High-Pressure Liquid Chromatographic Determination of Sulfamethazine Residues in Beef Tissues

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Abstract □ A high-pressure liquid chromatographic procedure for the determination of sulfamethazine residues in beef tissues is presented. Tissue samples are extracted and analyzed utilizing normal-phase chromatography and a UV detector at 254 nm. Primary and confirmatory identification and quantitative determination are provided at the tolerance limit of 0.1 ppm of sulfamethazine in tissues. Recoveries obtained from liver, fat, kidney, and muscle tissues were 69, 82, 77, and 76%, respectively, when spiked at the tolerance limit.

Keyphrases □ Sulfamethazine—high-pressure liquid chromatographic determination, beef tissues □ High-pressure liquid chromatography—analysis, sulfamethazine, beef tissues □ Chemotherapeutic agents—sulfamethazine, high-pressure liquid chromatographic determination, beef tissues

Sulfamethazine (I), a chemotherapeutic agent, has been used successfully for more than 30 years in the treatment of infectious diseases in cattle. A tolerance limit of 0.1 ppm has been established for the drug residues in uncooked edible tissues of cattle[1]. The Bratton–Marshall (1) procedure and subsequent modifications (2, 3) were used to indicate the residue levels of I but, due to nonspecificity (4), these procedures no longer meet the rigorous requirements recently established by the regulatory agencies for residue assay procedures.

Several procedures that provide improved specificity for sulfamethazine residues in tissues using TLC (4), GLC (5), and high-pressure liquid chromatography (HPLC) (6) were reported recently. However, no procedure has been approved by the regulatory agencies for use in a tissue residue study. A study was conducted in this laboratory to develop a procedure that could be approved for the determination of sulfamethazine residues in beef tissues. The method employs HPLC to provide a qualitative (both primary and confirmatory) and quantitative assay of I in tissues at the tolerance limit.

EXPERIMENTAL

Materials—Methylene chloride[2], chloroform[2], methanol[3], isooctane[3], and acetic acid[3] were analytical reagent grade and were used as received. Sulfamethazine[4] (I), sulfadimethoxine[5] (III), sulfadiazine[6] (VI), and sulfachlorpyridazine[2] (V) were used as received. Sulfabromomethazine[7] (II) was dissolved in water, buffered to pH 5–6, and extracted into methylene chloride prior to chromatography. Sulfaethoxypyridazine[4] (IV) was obtained by direct dissolution of an equivalent amount of a bolus[8] into methylene chloride.

Apparatus and Chromatographic Parameters—A high-pressure liquid chromatograph[9] was equipped with a septumless injector, a normal-phase 25-cm column[10], a UV detector set at 254 nm, and a strip-chart recorder. Unless otherwise noted, a mobile phase of 65% chloroform, 30.5% isooctane, 4.0% methanol, and 0.5% acetic acid was used. The flow rate was 20 ml/hr, and the UV detector was operated at 0.002 aufs.

Working Standards—Solutions of each sulfonamide were prepared at 8 μg/ml in methylene chloride.

Analytical Standards—Standards equivalent to 0.1, 0.2, and 0.4 ppm of I in tissues were prepared by pipetting 0.5, 1.0, and 2.0 ml, respectively, of a 4-ppm solution of I in methylene chloride into tared 30-ml beakers. The weight of each solution was adjusted to 1 ± 0.02 g by evaporation or addition of methylene chloride immediately prior to injection of a 10-μl aliquot into the liquid chromatograph. *Standards were not allowed to evaporate to dryness.*

Acetylation Technique—One milliliter of methanol and 2 drops of acetic anhydride were added to each sample to be acetylated. The samples then were heated on a hot plate at 80–90° until acetic acid fumes could not be detected. The residue was dissolved in 1 g of methylene chloride, and 10-μl aliquots were injected.

Residue Assay Procedure—The tissues were cut into small pieces (200–300 mg), and 20 ± 0.1 g was weighed into 100-ml beakers. Following the addition of 25 ml of methylene chloride, the samples were mixed with a glass stirring rod and allowed to stand for 20–30 min with frequent stirring. The methylene chloride was decanted into 125-ml separators through a funnel plugged with glass wool. The tissues were extracted with two additional 25-ml portions of methylene chloride, and the aliquots were pooled in their respective separators.

After the addition of 10 ml of 3 *N* H_2SO_4, the samples were extracted for 15 min, and the lower phase was discarded completely. The remaining aqueous phase was washed twice by adding 10 ml of methylene chloride, shaking for 10 min, and discarding the lower phase.

One milliliter of 10% K_2HPO_4 and 3 ml of 40% NaOH were added to each separator. The samples were mixed well and allowed to cool, and the pH was measured with pH paper. A pH of 5–6 was obtained by dropwise addition of 3 *N* H_2SO_4 or 40% NaOH. The final pH was measured potentiometrically.

After the addition of 5 ml of methylene chloride, the samples were extracted for 15 min. The lower phase was dried by draining through a bed of anhydrous sodium sulfate and collected in previously weighed 50-ml beakers. The extraction was repeated, and the extracts were collected in their respective beakers.

The samples were placed on a hot plate at 60–70°, and the weight of the solution was adjusted to 1 ± 0.02 g by evaporation immediately prior to injecting 10 μl into the liquid chromatograph.

Evaporation to dryness of either samples or standards was avoided because of resulting sulfamethazine losses.

After the samples were chromatographed satisfactorily, the samples were acetylated and reinjected.

RESULTS AND DISCUSSION

Qualitative Assay—Ten-microliter samples of each working standard were injected into the liquid chromatograph stabilized at the specified parameters. Retention times of each sample are given in Table I. Primary identification of an unknown sulfonamide can be made by comparison of retention times for the sample to those of authentic standards.

Table I—Sulfonamide Retention Times

Sulfonamide	Retention Time, min
Sulfabromomethazine	3.56
Sulfadimethoxine	3.99
Sulfamethazine	4.34
Sulfaethoxypyridazine	4.60
Sulfachlorpyridazine	5.60
Sulfadiazine	5.55

[1] 21 CFR 556.670.
[2] Mallinckrodt Chemical Co., St. Louis, Mo.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] American Cyanamid Co., Princeton, N.J.
[5] Hoffmann-La Roche Inc., Nutley, N.J.
[6] K & K Laboratories, Plainview, N.Y.
[7] Merck and Co., Rahway, N.J.
[8] SEZ C-R bolus, American Cyanamid Co., Princeton, N.J.
[9] Aerograph 4100 series, Varian Associates, Palo Alto, Calif.
[10] MicroPak CN-10, Varian Associates, Palo Alto, Calif.

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Table II—Acetylated Sulfonamide Retention Times

Acetylated Sulfonamide	Retention Time, min
Acetylsulfabromomethazine	4.16
Acetylsulfadimethoxine	4.70
Acetylsulfamethazine	5.34
Acetylsulfaethoxypyridazine	5.23
Acetylsulfachlorpyridazine	6.68
Acetylsulfadiazine	6.78

Table III—Retention Times of Sulfadimethoxine, Sulfamethazine, Sulfaethoxypyridazine, and Their Acetylated Derivatives Using the Alternative Solvent System of 97% Chloroform, 2% Acetic Acid, and 1% Ethanol

Sulfonamide	Retention Time, min
Sulfadimethoxine	3.5
Sulfamethazine	4.4
Sulfaethoxypyridazine	5.3
Acetylsulfadimethoxine	8.0
Acetylsulfamethazine	10.2
Acetylsulfaethoxypyridazine	13.5

One-milliliter portions of each working standard were acetylated, and 10-μl aliquots were injected as described. Retention times of each acetylated sulfonamide are given in Table II. Confirmatory identification of an unknown sulfonamide can be made by comparison of its retention time after acetylation to that of acetylated authentic standards.

Improved resolution of I, III, and IV was obtained using an alternative solvent system of 97% chloroform, 2% acetic acid, and 1% ethanol (Fig. 1). Retention times of these three sulfonamides and their acetylated derivatives are given in Table III.

Solutions containing mixtures of sulfamethazine and any of the other sulfonamides examined could be resolved completely with the two solvent systems. Due to column variation, slight adjustment of the mobile phases may be necessary for adequate resolution.

The specificity of the technique, primarily due to the retention times of sulfamethazine and its acetylated derivative, is increased by the tissue extraction procedure. Only compounds possessing amphoteric properties very similar to I are recovered to any significant degree. Many potentially interfering, naturally occurring components of tissue and nearly all drugs other than sulfonamides currently being used in cattle are eliminated prior to chromatography. Although recoveries of sulfonamides other than I have not been confirmed, the potential interference of many compounds (II–VI) is eliminated by the resolutions obtained with the technique. Sulfonamides other than the ones mentioned previously that have been examined and found not to interfere include sulfamerazine, sulfathiazole, sulfanilamide, sulfapyridine, and sulfaquinoline. No compound has been found to date that interferes with the identification of residues of I in tissues using this method.

Quantitative Assay—Analytical standards containing amounts of I equivalent to 0.1, 0.2, and 0.4 ppm in tissues were prepared. Ten-microliter injections of these standards after weight adjustment of the solution to 1 ± 0.02 g gave peak heights proportional to the amounts of I injected. Reproducibility and linearity of the technique are shown by replicate injections of standards at the various concentrations (Table IV). Unknown amounts of I in solution are determined by comparison of sample and standard peak heights obtained from 10-μl injections after weight adjustment to 1 ± 0.02 g.

Residues of I in tissues are calculated from:

$$\frac{\text{sample peak height}}{\text{standard peak height}} \times \frac{\text{micrograms of I in standard}}{\text{weight of sample, g}} = \text{ppm of I} \quad \text{(Eq. 1)}$$

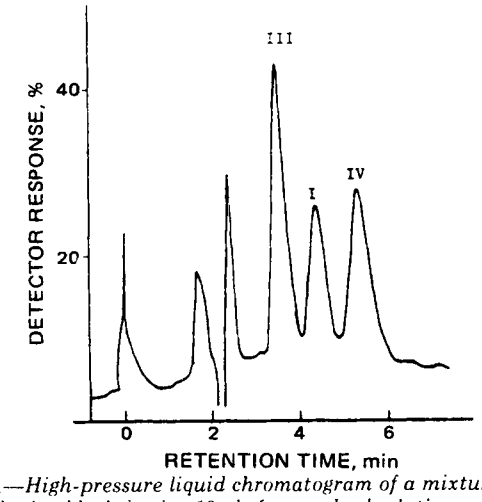

Figure 1—*High-pressure liquid chromatogram of a mixture of I, III, and IV obtained by injecting 10 µl of a standard solution equivalent to 0.1 ppm of each in tissue. The solvent system was 97% chloroform, 2% acetic acid, and 1% ethanol.*

Table IV—Reproducibility and Linearity of the Quantitative Technique

Concentration, ppm	Peak Height[a] (±*SD*)
0.1	29.2 (±1.16)
0.2	56.6 (±0.58)
0.4	103.4 (±3.3)

[a] Average of four injections.

Table V—Percent Recovery Data[a] Obtained by Applying the Residue Assay Procedure to Spiked Beef Tissues

Concentration Added, ppm	Percent Recovered			
	Liver	Kidneys	Muscle	Fat
0.1	73, 71	84, 91	76, 83	80, 87
0.1	66, 66	73, 71	76, 76	80, 86
0.1	59, 65	67, 75	76, 79	84, 83
0.1	75, 74	69, 73	65, 65	80, —[b]
0.1	73, —[b]	83, 81	81, 81	75, 83
Average	69.1	76.7	75.8	82.0
SD	5.4	7.7	6.2	3.7
0.2	68, 69	87, 84	61, 96	81, 83
0.2	64, 71	81, 78	76, 73	82, 87
0.2	68, 67	72, 72	76, 77	86, 80
0.2	68, 69	66, 74	67, 68	77, —[b]
0.2	68, 70	82, 79	79, 86	—[b], 76
Average	68.2	77.5	75.9	81.5
SD	1.9	6.4	9.9	3.9
0.4	82, 82	84, —[b]	91, 86	74, 83
0.4	72, 76	79, 79	87, 82	82, 72
0.4	71, 69	78, 78	79, 85	75, 81
0.4	63, 70	65, 72	70, 68	61, 86
0.4	74, —[b]	80, 82	90, 100	81, 86
Average	73.2	77.4	83.8	78.1
SD	6.1	5.7	9.6	7.7

[a] Duplicate samples. [b] Sample lost.

Recovery of I from Tissues—Percent recovery data (Table V) were obtained by taking beef tissues spiked at 0.1, 0.2, and 0.4 ppm of I through the tissue assay procedure and comparing the peak heights obtained to those of the appropriate analytical standards. These data were obtained from five samples of each tissue, in duplicate, at each concentration. Analyses of the data showed >70% average recovery in every tissue at the

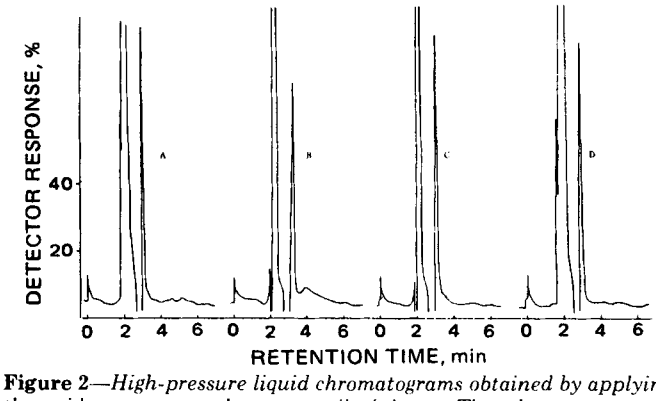

Figure 2—*High-pressure liquid chromatograms obtained by applying the residue assay procedure to unspiked tissues. The solvent system was 65% chloroform, 30.5% isooctane, 4.0% methanol, and 0.5% acetic acid. Key: A, liver; B, kidneys; C, muscle; and D, fat.*

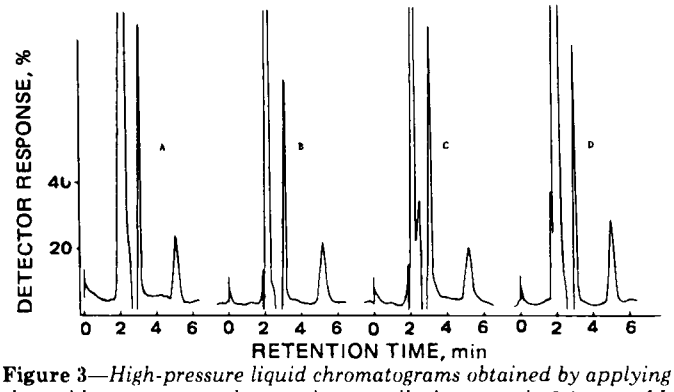

Figure 3—*High-pressure liquid chromatograms obtained by applying the residue assay procedure to tissues spiked to contain 0.1 ppm of I. The solvent system was 65% chloroform, 30.5% isooctane, 4.0% methanol, and 0.5% acetic acid. Key: A, liver; B, kidneys; C, muscle; and D, fat.*

tolerance level except liver, which had a recovery of 69% and a relative standard deviation of ~10% (Table V).

Unspiked tissues typically showed no peak other than random baseline noise in the region where I eluted (Fig. 2). The magnitude of the sulfamethazine signal obtained from tissues spiked at the tolerance limit was ~20% of full scale (Fig. 3). A favorable signal-to-blank ratio at the tolerance limit was obtained with this procedure, allowing low detection limits. The tolerance limit of 0.1 ppm of I in tissues was approximately five times the detection limit of the method.

With slight modifications, the method should be applicable to several sulfonamides currently used in veterinary medicine. Recoveries of sulfamethazine only have been demonstrated to date.

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Teratogenic Potential of Cocaine Hydrochloride in CF-1 Mice

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Abstract □ This investigation revealed that cocaine hydrochloride was teratogenic when administered in nontoxic doses to gravid CF-1 mice on Days 7–12 of gestation. The teratogenic susceptibility of the CF-1 mouse fetus to cocaine hydrochloride was evident throughout this portion of the gestation period. The early appearance of eye defects and the occurrence of skeletal defects later in gestation after cocaine hydrochloride challenge paralleled the sequence of ontogenesis.

Keyphrases □ Cocaine hydrochloride—evaluation as potential teratogen, effect on catecholamine levels in placenta, skeletal and soft tissue malformations in fetal mice □ Teratogenic drugs—cocaine hydrochloride, effect on fetal mice □ Inhibition, competitive—cocaine hydrochloride and norepinephrine, effect on fetal mice

Cocaine (benzoylmethylecgonine), the first local anesthetic discovered, not only inhibits nerve conduction but also prevents norepinephrine reuptake at nerve terminals (1). Because of the latter action, norepinephrine levels are augmented and vasoconstriction occurs (1).

BACKGROUND

Previous studies detected catecholamines in human amniotic fluid (2) and placenta (3). If the mechanisms for storage and release of norepinephrine in the placenta are comparable to those at nerve terminals, increased catecholamine levels induced by cocaine could cause placental vasoconstriction.

A relationship between placental vasoconstriction and fetal anomalies was demonstrated in studies involving mechanical obstruction of uterine and placental vascular flow by clamping, which probably causes fetal malformations through decreased oxygen availability (4–7), and by drugs such as serotonin (8) and morphine (9), which accomplish similar effects pharmacodynamically.

With respect to the autonomic neural involvement of cocaine as a potential teratogen, Furchgott *et al.* (10) detected a specific transfer site with which norepinephrine molecules combine before being transferred to storage or enzymatic inactivation loci. According to Johnson and Kahn (11), the potentiation of norepinephrine responses by cocaine results from competitive inhibition between the local anesthetic and norepinephrine molecules at these transfer sites. Thus, in pregnant mice, cocaine conceivably could induce fetal malformations by raising catecholamine levels which, in turn, cause placental vasoconstriction *via* competitive inhibition.

The purpose of this study was twofold: (*a*) to test the hypothesis that cocaine is a potential teratogen in mice, and (*b*) if the results were affirmative, to ascertain the precise days of gestation when fetal susceptibility to the teratogen is evident.

EXPERIMENTAL

Animals—The test animals were CF-1 mice[1], 25–30 g. Females were placed in aggregate cages, each holding 10 animals. Two weeks after their arrival, the mice were bred if they weighed at least 25 g. Males were placed individually in metal cages (12.5 × 15 × 10 cm) with a wire-mesh front and floor[2]. All animals were maintained on laboratory food[3] and tap water *ad libitum*.

The room housing the animals was protected from exposure to natural sunlight and was equipped with an electrical lighting system[4], which al-

[1] Charles River Breeding Laboratories, Wilmington, Mass.
[2] RD-T unit, Norwich Wire Works, Norwich, N.Y.
[3] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.
[4] Model V-45073 astronomic dial time switch with "skipper," International Register Co., Spring Grove, Ill.

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