

Evaluation of Commercial Immunochemical Assays for Detection of Sulfamethazine in Milk

ABSTRACT

Sulfamethazine (SMZ) is effective in the treatment of bacterial infections in food producing animals but its use is prohibited in dairy cows. Nevertheless, a 1988 survey of milk in ten cities conducted by the Food and Drug Administration revealed the presence of SMZ. Therefore, it was apparent that there was a need for rapid screening methods for SMZ. We evaluated commercial immunochemical test kits for SMZ with detectabilities of 1-10 parts per billion (ppb). Manipulations are suggested to effectively optimize immunochemical detection of SMZ in raw and processed fluid milk. The performances of the enzyme immunochemical test kits were evaluated by studying the effects of sample preparation, sample matrix, calibration and detection range of the kits using raw and processed milk samples. Immunochemical results were compared to quantitative high performance thin layer chromatography and high performance liquid chromatography with electrochemical detection. Both chromatographic methods had detectabilities in the low parts per billion range.

In 1988, the detection of sulfamethazine (SMZ) in market fluid milk (1-4) alarmed the dairy industry. SMZ levels in milk dropped in milk from 1988 to 1990 (5) and a recent survey (6) in the first quarter of 1991 showed no SMZ residues in milk at 5 ppb (5 ng/ml) limits of detection. Sulfamethazine is not approved for use in lactating dairy cattle but was available to farmers for treatment of bacterial infections in cattle, swine, sheep, and poultry. Since the consumers and the dairy industry are sensitive to residue issues, our role was to assess existing commercial immunochemical assays to determine if they can be used on the farm or at the plant to screen for the presence of SMZ in milk at the 10 ppb (10 ng/ml) level. In our study, milk from an experimentally dosed lactating cow and processed fluid milk purchased from local markets were analyzed for SMZ. In an initial 4-month study, from May until August 1988,

three immunochemical test kits were evaluated and results were compared to those obtained from quantitative high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) with electrochemical detection (13). In late 1988 and early 1989, two more immunochemical kits were marketed and were also evaluated in our laboratory. This study presents the results and describes conditions that were required for optimum detection of sulfamethazine in raw and processed milk.

MATERIALS AND METHODS

Sample collection and preparation

Raw milk was obtained from two lactating cows which had been given single oral doses of sulfamethazine (1.5 mg/kg) with a balling gun. Milk samples were collected at 12 h before treatment and at 12, 36, and 60 h post-treatment in the first study. In the second study, 84- and 156-h samples were also collected. These treatments were carried out at the Food Animal Protection Laboratory, Agricultural Research Service, USDA, at College Station, Texas. Raw milk samples were analyzed in Texas using Signal ELISA (SE) immediately after being collected and were analyzed in Philadelphia within 5 d of sampling. Fresh raw milk samples were also obtained from untreated cows from Delaware Valley College (Doylestown, PA) and were used as control samples at the duration of these studies. Commercial shelf milks (whole milk, low fat milk and skim milk) samples were purchased from local markets in the Greater Philadelphia area from May 1988 through June 1989 and sparingly in October 1990.

Immunochemical assays

In an initial 4-month study, these milk samples were analyzed in our laboratory by enzyme immunoassays such as: E-Z Quik Card (QC) from Environmental Diagnostics, Burlington, North Carolina, Signal ELISA (SE) from Smith Kline Animal Products, Westchester, Pennsylvania and Cite Cup (CC) from Idexx, Portland, Maine. The raw milk samples with incurred sulfamethazine were sent to Idetek (San Bruno, CA) and Neogen (St. Louis, MO) and were analyzed by LacTek (LT) and Agri-Screen (AS) ELISA techniques. These commercial immunochemical test kits are based on the principle of solid phase enzyme immunoassay (Fig. 1). These materials were configured and supplied by the test manufacturers. The typical procedure are shown in Fig. 1 and 2. Sufficient binding time of 3 to 20 min (at room temperature, 20°C) allowed SMZ-peroxidase and free SMZ to

compete with the limited amount of binding sites on the antibody. After washing-out the excess sample and reagents, either TMB (3,3', 5,5'-tetramethylbenzidine) or ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate was added. Chromogens formed and their color intensities were visually compared to a standard control. A positive or negative response is determined by visual observation 5-15 min (at room temperature) after addition of substrate. By using a calibration standard (1-10 ppb with Signal ELISA and LacTek, 10-150 ppb with Agri-Screen), ELISA kits were used quantitatively for analysis of SMZ in the milk samples. SMZ calibration standard was obtained from Sigma (St. Louis, MO). ELISA Reader (Dynatech MR650, Alexandria, VA) was used with SE and AS kits while an Idetek photometer was utilized for LT kit. Amounts of SMZ were determined from linear plots of B_0/B vs SMZ concentration (ppb) where B_0 is the absorbance of control milk with "0" SMZ and B is the absorbance of the sample at 630 or 405 nm. We have previously reported that results from linear plots were not significantly different to results obtained from hyperbolic curves (7).

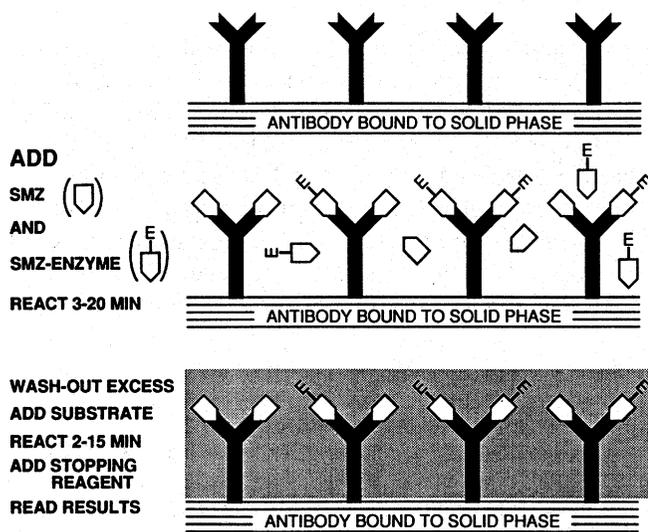


Figure 1. Schematic diagram of solid phase enzyme immunochemical assay principle.

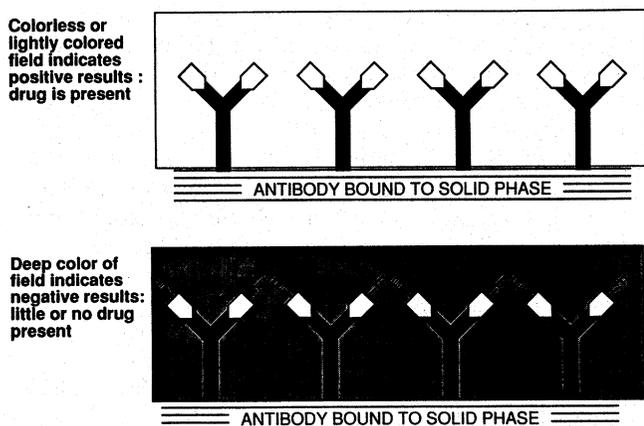


Figure 2. Interpretation of immunochemical results.

Sample preparation

Raw milk was analyzed directly with the SE, CC, and LT enzyme immunochemical assays. Agri-Screen ELISA test was used as specified by the manufacturer for sample preparation. Pretreatment involved precipitation and centrifugation of raw milk samples. However, particulate materials in milk clogged the glass fiber support of the QC and could not be used for direct analysis of raw milk. Therefore, effects of various treatments on detection

of incurred SMZ were compared using the QC and SE. Such treatments were as follows: a) freezing milk and thawing followed by warming at 37°C for 15 min and centrifugation at 4,000 or 6,000 x g for 15 min; b) warming at 37°C for 15 min followed by centrifugation at 1,000, 6,000, or 13,000 x g for 15 min using a table top microcentrifuge (Model mv 13, Hill Scientific, Derby, CT); and c) precipitation with 1 M ammonium sulfate (1.3 g/10 ml milk) followed by centrifugation at 500 or 1000 x g (#4 or #6 setting) using a table top Model CL clinical centrifuge (International Equipment Centrifuge, Needham, MA).

Homogenized and pasteurized whole, low fat and skim milks were analyzed without further treatment by SE, CC, and LT tests. Tween 20 (2 drops/25 ml milk) was added to milk prior to analysis by QC to facilitate wetting of the glass fiber. The manufacturer's directions were followed with the use of AS.

Assay confirmation

Quantification of sulfamethazine was also carried out with methods developed in our laboratory (5) which included HPTLC and high performance liquid chromatography equipped with oxidative electrochemical detection (HPLC-EC). A 3-column solid phase clean-up procedure utilizing C-18, acid alumina and ion-exchange (buffered at pH 7.9) columns was used in both procedures. HPLC and HPTLC were utilized to validate accuracies of the immunoassays. Standard statistical tests (standard deviations, coefficient of variability, and Student's t test) were utilized to determine precision of assays.

RESULTS AND DISCUSSION

Detection levels of enzyme immunoassay kits

The minimum detectable amounts of SMZ measured by the kits shown in Table 1 were determined by analyzing varying concentrations of SMZ (fortified) in raw or processed milk. The minimum detectable amounts were determined by comparing color to zero control and color of sample containing 1.5-2X concentration of zero detectability. Positive results were indicated by no color or light color formation when compared to standard controls provided by the manufacturers for the screening technique. SE and QC had optimal detectability of 5 ppb when used for qualitative screening tests. LT and AS kits utilized 2 and 10 ppb SMZ concentrations, respectively, as reference standards. Positive results were indicated by samples yielding lighter color than the reference standard. The CC test had a built-in standard at 10 ppb concentration of SMZ giving negative results for milk containing <10 ppb SMZ. CC was calibrated against 10 ppb of incurred parent SMZ in milk of treated infected cows while other test kits were calibrated against fortified amounts of SMZ. Other immunochemical tests would detect 2-3 times less the amount of SMZ compared to the CC test as antigenic metabolites in milk from infected cows treated with SMZ can also bind with the antibody utilized in above kits. The ELISA assays can be used quantitatively using a 4-5 point standard curve with one standard point exceeding the maximum binding concentration. SE and LT were most reliable at the 2-5 ppb range while AS was most reliable above 10 ppb.

Better precision and accuracy were also observed with quantitative use of SE or LT when sample dilution was less than 1:5 yielding less than 5 ppb SMZ per well or per tube. Underestimation of measured SMZ occurs if samples analyzed contain greater than 5 ppb per well. Samples contain-

TABLE 1. Minimum detection levels of sulfamethazine by immunochemical and chromatographic analyses

| | | Visual (ppb) ¹ | Instrumental ² (ppb) |
|-------------------|------|---------------------------|---------------------------------|
| Signal ELISA™ | (SE) | ≈ 5 | <1 |
| Quik Card™ | (QC) | ≈ 5 | |
| Cite™ | (CC) | ≈ 10 | |
| LacTek™ | (LT) | ≈ 2 | <1 |
| Agri-Screen™ | (AS) | ≈ 10 | <5 |
| HPLC ³ | (8) | | <1 |
| TLC ³ | (8) | | <1 |

¹ Parts per billion (ppb) = ng/ml = pg/μl.

² ELISA Reader used with SE and AS. Photometer used with LT.

³ HPLC and TLC were equipped with electrochemical and fluorescent detectors, respectively. Immunochemical results are based on summaries of all tests performed in this laboratory.

ing greater than 25 ppb (>5 x dilution of samples) are better analyzed quantitatively by AS which has a wider range of calibration standard concentration (e.g., 0-150 ppb) due to a higher antibody capacity compared to 0-5 ppb with SE or LT. Quantitative analysis of samples with >5 ppb amounts is at maximum 5 ppb reading unless diluted and analyzed again. These kits were designed for visual positive/negative responses except that AS has a quantitative line in addition to visually evaluated kits. The precision and accuracy of these kits were evaluated quantitatively where instrumental analysis was applicable.

Sample preparation

Pretreatment of raw milk was necessary to eliminate clogging of the fiber glass supports of the QC wells and the solid phase columns used for sample clean-up prior to analysis by thin layer chromatography (TLC) and HPLC. This blockage can be attributed to presence of particulate materials (somatic and microbial cells, colloidal, or micellar proteins) in unprocessed milk. Casein micelles and fat globules in raw milk can also aggregate at the surfaces of the support, thus inhibiting flow of the sample. Such aggregation is prominent in milk samples stored below 0°C. The use of the QC was possible after warming the milk at 37°C and centrifuging at 4,000, 6,000, or 13,000 x g or freeze-thaw cycle and centrifugation which showed similar results at all levels of centrifugation. The skim fraction of raw milk precipitated with 1.5 M ammonium sulfate (2 g/10 ml) and centrifuged in a clinical centrifuge (approximately 1000 x g) showed more intense blue color (indicating more negative results) than samples warmed at 37°C and centrifuged at 6000 or 13,000 x g. This suggests that borderline samples (containing >10 and <20 ppb) can give false-negative response to an untrained person (e.g., farmer, bulk tank driver, etc.) when ammonium sulfate is used for pretreatment. Therefore, tests can not be reliably used for "on-site" detection of low level of SMZ. Precipitation of 16 fortified milk and raw milk samples containing incurred SMZ (36 and 60 h) in Study I and II showed that 5 and 10 ppb fortified milk had equal intensity of blue color but darker than 10 ppb SMZ in water. The 15 ppb fortified milk also exhibited more blue color than 10 ppb SMZ in water

but lighter than control milk with "0" SMZ, indicating positive result of <10 ppb. The 36- and 60-h samples showed equal and darker color than samples warmed at 37°C and centrifuged at 13,000 x g indicating interference from whole milk components. In a laboratory setting this centrifugation is desirable. Prewarming of milk at 37°C or pasteurization at 145°C (30 min) followed by centrifugation at 13,000 x g showed no difference in detectability between samples. HPLC analysis confirmed these observations. However, after the conclusion of this study, QC was modified with a prefilter for raw milk resulting from our suggestions. A reminder to those not familiar with indirect enzyme immunoassay that color formation indicates a negative result and absence of color is indicative of positive result.

Using signal ELISA, various sample preparation techniques were compared using SMZ containing raw milk ("incurred SMZ") taken 12 and 36 h after SMZ medication. Results in Table 2 showed that the 12-h sample, which contained a high level of SMZ, showed variability of 25-35% when samples were subjected to the same sample preparation treatment and only 11-27% in the 36 h sample which contained a lower amount of SMZ. These variabilities were greater when compared to the 14-16% variabilities among different sample preparation techniques. Statistical analysis using Student's t test on the mean SMZ values from these four sample preparation treatments showed that the confidence limits at 95% were 152 ± 40.0 and 15.2 ± 3.5 for the 12- and 36-h samples, respectively. The mean SMZ results from these four treatments were not significantly different in the 12- and 36-h samples. However, results (mean of duplicates) from separate assays showed that 29% of the 12-h and 10% of the 36-h samples were outside the confidence intervals of 95%. This observation led us to study the variabilities in antibody binding capacities shown by the absorbance values of antibody coated wells from the same lot and wells from different lots of the ELISA kits.

TABLE 2. Effects of sample preparation on SMZ concentration in raw milk¹ measured by Signal ELISA (SE).

| Time after treatment (H) | | SMZ amounts (ppb) | | | | X Means (ppb) |
|--------------------------|----------------|-------------------|--------------------------------------|-------------------------------|------------------------------------------------------------------------|---------------|
| | | Untreated | Pasteurized centrifuged (13,000 x g) | 37°C centrifuged (13,000 x g) | (NH ₄) ₂ SO ₄ centrifuged (1000 x g) | |
| 12 | n ² | 4 | 2 | 3 | 5 | 4 |
| | x | 123 | 170 | 157 | 158 | 152 |
| | SD | 41 | 57 | 55 | 42 | 49 |
| | CV | 33% | 33% | 35% | 27% | 32% |
| | | | | | | |
| 36 | n | 2 | 2 | 2 | 4 | 4 |
| | x | 16.5 | 18.5 | 13.5 | 12.25 | 15.2 |
| | SD | 2.1 | 4.9 | 1.5 | 3.1 | 2.2 |
| | CV | 13% | 27% | 11% | 24% | 14% |
| | | | | | | |

¹ Study II.

n² = number of samples analyzed in duplicate; ppb = ng/ml; x = mean; SD = standard deviation and CV = coefficient of variation (100 SD/x). Zero time samples were obtained 12 h prior to dosing.

Five lots of kits (received in the initial 4-month period of the study) were evaluated using raw milk from an untreated cow. Absorbances of control samples without SMZ and after fortification with 2.5 ppb were measured using an ELISA reader at 630 nm. Percent variability ($CV: \frac{100s}{x}$) of absorbance values on individual wells within a lot were 9.2, 14.0, and 7% when tested with raw milk without SMZ fortification. Two batches or lots of kits were used to analyze milk after fortification with 2.5 ppb SMZ. Variabilities of 9 and 27% were found when analyzed repeatedly using 3-7 replicates per lot. The mean variability of five lots was 22% at 0 ppb and 19% at the 2.5 ppb levels. These variabilities may be attributed partly to deterioration of antibody or enzyme-conjugate caused by transport or storage of the kits at temperatures above 20°C. Other batches of kits left accidentally at room temperature overnight exhibited significant loss of binding capacity (i.e., >20%). Experiments also showed that SMZ conjugate can lose binding ability with no apparent loss of enzymatic reactivity of SMZ-conjugate enzyme. The latter observation suggests that SMZ may be breaking off from the enzyme conjugate. Released SMZ, in turn, may bind with the antibody coated in wells resulting in decreased binding of SMZ-enzyme conjugate to the antibody coated in wells shown by lower absorbance values due to lower substrate-enzyme interaction. The occurrence of such a phenomenon can lead to false-positive results, especially when kits are used for visual screening purposes. This problem can occur in all enzyme immunochemical kits unless such reagents are treated with stabilizers or chemically stable conjugates are employed. Immunochemical kits should also be packaged in small units to avoid numerous cycles of chilling and warming at room temperature. To ascertain quality of performance of the test kits, manufacturers must recommend an absorbance range for the "zero" control. For quality control purposes, users should reliably use those kits within the recommended absorbance range. In addition, periodic measurements of control standards at 25, 50, and 75% binding of the standard curve should be performed. Reliable results can be attained if variabilities are less than 20%. However, visual users of these test kits cannot use these measurements and must rely on manufacturers' quality control so long as proper temperature handling (in transport and storage) of these test kits are properly adhered to. Results and observations from this study and previous experience in development of immunoassay methods (7,8) led us to develop a short but comprehensive guideline (shown in Table 3) for evaluation of immunoassays which can be helpful to users of commercially available kits. Adherence to these criteria for assessment and quality control of immunochemical kits can be an assurance for reliable results.

SMZ from milk in SMZ-treated cows

In the first study (Table 4a), milk collected 12 h before SMZ administration showed trace levels of SMZ by SE, TLC, and HPLC. Contamination from our laboratory was ruled out and other possible sources of the contamination were not verified. It was reported in the literature (10,11) that SMZ peaked at 6-12 h when dairy cows were dosed

TABLE 3. Criteria for evaluation of test kits.

1. Determine "cut-off" concentration (minimum/maximum) for positive/negative screening test using fortified milk samples and known incurred SMZ.
2. Compare results of manufacturer's reference standard with in-house reference standard.
3. Determine dose concentration range in ELISA assays using an ELISA reader or other optical reader.
4. Determine well-to-well and lot-to-lot variabilities of absorbances at "zero" SMZ and at SMZ concentration equivalent to 50% of absorbance value at "zero" using fortified milk.
5. If absorbance value at "zero" is unusually low (<50% of zero reference standard), determine binding ability of antibody adsorbed to wells or matrices. Also determine binding ability of SMZ enzyme conjugate and lastly, evaluate enzymic activity of conjugate.
6. Determine binding/cross-reactivity of structurally related sulfonamides and major metabolite/s, e.g., N-4-acetyl-SMZ.
7. Determine effects of sample preparation techniques on assay performance.
8. Reproducibility of the assay can be determined by repeated analysis of incurred and fortified SMZ in milk.
9. Accuracy of the assay can be determined by comparison to chromatographic results or to chromatographically validated ELISA results.

orally. In our studies (Tables 4a, 4b), samples collected 12 h after dosing had mean SMZ concentrations of 53 ppb in Study I and 158 (SD=55) ppb in Study II. QC and CC also gave positive results. Thirty and 22 ppb were found by TLC while 24 and 60 ppb were detected by HPLC in Studies I and II, respectively. SMZ concentrations dropped below 10 ppb after 36 h and were detectable in trace amounts (<1 ppb) after 84 h. SE and AS detected trace amounts (less than one ppb) in the 156-h sample and these results can be confirmed by lowering the minimum calibration standard concentration below 1 ppb. The disappearance of SMZ in milk collected 84 h after dosing with a balling gun is similar to observations previously reported (8,10) for oral dosing with SMZ. In Study II, the observed SMZ levels were 2 1/2 times the concentration detected in Study I. This difference can be due to variation in physiology of the animals since the first cow produced twice as much milk during the collection period as the second cow. This study shows an apparent "concentration effect" of the residue and a slower clearance of SMZ in the second cow.

TABLE 4a. SMZ level (ppb) in raw milk from treated cows.¹

| Hours from treatment | Immunochemical methods | | | |
|----------------------|------------------------|--------------|--------------------|----------|
| | Quik Card | Signal ELISA | | Cite |
| | Visual | Visual | Dynatech Reader | Visual |
| -12 | ND ² | ND | TRACE ³ | ND |
| 12 | ++(>>10) | ++(>>10) | 53 | ++(>>10) |
| 36 | +(<5) | +(<5) | 3.4 | ND |
| 60 | ND | ND | TRACE | ND |

¹ Study I.

² ND = Not detectable.

³ Trace equivalent to <1ppb (<1pg/μl).

-12 h = zero time samples.

TABLE 4b. SMZ levels in raw milk from treated cow.¹

| Hours from treatment | Signal ELISA | | | Quick Card | Cite | LacTek ⁵ | Agri-Screen ⁵ |
|----------------------|--------------------|--------------------|--------------------|------------------|------------------|---------------------|--------------------------|
| | Untreated | 37°C 13,000 x g | 37°C 13,000 x g | Untreated Raw | Untreated Raw | Untreated Raw | Untreated Raw |
| | | | | | | | |
| -12 | -(ND) ² | 1 | -(ND) | -(ND) | -(ND) | -(ND) | <5 |
| 12 | ++(>>10) | 123 (41) | 158 (55) | ++(>>10) | ++(>>10) | 78.9 | 100 |
| 36 | +(>10) | 16.5 (2.1) | 13.5 (1.5) | +(>10) | +(>10) | 5.8 | 4.5 |
| 60 | +(<10) | 4.3 (1.7) | NA ⁴ | TRACE | -(ND) | 1.1 | <5 |
| 84 | TRACE ³ | 1.2 (0.2) | NA | -(ND) | -(ND) | NA | <5 |
| 156 | -(ND) | TRACE | NA | -(ND) | -(ND) | -(ND) | <5 |

¹ Study II.² ND = not detectable at sensitivity limits of kit.³ Trace = <1 ppb (<1 pg/μl).⁴ NA = not analyzed.⁵ Analysis performed in Idetek and Neogen Laboratories by their staff.

HPTLC analysis (Table 5) of the 36 h milk (Study II) yielded 11 ppb of SMZ, which was close to the lower limits of detection of 11.7-18.7 ppb by ELISA assay when analyzed with Student's t test 95% confidence limits. However, HPLC (or LC-EC) gave SMZ levels that were 1/3 of the concentrations detected by ELISA in the 12- and 36-h samples which were pretreated by warming samples at 37°C for 15 min and centrifuged at 13,000 x g. This discrepancy may be due to presence of antigenic metabolites in milk from animals medicated with SMZ which also bound with the anti-SMZ. Our tests showed that N₄-acetyl-SMZ metabolite binds to the antibody at same concentration as SMZ. A 100% cross-reactivity with this metabolite was observed in our laboratory. This compound was reported by Nouws and co-workers (9) as one of the major metabolites in bovine milk from cows treated orally or intravenously with SMZ.

SMZ in market fluid milk

Shelf milk has undergone centrifugation, pasteurization, and homogenization processes before it reaches the market and, therefore, analysis of the processed milk by QC required only an addition of a wetting agent (Tween 20). No treatment was necessary for analysis by CC, SE, and LT. Milk analyzed by Agri-Screen was pretreated with a precipitant and subsequently centrifuged before analysis as suggested by the manufacturer. Shelf milks in the Philadelphia area were randomly sampled from May, 1988 through June, 1989 (Table 6). Samples collected from May through June, 1988 contained greater than 10 ppb in 11% of the samples. Thirty percent of the samples screened by SE and QC had less than 10 ppb. Using an ELISA reader for SE, LT, and AS, quantitative concentrations of SMZ in milk (Tables 4a, 4b, and 7) were estimated from linear plots

TABLE 5. Chromatographic analysis of SMZ in raw milk from treated cows.

| Hours from treatment | Study I ¹ | | Study II | |
|----------------------|----------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| | HPTLC 140°C homogenized (ppb) | LC-EC 37°C 13,000 x g (ppb) | HPTLC 37°C 13,000 x g (ppb) | LC-EC 37°C 13,000 x g (ppb) |
| | -12 | TRACE ² | ND ³ | ND |
| 12 | >30 | 24 | 221 | 60 |
| 36 | TRACE | TRACE | 11 | 4 |
| 60 | TRACE | ND | ND | 2 |
| 84 | | | TRACE | NA ⁴ |
| 156 | | | ND | NA |

¹ Milk collected only up to 60 h in Study I.² Trace = < 1 ppb (<1 pg/μl).³ ND = not detectable.⁴ NA = not analyzed.

of absorbance ratios of zero control/unknown samples (B₀/B) vs SMZ concentration (ppb). HPLC and TLC results confirmed the ELISA results. Detectable SMZ levels were below 10 ppb in 65% of the shelf milk samples taken during July and August, 1988 and tested by SE and TLC. Fifty-nine percent of the samples had levels below 10 ppb as determined by HPLC and only 47% were detectable by QC. In this period, all samples showed negative results by CC test which had minimum detectability of 10 ppb. In the period of December, 1988 to January, 1989, Schwartz (9) analyzed 37 milk samples with chromatographic methods using micro columns and reported that 43% of shelf milk had less than 10 ppb but only one sample containing >10 ppb was confirmed by quantitative SE. Again, from March to June, 1989 milk samples were collected and tested by

TABLE 6. Occurrence of SMZ in shelf milk as determined by immunochemical and chromatographic analysis.

| I. May - June 1988: 27 samples | | |
|------------------------------------|--------------|------------------------------------------|
| Amounts (ppb) | % Occurrence | Methods |
| > 10 | 11% | Immunochemical (SE, QC), HPTLC, LC-EC |
| < 10 | 30% | Immunochemical (SE, QC), HPTLC, LC-EC |
| Negative | 59% | Immunochemical (SE, QC), HPTLC, LC-EC |
| II. July - August 1988: 17 samples | | |
| Amounts (ppb) | % Occurrence | Methods |
| > 10 | None | |
| < 10 | 65% | SE |
| | 47% | QC |
| | 59% | HPLC |
| | 65% | HPTLC |
| Negative | 100% | CC |
| III. March - June 1989: 25 samples | | |
| Amounts (ppb) | % Occurrence | Methods |
| > 5 | 4% | SE |
| < 5 | 32% | SE |
| > 2 | 24% | LT |
| Negative | 100% | AS |

¹ Immunochemical results were in agreement with chromatographic results.

SE, LT, and AS. Only the samples that contained detectable amounts of SMZ by SE were analyzed by AS and LT kits due to insufficient numbers of the latter kits. The LT test gave more false-negative results (2 out of 3) than SE when screened visually. Samples analyzed by the AS test showed more intense blue color when milk samples were prepared according to manufacturer's protocol compared to samples analyzed without pretreatment. Therefore, it must be emphasized that standards and samples must be analyzed by precisely following the manufacturer's protocol. In summary (Table 7), SE detected SMZ in 36% of the shelf milks at less than 10 ppb levels. Of these, 89% had less than 5 ppb. Quantitative SE showed trace amounts (<1 ppb) in all other samples. LT analysis showed that 25% of the samples had SMZ levels greater than 2 ppb. Samples with >5 ppb SMZ by either LT or SE tests were also analyzed by TLC showing similar concentrations of SMZ as those found by LT or SE. The AS test yielded negative results in all samples containing <10 ppb SMZ. The SMZ concentration in shelf milks in the Philadelphia area appeared to decrease in concentration over the duration of the studies. Shelf milk sampled in 1990 showed negative results with SE. In later analysis, samples containing >10 ppb SMZ were validated by chromatographic methods as these levels are of interest to United States regulatory agencies and the milk producers.

TABLE 7. Summary analysis of shelf milk for SMZ by immunoassays.¹

| Sample # ² | SE | SE | SE | LT | Sample # | SE | SE | LT | LT |
|-----------------------|--------------|-----------------------|--------------|--------------------------------------|----------|--------------|-----------------------|--------------|-------------------------|
| | Visual (ppb) | Dynatech Reader (ppb) | Visual (ppb) | Idetek Photometer ⁵ (ppb) | | Visual (ppb) | Dynatech Reader (ppb) | Visual (ppb) | Idetek Photometer (ppb) |
| | | | | | | | | NA | 2.0 |
| 46 | +(<5) | 4.2 | +(>2) | >2 | 63 | - | 0.9 | NA | NA |
| 47 | - | 0.9 | - | <2 | 64 | +(<5) | 1.1 | NA | NA |
| 48 | +(<5) | 3.9 | +(>2) | >2 | 66 | - | NA | NA | NA |
| 49 | +(<5) | 3.7 | +(>2) | >2 | 67 | - | NA | NA | NA |
| 50 | - | 0.8 | - | <2 | 68 | - | NA | NA | NA |
| 51 | - | 1.0 | - | <2 | 69 | +(<5) | 1.2 | -(=2) | 4.1 |
| 52 | - | 0.7 | - | <2 | 70 | - | <0.5 | -(=2) | 2.5 |
| 53 | +(<5) | 1.4 | +(>2) | >2 | 71 | - | ND ⁴ | - | ND |
| 54 | - | 0.7 | - | 1.4 | 72 | | 1.1 | (=2) | >5 |
| 55 | +(<5) | NA ³ | - | NA | 75 | +(>5) | >5 | +(>2.5) | >5 |
| 56 | - | NA | + | 3.85 | 74 | - | 0.4 | - | 2.0 |
| 61 | +(<5) | 1.2 | - | NA | 75 | +(>5) | >5 | +(>2.5) | >5 |
| 62 | - | 0.65 | NA | 2.0 | | | | | |

¹ SE = Smith Kline ELISA, LT = LacTek; Agri-Screen as was also used to analyze Sample Nos. 61, 62, 63, 64, 69, and 75. All samples were negative except No. 75 which had >5 and <10 ppb.

² Samples collected March-June, 1989.

³ NA = not analyzed.

⁴ ND = not detectable.

⁵ Following LacTek protocol, a single point of 2 ppb was used as a standard. Therefore, 0 and <2 ppb SMZ cannot be determined quantitatively in samples 46-53.

CONCLUSION

This study demonstrated that immunochemical assays can be used to reliably detect SMZ in raw and processed milk at less than 10 ppb concentrations. The immunoassays have high sample through-put. They are fast, requiring less than 30 min to run an assay for 1-20 samples at a time. Raw milk could be potentially analyzed at the farm by the Cite Cup test which had lower detectability (10 ppb) than Signal ELISA and LacTek. The latter tests were designed with detectabilities of 1-5 ppb and required use of precision pipettes. The Quik Card test can be used for raw milk after a freeze-thaw cycle followed by centrifugation or treatment with ammonium sulfate and centrifugation. Agri-Screen procedure uses precipitation and centrifugation steps and has detectability of >10 ppb. All immunoassays detected free SMZ and possibly N₄-acetyl metabolite. Storage near 4°C is necessary for immunochemical kits unless specially stabilized to prevent loss of antibody binding ability.

Results from two cows given a single dose of SMZ indicated that the drug was cleared from milk after 3.5 d (detecting only trace levels of <1 ppb) which are in agreement with studies reported in literature. Concentration was at a maximum at about 12 h after dosing. The guidelines for quality assessment and quality assurance of the commercial test kits can enhance the reliability of immunochemical results.

ACKNOWLEDGMENTS

The authors acknowledge the National Milk Producers Federation (Arlington, VA) for their support of this study. We also thank all manufacturers for the enzyme immunoassay kits and Delaware Valley College for the supply of fresh raw milk.

REFERENCES

1. Brady, M. S., and S. E. Katz. 1988. Antibiotic/antimicrobial residues in milk. *J. Food Protection*, 51:8-11.
2. Cham, S. E., E. Zomer, and R. G. Salter. 1988. Confirmation of widespread sulfonamide contamination in Northeast U.S. Market Milk. *J. Food Protection* 51:920-924.
3. Food Chemical News. 1988. "Infant formula makers alerted to sulfamethazine problem," April 18. p.22.
4. Food Chemical News. 1988. "Sulfamethazine science review will determine tolerance," August 22. p. 38.
5. Food Chemical News. 1990. "FDA report indicates no milk risk from animal drug residues," April 16. pp. 57-63.
6. Food Chemical News. 1991. "No animal drug residues in milk found by FDA," April 29. p. 24.
7. Medina, M. B. 1986. Direct radioimmunoassay of 17, β -estradiol in ether extracts of bovine sera. *J. Agric. Food Chem.* 34:1046-1049.
8. Medina, M. B. 1988. Extraction and quantitation of soy protein in sausages by ELISA. *J. Agric. Food Chem.* 36:766-771.
9. Nouws, J. F., T. B. Vree, and H. J. Breulink. 1985. Dose dependent disposition of sulphamidine and of its N₄-acetyl and hydroxy metabolites in plasma and milk of dairy cows. *Vet. Quarterly* 7:177-186.
10. Nouws, J. F., D. Mevius, T. B. Vree, M. Baakman, and M. Degen. 1988. Pharmacokinetics, metabolism and renal clearance of sulfadiazine, sulfamerazine, and sulfamethazine and of their N₄-acetyl and hydroxy metabolites in calves and cows. *Am. J. Vet. Res.* 49:1059-1065.
11. Schipper, I. A., and D. F. Eveleth. 1959. Rates and routes of sulfonamide excretion in the cow 1. Milk levels following single intravenous and oral administration. *Am. J. Vet. Res.* 10:714-717.
12. Schwartz, D. P. 1989. Practical screening procedures for drug residues in some agricultural and biological matrices. Abstract. Workshop on Screening Methods for Veterinary Drugs and Natural Contaminants in Food Animal Production. Washington, DC. October 18-20, 1989. p. 15.
13. Unruh, J., E. Piotrowski, D. P. Schwartz, and R. A. Barford. 1990. Solid phase extraction of sulfamethazine in milk with quantitation at low ppb levels using thin layer chromatography. *J. Chromatography*, 519:179-187.