

DRUGS IN FEEDS

Rapid Screening Test for Sulfamethazine in Swine Feeds

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A simple, 10-min qualitative screening test for sulfamethazine (SM) in swine feeds is detailed. The method, which can be run in the field, uses 2 plastic tubes arranged piggyback style. The upper tube contains, from top to bottom, the feed sample (about 1 g), partially deactivated alumina, and an anion exchange resin buffered at pH 5.7. The bottom tube contains a small bed of anion exchanger buffered at pH 7.9, which traps the SM. After percolation of solvent through the system, the SM, if present, is eluted from the pH 7.9 resin and is reacted with Bratton-Marshall reagents to give a pink-to-lavender color. Feeds containing ≥ 0.15 ppm can be detected. A simple, additional dye concentration step allows for detection of 0.02 ppm, if desired. Only ampho-teric primary aromatic amino-containing compounds with a pKa close to that of SM can theoretically interfere. Preparation of permanent color solutions using cobalt and copper acetates in glacial acetic acid is described for the optional establishment of the minimum concentration of SM in the feed. The method offers a simple way to detect some cross-contaminated withdrawal feeds containing > 2 ppm SM, which can lead to violative (≥ 0.1 ppm) residues in swine liver.

The increased supplementation of animal feeds with growth-promoting and disease-allaying drugs has created a parallel demand for adequate methods to quantitate the drugs both in the feed and in the tissue or fluids of consuming animals (1). When large numbers of samples are to be analyzed, a great deal of time, effort, and expense can be saved if rapid screening methods are available, especially those that give low numbers of false positive tests. In this initial effort, we report such a method, namely, a screen for sulfamethazine, by far the major sulfa drug added to swine feeds (2, 3). The method has been specifically designed so that it can be run in the field and in the laboratory with equal facility, using simple, disposable equipment. The highly sensitive Bratton-Marshall (B-M) color test forms the basis for the detection method. The well

known nonspecificity of the reaction due to the presence of B-M-positive compounds in feeds has been circumvented by using small, buffered columns of anion exchange resins. Feed samples containing ≥ 0.15 ppm sulfamethazine can be detected in about 10 min and a simple, optional step allows detection of feeds containing 0.02 ppm sulfamethazine.

There are no screening tests for SM in swine feeds that can be readily used in both the laboratory and the field. However, a rapid screening test designed primarily for sulfadimethoxine in poultry feeds is available, which uses the non-specific reagent *p*-dimethylaminocinnamaldehyde (4).

METHOD

Apparatus and Reagents

All reagents were stored at room temperature without precaution to exclude light. Deionized or distilled water was used throughout the study.

(a) Sodium nitrite.—0.12% in water.

(b) Ammonium sulfamate.—0.8% in water.

(c) *N*-1-(Naphthyl)ethylenediamine dihydrochloride (NED).—(Sigma Chemical Co., St. Louis, MO 63178) 0.8% in water containing 0.1% ethylenediaminetetraacetic acid (EDTA).

Solutions (a), (b), and (c) were stored in, and dispensed from, drop dispenser bottles (Nalge 2411 Series, A. H. Thomas Co., Philadelphia, PA 19105). Solutions were usable for 2 months.

(d) Potassium dihydrogen phosphate.—0.2M. Dissolve 27.8 g KH_2PO_4 in 1 L water.

(e) Dibasic sodium phosphate.—0.2M. Dissolve 71.1 g $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ in 1 L water.

(f) pH 5.7 buffer.—Using pH meter, add (e) to 50 mL of (d) until pH = 5.7 ± 0.05 pH unit is obtained. If no pH meter is available, add 4.5 mL of (e) to 50 mL of (d).

(g) pH 7.9 buffer.—Using pH meter, add (d) to 50 mL of (e) until pH 7.9–7.95 is obtained. If no pH meter is available, add 4.0 mL of (d) to 50 mL of (e).

(h) Acidic alumina.—Add 10 mL pH 5.7 buffer

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to 90 g alumina (Fisher, No. A-948) in screw-cap bottle and shake until all lumps are broken. Do not substitute any other alumina.

(i) *Resin*.—Dowex 1X2, 100–200 mesh (Sigma).

(j) *Transfer pipets*.—Polyethylene. Pasteur-pette, bulb-type (Centaur Chemical Co., 180 Harvard Ave, Stamford, CT 06902).

(k) *Pipet tips*.—5 mL (Rainin Instrument Co., Mack Rd, Woburn, MA 01801).

Optional Apparatus and Reagents

(a) *Dowex 50-cellulose powder*.—Weigh equal amounts of Dowex 50X4 (100–200 mesh) (Sigma) and cellulose powder CF-11 (Whatman, Inc., Clifton, NJ 07014) and grind lightly (mortar and pestle) until light tan.

(b) *Cobaltous acetate solution*.—2%. Weigh 2 g \pm 5 mg $\text{Co}(\text{OAc})_2$ and dissolve in 80–90 mL glacial acetic acid in 100 mL volumetric flask. When completely dissolved, dilute to volume with glacial acetic acid and mix.

(c) *Cupric acetate solution*.—0.015%. Weigh 150 mg $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$ and dissolve in 100 mL glacial acetic acid as above.

(d) *Alumina scoop*.—To obtain ca 1 g alumina. Laboratory-made as follows: Mark Pasteur-pette 1 and 3 cm from bulb end. Insert point of single-edge razor blade at one seam and cut along mark to other seam. Make similar cut at other mark. With scissors, cut out a "window" along the seams from end of one razor cut to other. Cut off tip of pipet to facilitate transfer of alumina. After scooping up alumina, hold scoop vertically and tap at top with index finger to dislodge excess alumina. Then transfer alumina by letting it slide down barrel.

(e) *Feed scoop*.—For transferring ca 1 g feed samples. Make as described above except make first cut 1.5 cm from end of bulb.

(f) *Pressure bulb*.—Convenient apparatus to apply air pressure (A. H. Thomas, No. 1957-K10).

Procedure

Preparation of pH 5.7 and 7.9 resins.—Place 10 g (as received) Dowex 1X2 resin in 30 mL coarse sintered glass funnel. Wet resin with water to settle and let drain. Add 50 mL pH 5.7 phosphate buffer and let solvent percolate by gravity flow. After all of buffer has entered bed, force excess out by pressure or vacuum application and wash resin with ca 50 mL water until effluent emerges neutral (pH paper). Force out excess water and transfer the equilibrated resin to suitable container such as 60 mL narrow-mouth

polyethylene bottle with screw cap. Add 50 mL 95% ethanol. Prepare pH 7.9 resin in similar fashion using pH 7.9 phosphate buffer.

Preparation of pH 7.9 tube.—Cut 3 cm off tip and 0.5–1.0 cm off top of bulb portion of transfer pipet. Insert small wad of fine glass wool into bottom of barrel portion. Transfer 1 mL of a magnetically stirred suspension of pH 7.9 resin to tube, using pipet with relatively large tip opening. Let excess alcohol drain and wash down pipet walls with alcohol. Place small wad of glass wool on top of resin bed, but do not tamp. If no magnetic stirrer is available, suspend resin by shaking and pipet immediately.

Preparation of pH 5.7-alumina-feed tube.—Plug bottom of a 5 mL pipet tip with small wad of glass wool and transfer 1 mL of a magnetically stirred suspension of pH 5.7 resin and let alcohol drain. Place 1 g alumina on top of resin bed. Then add 1 g of feed sample. Feed sample should be homogeneous, representative of the lot or batch, and preferably ca 20–200 mesh. (An inexpensive (<\$20) coffee mill (Waring Products, Route 44, New Hartford, CT 06057, also available in some retail stores) is used in our laboratory to grind feed 10 s.)

Extraction of sulfamethazine.—Place tip of prepared pH 5.7-alumina-feed tube piggyback in pH 7.9 tube (Figure 1) and add 5 mL ethyl acetate-methanol-water (7 + 2.7 + 0.3). After solvent has completely passed through beds in both tubes, remove and discard upper tube; then pipet (or squirt to a premark) ca 1 mL 95% ethanol into bottom tube. Let ethanol drain and add 2 mL water and let drain. Place 1.9 mL ($\frac{1}{2}$ dram) lipless (shell) vial under tube and add 0.8 mL 3.5N aqueous HCl containing 0.01% Triton X-100 (Sigma) to elute sulfamethazine from pH 7.9 resin. Add 1 drop of reagents (a), (b), and (c) in sequence to the effluent, shaking vial 10–15 s between additions. Pink-to-lavender color indicates presence of sulfamethazine. Feeds containing ≥ 0.15 ppm will give positive response. If color is not apparent in vial, lower limit of detectability can be increased ca 7.5 times (to 0.02 ppm) by following procedure: Cut 3 cm off tip of a disposable glass Pasteur pipet ($5\frac{3}{4}$ in. long) and dab new tip into Dowex 50-cellulose powder mixture until short (0.2–0.4 cm) bed of powder is retained in tip. Wipe excess powder from outside and push tip into a tightly compacted bed of fine glass wool contained in a vial. Gently twist pipet until small plug of glass wool has been retained to support bed. Tap tip on solid surface to settle powder and give an even bed surface. Add 0.5 mL methanol to reaction vial,

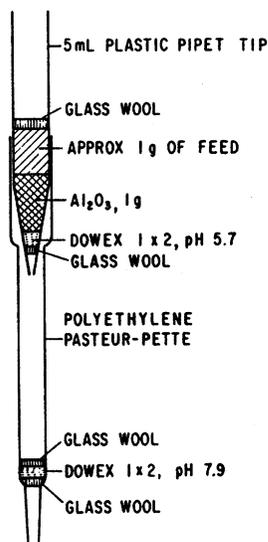


Figure 1. Setup of tubes for sulfamethazine screening test.

stir, and transfer to Pasteur pipet. Presence of narrow lavender band forming at top of bed as part or all of solution passes over bed indicates presence of sulfamethazine.

Preparation of Permanent Color Standards

Accurately pipet 1.7 parts cobaltous acetate solution, 0.5 part cupric acetate solution, and 2.8 parts glacial acetic acid, and mix. This solution will then contain color intensity equivalent to color obtained when running Bratton-Marshall (B-M) reaction on 2 μg sulfamethazine/mL reagents used in this study (3.5N HCl and 1 drop each of reagents (a), (b), and (c)). This would be equivalent to 2 ppm in feed, assuming quantitative recovery. To prepare solutions with colors equivalent to lower concentrations of (B-M) dye, dilute colored solution with glacial acetic acid, e.g., for 1 $\mu\text{g}/\text{mL}$ dilute 1:1, for 0.25 $\mu\text{g}/\text{mL}$ dilute 0.25:1.75, etc. To ensure against subtle changes in color of standards due to evaporation of acetic acid, seal 1 mL volumes in 2 mL glass ampules (Ace Scientific Supply Co., PO Box 127, Linden, NJ 07036, No. 10-1248-17).

Results and Discussion

Approximately 100 different feed samples containing various amounts of sulfamethazine were screened using the procedure. At least 3 analyses were made on each feed because of modifications made to the original procedure. The feed samples were primarily corn- or alfalfa-based or were mixtures of the two. A few

samples were reddish in color, were heavy, and apparently contained CaCO_3 because they released gas when acidified. The majority of samples contained less than 0.5 ppm as determined by specific quantitative and semiquantitative chromatographic methods in other laboratories that routinely conduct analyses, or which were spiked with levels of sulfamethazine ranging from 0.05 to 0.5 ppm in this and in other laboratories. Samples that were reported to contain no sulfa drug were always negative in our screening procedure or showed just a trace (using the micro Dowex-cellulose column) of sulfa drug. A clear, colorless effluent was always obtained from the pH 7.9 resin and the resultant color with the B-M reagents was always true, thereby reducing doubt as to the positive nature of the sample. Samples spiked with varying amounts yielded color intensities in the procedure that could be ranked correctly as to relative concentration.

The recovery of sulfamethazine from the feed sample is not quantitative and can vary from one type of feed to another and also with the concentration. The efficiency of the extraction procedure appears to be the major drawback in making the procedure quantitative. Therefore, the B-M color that is obtained can not be completely correlated with the actual amount of sulfamethazine in the sample. If one wishes to match the color obtained with standards prepared each day, or, more conveniently, with the permanent color standards, then an approximate *minimum* concentration of sulfamethazine in the feed can be established. Using the procedure, cross-contaminated withdrawal feeds containing >2 ppm, which can lead to >0.1 ppm violative residue in swine liver (5), can be readily ascertained. The eye cannot differentiate the intensity of the dye produced by the B-M reaction when the concentration of sulfamethazine is above about 2 $\mu\text{g}/\text{mL}$, so dilution is necessary in these instances.

Single samples of feed can be screened in about 10 min unless the feed sample has been too finely ground, in which case it will take longer. When properly set up (with vial racks and tube supports) over 100 samples can be run easily by one person in an 8 h day. Moreover, this can be accomplished in about 1 sq. ft of space. If many samples are to be run simultaneously, speed of analysis will not be an important factor.

Some sensitivity has been sacrificed to reduce analysis time. If the pH 7.9 column is made to run slower, quantitative exchange of sulfamethazine will occur (as opposed to about 90%),

resulting in about 10% increase in sensitivity. Sensitivity can also be increased if the resin is eluted with a smaller volume of acid, e.g., 0.4 mL instead of 0.8 mL, thereby doubling the color intensity and increasing the lower limit of detection from 0.15 ppm to between 0.07 and 0.08 ppm in the vial. Both of these parameters can be accomplished by using 0.5 mL of the pH 7.9 resin suspension instead of 1 mL, and making the column in the narrower diameter tip portion of the Pasteur-pette instead of in the barrel portion. This will reduce the flow rate from under 10 min to between 20 and 40 min, depending on how far down in the tapered tip the resin bed is located. This option is available to the analyst depending on assay needs; the micro Dowex 50-cellulose column described earlier may be used to detect even lower concentrations.

Specificity of the Method

Theoretically, only amphoteric compounds which contain a primary amino functional group on a benzene ring can interfere in the analysis and give a false positive reaction. Unlike the procedure that Tishler et al. (6) designed, in part, to limit interferences to ampholytes by using strong acid and strong base extractions, in our procedure a narrow pH range (5.7-7.9) is used for the exchange of sulfamethazine onto the resin. Thus only qualifying ampholytes with acid dissociation constants very close to sulfamethazine can possibly interfere. This range was established by equilibrating the resin against buffers of varying pH values and determining the amount of SM exchanged onto the resin. No exchange occurred below pH 5.7, and the amount exchanged increased to a maximum (100%) at or above pH 7.9. Although a number of sulfa drugs have a pKa very close to that of sulfamethazine (7, 8) and will exchange in this range, others, such as sulfathiazole, will exchange at pH 5.7 or below and will be removed in the upper trap column. Sulfamethazine and sulfathiazole, the major sulfa drugs used in swine feeds, were put through the entire system in the absence of feed at the 1-2 μg level and were recovered at 91 and 0%, respectively. Some naturally occurring ampholytes which qualify as potential false positive reactors in the analysis, e.g., *p*-aminobenzoic acid, anthranilic acid (*o*-aminobenzoic acid), and kynurenine (3-anthraniloylalanine) as well as sulfanilic acid, a possible breakdown product of sulfa drugs, will also be removed in the upper trap column. Alumina (deactivated to a point where sulfamethazine will not adsorb) is used primarily to adsorb some interfering

plant pigments. It also has a strong affinity for relatively strong carboxylic (and sulfonic) acids, thereby further reducing the number of compounds qualifying as potential false positive B-M reactors.

Triton X-100 (Sigma) was incorporated in the 3.5N HCl after we noted that some feed samples contained lipids (presumably water-insoluble acids) that coated the resin and made elution of SM slow and difficult. Triton X-100, a non-ionic wetting agent which facilitates the wetting and subsequent exchange of Cl^- for SM anions on the resin, has no effect on the diazotization of SM and subsequent coupling with NED.

Permanent Color Standards

There is an obvious need, especially in field screening, for permanent color standards to simulate the colors obtained in the B-M reaction and to give the analyst at least an approximation of the minimum concentration of SM present in a feed sample. These permanent color standards should find greater utility, however, in more quantitative procedures.

Attempts to prepare permanent color standards using the B-M reaction product with sulfamethazine were unsuccessful due to fading of the dye. This occurred even when the dye solution was sealed in an inert atmosphere and in the presence or absence of ascorbic acid and/or isoascorbic acid as antioxidants and with the solutions stored in the dark at 4°C. The colors could be simulated very well by using some organic dyes such as alkaline solutions of phenol red, but these also faded rapidly even under inert conditions. The use of cobaltous and cupric acetates in glacial acetic acid in the recommended ratios gives colors that are virtually indistinguishable to the eye from the corresponding B-M dye. These colors are unaffected by light, time, and oxygen, and, barring losses of acetic acid from evaporation, should remain unchanged for prolonged periods. Glacial acetic acid was the only solvent found for the salts in which the desired color could be obtained; water, methanol, and 95% ethanol were unsatisfactory.

Stabilization of NED

One of the anticipated problems in using the screening procedure in the field was the known instability of NED solutions. Thus, analysts with no access to an analytical balance would have to resort to a more inaccurate way to prepare fresh NED solutions. The stability of NED solutions prepared with water from various sources was studied. We found that tap water, or distilled

water that had been in contact with metal, gave NED solutions that began to darken within hours after preparation. Solutions of NED in deionized water, however, did not begin to darken for several days. The addition of EDTA to water from any source stabilized the NED (presumably against metal-catalyzed oxidation) by delaying the onset of darkening for 3-4 weeks and subsequent deterioration of the NED which is manifested by precipitation. Concentrations of EDTA (from 0.05 to 0.20%) were equally as effective in stabilizing NED in the 3 types of water studied. Even after 2 months, the addition of EDTA-stabilized NED to diazotized sulfamethazine gave the same color yield as did freshly prepared NED in deionized water.

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