

Practical Screening Procedures for Sulfamethazine and *N*⁴-Acetylsulfamethazine in Milk at Low Parts-Per-Billion Levels

Relatively simple and inexpensive procedures for screening milk for sulfamethazine (SMZ) and one of its metabolites, *N*⁴-acetylsulfamethazine (ASMZ), are detailed. Both methods detect at the low parts-per-billion level and are suitable for both field and laboratory use. Milk is passed over Chromosorb 102, which adsorbs SMZ. The drug is eluted and purified by direct passage of the effluent over small beds of buffered anion-exchange resins and alumina and is finally isolated and detected colorimetrically. For ASMZ, the procedure is modified so that SMZ is removed in the purification steps. The isolated ASMZ is then hydrolyzed to SMZ for detection. Application of the methods 5 years apart (1988 and 1993) shows that SMZ is still being used but to a lesser extent in 1993. Of over 250 samples screened in the 2 studies, only 2 were estimated to contain SMZ at 10 ppb, and the majority contained SMZ at 1 ppb. ASMZ was detected in a number of samples that were negative for SMZ.

Sulfamethazine (SMZ) is an effective and inexpensive drug for maintaining the health of farm animals. Several reports in 1988 and 1989 showed that SMZ was present in market milk (1, 2), and another report (3) indicated that sulfonamides were present in milk but the specific drugs were not identified. After learning of these studies, this center undertook development of both a quantitative method (4) and a qualitative

screening procedure for SMZ in milk at the low parts-per-billion level. This paper details the screening method—a relatively simple, inexpensive technique applicable for both field and laboratory use. In addition, a method is described for screening milk for *N*⁴-acetylsulfamethazine (ASMZ), a prominent metabolite of SMZ in milk (5). The screening procedures were first applied to a fairly large number of milk samples in 1988–1989 and then again to a smaller number of milk samples in 1993. In the interim, immunoassay screening test kits have proliferated and have been evaluated (6).

METHOD

Reagents and Apparatus

Distilled water was used. All solutions were aqueous unless otherwise specified.

(a) *Sodium nitrite*.—0.12%.

(b) *Ammonium sulfamate*.—0.8%.

(c) *N-1 (Naphthyl)ethylenediamine dihydrochloride*.—0.8% in 0.1% ethylenediaminetetraacetic acid, di- or tetrasodium salt (Sigma Chemicals, St. Louis, MO). This solution was contained in a 2 mL screw-capped vial. Nitrogen was bubbled through the solution for 1 min, and the vial was immediately sealed with an open-top screw cap containing a Teflon–silicone

8 mm disk (Pierce Chem., Rockford, IL). Solutions (a) and (b) were contained in 15 mL squeeze-type polyethylene dispensing bottles. All solutions were stored at 4°C when not in use. Solutions (a) and (b) were made fresh every 2 months, and solution (c) was renewed once a month.

(d) *Potassium dihydrogen phosphate*.—0.2M (27.8 g $\text{KH}_2\text{PO}_4/\text{L}$).

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

(f) *pH 7.9 buffer*.—Add (d) to 300 mL of (e) until pH 7.90 to 7.95 is obtained, as measured with pH meter.

(g) *pH 5.7 buffer*.—Add (e) to 300 mL of (d) until pH 5.65 to 5.75 is obtained, as measured with pH meter.

(h) *AGMP-1 anion-exchange resin*.—100–200 mesh, chloride form (Bio-Rad Laboratories, Richmond, CA).

(i) *Alumina, acidic*.—Alfa Products, Danvers, MA.

(j) *Dowex*.—50 × 4, 200 RH⁺ (Sigma).

(k) *Coarse sand*.—ASTM (A.H. Thomas G, Swedesboro, NJ).

(l) *Fine sand*.—Washed (J.T. Baker Inc., Phillipsburg, NJ).

(m) *Chromosorb 102*.—100–120 mesh (Sigma).

(n) *Benzenesulfonic acid silica cation-exchange resin*.—United Chemical Technologies, Bristol, PA.

(o) *N⁴-Acetylsulfamethazine*.—Reagent was prepared (7) and recrystallized from dioxane–H₂O (1 + 1) and then from absolute ethanol [melting point, 258°–260°C; literature value, 254°–256°C (7)]. Thin-layer chromatography on silica gel G with ethyl acetate as mobile phase showed one spot by *N*-chlorination (8).

(p) *Pipet tips for Oxford pipettor*.—0.6–1.0 mL (Thomas).

(q) *Disposable filter columns*.—(Fisher, King of Prussia, PA).

(r) *Luer-Lok syringe needles*.—26 gauge × 1/2 in. (Popper & Sons, New Hyde Park, NY). Do not substitute.

(s) *Adapter*.—Made by cutting off the rounded top of the bulb portion and ca 2 cm of the stem portion (below the bulb) of a 7 mL polyethylene Pasteur pipet (Fisher).

(t) *Homemade scoops*.—To hold ca 50 mg Chromosorb 102 and ca 100 mg alumina.

Preparation of pH 5.7 and pH 7.9 Resins

Stir magnetically 10 g AGMP-1 resin with 300 mL pH 5.7 (or pH 7.9) buffer for 1 h. Let stand for 10 min. Decant fine materials and transfer remainder to 60 mL coarse sintered-glass funnel. Wash with water until filtrate is neutral. Remove excess water by applying vacuum. Transfer resin to suitable container with 100 mL water and 100 mL 95% ethanol. Store tightly closed at 4°C when not in use.

Preparation of Cation-Exchange Resin

Wash Dowex 50 resin on filter funnel with water until effluent is colorless. Remove excess water by applying vacuum. Transfer 1 g damp resin to suitable container and add 10 mL water.

Preparation of Chromosorb 102 Column

Add 50 mg Chromosorb 102 to a disposable filter column and tap to settle powder. Place in a 1-hole stopper in a filter flask and apply vacuum. Wash any particles from sides with

water. Add ca 0.5 cm coarse sand. Remove column and fill it with water. Apply vacuum momentarily to filter column to start flow and then let drain by gravity. Fill with water again and let drain. Fill with acetone and twice more with water all by gravity flow. Washing procedure should be done within 1 h of screening milk sample. Attach 26-gauge needle to column tip to regulate milk flow.

Preparation of pH 5.7 Resin–Alumina Column

Plug a pipet tip with fine sand to a height of ca 1 cm. Sand is conveniently dispensed from squeeze-type dispensing bottle. Pipet 0.25 mL of well-shaken pH 5.7 suspension onto sand and let drain by gravity or, more quickly, by applying vacuum. Add ca 100 mg alumina.

Preparation of pH 7.9 Resin Column

Plug a pipet tip with fine sand and pipet 0.5 mL of pH 7.9 resin suspension. Let drain as above and then add ca 3 mm fine sand.

Preparation of Cation-Exchange Resin–Alumina Column

Plug pipet tip as described above and pipet 0.5 mL of suspended Dowex 50 resin. Let drain and add ca 100 mg alumina.

Preparation of Color Concentrating Tip

Dab tip of glass Pasteur pipet (5³/₄ in.) into benzenesulfonic acid silica until 5–10 mm is retained. While holding pipet approximately horizontal, push tip into a vial stuffed tightly with fine glass wool. Twist the pipet until a plug of glass wool is retained. Tap pipet gently to settle powder. Cut pipet ca 2 cm above taper.

Screening Milk for SMZ

(a) *Principle*.—Milk is passed over Chromosorb 102, which adsorbs SMZ. After bed is washed with water, it is eluted, and the effluent is passed directly over tandem columns containing both a pH 5.7 anion-exchange resin–alumina (to remove some organic acids, some sulfa drugs, and riboflavin) and a pH 7.9 anion-exchange resin, which retraps SMZ. After bed is washed, it is eluted with dilute acid. SMZ in the effluent is diazotized and coupled. If the dye is not visible, the solution is passed over a microbed of benzenesulfonic acid silica to concentrate the dye.

(b) *Procedure*.—Add milk sample (3.5 mL) to Chromosorb 102 filter column with attached 26-gauge needle and permit effluent to go to waste. Remove needle and wash sides with ca 1.5 mL water. Fill tube with water and let drain by gravity. Remove excess water by applying vacuum or positive pressure. Place filter column in an adapter and fit adapter into pH 5.7 resin–alumina tip and then place the latter in pH 7.9 resin tip. Wedge a wire between the 2 pipet tips (Figure 1). Elute Chromosorb 102 with 0.75 mL acetone–methanol–water (85 + 10 + 5) and let solvent drain through all beds. Remove filter column and adapter and wash sides of pH 5.7 resin–alumina tube with 0.25 mL of above solvent. Let drain through both tips. Remove upper tip and wash pH 7.9 bed with 0.25 mL

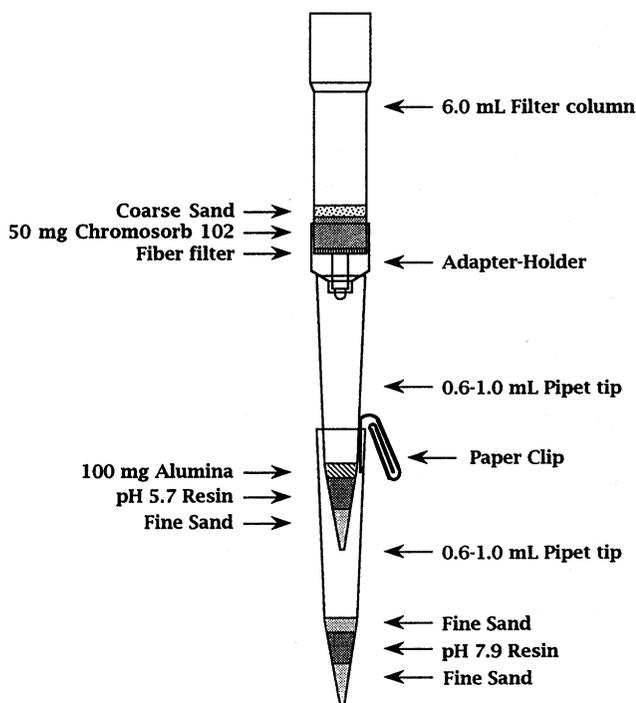


Figure 1. Tandem setup for isolation of sulfamethazine from milk.

water and then elute bed with 0.25 mL 2N HCl. Collect effluent in a 2 mL shell vial. Add 1 drop each of solutions (a) and (b) and 3 μ L solution (c) in that sequence at ca 15 s intervals, shaking after each addition. Place vial in the dark for ca 15 min and examine for color (along with a reagent blank) against a white background. If no color is evident (which was almost always the case with milk samples we screened), transfer solution to a color-concentrating tip and place in dark. After the solution has drained, examine for a lavender-violet zone at top of bed. A lens with a $\geq 5\times$ magnifying power is very helpful in discerning whether color is present. Flow through the color-concentrating tip is relatively slow and variable and may take ≥ 1 h. Flow rate may be speeded up by placing the tip on absorbent paper. There is also no problem in allowing this step to proceed overnight, as the color is stable on the benzenesulfonic acid silica for at least 48 h.

Screening Milk for ASMZ

(a) *Principle.*—Milk is passed over a bed of Chromosorb 102, which adsorbs both SMZ and ASMZ. After a water wash, both compounds are eluted, and the effluent is passed directly over tandem columns of cation-exchange resin–alumina to remove SMZ and interfering compounds and pH 7.9 anion-exchange resin to retrap ASMZ. ASMZ is eluted and hydrolyzed to SMZ, and SMZ is detected as described above.

(b) *Method.*—Follow procedure described for isolation of SMZ on Chromosorb 102. Place Chromosorb 102 column in tandem setup as described for SMZ, except that the pH 5.7 alumina pipet tip is replaced by a Dowex 50–alumina pipet tip. Elute and wash as described for SMZ, except that the pH 7.9

bed is washed with 0.25 mL methanol instead of water. Elute ASMZ with 0.25 mL 10% acetic acid in methanol and collect effluent in a 2 mL screw-capped vial. Remove solvent under a stream of nitrogen at room temperature. Add 1 drop 2N NaOH, seal, and heat at 100°C for 30 min. After the vial has cooled, add 0.25 mL 2N HCl. Follow remainder of procedure for detecting SMZ.

Results and Discussion

Specificity of SMZ Method

The behavior in the isolation scheme of a number of sulfonamides, some widely used in animal health maintenance, and a number of other aromatic primary amines was studied to establish method specificity. The following compounds are not retained by Chromosorb 102 and thus will not interfere: arsanilic, *p*-aminobenzoic, *o*-aminobenzoic (anthranilic), and sulfanilic acids; sulfanilamide; sulfadiazine; sulfacetamide; DL-kynurenine; and procaine penicillin. The following compounds were adsorbed (from water) onto Chromosorb 102 but were trapped by either the alumina or the pH 5.7 resin and also did not interfere: sulfadimethoxine, sulfapyridine, sulfathiazole, sulfaquinoxaline, and sulfamethizole. All of the aforementioned compounds were tested at the 0.5–2.0 μ g level. Three sulfa drugs were found to interfere: sulfamerazine, sulfaethoxy-pyridazine, and sulfabromomethazine. Sulfabromomethazine has not been manufactured for many years (5). The tolerance for sulfaethoxy-pyridazine in milk is zero (1). Sulfamerazine, as sulfamethazine, is not approved for use with lactating animals (1).

Specificity of ASMZ Method

Aromatic primary amines are the only class of compounds that can be diazotized and coupled under the conditions used. They would be removed by the Dowex 50 cation exchanger in the H^+ form and thus cannot interfere. Moreover, if any aromatic primary amine should pass through the Dowex 50–alumina bed, it would have to possess an acidic functional group of the proper strength to exchange onto the pH 7.9 anion-exchange resin. These facts make the likelihood of interference by an aromatic primary amine very remote. However, interference by an *N*-acetylated aromatic amino acid might be anticipated.

All of the sulfa drugs listed above that were adsorbed by Chromosorb 102 were completely exchanged (from water) onto the Dowex 50 resin when approximately 200 ng of each was tested separately. More extensive studies on removal of SMZ by Dowex 50 were conducted. SMZ in amounts ranging from a few parts per billion to 0.7 ppm was completely removed from water or milk by the resin.

Quantitative Aspects

Although the primary intention was to develop a screening (yes-or-no) procedure, a limited study of the quantitative aspects of the method was also undertaken. Spiking of 100 ng amounts of SMZ into 3.5 mL milk gave recoveries averaging 80–90%. Quantitation was done spectrophotometrically at

540 nm on a 1 mL solution of the dye. Higher concentrations (e.g., 200 ng SMZ/3.5 mL milk) gave lower (60–70%) recoveries. This suggests that at some step in the isolation scheme one or more of the beds were being overloaded. Concentrations of SMZ in milk as high as 100 ng/3.5 mL (approximately 29 ppb) were not anticipated to be encountered, on the basis of literature values and our analysis of milk samples screened up to that point. As a consequence, the lower recoveries obtained with the 200 ng level of SMZ were of little concern and even indirectly suggested that concentrations of SMZ lower than approximately 29 ppb might give recoveries higher than 80–90%, although this possibility was not investigated. Estimating the very low amounts of SMZ isolated from milk was considered more important than obtaining higher recoveries. To this end, increments of SMZ from zero to 10 ppb were spiked into SMZ-free milk, and the resulting rings of dye on the concentrating columns were observed. Experience gained this way allowed estimation of SMZ concentration with good accuracy, which was confirmed by comparing results with those obtained by a quantitative thin-layer chromatographic method (5). Several attempts to prepare artificially colored ring standards on the color-concentrating bed to simulate the dye rings were unsuccessful. Limited quantitative studies on ASMZ were also conducted. Spiking 200 ng amounts of ASMZ in 3.5 mL water or milk gave recoveries of approximately 85%.

Application to Market Milk

In the initial study (June 1988 to March 1989), the SMZ screen was applied to 187 samples of homogenized or skim milk purchased at market outlets in the Philadelphia, PA, area. Of the 187 samples, SMZ was detected in 91 (48%). Of the 91 positives, 38 (42%) were estimated to contain SMZ at ≤ 1 ppb. Only one sample was estimated to contain SMZ at ≥ 10 ppb. Twelve samples (13% of positives) were judged to have SMZ at >5 to <10 ppb, and the remainder (45% of positives) had SMZ at 1–5 ppb.

The SMZ screen was applied also to other dairy products in the initial study. Skim milk powder (11 samples) and evaporated whole and skim milk (11 samples) were purchased locally. The powdered skim milk samples were reconstituted to the normal (9%) solids-not-fat content of fluid skim milk. The evaporated milk samples were diluted 1:1 with water. Eight (73%) of the powders screened positive for SMZ. Three (27%) contained SMZ at 1 ppb, 2 had SMZ at 2–3 ppb, one had SMZ at 3–5 ppb, and the other had SMZ at 5–10 ppb. In the evaporated milks, 4 (36%) were positive. Three of these had SMZ at ≤ 1 ppb, and the other had SMZ at ≥ 10 ppb.

Infant formulas containing milk constituents (3 samples) were screened and were negative.

Toward the end of the initial screening study (March 1989) for SMZ in milk, the screening method for ASMZ was perfected, and 74 new samples of milk were then screened simul-

taneously for SMZ and ASMZ. Forty-five samples (61%) were positive for SMZ. Of these, 32 (71%) contained SMZ at ≤ 1 ppb and no samples had SMZ at >5 ppb. For ASMZ, 59 samples (80%) screened positive. All but 4 samples had ASMZ at ≥ 1 ppb and these had 1–3 ppb.

In the most recent study of SMZ and ASMZ in market milk (June to September 1993), 33 samples were tested. Seven (21%) contained SMZ, and 18 (54%) were positive for ASMZ. No milk had >2 ppb of either the drug or its metabolite, and the majority had <0.5 ppb of SMZ or ASMZ.

The screening method for ASMZ is the first that we know of for any metabolite of SMZ. The presence of ASMZ in milk is unequivocal evidence that SMZ was used on the cow even when SMZ cannot be detected. For example, 16 and 12 milks that tested negative for SMZ in the initial and recent studies, respectively, were positive for ASMZ.

The milk from a cow treated with a single dose (1 g/1400 lb) of SMZ given as a bolus was screened over a 132 h period after administration. Estimates (in ppb) of the concentration of SMZ in her milk were as follows: 12 h, 100–200; 36 h, ≥ 10 ; 84 h, ≤ 0.5 ; 132 h, not detected. The dose given to this cow was relatively low. It is possible that cows given higher doses might secrete the drug into their milk for longer periods.

Twelve samples of milk screened simultaneously could be brought to the point of applying the solution to the color concentration step in 3–3.5 h. An analyst may be able to screen 24 samples in a working day because the color-concentrating step can be run overnight.

The screening procedures normally run smoothly. Occasionally, flow in the pipet tip may stop because of an air bubble. Finger pressure applied to the tip usually is sufficient to restart flow. If not, the bubble can be eliminated by gently stirring the bed.

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