EIA Standard Curve

sulfamethazine sample,[a] μg/mL	EIA, μg/mL		
	mean	SD	% CV
0.1	0.104	0.010	9.6
0.05	0.055	0.005	9.0

[a] Each sulfamethazine sample was assayed in quadruplicate on 10 different days. Amount of sulfamethazine in the samples was determined from the standard curve prepared on that particular day.

Precision of the Assay. Precision of the EIA was analyzed by repeated determinations of carefully prepared plasma samples. Interwell coefficient of variation (CV) of the absorbance at three different sulfamethazine concentrations ranged from 4.9 to 9.1% with a mean of about 7% (Table III). Interassay CV of the percent binding of the standards on 14 different days ranged from 2.2% at 0.016 μg/mL to 11.4% at 1 μg/mL with a mean of 5.4%. CV, in general, increases with an increase in sulfamethazine concentration due to subsequent decrease in absorbance value (Table IV). Interassay CV of the sulfamethazine value determined from an EIA standard curve for two samples on 10 different days was less than 10% (Table V). Low CV of the day-to-day variabilities indicated the stability of the standard curve over an extended period of time. Average intraassay CV of four samples determined

Table VI. Intraassay Variability (μg/mL) of Sulfamethazine ETA

sample no.[a]	mean SD	% CV
1	0.389 ± 0.031	7.95
2	0.241 ± 0.035	14.52
3	0.119 ± 0.013	10.92
4	0.072 ± 0.013	18.06

[a] Each sample was assayed in replicates of 32. Individual absorbance was used to determine the amount of sulfamethazine from the standard curve.

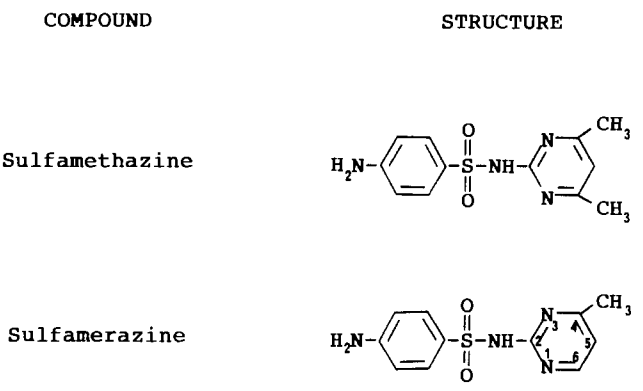

Figure 3. Structure of sulfamethazine and the analogue sulfamerazine showing cross-reactivity with anti-sulfamethazine antibody.

on a single day was 12.8% (Table VI). The precision and accuracy of the sulfamethazine EIA were superior to other EIAs described for haptens (Ram et al., 1986).

SPECIFICITY

Thirty different sulfonamide analogues were assessed for cross-reactivity with anti-sulfamethazine antibody (Table VII). All analogues tested, except for sulfamerazine, showed insignificant (<1%) cross-reaction. It appears that epitopes for antibody recognition lie in the 2-pyrimidinyl portion of the sulfamethazine molecule. Sulfamerazine, with the only difference of one methyl group on the pyrimidinyl C-4 position, showed 12% cross-reactivity (Figure 3). In terms of specificity, this antibody preparation was superior to a previously reported preparation (Fleeker and Lovett, 1985).

DISCUSSION

Various chromatographic methods have been described for analysis of sulfamethazine residue in tissues and body fluids of pork (Vilim et al., 1980; Manuel and Steller, 1981; Cox and Krzeminski, 1982; Malanoski et al., 1981; Beville et al., 1978). These methods involve prior extraction of samples with organic solvents and often require costly equipment to run the tests. Their lengthy procedures result in slow turnaround time for the sample analysis. Furthermore, these methods cannot be readily automated and hence are cost ineffective for routine analysis of a large number of samples. More recently, an enzyme immunoassay for sulfamethazine has been reported by Fleeker (1986). This method, unfortunately, was unable to solve major drawbacks of the chromatographic methods in that this also required sample extraction before actual testing and took overnight to complete the analysis reflecting poor sensitivity. We have addressed these problems by increasing sensitivity of our EIA. This was achieved by using superior anti-sulfamethazine antibodies and the sulfamethazine–HRP conjugate in the assay.

The direct competitive EIA described here is based on competition of free sulfamethazine in the plasma and the sulfamethazine–HRP conjugate for a limited amount of anti-sulfamethazine antibody immobilized on microtitration wells through protein-A precoating. We have used protein-A as a precoat on wells because direct immobilization of antibodies to the surface limits the usefulness of solid-phase assays for several reasons: (1) Variations in physical structure of surfaces are extended to the analytical system, requiring careful selection of batches of microtiter plates in immunosorbent assay (Shekarachi et al., 1984). This can cause concern regarding quality control and continuity of analytical procedures when a particular batch has been used. (2) Lateral surface interaction of the adsorbed antibody distorts the molecule (Morrissey, 1977). (3) Interaction of lipophilic domains of the antibody with the solid matrix might either include the antigen binding site directly or contribute to the steric binding hinderance for optimal antigen access and/or binding (Schramm et al., 1987). Further, as protein-A binds through the Fc portion of immunoglobulin, it may not as easily disassociate as the case may be with the direct antibody absorption to the surface (Deisenhofer, 1981). The high-level performance and precision of our EIA procedure may be due partly to this special immobilization procedure of anti-sulfamethazine antibody through protein-A molecules. On the basis of our preliminary observations, immobilized antibodies are stable for at least 1 year at 4 °C, giving consistent EIA performance.

Accuracy of the EIA method as measured by the recovery experiment is close to 100%, a level not achieved with most other analytical procedures (Vilim et al., 1978; Cox and Krzeminski, 1982; Goodspeed et al., 1978). In addition, the test is highly sensitive in that it can accurately measure sulfamethazine up to 0.01 μg/mL. This level of sensitivity is at least 5–10 times better than the sensitivities of 0.05–0.1 μg/mL reported by other investigators (Beville et al., 1978; Manuel and Steller, 1981;

Table VII. Cross-Reactivity of Anti-Sulfamethazine Antibodies toward Sulfamethazine Analogues

analogue	% cross-react.[a]
sulfamethazine	100.0
sulfamerazine	12.10
sulfacetamide	0.66
5-methoxysulfadiazine	0.34
2-amino-4,6-dimethylpyrimidine	0.07
sulfathiazole	0.07
sulfadimethoxine	0.06
sulfachloropyridazine	0.0
N′-(6-indazolyl)sulfanilamide	0.0
sulfamethoxazole	0.0
sulfanilyl fluoride	0.0
sulfabenzamide	0.0
sulfamethizole	0.0
sulfisomidine	0.0
sulfaguanidine	0.0
sulfanitran	0.0
sulfanilamide	0.0
sulfasalazine	0.0
sulfaquinoxaline	0.0
thymine	0.0
sulfinpyrazone	0.0
sulfapyridine	0.0
sulfamethoxypyridazine	0.0
sulfadiazine	0.0
4-carboxybenzenesulfonamide	0.0
N′-(4,5-dimethyloxazol-2-yl)sulfanilamide	0.0
sulfanilic acid, sodium salt hydrate	0.0
sulfisoxazole	0.0
2,6-di-*tert*-butyl-4-methylphenol	0.0
sulfisomidine	0.0
sulfamide	0.0

[a] Cross-reactivity defined as (nanomoles of sulfamethazine for 50% binding)/(nanomoles of sulfa analogue for 50% binding) × 100.

Phillips and Trafton, 1975).

A distinct advantage of using plasma in the EIA is the convenience of collection of blood in abattoirs. This should provide valuable information as regards to sulfamethazine residue problem in swine before or after their slaughter. Since the procedure takes less than 2 h for analysis, it should help management in making quick decisions whether to retain or condemn a carcass the same day, thereby avoiding loss of time and freezer space.

Since the test requires only 50 μL of plasma sample, readily obtainable from blood samples collected from the ear vein of animals, the current method could be easily adapted for random checking of hogs for drug residues before animals are slaughtered. This should avoid the problem of a litigation suit by the government, saving money and anxiety to the producers.

In order for the plasma to become a valuable screening medium, its sulfamethazine level should be established relative to the tissue tolerance level of 0.1 ppm set by the government. Two studies on the analysis of sulfamethazine in hog plasma/serum by TLC procedure have provided an excellent index for predicting the corresponding tissue concentration (Bourne et al., 1977; Beville et al., 1978; Ashworth et al., 1985; Randecker et al., 1987). In one such study, the presence of sulfamethazine in serum at 0.149 and 0.787 ppm level will make liver and muscle violative, respectively (Ashworth et al., 1985), whereas in the second study the corresponding violative levels are 0.111 and 0.416 ppm for liver and muscle, respectively (Randecker et al., 1987). The EIA procedure described here is capable of analyzing sulfamethazine accurately in the above ranges.

In conclusion, we have developed a sensitive, specific, and convenient EIA procedure for routine screening of sulfamethazine in swine plasma. The test does not involve extraction of sulfonamides from the plasma common with most analytical procedures. Over 80 plasma samples can easily be screened for sulfamethazine in less than 2 h. This test is equally useful for analysis of the drug in hog sera. With use of a robotic sample handler, the testing efficiency can be further improved, allowing the analysis of well over 1000 hog plasma samples/day (Allison, unpublished work).

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LITERATURE CITED

Allison, D., personal communication, 1988.

Anton, A. H. Drug-induced changes in distibution and renal excretion of sulfonamides. *J. Pharmacol. Exp. Ther.* **1961**, *134*, 291–303.

Ashworth, R. B.; Epstein, R. L.; Thomas, M. H.; Frobish, L. T. Sulfamethazine blood/tissue correlation study in swine. *Am. J. Vet. Res.* **1985**, *47*, 2596–2603.

Beville, R. F.; Schemske, K. M.; Luther, H. G.; Dzierzak, E. A.; Limpoka, M.; Felt, D. R. Determination of Sulfonamide in Swine Plasma. *J. Agric. Food Chem.* **1978**, *26*, 1201–1203.

Bourne, D. W. A.; Beville, R. F.; Sharma, R. M.; Gural, R. P.; Dittert, L. W. Disposition of sulfonamide in food producing animal; Pharmacokinetics of sulfamethazine in lambs. *Am. J. Vet. Res.* **1977**, *38*, 967–972.

Bratton, A. C.; Marshall, E. K., Jr.; Babbit, D.; Hendrickson, A. R. A new coupling component for sulfanilamide determination. *J. Biol. Chem.* **1939**, *28*, 537–550.

Code of Federal Regulations. *New Animal Drugs for Use in Animal Feeds*; U.S. Government Printing Office: Washington, DC, 1983; No. 21, Parts 558.145.

Cox, B. L.; Krzeminski, L. F. High pressure liquid chromatographic determination of sulfamethazine in pork tissues. *J. Assoc. Off. Anal. Chem.* **1982**, *65*, 1311–1315.

Deisenhofer, J. Crystallographic refinement and atomic models of human Fc fragment and its complex with fragment B of Protein A from *Staphylococcus aureus* at 2.9- and 2.8-A resolution. *Biochemistry* **1981**, *20*, 2363–2670.

Fed. Regist. Sulfamethazine containing products for use in swine feed or drinking water; fifteen day withdrawal period prior to slaughter. **1977**, *42*, 62211.

Fleeker, J. R. Sulfamethazine residue in animal blood. In *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Bergmeyer, J., Grassl, M., Eds.; VCH Verlagsgesellschaft: FRG, 1986; Vol. XII, pp 391–401.

Fleeker, J. R.; Lovett, L. J. Drug residues in animal tissues. Enzyme immunoassay for screening sulfamethazine residues in swine blood. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 172–174.

Garvey, J. S.; Cremer, N. E.; Sussdorf, D. H. Ammonium sulfate precipitation. In *Methods in Immunology*, 3rd ed.; Benjamin/Cummings: Reading, MA, 1977; pp 218–219.

Goodspeed, D. P.; Simpson, R. M.; Ashworth, R. B.; Shafer, J. W.; Cook, H. R. Sensitive and specific gas-liquid chromatographic-spectrophotometric screening procedures for trace levels of five sulfonamides in liver, kidney, and muscle tissue. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 1050–1053.

Lehmann, R. P. Implementation of the recommendations contained in the report to the commissioner concerning the use of antibiotics in animal feed. *J. Anim. Sci.* **1972**, *35*, 1340–1345.

Malanoski, A. J.; Barnes, C. J.; Fazio, T. Comparison of three methods of determination of sulfamethazine in swine tissues: collaborative study. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 1386–1391.

Manuel, A. J.; Steller, W. A. Gas-liquid chromatographic determination of sulfamethazine in swine and cattle tissues. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 794–799.

Mercer, D. H. *Antimicrobial Drugs in Food Producing Animals. Veterinary Clinics of North America*; W. D. Saunders: Philadelphia, 1975; Vol. 5.

Morrissey, B. M. The absorption and conformation of plasma proteins: a physical approach. *Ann. N.Y. Acad. Sci.* **1977**, *283*, 50–64.

MPI (Meat and Poultry Inspection). *1984 Report of the Secretary of Agriculture to the U.S. Congress*; U.S. Department of Agriculture, Food Safety and Inspection Service: Washington, DC, 1985; p 49.

Murrell, K. D. Strategies for the control of human trichinosis transmitted by pork. *Food Technol. (Chicago)* **1985**, *39*, 65–68.

Phillips, W. F.; Trafton, J. E. A screening method of sulfonamides extracted from animal tissues. *J. Assoc. Off. Anal. Chem.* **1975**, *58*, 44–47.

Ram, B. P.; Hart, L. P.; Shotwell, O. L.; Pestka, J. J. Enzyme-linked immunosorbent assay of aflatoxin B1 in naturally contaminated corn and cotton seed. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 904–907.

Randecker, V. W.; Reagan, J. A.; Engel, R. E.; Soderberg, D. L.; McNeal, J. E. Serum and urine as predictors of sulfamethazine levels in swine muscle, liver and kidney. *J. Food Protect.* **1987**, *50*, 115–122.

RAP (Residue Avoidance Program), FSIS 20. U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, DC, 1982.

Schramm, W.; Yang, T.; Midgley, A. R. Surface modification with Protein A for uniform binding of monoclonal antibodies. *Clin. Chem.* **1987**, *33*, 1338–1342.

Shekarachi, I. C.; Sever, J. L.; Lee, Y. J.; Castellano, G.; Madden, D. L. Evaluation of various plastic microtiter plates with measles, toxoplasma, and gamma globulin antigens in enzyme-linked immunosorbent assays. *J. Clin. Microbiol.* **1984**, *19*, 89–96.

Singh, P. Lidocaine antigens and antibodies. U.S. Patent 4,069,105, Jan 17, 1978.

SOS. Sulfa on Site Test, A Handbook. U.S. Department of Agriculture.

Van Houweling, D. D.; Kingma, F. J. The use of drugs in animals raised for food. *Am. J. Vet. Res.* **1969**, *155*, 2197–2202.
Vilim, A. B.; Larocque, L.; MacIntosh, A. I. A HPLC screening procedure for sulfamethazine residues in pork tissues. *J. Liq. Chromatogr.* **1980**, *3*, 1725–1736.
Williams, S., Ed. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984; pp 785–786.

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Ferulic Acid Esters from Bark of *Pseudotsuga menziesii*[1]

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A mixture of ferulic acid esters was isolated from the *n*-hexane solubles (*n*-hexane wax) of the bark of *Pseudotsuga menziesii* (Mirb.) Franco. Saponification of the isolated esters yielded 1-docosanol, 1-tetracosanol, and ferulic acid. These esters may be related to suberin formation in bark cork cells and in cut potato tubers.

Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) is the most important commercial timber species on the west coast of North America. How to use the large amount of bark that accumulates after lumber and wood products have been manufactured from *P. menziesii* has always been a concern to the timber industry. For about 40 years there has been commercial interest in the wax from the bark (Hall, 1971). During our studies on the bark of this species, we have investigated *n*-hexane wax (*n*-hexane solubles) and benzene wax (benzene solubles extracted from the *n*-hexane insoluble residue). Previous papers (Laver et al., 1971; Loveland and Laver, 1972a,b; Laver and Fang, 1986) have been concerned with sitosterol, campesterol, fatty acids, wax alcohols, and chemically intact sterol and wax esters. In this paper we report the isolation of chemically intact ferulic acid esters from the *n*-hexane wax and discuss their possible significance in suberin formation in bark cork cells and in cut potato tubers.

EXPERIMENTAL SECTION

Melting points are uncorrected. Thin-layer chromatography (TLC) and preparative TLC (1.0 mm) were performed on silica gel G with the following solvent systems: (A) diethyl ether–*n*-hexane (1:4, v/v); (B) chloroform–carbon tetrachloride (6:1, v/v); (C) diethyl ether–*n*-hexane–methanol (10:40:1, v/v/v). Detection method: ultraviolet light (UV) followed by iodine vapors. Gas–liquid chromatography (GLC) utilized flame ionization detectors and the following column systems: (1) 5% SE-30 on Gas-Chrom Q packed in 1.50 m × 2.159 mm stainless steel, isothermal 250 °C, helium at 30 mL/min, injector heater 245 °C, detector heater 250 °C; (2) 3% OV-17 on Gas-Chrom Q packed in 1.829 m × 2.159 mm, stainless steel, isothermal 210 °C, helium at 30 mL/min, injector heater 240 °C, detector heater 255 °C. Preparative GLC utilized the conditions of column system 2, except the column was 1.829 m × 5.334 mm and the effluent was split 1/6 to detector and 5/6 to collection trap (liquid nitrogen). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained at 100 MHz with tetramethylsilane (TMS) as internal standard. Electron impact mass spectra (MS) (probe) were obtained on a quadrupole instrument at 70 eV. Field desorption mass spectra (FD/MS) were obtained on a magnet instrument equipped with a carbon whisker emitter, +1.8 kV to the field anode (FD emitter) and −6.8 kV to the cathode, emitter heating current 0.0 mA. GLC/MS spectra were obtained on column system 1 and a quadrupole instrument at 70 eV.

Plant Material and Extraction. Bark 3.8–5.1 cm thick was collected from a freshly cut, dominant, 58-year-old Douglas-fir tree in the George T. Gerlinger Experimental Forest near Falls City, OR. The bark (1974.76 g, moisture content 11.2%, ground to pass a screen with holes 1.3 cm square) was Soxhlet extracted with *n*-hexane for 36 h. Solvent evaporation left a light yellow, waxlike solid (100.91 g).

Isolation of Ferulic Acid Esters. An aliquot (17.00 g) of the wax was separated on a silica gel G column with chloroform–*n*-hexane (3:1, v/v). Ten bands were observed under UV light. The second fastest moving band was collected. The solids (4.17 g) were recovered by solvent evaporation and purified by preparative TLC (solvent A, UV detection); mp 67–70 °C. TLC (solvent B) showed a single spot, R_f 0.19. GLC (column systems 1 and 2) showed no peaks either before or after silylation [hexamethyldisilazane and trimethylchlorosilane in pyridine (2:1:10, v/v/v)]. IR (ν_{max}, cm^{-1}; $CHCl_3$): 3500, 1730, 1315, 1269, 1175, 719.

Saponification of Ferulic Acid Esters. Saponification was accomplished by refluxing for 3.75 h in 10% ethanolic potassium hydroxide, extraction with *n*-hexane (unsaponifiables), acidification, and extraction with diethyl ether (saponifiables).

1-Docosanol and 1-Tetracosanol. TLC (solvent C) of the unsaponifiables showed one spot only (R_f 0.70) identical with 1-docosanol and 1-tetracosanol on cochromatography. The unsaponifiables were silylated, and GLC (column systems 1 and 2) showed spectra with two peaks only (retention times 14.0 and 26.5 min, and 6.5 and 12.5 min, respectively), identical with those of silylated authentic 1-docosanol and 1-tetracosanol. GLC/MS of the GLC peak with 14.0-min retention time showed peaks of m/z 383 $[M - CH_3]^+$, 247, 125, 111, 99, 98, 97, 96, 85, 84, 83, 71, 70, 69, 58, 56, 55, 43, 42, 41, and 29. GLC/MS of the GLC peak with 26.5-min retention time showed peaks of m/z 411 $[M - CH_3]^+$ plus the lower mass peaks as for

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