Confirmation of Widespread Sulfonamide Contamination in Northeast U.S. Market Milk

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ABSTRACT

Seventy percent of "off the shelf" and tanker milk tested in the northeast U.S. was contaminated with sulfonamide at above 5 ppb with 35% having more than 25 ppb (equivalent sulfamethazine). In this study milk was screened with an A.O.A.C. receptor assay and with a newly developed growth inhibition assay for sulfonamides with sensitivity of 10-20 ppb. Sulfamethazine was found as the major sulfonamide contaminant but not the only one as determined by high pressure liquid chromatography and mass spectrometry. An incurred residue study with long acting sulfamethazine boluses showed residue persisting in milk above 25 ppb after 7 d. Sulfonamides are readily available as "over the counter" drugs in agricultural stores and are frequently used for treatment by veterinarians. The regulatory disc assay which is insensitive to sulfonamides failed to protect the milk supply in the Northeast against sulfonamide contamination.

Sulfonamides, a therapeutically important group of antimicrobial drugs, are widely used to treat and prevent acute systemic and skin infections in farm animals. They are often used as a food supplement in combination with other antimicrobials or in drinking water to promote growth. Although sulfonamides are available without prescription, they are not supposed to be used with lactating dairy animals except with the exception of sulfadimethoxine, sulfabromomethazine, and sulfaethoxypyridazine (7). The latter drug has zero tolerance and the former two have 10 ppb tolerance. About 3.4% of the population is known to be allergic to sulfonamides with incidence of sensitivity similar to those found with penicillin-type drugs (2). Sulfonamides also show abnormal mitogenic activity in tissue culture (1). Dapsone, a closely related p-aminobenzoic acid (PABA) analog, is suspected of being a carcinogen (11). A recent preliminary report of the FDA suggests that sulfamethazine is carcinogenic (9). Sulfamethazine has been used commonly by veterinarians to treat dairy animals after calving.

Recent surveys noted a widespread contamination of milk with sulfonamides and other drugs (3,8,17). These studies used the radio receptor assay, which can detect seven families of antibiotics/antimicrobials and is a First Action A.O.A.C. method (5,6). In this study, in addition to the receptor assay, a newly developed microbial inhibition assay for sulfonamides sensitive to 10-25 ppb was used. Both high pressure liquid chromatography (HPLC) and mass spectrometry were used to confirm and identify contaminating molecular species.

The B. stearothermophilus disc assay method used by regulatory agencies to monitor antimicrobial drugs in milk is insensitive to sulfonamides as it is for certain other drugs (12). This together with the facts that sulfonamides are available to producers without prescription and are used commonly by veterinarians could generate conditions for widespread contamination.

MATERIALS AND METHODS

Milk sampling

Frozen raw milk samples were obtained from dairies and included milk from producers from six New England States, New York, and Pennsylvania. Milk samples were assayed for sulfonamides using both the receptor and inhibition assays within 1 week of being collected and frozen at the dairy. Fresh milk was purchased from local markets and assayed within 1 d of purchase. Portions were frozen for later assay using a microbial inhibition assay, HPLC, and mass spectrometry.

Inhibition assay

B. stearothermophilus, ATCC 10149, was cultured in Mueller Hinton Broth (MHB) (Difco), containing 100 ppb trimethoprim (TMP), (a stock solution 1 mg/1 in ethanol is stable for 1 month at 4°C). The cells were grown for 6 h at 60°C and harvested at mid log phase, (0.7 O.D. 585 nm) or about 10⁸ CFU/ml. Either frozen or lyophilized, the cells were stored at -20°C for up to 1 month.

Mueller Hinton agar was prepared containing 100 ppb TMP and 6 ppm bromocresolpurple (BCP). Half the tubes had 1 mg/1 PABA for specific reversibility of sulfa inhibitor. Volumes of 1 ml were dispensed into 13x100 test tubes and autoclaved. Medium prepared this way could be stored at -20°C for up to 1 month. Agar was melted before use by heating to 90°C.

The pH of milk samples was adjusted to pH 7.4 with 5M NaOH, sterilized for 20 min at 121°C and the pH readjusted to 6.8. Frozen or reconstituted lyophilized cells were held at 25°C
in MHB for 15 to 30 min. Reconstituted cells were diluted to about 3000 CFU/ml for use as the assay inoculum. The dilution was stable for up to 1 week. One ml inoculum was mixed with 9 ml sterile milk. One ml of inoculated milk was added to each tube containing assay medium. Capped tubes were incubated for 18 h at 64°C. Inhibition assays were performed in triplicate.

A change of BCP in the agar from purple to yellow indicated growth, while no change from purple indicated inhibition. Growth in PABA containing tubes and inhibition of growth in tubes without PABA indicated presence of sulfonamide (16). The minimum sensitivity of the assay was 10 ppb for sulfadimethoxine and 25 ppb for sulfamethazine (see Table 1).

Receptor assay
The receptor assay for sulfonamides was published in detail elsewhere (5).

Two hundred μl H₂ sulfamethazine reagent were added to a 13x100 mm test tube. Five ml milk and 200 μl Reagent R (a microorganism containing receptor sites for sulfonamides) were added, mixed on shaker, and incubated 5 min in a dry well heater at 45°C. The tube was centrifuged 3 min at 1200g to pellet Reagent R, and milk decanted. Three hundred μl H₂O were added to resuspend the pellet, and 3 ml "Optifluor" scintillation fluid added. The tube was mixed and the radioactivity counted 1 min in a scintillation counter. Measurements were correlated with standard sulfamethazine standards. Assays were performed in triplicate.

Standards
Zero milk standard (ZMS). Pasteurized or raw milk was passed through a charcoal column (6) to remove any antibiotic/antimicrobial residues. The ZMS was prepared using high temperature short time (HTST) sterilization, 160°C, 0.02 s.

Positive sulfonamide standards. Dried standard sulfonamides were obtained from U.S. Pharmacopeial Convention Inc., Rockville, MD 20852. Dilutions were made with the zero milk standard.

Extraction and chromatography

High Performance Liquid Chromatography (HPLC)
All solvents and buffer used were HPLC grade. Ten ml lyophilized milk were extracted with 100 ml ace tone-methanol (85:10). Sulfonamides were purified on an anionic resin MP-1, 1 gm, (Bio-Rad) pre-equilibrated with 0.2 M phosphate buffer pH 7.8. The resin was eluted with acetonitrile 10mM ammonium acetate buffer pH 4.6 (1:3) which was also used for the HPLC. A reverse phase column (Lichrosorb RP8) with isocratic elution was used to separate six common sulfonamides, (18). Positives were identified using Waters 990 photodiode array detector to monitor the spectrum of the peaks and they were confirmed with a library of UV spectra. Sensitivity of assay was about 20 ppb for individual sulfonamides.

Gas Chromatography - Mass Spectrometry (GC-MS)
The eluted HPLC peak was dried and derivitized with diazomethane (Diazald diazomethane generator, No. 210, Aldrich Chemical Co.) according to manufacturer’s instructions. The MS was done using capillary GC column DD-5 fused silica combined with multiple ion detector Finnigan 4500 mass spectrometry using the method described by Simpson (15) with monitored Ions m/z 92, 227, and 228 for the confirmation of sulfamethazine (10).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Receptor assay</th>
<th>Inhibition assay</th>
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<tbody>
<tr>
<td></td>
<td>Growth without PABA (CPM) ± 10%</td>
<td>Growth with PABA</td>
</tr>
<tr>
<td>T-21</td>
<td>3700</td>
<td>+</td>
</tr>
<tr>
<td>T-22</td>
<td>1066</td>
<td>-</td>
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<tr>
<td>T-24</td>
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<td>1031</td>
<td>-</td>
</tr>
<tr>
<td>T-19R</td>
<td>4177</td>
<td>+</td>
</tr>
<tr>
<td>T-24</td>
<td>1015</td>
<td>-</td>
</tr>
<tr>
<td>T-11</td>
<td>3893</td>
<td>+</td>
</tr>
<tr>
<td>T-18F/T-18R</td>
<td>870</td>
<td>-</td>
</tr>
<tr>
<td>T-9C/T-14W</td>
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<tr>
<td>12W</td>
<td>928</td>
<td>-</td>
</tr>
<tr>
<td>Supermarket</td>
<td>790</td>
<td>-</td>
</tr>
<tr>
<td>December 5</td>
<td>1050</td>
<td>-</td>
</tr>
<tr>
<td>November 30</td>
<td>850</td>
<td>+</td>
</tr>
<tr>
<td>Pennsylvania silo sample</td>
<td>650</td>
<td>+</td>
</tr>
</tbody>
</table>

Sulfonamides standards
Sulfamethazine - SMZ
Sulfadimethoxine - SDM
Zero
SDM 5 ppb: 5829, 5545, 5902, +
SMZ 5 ppb: 3054, 3236, 3111, +
SMZ 10 ppb: 2118, 2179, 2259, +
SDM 10 ppb: 1179, 1218, 1237, +
SMZ 25 ppb: 1201, 1241, 1270, +
SDM 25 ppb: 887, 892, 1051, +
SMZ 50 ppb: 778, 801, 809, +
SMZ 25000 ppb: 492, 476, --

*Inhibition assay was done without dilution and was not reversed by PABA because of high sulf± in sample.

*Inhibition assay not sensitive to sulfamethazine less than 20 ppb without concentration of milk sample.

*For sulfonamide concentrations greater than 400 ppb, the PABA concentration was insufficient to reverse inhibition.
INCURRED RESIDUE STUDY FOR LONG ACTING SULFAMETHAZINE BOLUS

Six late lactating dairy cows with no history of recent antibiotic therapy were divided into a treatment group (four cows) and control group (two cows). All six cows were sampled before the study to establish the zero control background (see Table 3). Treated cows received “Span Bolet II” sustained-released tablets orally at the rate of 1 bolus (27 g of sulfamethazine) per 70 kg body weight, (Norden Laboratories Inc., Lincoln, NE 68501). Composite milk samples from all four quarters were collected from each cow prior to treatment and at each milking thereafter for a period of 10 d. Milk samples for assay were frozen immediately following collection (4).

RESULTS AND DISCUSSION

During the Spring and Fall of 1987, of 120 samples of milk screened with receptor and with growth inhibition assays, 29% had less than 5 ppb, 36% had between 5 and 25 ppb, and 36% had 25 ppb or more sulfonamide contamination (see Figure 1).

Seventeen samples shown in Table 1 were representative of the 120 samples in the study. Of these 17 samples from dairies and markets, 13 showed positive at 25 ppb and higher using the receptor assay and the growth inhibition tests (see Table 2). Inhibition reversed by PABA indicated the presence of sulfonamide or other PABA analog (12,16). The receptor assay detected PABA as well as sulfonamides and other PABA analogs (5). The agreement between the inhibition and receptor assays confirmed that PABA was not causing receptor assay false positives. In addition sulfonamides were the only PABA analog that had a direct path into the milk supply. The sensitivity of the inhibition method described here was about 20 ppb while similar inhibition methods for sulfonamides were limited to 100 - 200 ppb (12). Thus only those samples in Figure 1 with more than 20 ppb sulfamethazine were confirmed positive with the inhibition method.

One sample (farm tank) showed 15,000 to 20,000 ppb (1/26/88, Table 2 and Table 3). This milk was from a New York herd treated for pneumonia.

| TABLE 2. Confirmation of sulfamethazine in positive milk samples by HPLC. Milk from tankers, “off the shelf and the incurred residue study (Tables 1, 3) that gave positive results with both the receptor and inhibition assays were lyophilized, extracted, and analyzed with HPLC at 275 nm. |
|------------------|------------------|------------------|---------------|
| Sample           | Radio receptor assay | Sulfamethazine by HPLC area (RT = 4.80 min) | |
|                  | Dilution          | CPM              | Estimated PPB | Au/oMIN (10-3) | PPB   |
| COW A*           |                  |                  |               |               |
| Pre Admin.       | None             | 5622             | 0             | 0.34           | No Peak |
| Day 1            | 1:1000           | 1276             | 2500          | 424.50         | 42200  |
| Day 2            | 1:1000           | 1220             | 2500          | 209.40         | 20820  |
| Day 3            | 1:1000           | 2009             | 1200          | 102.40         | 10180  |
| Day 4            | 1:100            | 1753             | 1500          | 11.40          | 1130   |
| Day 5            | 1:10             | 2000             | 120           | 0.90           | 90     |
| Day 6            | None             | 1489             | 15            | 0.30           | No Peak |
| Sulfamethazine 50 ppb | None         | 800              | --            | 0.38           | --     |
| Zero milk        | None             | 5700             | 0             | 0.28           | 0      |
| Pennsylvania silo sample | None | 650             | 50            | 0.36           | 10     |
| Supermarket December 5th | None | 790             | 40            | 0.44           | 30     |
| Supermarket January 9th | None | 1300            | 20            | 0.13           | 10.2   |
| New York farm tank       | 1:1000          | 1400             | 15000 to 20000 | 160.50         | 16200  |

*Cow A (See Table 3).
bSample was confirmed by GC-MS to contain 30 ppb sulfamethazine (See Fig. 4).
Sample contained a second HPLC peak identified as sulfapyridine.
HPLC identified six common sulfonamides. Selected samples from an incurred residue study as well as positive raw silo milk and market milk subjected to HPLC analysis showed peaks that correspond with underivatized sulfamethazine (see Table 2, and Fig. 2).

In addition, the contamination in one supermarket sample (Dec. 5, 1987, Table 2) with 40 ppb by receptor assay was identified as sulfamethazine by HPLC (Table 2, Fig. 2 and 3). This sample, subjected to confirmation of sulfamethazine by mass spectrometry, was found to contain 30 ppb sulfamethazine (see Fig. 4). A second market sample, January 9, was found to contain 15 ppb sulfamethazine and a second smaller peak with a retention time and UV spectrum similar to sulfapyridine (see Table 2 and Fig. 2). The receptor assay showed the sample contained about 30 ppb sulfonamide. Two different sulfonamides were detected in this case.

Figure 2. Separation of six sulfonamide standards by HPLC. Eluate was continuously monitored by UV absorption at 275 nm. The peaks are: #1. sample injection, #2. p-aminobenzoic acid, #3. sulfapyridine, #4. sulfamethazine, #5. unknown, #6. sulfisoxazole, #7. sulfamethoxazole, #8. sulfadimethoxine.

Figure 3. Detection of sulfamethazine in "off the shelf" milk by HPLC. Milk sample from December 5, 1987, (see Table 2) was extracted and analyzed on HPLC as described in methods. The peaks are: #1. sample injection, #2. peak has the same retention time as authentic standard sulfamethazine, #3. unknown.

Figure 4. Confirmation of sulfamethazine in "off the shelf" milk by GC-MS. Market sample from December 5, 1987, was screened at 40 ppb by receptor assay and 30 ppb by HPLC (see Table 2) shows sulfamethazine peak on the GC and was confirmed as sulfamethazine by the MS multi ion detector. (Standards sulfamethazine was eluted at 3270 Scan on the GC. Ions elz of 92, 227, and 228 are monitored by mass spectrometry.)

Mass spectrometry was the method used by FDA to identify contaminating molecular species in confirming a CFR violation. The December 5 market milk was a confirmed violation of the CFR since there was no tolerance for sulfamethazine (7).

The incurred residue study employing two control cows and four cows with long acting sulfamethazine boluses (Span Bolet II) showed positive milk after 6 d at the 15 to 30 ppb level (Tables 3 and 4). All six cows were sampled before the study to establish the zero control background (see pre-admin. in Table 3). Although cows E and F were zero controls there appeared to be a fluctuation between 0 and 5 ppb. The reason for this was not known but may be due to a metabolite that interferes with the receptor assay or sulfonamide contamination in feed. The PABA at 0.1 ppb gives a false positive equivalent to 3 ppb sulfamethazine. Feed containing yeast could be a source of PABA. The growth inhibition assay, HPLC, and mass spectrometry showed that above 5 ppb the receptor assay accurately measured sulfonamides in milk and was not measuring PABA. The sensitivity of these confirming methods was increased to measure 5 ppb by concentrating the milk samples with freeze drying.

A recent study of 10 cities in the U.S. by FDA using an HPLC method confirmed sulfonamide contamination primarily in the Northeast and Northwest. Seattle had the worst combination with Boston second and New York City third worst (9). Of 49 samples tested, 36 were contaminated with sulfonamide. In contrast, 1000 tanker samples of milk tested on Prince Edward Island, Canada showed no sulfonamide contamination. Sulfonamides were not used "over the counter" on Prince Edward Island (13).

It is concluded that the disc assay required by the Pasteurized Milk Ordinance (14) for monitoring antibiotics in
This requires a regulatory test that detects sulfonamides in plants. The screening of silos and milk tankers at processing facilities can be achieved by removing sulfonamide drugs from use, or in the case of milk, not protecting the milk supply from sulfonamide contamination.

Control of sulfonamide contamination in the U.S. may be achieved by removing sulfonamide drugs from use, or controlling use of the way beta lactam drugs are controlled. This requires a regulatory test that detects sulfonamides and the screening of silos and milk tankers at processing plants.

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**REFERENCES**