Development and Evaluation of an Enzyme Immunoassay for Sulfamerazine in Milk

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Competitor conjugates for sulfamerazine enzyme immunoassays (SMR EIAs) were prepared using azo and hemisuccinate linking of SMR to BSA. Hapten amounts for the azo-linked conjugates were best estimated using a [^{14}C]sulfamethazine–BSA standard curve, and amino acid analysis indicated that SMR was predominantly linked to histidine residues. Hapten amounts for the hemisuccinate-linked conjugates were determined by quantification of sulfanilic acid after acid hydrolysis. The best azo- and hemisuccinate-linked conjugates (hapten–protein molar ratios of 4.3 and 6.4, respectively) were compared to the Charm II and CITE sulfa trio tests using milk samples from cows treated with oral doses of sulfonamide containing 3-Sulvit. Milk samples were collected over 7 days and 10 ppb cutoff dates determined. The developed SMR EIAs with detection ranges of 0.15–16.6 ppb of SMR predicted the earliest cutoff dates, closely followed by both commercial tests.

INTRODUCTION

Antimicrobial agents are important agricultural tools producers use to keep livestock healthy and their businesses economically viable. Unfortunately, the therapeutic treatment of livestock with a group of antimicrobial agents known as sulfonamides has become a recent concern as sulfonamide residues have been discovered in cows' milk destined for human consumption (Brady and Katz, 1988; Charm *et al.*, 1988; Collins-Thompson *et al.*, 1988; Larocque *et al.*, 1990). Sulfonamides endanger human health through allergic reactions (Huber, 1986) and their suspected carcinogenicity (Littlefield *et al.*, 1990). They also pose an economic hazard through inhibition of growth of dairy starter cultures (Schiffmann *et al.*, 1992).

Many commercial screening tests for sulfonamides have been developed for milk. Some tests, such as the Charm II microbial receptor assay and microbial inhibition tests, are able to detect the majority if not all of the sulfonamides. However, Cullor (1992) and Tyler *et al.* (1992) have suggested that the immunological response of cows could result in the reporting of false positives for many of these microbial assays, and the only test free of this interference was a competitive enzyme immunoassay (Tyler *et al.*, 1992). Enzyme immunoassays (EIAs) are sensitive and reliable analytical techniques, and tests such as the LacTek (Idetex Inc., Sunnyvale, CA) are capable of a 10 parts per billion (ppb) detection limit for sulfamethazine (SMT). However, commercial EIAs detect only individual sulfonamides, and most detect only SMT. With the U.S. Food and Drug Administration's (FDA) establishing a safe level for all sulfonamides at 10 ppb (Zomer *et al.*, 1992), there is a need for EIAs that detect sulfonamides other than SMT. An FDA report has indicated that one of the major sulfonamide contaminants in milk is sulfamerazine (National Survey of Shelf Milk for Sulfonamides and Tetracyclines, April 3, 1990). While sulfamerazine (SMR) is very similar in chemical structure to SMT, EIAs are so specific that a SMT EIA would be considerably less sensitive for SMR. To our knowledge, there have not been any screening tests developed specifically for SMR in milk.

In the development of a SMR EIA it is important to determine which hapten–protein linking method, and what hapten–protein molar ratio, will produce the optimal EIA competitor conjugate used for coating the wells of the microtiter plate. Reliability of a developed SMR EIA is best indicated using milk from sulfonamide-treated cows. The potential of a SMR EIA is suitably determined through comparison of EIA performance with commercially available sulfonamide tests.

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EXPERIMENTAL PROCEDURES

Instrumentation. The EIA analyses were performed in 96-well, Immulon 2, flat-bottom microtiter plates from Dynatech Laboratories Inc. (Chantilly, VA) with Linbro nonsterile, acetate plate sealers manufactured by ICN Biomedicals, Inc. (Costa Mesa, CA) to prevent evaporation. Absorbance measurements were made using a Model EL 309 microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). Spectra/Por 2 membrane tubing (12 000–14 000 molecular weight cutoff) from Spectrum Medical Industries Inc. (Los Angeles, CA) was used for dialyzing the conjugates and a Virtis 5L freeze-drier (The Virtis Co. Inc., Gardiner, NY) for lyophilization. Spectrophotometric analysis was carried out with the use of an HP 8541A diode array spectrophotometer from Hewlett-Packard, Canada Ltd. (Mississauga, ON). Solvents were removed from samples using a Büchi Rotavapor from Fisher Scientific (Ottawa, ON). PE SIL G/UV TLC plates with polyester backing obtained from Whatman Ltd. (Maidston, England) and a Model UVS-54 lamp (254-nm light emission) produced by Ultra Violet Products Inc. (San Gabriel, CA) were used for thin-layer chromatography (TLC). Carbon-14 radioactive decay was measured in 20-mL plastic scintillation vials from Fisher Scientific, using EcoLite+ scintillation cocktail from ICN Biomedicals Inc. (Irvine, CA) in an LS 1801 liquid scintillation counter from Beckman Instruments Inc. (Mississauga, ON).

Nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared spectroscopy (IR), and microanalysis were all performed by the laboratories of University of Alberta Chemistry Services. NMR spectra were measured using a Bruker WH-400 instrument and MS spectra using a Kratos AEI MS-50 (high-resolution, electronic impact ionization) for exact mass determination. IR analyses were measured as a Nujol mull using a Nicolet 7199 FT-IR spectrophotometer.

Amino acid analyses were carried out at the University of Alberta Biochemistry Department on a Beckman 6300 system, with a cation-exchange resin and a postcolumn ninhydrin detection system. Integration of peaks was performed using Beckman System Gold data analysis software.

Reagents. Sulfamerazine (SMR), sulfamethazine (SMT), sulfathiazole (STZ), sulfadimethoxine (SDX), [*phenyl*-ring-UL-

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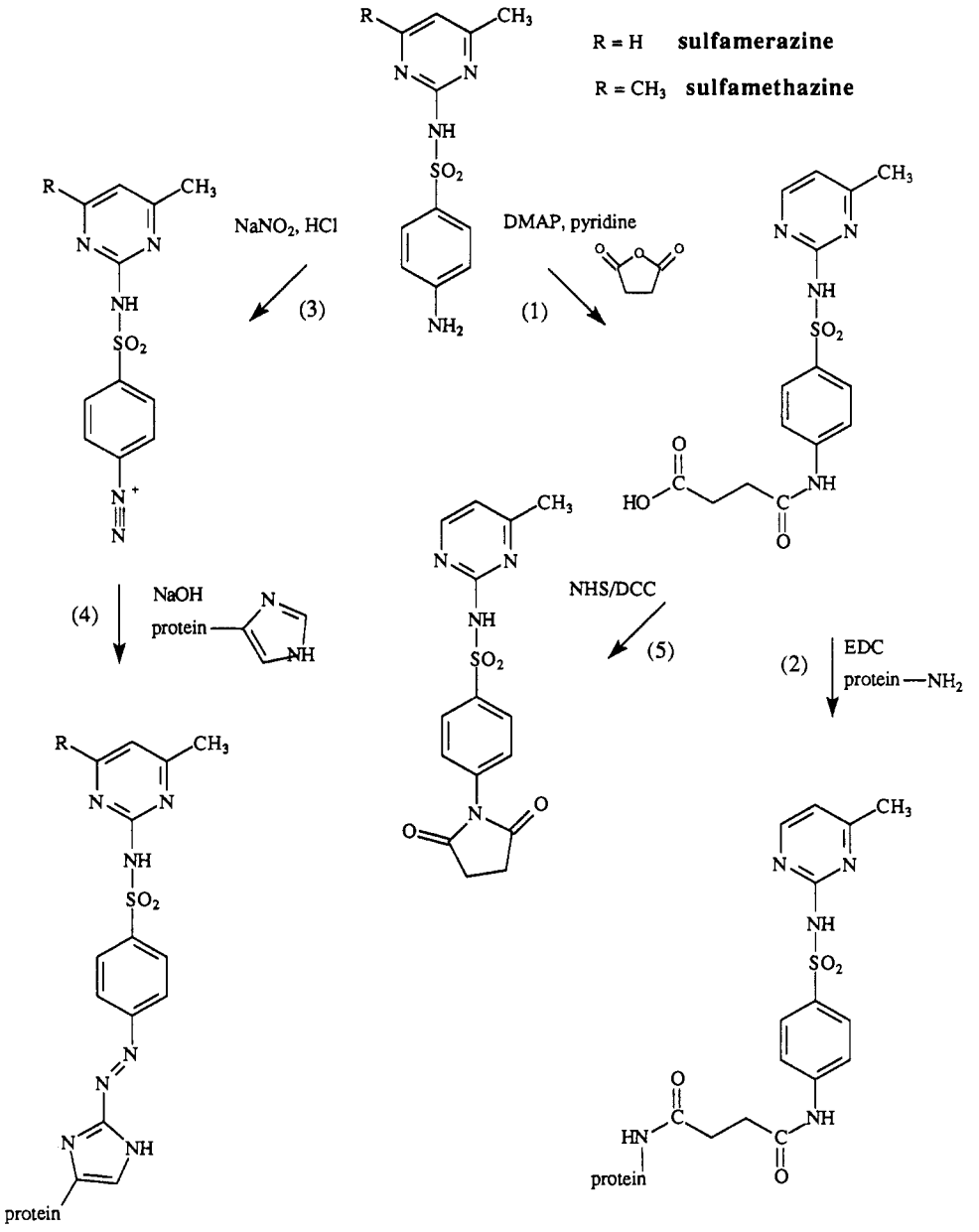

Figure 1. Preparation of azo- and hemisuccinate-linked conjugates.

^{14}C]sulfamethazine (^{14}CSMT), bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), 4-(dimethylamino)-pyridine (DMAP), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), *N*-(chloroacetyl)-L-tyrosine (NCAT), *N*-α-acetyl-L-histidine (NAH), 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB), ammonium sulfamate, thimerosal, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium nitrite was obtained from Fisher Scientific and sulfanilic acid from Terochem Laboratories Ltd. (Edmonton, AB). Succinic anhydride was supplied by BDH Chemicals (Edmonton, AB) and anhydrous *N*,*N*-dimethylformamide by Aldrich Chemical Co. Inc. (Milwaukee, WI). *N*-1-Naphthylethylenediamine dihydrochloride (NED) was obtained from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI). Urea peroxide and goat anti-rabbit peroxidase-conjugated antibodies were supplied by Calbiochem Co. (San Diego, CA). 3-Sulvit was produced by Sanofi Santé Animale (Victoriaville, PQ). Homogenized milk (3.25% milk fat) obtained in 1-L containers was produced by Nu-Maid Dairy Products (Northern Alberta Dairy Pool Ltd., Edmonton, AB). All other chemicals were of reagent grade or better, and solutions were prepared using water purified by a Millipore Milli-Q system (Millipore Corp., Milford, MA).

Preparation of Succinylsulfamerazine (SMR-SA). To prepare SMR-SA (reaction 1, Figure 1), 1.33 g (5.05 mmol) of the acid form of SMR, 2.67 g (26.7 mmol) of succinic anhydride, and 0.0588 g (0.481 mmol) of DMAP were dissolved in 15 mL of pyridine. The solution was heated at 55 °C for approximately 3 h in an oil bath and the endpoint of the reaction determined by TLC. The TLC solvent contained acetone/*n*-heptane/methanol/ammonia/1-butanol in a ratio of 72:21:9:10:10 (v/v) (Bregha-Morris, 1979).

After cooling, the pyridine containing SMR-SA was transferred to a separatory funnel with 20 mL of water. This solution was washed three times with 10 mL of methylene chloride. The combined methylene chloride washings were then washed three times with 10-mL additions of 0.1 N hydrochloric acid and then once with 10 mL of water. The methylene chloride extract was dried over anhydrous sodium sulfate and the solution filtered through Whatman No. 4 filter paper into a round-bottom flask. The methylene choride was codistilled with toluene on a rotary evaporator with water bath (45–55 °C) until a beige powder (SMR-SA) was obtained. The SMR-SA powder was then recrystallized

Table 1. Concentrations of Reactants Used in the Synthesis of Azo- and Hemisuccinate-Linked Conjugates

azo-linked conjugates			hemisuccinate-linked conjugates		
conjugate	diazonium ion (mmol)	protein (mg)	conjugate	SMR-SA (mmol)	protein (mg)
SMR-BSA(L)	0.0267	257.5	SMR-SA-BSA(L)	0.0285	75.12
SMR-BSA(M)	0.0522	207.0	SMR-SA-BSA(M)	0.0832	75.12
SMR-BSA(H)	0.376	255.8	SMR-SA-BSA(H)	0.101	53.4
SMR-LPH	0.0261	106.9			

from water with a 46% yield of purified crystalline material. The structure of the purified SMR-SA powder was confirmed by NMR, MS, and IR: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.88 (very br s, 2 H, N*H*), 10.34 (s, 1 H, COO*H*), 8.29 (d, 1 H, 5.1 Hz, Ar *H*), 7.90 (d, 2 H, 8.5 Hz, Ar *H*), 7.72 (d, 2 H, 8.5 Hz, Ar *H*), 6.88 (d, 1 H, 5.1 Hz, Ar *H*), 2.57 (t, 2 H, 6.0 Hz, CH_2), 2.51 (t, 2 H, 6.0 Hz, CH_2), 2.27 (s, 3 H, CH_3); exact mass $(M - HSO_2)^+$ m/z 299 (2.42%) calcd for $C_{15}H_{15}N_4O_3$, 299.1144 (measured 299.1148); exact mass $(M - H_2SO_3)^+$ m/z 281 (52.78%) calcd for $C_{15}H_{13}N_4O_2$; 281.1038 (measured 281.1040). The IR of SMR-SA showed two strong absorption bands at 1720.95 and 1673.50 cm^{-1} which were not present in the IR of SMR, indicating the hemisuccinate linkage had been formed.

Preparation of Hemisuccinate-Linked Competitor Conjugates. A general procedure was used for the synthesis of all the hemisuccinate-linked competitor conjugates (reaction 2, Figure 1). The synthesis of the lightly loaded, hemisuccinate-linked, sulfamerazine–bovine serum albumin conjugate [SMR-SA–BSA(L)] involved dissolving 10.4 mg (0.0285 mmol) of SMR-SA and 11.0 mg (0.0573 mmol) of EDC in 1 mL of anhydrous *N*,*N*-dimethylformamide. A molar ratio of approximately 2:1 (EDC:SMR-SA) was used in the synthesis of all hemisuccinate conjugates. The SMR-SA/EDC solution was added dropwise to a rapidly stirring solution composed of 75.12 mg of BSA, 1 mL of water, and 20 μL of 6 N sodium hydroxide. Six hours later, 10.7 mg of EDC (approximately 2 times the initial molar concentration of SMR-SA) was added to the reaction mixture, which was left to stir overnight at room temperature. Table 1 provides the molar concentration of reactants used for the medium and heavily loaded conjugates, SMR-SA–BSA(M) and SMR-SA–BSA(H), respectively.

The next day the SMR-SA–BSA conjugates were transferred from the reaction flask to Spectra/Por 2 membrane tubing with washings of 8 M urea and dialyzed against 1 L of 8 M urea for 16 h. The dialysis tubing and contents were then transferred to a 4-L beaker containing 50 mM ammonium bicarbonate and dialyzed for 12-16 h, followed by dialysis against 25 mM ammonium bicarbonate for a further 15-16 h. Finally, the conjugate was removed from the dialysis bag and freeze-dried. These dialysis and freeze-drying procedures were common to all sulfonamide–protein conjugates.

Preparation of Azo-Linked Competitor Conjugates. A general procedure was used for the synthesis of all azo-linked conjugates. The synthesis involved the production of a SMR diazonium ion (reaction 3, Figure 1), which was further reacted with a protein to form an azo-linked conjugate (reaction 4, Figure 1).

During the synthesis of the lightly loaded, azo-linked, sulfamerazine–bovine serum albumin conjugate [SMR-BSA(L)], 7.65 mg (0.0267 mmol) of SMR was dissolved in 1 mL of 3.5 N hydrochloric acid. This was followed by the addition of 0.4 mL of 1% aqueous sodium nitrite (0.0580 mmol) and then 19.6 mg (0.172 mmol) of ammonium sulfamate; the solution was vortexed well between each addition. This produced the SMR diazonium ion solution required for protein conjugation.

The SMR diazonium ion solution was added dropwise (with rapid mixing) to 257.5 mg of BSA dissolved in a 4 mL of PBS (pH 7.3) in a 10-mL pear-shape flask. The solution was left to mix for 5 min, and then 20 drops of 6 N sodium hydroxide was added from a Pasteur pipet to raise the pH to >8. The flask and contents were wrapped in foil and left to stir overnight at 4 °C and then dialyzed and freeze-dried in the same manner as the hemisuccinate-linked competitor conjugates.

For the synthesis of immunogen (SMR-LPH) and the medium and heavily loaded competitor conjugates [SMR-BSA(M) and SMR-BSA(H), respectively], the amounts of SMR diazonium ion and BSA used are given in Table 1.

Preparation of the *N*-(Chloroacetyl)-L-tyrosine (NCAT) and *N*-α-Acetyl-L-histidine (NAH) Standard Curves. For the NCAT standard curve, SMR dilutions were prepared using 0.1 N sodium hydroxide and ranged from 3.510 to 0.4712 μmol of SMR/mL. For the NAH standard curve, SMR dilutions were prepared with 0.1 N sodium hydroxide and ranged from 175 to 23.6 nmol of SMR/mL.

The NCAT and NAH standard curves were produced by reacting the SMR dilutions under the following conditions. A 0.75-mL aliquot of a SMR dilution was added to 0.75 mL of 3.5 N hydrochloric acid and vortexed. Next, 0.23 mL of a 1% aqueous solution of sodium nitrite was added, followed by 0.35 mL of a 0.8% aqueous solution of ammonium sulfamate; the mixture was vortexed well between each addition. Depending on the standard curve, the next addition was either 0.75 mL of a 13.52 mg of NCAT/mL of 0.1 N sodium hydroxide (52.46 μmol/mL) solution or 0.75 mL of a 10.34 mg NAH/mL of 0.1 N sodium hydroxide (52.43 μmol/mL) solution. Finally, 0.75 mL of 6 N sodium hydroxide was added, and the solutions were left stirring overnight at 4 °C. This reaction produced an azo link between either SMR and NCAT (SMR–NCAT) or SMR and NAH (SMR–NAH), depending on the standard curve. A blank solution was prepared by substituting 0.75 mL of 0.1 N sodium hydroxide for the SMR dilution in the first step of the reaction. The following day the absorbances of all the standard curve solutions (SMR–NCAT and SMR–NAH) were measured at 492 nm (the absorbance maximum of the SMR–NCAT solutions) and at 436 nm (the absorbance maximum of the SMR–NAH solutions). The absorbance values at 492 and 436 nm for both SMR–NCAT and SMR–NAH solutions were plotted against the molar concentration of the SMR dilution. From these plots linear relationships were obtained, with each having a coefficient of determination (r^2) of 0.98 or greater. The slopes of the lines were used to calculate eqs 1 and 2, which represent the molar concentrations of SMR–NCAT ($M_{SMR-NCAT}$) and SMR–NAH ($M_{SMR-NAH}$), respectively.

$$M_{SMR-NCAT} = [12.0(A_{492nm}) - 3.83(A_{436nm})] \times 10^{-4} \quad (1)$$

$$M_{SMR-NAH} = [5.53(A_{436nm}) - 3.98(A_{492nm})] \times 10^{-5} \quad (2)$$

An accurately weighed sample of each of the azo-linked competitor conjugates was dissolved in 0.6 N sodium hydroxide in a 10-mL volumetric flask. Absorbance readings for each of the azo-linked conjugate solutions were measured at 492 and 436 nm. The number of moles of SMR per mole of protein calculated using eqs 1 and 2 are reported in Table 2.

Preparation of the Azo-Linked, ^{14}Carbon-Labeled Sulfamethazine–Bovine Serum Albumin (^{14}CSMT–BSA) Standard Curve. A "hot stock" was prepared by dispersing 50 μCi of lyophilized ^{14}CSMT in 200 μL of PBS (adjusted to pH 8.2). "Hot and cold stock" was prepared by combining a 50-μL aliquot of the hot stock with 250.84 mg of SMT in a 5-mL volumetric flask (diluted to volume with 0.1 N sodium hydroxide). BSA (1.5093 g) dissolved in 10 mL of PBS (pH 7.3) was used as the "BSA stock" solution. The hot and cold stock (50.17 mg of SMT/mL of 0.1 N sodium hydroxide) and the BSA stock (150.93 mg of BSA/mL of PBS) were used to synthesize each of the ^{14}CSMT–BSA conjugates for the standard curve.

The synthesis of the ^{14}CSMT–BSA conjugates was performed following the same series of reactions (reactions 3 and 4, Figure 1) used to prepare the azo-linked competitor conjugates. To produce the diazonium ion for the most heavily loaded ^{14}CSMT–BSA conjugate, 0.4 mL of hot and cold stock and 0.4 mL of 3.5 N hydrochloric acid were combined in a test tube. This was followed by the addition of 0.5 mL of 1% sodium nitrite and then 16.6 mg of ammonium sulfamate; the solution was vortexed well

Table 2. Hapten–Protein Molar Ratios Determined for Azo-Linked Competitor Conjugates

method of analysis		azo-linked competitor conjugates (mol of hapten/mol of protein)		
		SMR–BSA(L)	SMR–BSA(M)	SMR–BSA(H)
NCAT/NAH standard curves	$M_{SMR-NCAT}$[a]	8.1	17.6	90.0
	$M_{SMR-NAH}$[b]	1.3	3.0	5.5
	total	9.4	20.6	95.5
NAH standard curve (A_{436nm})		1.5	3.6	7.9
^{14}CSMT–BSA standard curve		4.3	10.0	22.2
amino acid analysis (glycine)	azotyrosines	0.3	0.6	nd[c]
	azohistidines	2.1	7.7	nd
	total	2.4	8.3	
amino acid analysis (arginine)	azotyrosines	0.2	−0.1	nd
	azohistidines	2.0	7.3	nd
	total	2.2	7.3[d]	

[a] Values for moles of hapten per mole of protein were calculated for each conjugate using the molar concentration of SMR derived from eq 1 and the known concentration of protein used for absorbance measurements. [b] Values for moles of hapten per mole of protein were calculated for each conjugate using the molar concentration of SMR derived from eq 2 and the known concentration of protein used for absorbance measurements. [c] Not determined for this conjugate. [d] Negative value not considered in total.

between each addition. To prepare other ^{14}CSMT–BSA conjugates for the standard curve, the ratio of ^{14}CSMT to BSA was varied by decreasing the volume of hot and cold stock that was used. Sodium hydroxide (0.1 N solution) was added before the addition of 3.5 N hydrochloric acid, as required to keep the total volume in the test tube constant from conjugate to conjugate.

The ^{14}CSMT–BSA diazonium ion solution from the test tube was added dropwise, with rapid mixing, to a pear-shape flask containing 1.0 mL of the BSA stock solution and 1.3 mL of PBS (pH 7.3). Finally, 0.4 mL of 0.6 N sodium hydroxide was added to the reaction mixture in the pear-shape flask to raise the pH above 8. The flask was then wrapped in foil and left to stir overnight at 4 °C.

The next day the contents of the pear-shape flask containing the ^{14}CSMT–BSA conjugate were transferred to Spectra/Por 2 membrane tubing and dialyzed and freeze-dried as described for the hemisuccinate-linked competitor conjugates. After freeze-drying, a precisely weighed portion of the conjugate was dissolved in 0.6 N sodium hydroxide in a 10-mL volumetric flask. The solution was used for spectrophotometric analysis and for radioactive decay measurements.

The ^{14}CSMT–BSA standard curve was created by plotting the absorbance value at 430 nm (A_{430nm} per milligram of protein conjugate per milliliter of 0.6 N sodium hydroxide) vs the moles of SMT per mole of BSA (determined by radioactive decay) for each of the ^{14}CSMT–BSA conjugates that were synthesized. From the ^{14}CSMT–BSA standard curve, eq 3 was derived ($r^2 = 0.99$).

$$m = A_{430nm}/0.123 \quad (m = \text{mol of sulfonamide/mol of protein}) \quad (3)$$

Using eq 3, values were calculated for the moles of sulfonamide per mole protein for the azo-linked SMR–BSA competitor conjugates and reported in Table 2.

Amino Acid Analysis of the Azo-Linked Competitor Conjugates. To hydrolyze the azo-linked competitor conjugates for analysis, approximatley 0.1 mg of the conjugate was dissolved in 5 drops of boiling hydrochloric acid (containing 0.1% phenol) in a 10 × 75 mm borosilicate culture tube. The top of the tube was elongated in an oxygen flame, the tube evacuated using a vacuum pump, and the neck of the tube sealed. The tube was placed in a 100 °C oven overnight. The next day the top of the tube was scored and removed, and the tube placed in a vacuum dessicator with sodium hydroxide overnight. The tube contents were dissolved in 100 μL of 0.2 M citric acid buffer (pH 2.2) and samples taken for analysis on the Beckman 6300 system.

The retention times and the response factors of each of the amino acids were confirmed by analyzing a set of amino acid standards before the analysis of the hydrolyzed protein conjugates. To provide a protein standard for comparison, BSA was hydrolyzed and the amino acid composition measured in the same manner as the azo-linked competitor conjugates. Data from the amino acid analysis were examined, and the modifications to each of the azo-linked competitor conjugates (using BSA as a standard) were calculated using

$$R = \left(\frac{[A]_{BSA} - [A]_{conj}([C]_{BSA}/[C]_{conj})}{[A]_{BSA}} \right) \times N \quad (4)$$

where R is the number of azo-linked amino acid residues/BSA, A represents tyrosine or histidine, C represents glycine or arginine, N is the number of tyrosine or histidine molecules per molecule of BSA (tyrosine, 20; histidine, 17), $[A]_{BSA}$ is nanomoles of the amino acid in BSA, and $[A]_{conj}$ is nanomoles of the amino acid in the azo conjugate.

Equation 4 was used to determine the number of azotyrosine and azohistidine residues (using glycine and arginine as internal standards) for azo-linked competitor conjugates. The results of these determinations are reported in Table 2.

Preparation of the Sulfanilic Acid Standard Curve. The sulfanilic acid standard curve was prepared using a modified Bratton–Marshall (B–M) procedure similar to that described by Low *et al.* (1989). Aqueous serial dilutions of sulfanilic acid used to prepare the curve ranged from 50 to 5 μg/mL. An aliquot of each sulfanilic acid dilution (1 mL) was added to 4 mL of 6 N hydrochloric acid, and 1 mL of each of these sulfanilic acid/hydrochloric acid solutions was used in the modified B–M reaction. The 0.8% aqueous NED solution was prepared the day of the assay, as the solution blackened with time.

Modified B–M Method. To 1 mL of each of the sulfanilic acid/hydrochloric acid solutions was added 200 μL of a 0.2% aqueous dilution of sodium nitrite, and the solution was vortexed. This was followed by the addition of 200 μL of a 3.3% aqueous ammonium sulfamate solution, 500 μL of 98% ethanol, and 200 μL of the 0.8% aqueous NED; the solutions were vortexed well between each of the additions. After 15 min in the dark, the absorbance values of the solutions were measured at 554 nm. A standard curve was created by plotting the A_{554nm} against the molar concentration of the sulfanilic acid in each dilution. The slope of the curve was 5082 (absorbance units/M) with $r^2 = 1.00$.

Hydrolysis of Hemisuccinate-Linked Competitor Conjugates. Each of the hemisuccinate-linked competitor conjugates was hydrolyzed, and the hapten–protein molar ratios were determined, under the following set of reaction conditions.

An aqueous solution was prepared containing between 2 and 7 mg of a freeze-dried hemisuccinate-linked competitor conjugate, in a 10-mL volumetric flask. A 1-mL sample was taken from the volumetric flask and added to 4 mL of 6 N hydrochloric acid. This solution was refluxed for 2.5 h. Once cooled, 1 mL of the competitor conjugate hydrolysate was used in the modified B–M reaction in place of the sulfanilic acid/hydrochloric acid dilutions. For each hemisuccinate competitor conjugate, an absorbance value at 554 nm was obtained, and this value converted to a molar concentration of SMR-SA in the conjugate using the sulfanilic acid standard curve. The molar concentration of BSA in the conjugates could be calculated by accounting for the weight of SMR-SA for each of the competitor conjugates. The molar

Table 3. Hapten–Protein Molar Ratios Determined for Hemisuccinate-Linked Competitor Conjugates

	hemisuccinate-linked competitor conjugates (mol of hapten/mol of protein)		
method of analysis	SMR-SA–BSA(L)	SMR-SA–BSA(M)	SMR-SA–BSA(H)
sulfanilic acid curve	6.4	18.6	22.0

Table 4. Composition of 3-Sulvit

active ingredient	amount per package	% wt based on package wt (450 g)
sulfamethazine, sodium	170.50 g	37.9
sulfamerazine, sodium	67.40 g	15.0
sulfathiazole, sodium	136.30 g	30.3
vitamin A	240 000.00 IU	
vitamin D_3	80 000.00 IU	
riboflavin	200.00 mg	
niacinamide	3.00 g	
d-pantothenic acid	700.00 mg	
vitamin B_{12}	2.30 mg	
magnesium sulfate	200.00 mg	
sodium acetate	2.80 g	
sodium chloride	3.15 g	
calcium chloride	250.00 mg	
potassium chloride	250.00 mg	

Table 5. Statistical Data of Treated Cows

cow ID no.	approx wt (kg)	stage of lactation	3-Sulvit administered (g)	av milking in kg/day[a] (days 1/a.m.–7/a.m.)
8421	630	7	60.8	25.3
8881	580	2	60.3	17.6
9020	525	1	60.1	25.2

[a] Daily milkings varied by no more than ±3 kg.

ratios of SMR-SA to BSA for each of the hemisuccinate-linked competitor conjugates are reported in Table 3.

Preparation of Sulfonamide-Treated Bovine Milk. A herd of 55 Holstein dairy cows managed by the University of Alberta Dairy Unit was the source of raw milk for this study. The herd was milked twice daily using a single eight herringbone milking parlor and the milk stored in a refrigerated bulk tank. At the time of this study, the cattle were not undergoing any antimicrobial treatment, and the bulk tank was the source of sulfonamide-free raw milk. Three cows with identification numbers 8421, 8881, and 9020 were chosen for treatment with 3-Sulvit (composition found in Table 4) on the basis of their stage of lactation and the number of days into their milking cycle. Each of the cows had been milked for 150–200 days, well into their milking cycle, which eliminated any problems with colostrum milk. Data on the three cows are given in Table 5. Pretreatment milk samples (day 1/a.m., Tables 6–8) of 100–200 mL were taken from the three cows prior to the administration of 3-Sulvit and provided the background response level for each animal.

3-Sulvit treatment was administered to each cow (approximately 5 h after the day 1/a.m. milkings) by means of a stomach drench. Stomach drenching involved pumping, from a plastic container, an aqueous solution (approximately 2 L) of the 3-Sulvit through a hose directly into the cow's stomach. This was immediately followed by pumping 1–2 L of water to wash any remaining 3-Sulvit into the stomach from the plastic container and hose.

The level of 3-Sulvit chosen for treatment of the cows was based on package recommendations with some modification. The package suggested dissolving one pouch (450 g) in 1350 L of drinking water, when administered to cattle. With each Holstein cow drinking, on average, 120 L of water a day (estimated by the University of Alberta Dairy Unit manager), a cow would then be expected to ingest 40 g of 3-Sulvit/day. For this study, a treatment of 60 g/stomach drench of 3-Sulvit was chosen (1.5 times the calculated daily intake) to ensure that an adequate dose of the sulfonamides was excreted by the cow into her milk. With sulfonamides composing approximately 83% of the package weight of 3-Sulvit, the oral dose that each cow received would be (on average) 87 mg of sulfonamides/kg of body weight. Other authors who have orally treated cows with sulfonamides have used from 26.4 to 220 mg of sulfonamide/kg of body weight (Paulson *et al.*, 1992a). Intravenous doses of 160 mg/mL of SMR, SMT, and sulfathiazole (STZ) have been administered to dairy cows with minimal ill effects (Rehm *et al.*, 1986).

3-Sulvit-treated cows were tagged and milked separately from the rest of the herd. Samples of their milk (100–200 mL) were collected from the total morning (a.m.) and evening (p.m.) milkings and immediately frozen for later analysis. The approximate hours between the a.m. and p.m. milkings, from day 1/a.m. to day 7/a.m. are given in Tables 6–8. After sampling, the remaining sulfonamide-contaminated milk was discarded. This process was continued until the treated cows' milk contained less than 10 ppb of sulfonamide residue as determined by Charm II analysis. It was then determined to be safe to put the cows back on-line with the rest of the herd. Treated cows demonstrated no apparent ill aftereffects from either the stomach drenching procedure or the 3-Sulvit treatment.

Testing of the pre-3-Sulvit and 3-Sulvit-treated cows' milk samples, for each cow, was run on three consecutive days. The morning of testing, the milk samples from one cow were thawed, and all three test formats (Charm II, SMR EIA, and CITE sulfa trio) were performed on the milk samples that day, while the samples were kept in an ice bath. The day 6/a.m. sample for cow 8412 (Table 6) was inadvertently discarded prior to analysis.

Preparation of Sulfonamide-Spiked Homogenized Milk Samples. Commercially produced homogenized milk was used for the preparation of SMR standard curves and for cross-reactivity studies with SMT, STZ, and sulfadimethoxine (SDX). Each of the sulfonamides (sodium salt form) was first dissolved in water, and then a 10-fold dilution of the aqueous solution was

Table 6. Results of 3-Sulvit Treatment of Cow 8421

milking		SMR–BSA(L)	SMR-SA–BSA(L)	CITE sulfa trio[a]	Charm II assay	
day	hours	EIA (ppb)	EIA (ppb)	(ppb)	cpm[b]	ppb[b]
1/a.m.	0	*c*	*c*		1431	<10
1/p.m.	9	>100	>100	>10	789	>10
2/a.m.	24	>100	>100		828	>10
2/p.m.	33	>100	>100		959	>10
3/a.m.	48	>100	>100		856	>10
3/p.m.	57	~29[d]	~73	>10	926	>10
4/a.m.	72	5.2	5.4		857	>10
4/p.m.	81	3.5	3.7	>10 (SMT only)	996	>10
5/a.m.	96	1.1	1.1	≈10	1056	>10
5/p.m.	105	0.64	0.73	<10	1062	>10
6/a.m.	120	*e*	*e*	*e*	*e*	*e*
6/p.m.	129	0.41	~0.24		1826	<10
7/a.m.	144	0.16	~0.10		1700	<10

[a] The CITE sulfa trio test is designed as a 10 ppb cutoff test. [b] 1100 cpm ≈ 10 ppb concentration of sulfonamide in the milk. [c] Sulfonamide-free milk samples for SMR EIA. [d] "~", quantifiable results that fall outside the linear region of the sigmoidal standard curves. [e] No sample was taken.

Table 7. Results of 3-Sulvit Treatment of Cow 8881

milking		SMR-BSA(L)	SMR-SA-BSA(L)	CITE sulfa trio[a]	Charm II assay	
day	hours	EIA (ppb)	EIA (ppb)	(ppb)	cpm[b]	ppb[b]
1/a.m.	0	c	c		2504	<10
1/p.m.	9	>100	>100		946	>10
2/a.m.	24	>100	>100		976	>10
2/p.m.	33	>100	>100		864	>10
3/a.m.	48	>100	>100		931	>10
3/p.m.	57	~38[d]	>100		934	>10
4/a.m.	72	~20	~40	>10	987	>10
4/p.m.	81	~11	~18	>10 (SMT only)	1024	>10
5/a.m.	96	5.9	6.5	≈10	1052	>10
5/p.m.	105	6.9	6.8	<10	1175	<10
6/a.m.	120	1.7	2.4		1156	<10
6/p.m.	129	1.4	2.2		1270	<10
7/a.m.	144	0.31	~0.46		1373	<10

[a] The CITE sulfa trio test is designed as a 10 ppb cutoff test. [b] 1100 cpm ≈ 10 ppb concentration of sulfonamide in the milk. [c] Sulfonamide-free milk samples for SMR EIA. [d] "~", quantifiable results that fall outside the linear region of the sigmoidal standard curves.

Table 8. Results of 3-Sulvit Treatment of Cow 9020

milking		SMR-BSA(L)	SMR-SA-BSA(L)	CITE sulfa trio[a]	Charm II assay	
day	hours	EIA (ppb)	EIA (ppb)	(ppb)	cpm[b]	ppb[b]
1/a.m.	0	c	c		2234	<10
1/p.m.	9	>100	>100		814	>10
2/a.m.	24	>100	>100		923	>10
2/p.m.	33	>100	>100		940	>10
3/a.m.	48	~25[d]	>100	>10	837	>10
3/p.m.	57	~11	~44	>10	936	>10
4/a.m.	72	3.3	8.2	>10	1023	>10
4/p.m.	81	2.8	7.7	≈10	1218	<10
5/a.m.	96	0.89	2.1	<10	1239	<10
5/p.m.	105	1.2	2.1		1334	<10
6/a.m.	120	0.24	1.4		1918	<10
6/p.m.	129	0.19	~0.59		1993	<10
7/a.m.	144	~0.14	~0.58		2529	<10

[a] The CITE sulfa trio test is designed as a 10 ppb cutoff test. [b] 1100 cpm ≈ 10 ppb concentration of sulfonamide in the milk. [c] Sulfonamide-free milk samples for SMR EIA. [d] "~", quantifiable results that fall outside the linear region of the sigmoidal standard curves.

made with milk. The sulfonamide/milk solutions were then used to make further 10-fold serial milk dilutions of each of the sulfonamides to be used in test evaluations. For the standard curves, SMR milk dilutions ranged from 1 μg/mL to 0.01 fg/mL, and for the cross-reactivity studies, sulfonamide milk dilutions ranged from 10 μg/mL to 0.01 ng/mL.

Charm II Microbial Receptor Assay Procedure. The Charm II analyses were performed at the Northern Alberta Dairy Pool processing plant (Edmonton, AB) using equipment and reagents purchased from Charm Sciences Inc. (Malden, MA). The procedure used in analyzing the milk samples was the "antimicrobial sulfa drug sequential assay", as outlined in the operator's manual for the Charm II assay. The Charm II assay, by the manufacturer's claims, should detect all three sulfonamides (SMT, SMR, and STZ) found in 3-Sulvit. In the sequential assay, sulfonamide from the milk sample was first given the opportunity to bind to the receptor molecule. Then a ^{3}H-labeled SMT drug tracer was added which bound to any remaining sites. The receptor/sulfonamide complex was centrifuged, any unbound radioactivity and free sulfonmide were decanted away, and the radioactivity of the remaining complex was measured after the addition of scintillation fluid. The amount of radioactivity in counts per minute (cpm) was inversely proportional to the amount of sulfonamide in the milk sample. Reconstituting a positive control (10 ppb of SMT) milk powder, supplied by Charm Sciences, allowed the establishment of a relationship between the cpm and a 10 ppb of SMT milk solution. Through numerous trials, a 10 ppb of sulfonamide control point was set when the Charm II analyzer gave a fixed display of 1100 cpm. Therefore, any milk samples with counts less than 1100 cpm contained in excess of 10 ppb of sulfonamide.

CITE Sulfa Trio Test Kit Procedure. The CITE sulfa trio test kits were obtained from IDEXX Laboratories, Inc. (Westbrook, ME). The CITE sulfa trio test is a membrane-immobilized, antibody-based test comprised of a control and three test spots specific for the sulfonamides SMT, STZ, and SDX. The test utilizes a competitive format in which the residual sulfonamide present in a milk sample and an enzyme-labeled sulfonamide standard (which is added to the milk sample) compete for antibody binding sites. After washing, and upon the addition of substrate, a blue precipitate is formed by the enzyme which is part of an enzyme–sulfonamide–antibody complex at the membrane surface. The formation of blue precipitate is inversely related to the amount of residual sulfonamide originally present in the milk sample. A visual inspection of precipitate in each of the sulfonamide-designated regions of the test cups was used to determine whether the milk samples contained greater than 10 ppb of SMT, STZ, or SDX. From analyses performed by SMR EIAs and Charm II analyses, the range of milk samples that could provide information using the CITE sulfa trio test was narrowed to four or five samples. Milk was tested until a sample was found that could be visually identified by the test as containing approximately 10 ppb of SMT or STZ.

Phosphate-Buffered Saline (PBS and PBST) Solutions. The PBS solution was prepared by dissolving sodium chloride (9.0 g), disodium hydrogen phosphate (1.108 g), potassium dihydrogen phosphate (0.3 g), and thimerosal (0.1 g) in 1 L of water. PBS, prepared without thimerosal, was autoclaved and used in the preparation of the immunogen (SMR–LPH). To prepare PBST, 0.5 g (0.05%) of Tween 20 was added to the PBS solution prior to dilution to volume with water. The pH of the PBS or PBST was adjusted to 7.3 with sodium hydroxide or hydrochloric acid as needed.

Polyclonal Antibody Antiserum Preparation. LPH was chosen for the preparation of the immunogen (SMR–LPH), as it was a protein foreign to the immune system of the experimental animals. The SMR–LPH conjugate had a hapten–protein molar ratio of 8.4, which was calculated using the $^{14}CSMT$–BSA standard curve. This was within the range of 8–25 hapten molecules/BSA as prescribed for strong antibody titers by Erlanger (1980). For the production of antiserum, two 12-week-old, female, prebled Flemish Giant × Dutch Lop Ear rabbits

were each injected with a mixture composed of 1 mg of SMR-LPH, dissolved in 1 mL of PBS (pH 7.3), emulsified in 1 mL of Freund's complete adjuvant. The rabbits were injected subscapularly (2 × 0.5 mL) and intramuscularly (2 × 0.5 mL). Twenty-eight days later, the rabbits were boosted using the same protocol as the initial injection except Freund's incomplete adjuvant replaced Freund's complete adjuvant. On day 41 after injection, the first bleed was taken for analysis (10 mL from the large artery of the ear of each rabbit). On day 64, both rabbits were boosted once again in the manner described earlier, and on day 77, an ear bleed from each rabbit was taken for analysis. Finally, 91 days after the initial injection, both rabbits were bled out via cardiac puncture (approximately 70 mL from each rabbit). Collected blood was allowed to clot for at least 1 h at 22 °C, and the antiserum was decanted and stored in 1.5-mL sealed containers at −20 °C. At the time of the bleed-out, the antisera of both rabbits were of equal strength and performance. Since strong titers were obtained with both rabbits, only the antiserum from one rabbit was used in all EIA experiments.

Competitive Indirect EIA Procedures. *EIA Procedure for Competitor Conjugate Evaluation.* Individual wells of a microtiter plate were coated with 200 μL/well of a 10 ppb solution (w/v) of one of the three azo-linked or one of the three hemisuccinate-linked competitor conjugates dissolved in PBS. The plate was protected from evaporation with an acetate plate sealer and left overnight at 4 °C. The next day the coating solution was shaken from the wells and the plate coated with a 1% BSA in PBS solution. The plate was then sealed with a plate sealer and left to incubate for 1 h at room temperature. After 1 h, the 1% BSA in PBS solution was shaken from the plate and the wells washed three times with PBST; the PBST from the plates was shaken and blotted onto paper towels between each washing. Aqueous sodium SMR dilutions (10 μg/mL–1 fg/mL) were added (100 μL/well) as a source of free hapten in solution. To some of the wells was added 100 μL of water as a control. Antiserum diluted 500 000 times with 0.05% BSA in PBST was added (100 μL/well) and the plate sealed and incubated 2 h. Following the incubation, the solution was shaken from the wells, and the plate was washed and blotted three times as previously described. Goat anti-rabbit peroxidase-conjugated antibodies diluted 3000 times with PBST were added to all wells (200 μL/well), and the plate was incubated with a plate sealer for a further 2 h at room temperature. After the plate was washed with PBST as before, 200 μL of a substrate solution [TMB (0.1 mg/mL) and urea peroxide (1.0 mg/mL) dissolved in 0.1 citrate buffer, pH 4.0] was added to each well. The enzyme–substrate reaction was stopped with the addition of 50 μL/well of 2 M sulfuric acid and the absorbance read at 450 nm.

EIA Procedure for Evaluation of Milk Samples. The competitive indirect EIA procedure used for sulfonamide-treated milk samples was the same as that used for the evaluation of competitor conjugates except for the following modifications. The wells of the 96-well microtiter plate were coated with 200 μL of a 10 ppb (w/v) solutions of SMR-SA–BSA(L) or a 20 ppb (w/v) solution of SMR–BSA(L) prepared with PBS. For the SMR standard curves and sulfonamide cross-reactivity studies, serial dilutions of sulfonamides prepared with homogenized milk were added to the wells (100 μL/well) as a source of free hapten. For the 3-Sulvit study, 100 μL of each of the medicated cows' milk samples was added to each well. Some wells received 100 μL of homogenized milk or premedicated cows' milk added as a control. Serum was diluted 500 000 times [SMR–BSA(L) competitor conjugate] and 1 000 000 times [SMR-SA–BSA(L) competitor conjugate] with 0.05% BSA in PBST before addition (100 μL) to the wells.

EIA Data Analysis. *Analysis of EIA Data for Competitor Conjugates.* The I_{50} value (ppb of SMR) was used as a comparison point, for each of the conjugates. The I_{50} value was the inflection point of a best-fit, four-parameter curve (eq 5, calculated by SOFTmax (version 2.01) software (Molecular Devices Corp., Menlo Park, CA)

$$y = \frac{a - d}{1 + (x/c)^b} + d \qquad (5)$$

where a and d are the upper and lower asymptotes, b is the slope of the linear portion of the curve, and c is the inflection point (middle) of the curve (I_{50}).

To prepare the data for the SOFTmax program, a blank value for the plate was subtracted from the measured absorbance value for each well. The blank value for the plate was measured by taking the average absorbance from at least 12 wells on the plate, where a 1% BSA in PBS solution was used for coating the wells instead of either an azo- or hemisuccinate-linked competitor conjugate solution. All other additions of solutions to the plate remained the same for the 1% BSA in PBS-coated wells as for the competitor conjugate coated wells. Once the blank value had been removed from the absorbance value of each well, a value of B (absorbance of a well containing SMR) divided by B_0 (absorbance of a well without SMR) could be calculated for each well. The B/B_0 values and their corresponding concentration of SMR (ppb) were then processed by the SOFTmax software.

Analysis of EIA Data for Milk Samples. Analysis of data for milk samples was the same as described for the competitor conjugates. This included the subtraction of a blank value (average absorbance of 1% BSA coated wells) from each of the sample and control wells. The value for B was the absorbance of a well containing sulfonamide-contaminated milk. The values of B_0 for the standard curves and cross-reactivity studies were obtained using homogenized milk with no sulfonamide added. The values of B_0 for the 3-Sulvit-treated cows' milk samples were obtained using the day 1/a.m. milk from each cow.

Data Processing for SMR EIA Standard Curves and Sulfonamide-Contaminated Raw Milk Samples. Sigmoidal standard curves were produced using SMR-spiked homogenized milk and the SOFTmax software, for both the SMR–BSA(L) and SMR-SA–BSA(L) competitor conjugates. The equations of the sigmoidal curves for each competitor conjugate were used to convert the absorbance values (B/B_0) from the 3-Sulvit-treated cows' milk samples into numerical (ppb) values. All values less than 100 ppb were reported for the milk samples. A 20–80% reduction from the upper asymptote of each of the sigmoidal standard curves represented the most linear region of the curves, and ppb values derived from this region were considered the most accurate. For the sigmoidal standard curve of the SMR–BSA(L) competitor conjugate, the concentration of SMR within the linear region was from 0.15 to 5.7 ppb, and for the SMR-SA–BSA(L) competitor conjugate, the linear region was represented by a 0.68–16.6 ppb of SMR concentration. Each of the points used for the standard curves and the cows' milk samples was performed in triplicate, and the coefficient of variation of the absorbance values (V_A) was calculated for the linear region of the curves. For the SMR–BSA(L) competitor conjugate standard curve, the V_A average was 5.4%, and that for the SMR-SA–BSA(L) competitor conjugate standard curve was 12.4%. For all the sulfonamide-contaminated cows' milk samples, the average V_A for values obtained using the SMR–BSA(L) competitor conjugate for day 3/p.m. to day 7/a.m. (3/a.m. to 7/a.m. for cow 9020) milk samples was 6.8%. The average V_A for the cows' milk samples obtained using the SMR-SA–BSA(L) competitor conjugate from day 3/p.m. to day 7/a.m. (4/a.m. to 7/a.m. for cow 8881) was 5.6%.

RESULTS AND DISCUSSION

Importance of Determining the Accurate Chemical Composition of Competitor Conjugates. Research has shown that the method chosen for hapten linkage is important for EIA performance (Sheth and Sporns, 1991; Wie and Hammock, 1984; Vallejo *et al.*, 1982). When an immunogen is injected into rabbits, antibodies are created that have affinity for the carrier protein, the hapten, and the region by which both are linked (the linking arm). By choice of a different protein for the EIA competitor conjugate and by changing the hapten–protein linking method, selection of specific antibodies in the sera allows for better competition and a decreased background.

Increased EIA sensitivity has also been found when the amount of hapten bound to the well of the microtiter plate is limited (Ekins and Chu, 1991; Manning, 1991; Porstmann and Kiessig, 1992). Theoretically, when the amount

of plate-bound hapten is limited, there is a requirement for less hapten in solution to compete for the hapten-specific antibody in the antiserum. A range of 3–25 molecules of hapten/molecule of protein has been suggested for a successful EIA of small molecules (Roe, 1991).

This study examined not only the effect of a change in the hapten (SMR) to protein carrier (BSA) linking method but also the effect that a change in the number of hapten molecules per mole of protein carrier (hapten–protein molar ratio) has on the performance of the EIA competitor conjugates. However, any relationship between hapten–protein molar ratios and EIA sensitivity can only be established if the hapten–protein molar ratios are accurately known. Although many EIAs have utilized the azo linkage for the preparation of competitor conjugates, a comparison of past methods for hapten–protein molar ratios determinations indicated that some of these methods are unreliable.

Determination of Hapten–Protein Molar Ratios for Azo-Linked Conjugates. For the azo-linked competitor conjugates, the following methodologies were explored to determine a reliabile technique for measuring the number of hapten molecules bound to the protein carriers.

Microanalysis Method. SMR contains 49.98% carbon, 4.58% hydrogen, 21.20% nitrogen, 12.13% sulfur, and 12.11% oxygen. Microanalysis of the conjugates led to unsatisfactory results since differences in the carbon, hydrogen, nitrogen, and sulfur percentages of the SMR–BSA conjugates, when compared to the analysis results of BSA alone, were less than or equal to the margin of error (±0.3%) expected from this analysis.

Immunological Method. The use of immunological methods for determining hapten–protein molar ratios has been described by Morgan *et al.* (1983) and Wie and Hammock (1984). In this study we employed a commonly used protocol for this form of determination. It involved the use of competitor conjugates as sources of plate-bound and solution SMR. With conjugates from two different hapten–protein linking methods available, it was possible to produce a number of EIA combinations of plate-bound and solution competitor conjugate. Comparisons were made of EIAs that used the same competitor conjugate for plate coating, but hapten in solution was in the form of the competitor conjugate or the SMR sodium salt. In every case the antibodies displayed a strong bias for hapten as part of the competitor conjugate over sodium SMR. No meaningful hapten–protein molar ratios could be determined using this form of competitor conjugate evaluation.

^{14}CSMT–BSA Standard Curve Method. The ^{14}CSMT–BSA standard curve approach involved the determination of hapten–protein molar ratios through the preparation of a radioactive protein conjugate from commercially available ^{14}C-labeled SMT. Radioactive SMT was used since radioactively labeled SMR was not available. The absorbance spectrum of an azo-linked conjugate prepared with SMR could safely be equated to that prepared with SMT for the following reasons. SMR is very similar to SMT in structure and absorbance spectrum. The diazo reaction used in the production of the sulfonamide chromophore utilizes the amino group at the opposite end of the molecule from where the difference of one methyl group distinguishes SMR from SMT (Figure 2). In addition, it has been reported that the molar absorptivities of the chromogenic products of all diazotized sulfonamides are approximately equal (Horwitz, 1981). Therefore, a standard curve was prepared relating the A_{430nm} of the ^{14}CSMT–BSA conjugates to the hapten–protein molar ratios determined for these conjugates by radioactive decay. From this standard curve, knowing the A_{430nm} we calculated the hapten–protein molar ratios for the azo-linked competitor conjugates, and the values are reported in Table 2. This method was determined to be the best for estimating the degree of hapten linkage, since hapten number was directly related to absorbance of the conjugate solutions without the necessity of determining the precise source of the chromophore.

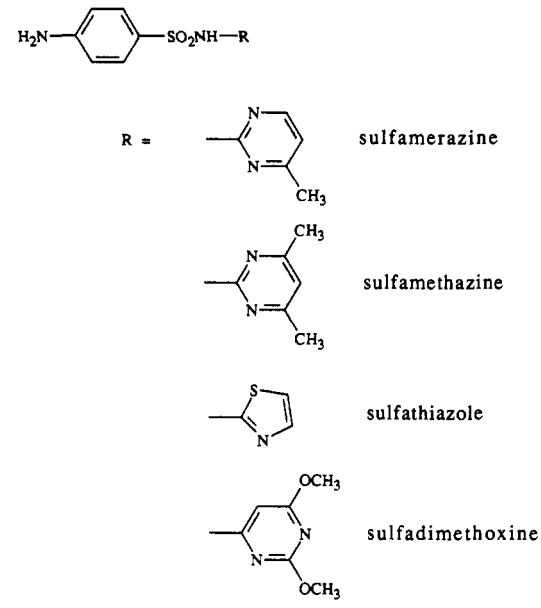

Figure 2. Structures of sulfonamides.

Amino Acid Analysis Method. Amino acid analyses were performed on SMR–BSA(L) and SMR–BSA(M). The hemisuccinate conjugates were not suitable for analysis since their hemisuccinate linking arm would be subject to hydrolysis during the sample preparation. The theory behind these analyses was that the conditions of hydrolysis required for sample preparation would not affect the azo linkage between SMR and the individual amino acids. This would result in a modified amino acid residue of either tyrosine or histidine which would elute from the cation-exchange column with retention times different from those of the unmodified amino acids. Equation 4 was used to quantitate the degree of amino acid modification through comparison of the amino acid composition found for BSA and the compositions found for the two azo-linked competitor conjugates. Individual calculations were performed using the concentration of either glycine or arginine as an internal standard. Glycine and arginine were chosen since their structures were not affected by the conditions of the acid hydrolysis and the peaks of these two amino acids bracketed the tyrosine and histidine region being examined. The values of N for tyrosine and histidine, 20 and 17, respectively, represent the number of tyrosine or histidine residues of the 583 amino acid residues that comprise a BSA molecule (Hirayama *et al.*, 1991). Examination of the results (Table 2) revealed that the primary amino acid involved in our azo-linked proteins was histidine and that there was minimal involvement of the tyrosine residues in the light and medium loaded azo conjugates examined. This preferential linkage to histidine in our conjugates was possibly due to the method of conjugate synthesis, where the conditions were not very basic. Assisting in the reaction of the diazonium ion with tyrosine is the deprotonation of the tyrosine's hydroxyl group (pK_a of 10.07; *The Merck Index*, 1989). In comparison, the pK_a of the ionizable nitrogen on histidine is 5.97 (*The Merck Index*, 1989). Since our reaction of the

sulfonamide diazonium ion with BSA was carried out at a pH greater than 8, the majority of the histidine residues, and not the tyrosine residues, would be ionized. The slightly lower values for the hapten–protein molar ratios obtained with amino acid analysis, compared with those reported by the ^{14}CSMT–BSA method, may result from the inability of amino acid analysis to account for the contribution of azo-linked tryptophan residues. Tryptophan residues represent 2 of the 583 amino acids in BSA (Hirayama *et al.*, 1991) and were considered by Howard and Wild (1957) to be possible sites for azo attachment in proteins. Tryptophan, however, is destroyed by the acidic conditions required to prepare the conjugates for amino acid analysis, and therefore the extent of involvement of this amino acid in the azo reaction is unknown.

NCAT/NAH Standard Curve Method. Spectrophotometric analyses were attempted with the azo-linked conjugates because we knew that the production of colored compounds primarily involved the amino acids tyrosine and histidine (Howard and Wild, 1957). This spectrophotometric method for determining hapten to carrier proteins ratios was reported by Fenton and Singer (1971) and was subsequently used by others (Dargar *et al.*, 1991; McAdam *et al.*, 1992) for similar azo reactions but different haptens. Hapten–protein molar ratios for each of the competitor conjugates were derived from standard curves prepared using NCAT and NAH. NCAT and NAH are the protected amino acid analogs of tyrosine and histidine, respectively. These analogs were used since they had the reactive amino group blocked, which prevented competition for the diazonium ion by the basic amino group (Tabachnick and Sobotka, 1959). In preparing the NCAT and NAH standard curves, we employed reaction conditions that closely mimicked those used to produce the azo-linked conjugates. However, this method for hapten–protein molar ratio determinations did not correlate well with results obtained using two earlier methods, the ^{14}CSMT–BSA standard curve and amino acid analysis (Table 2).

To discover why the NCAT/NAH standard curve method did not provide the expected values, the individual NCAT and NAH curves were more closely examined. Although suitable coefficients of determination ($r^2 \geq 0.98$) were obtained for the reaction of NCAT with SMR, the slope of the NCAT standard curve (1082 absorbance units/M) was approximately 10 times less than the extinction coefficient of 10 400 reported from a similar diazo reaction by Traylor and Singer (1967). This indicated that our reaction conditions may not have been suitable to produce the maximum amount of product from the initial quantities of the NCAT and SMR reactants. This would result in an overestimation by eq 1 and 2 of the contribution of azo-linked tyrosine residues in the conjugates. Also, it was determined from amino acid analyses that only azo-linked histidine residues were produced when a limited amount of diazonium ion was involved in the reaction. To discover whether an accurate estimation of the hapten–protein molar ratio could be determined spectrophotometrically, only the NAH standard curve was used. The slope of the NAH standard curve relating the A_{436nm} and the molar concentration of SMR was 22 460 absorbance units/M ($r^2 = 1.0$). This was very similar to the extinction coefficient of 22 600 reported by Traylor and Singer (1967) using a similar NAH–diazo reaction. Table 2 gives the values obtained by using the NAH standard curve at 436 nm. However, when compared to the ^{14}CSMT–BSA standard curve, the results using the NAH standard curve at 436 nm still underestimated the hapten–protein molar ratio for each competitor conjugate.

Table 9. Hapten–Protein Molar Ratio Determined for the ^{14}CSMT–BSA(2) Conjugate

method of analysis	^{14}CSMT–BSA(2) conjugate (mol of hapten/mol of protein)
radioactive decay	6.0
NCAT/NAH standard curve ($M_{SMR-NCAT}$)[a]	13.7
($M_{SMR-NAH}$)[b]	1.7
(total)	15.4
NAH standard curve (A_{436nm})	2.1
^{14}CSMT–BSA standard curve	5.2

[a] Values for moles of hapten per mole of protein were calculated using the molar concentration of ^{14}CSMT/SMT derived from eq 1 and the known concentration of protein used for absorbance measurements. [b] Values for moles of hapten per mole of protein were calculated using the molar concentration of ^{14}CSMT/SMT derived from eq 2 and the known concentration of protein used for absorbance measurements.

With direct measurements of radioactive decay as the best estimation of hapten–protein molar ratios, a radioactively labeled SMT conjugate [^{14}CSMT–BSA(2)], synthesized independently of those used to create the ^{14}CSMT–BSA standard curve, was used in a final evaluation of the spectrophotometric methodologies. The results obtained for the hapten–protein molar ratios using the ^{14}CSMT–BSA(2) conjugate are reported in Table 9. Amino acid analysis was not performed on this conjugate, as the containment of radioactivity could not be guaranteed. A trend in results, similar to that observed for the azo-linked competitor conjugates, was also found for the ^{14}CSMT–BSA(2) conjugate. The NCAT/NAH standard curves overestimated and the NAH standard curve (436 nm) underestimated the hapten–protein molar ratio for ^{14}CSMT–BSA(2). Only the ^{14}CSMT–BSA standard curve was able to provide an estimation of the hapten–protein molar ratio that was reasonably close to the value calculated from radioactive decay measurements of the ^{14}CSMT–BSA(2) conjugate. It was concluded that for our hapten the validity of comparing the absorbance of a chromophore derived from a single individual amino acid to that of the same amino acid as part of a larger protein structure is questionable and may lead to erroneous results.

Determination of Hapten–Protein Molar Ratios for Hemisuccinate-Linked Competitor Conjugates. Prior to the success of the EDC method for synthesis of the hemisuccinate-linked competitor conjugates, experiments were performed using the *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (NHS/DCC) reaction for active ester formation and succinylsulfamerazine linkage to BSA. However, in synthesis attempts made using the NHS/DCC method, only minute quantities, if any, of hemisuccinate-linked conjugate could be produced. Observed precipitation of the SMR active ester on addition to the BSA solution and subsequent poor yields of conjugate were probably the result of formation of a cyclic succinylsulfamerazine (reaction 5, Figure 1). The occurrence of such a cyclic form during the formation of hemisuccinate-linked conjugates has been described by Tatake *et al.* (1991) and Roseman *et al.* (1992).

Sulfanilic Acid Standard Curve. The sulfanilic acid standard curve was used to determine the values for the hapten–protein molar ratios of the hemisuccinate-linked competitor conjugates. This was accomplished by first digesting each of the conjugates in refluxing hydrochloric acid. The digestion served to decompose the hemisuccinate-linked conjugates, producing sulfanilic acid from

Table 10. I_{50} Values for Azo- and Hemisuccinate-Linked Competitor Conjugates

competitor conjugate	I_{50} value for SMR
azo-linked	
SMR-BSA(L)	3.4 (12)[a]
SMR-BSA(M)	7.7 (27)
SMR-BSA(H)	7.0 (25)
hemisuccinate-linked	
SMR-SA-BSA(L)	31 (108)
SMR-SA-BSA(M)	53 (185)
SMR-SA-BSA(H)	66 (231)

[a] ppb value followed by equivalent nM value in parentheses.

the bound hapten. The aromatic amino group of sulfanilic acid was then free to react under the conditions of the modified form of the B–M reaction to produce a quantifiable color. The length of digestion was optimized until an error of less than 2 μg/mL of the actual concentration was achieved with sulfanilic acid, SMR, and SMR-SA standards.

Comparison of Azo- and Hemisuccinate-Linked Competitor Conjugates in a Competitive Indirect EIA. *Assay Optimization.* The six competitor conjugates synthesized for this study were categorized as light, medium, or heavy depending on their hapten–protein molar ratios. The intracomparison of the azo- and hemisuccinate-linked competitor conjugates was performed on conjugates synthesized with the same hapten–protein linking method. The intercomparison of the azo- vs the hemisuccinate-linked competitor conjugates was permissible since the light, medium, and heavy loadings of hapten–protein for each linking method were approximately equal. To allow for the least bias in evaluation of the performance of all six conjugates, a common set of conditions was established for the competitive indirect EIA through the use of a checkerboard EIA. A 10 ppb dilution of competitor conjugate and a 500000-fold dilution of the antiserum were found to be the best compromise of conditions for the intra- and interconjugate comparisons.

Competitive Indirect EIA Results. The detection limits for each of the conjugates were compared using the I_{50} values (Table 10). The values indicated that the detection abilities of the azo-linked competitor conjugates were superior to those of the hemisuccinate-linked competitor conjugates. From within the two groups of conjugates, it was the lightly loaded conjugates, SMR-BSA(L) and SMR-SA-BSA(L), that outperformed their more heavily loaded counterparts. This was consistent with the theory that the least amount of hapten bound to the plate would provide the best competition for hapten in solution, for the limited number of antibody binding sites. Unexpectedly, the results also demonstrated that the azo-linked competitor conjugates were superior to the hemisuccinate-linked competitor conjugates. Since an azo-linked conjugate (SMR–LPH) was used for immunization, it was expected that the linking region would cause a shift in antibody binding preference toward the azo-linked competitor conjugates and away from hapten in solution. This should have led to higher detection limits, or greater I_{50} values, for the azo-linked competitor conjugates. However, the results of our study revealed that the azo-linked competitor conjugates still outperformed their hemisuccinate counterparts and that hapten–protein molar ratios had a substantial influence on minimizing EIA detection limits.

Sulfonamide-Contaminated Milk Samples. The azo-linked conjugate [SMR-BSA(L)] and hemisuccinate-linked conjugate [SMR-SA-BSA(L)] were determined to be the competitor conjugates with the lowest levels of detection for SMR. These competitor conjugates were used to detect SMR in contaminated milk from sulfonamide-treated cows.

SMR-Spiked Milk Samples. Preparation of the SMR standard curve was attempted using sulfonamide-free raw milk obtained from the bulk milk tank at the University of Alberta Dairy Unit. When 10-fold serial dilutions of SMR in the raw milk were tested in the EIAs, it was found that there were substantial fluctuations in the absorbance values of the highest dilutions. These fluctuations made it difficult to obtain a consistent standard curve and especially difficult to obtain an accurate I_{50} value from the sigmoidal curve. It was believed that the fluctuations were the result of the difficulty in spiking raw milk (a two-phase fat and aqueous system) with SMR at very low levels. The fluctuations in absorbance values of highly diluted SMR samples did not occur with homogenized milk. Although the fat content in homogenized milk is approximately the same as that of the raw milk tested, the homogenized milk, unlike the raw milk, did not separate out during the 2-h incubation step. Even though all of the samples were vortexed vigorously between dilutions, the hydrophobic SMR (Long *et al.*, 1990) was likely in greater concentrations in the fat portion of the separated raw milk. Minute changes in the fat-to-water ratios in raw milk could have contributed significantly to the dilution problem, especially in the very dilute SMR samples. Also unlike the homogenized milk system, the raw milk system contained a physically distinct fat layer which may have contributed to problems in the EIAs. Even with these problems noted, the standard curves obtained using raw milk were similar to those prepared using homogenized milk. The only difference in the standard curves was the high uncertainty found for the lowest levels of SMR. Other advantages to the use of homogenized milk were that it provided a consistent fat content in the milk from trial to trial and was a composite sample from many different cows.

Sulfonamide EIA Cross-Reactivity. Sulfonamide cross-reactivity studies were performed for each of the competitor conjugates [SMR-BSA(L) and SMR-SA-BSA(L)] using homogenized milk spiked with the sodium salts of SMR, SMT, STZ, and SDX (Figure 2). Although SDX was not present in the 3-Sulvit, it is the only sulfonamide that is approved for use in lactating cows (Cullor and Chen, 1991). It was also interesting to evaluate SDX for SMR EIA cross-reactivity since it was a sulfonamide that could be detected by both the CITE sulfa trio test and the Charm II assays. The results of the EIA cross-reactivity tests are reported in Table 11.

Both competitor conjugates demonstrated that the polyclonal antibodies had a strong specificity for SMR.

Table 11. Cross-Reactivity of Sulfonamides with EIA Competitor Conjugates

sulfonamide	SMR-BSA(L) I_{50} (nM)	SMR-BSA(L) cross-reactivity (rel %)	SMR-SA-BSA(L) I_{50} (nM)	SMR-SA-BSA(L) cross-reactivity (rel %)
sulfamerazine	5.2	100	11	100
sulfamethazine	97	5.4	227	4.8
sulfathiazole	1655	0.31	1803	0.61
sulfadimethoxine	>10000	<0.02	4816	0.23

Little affinity for STZ, and especially SDX, was demonstrated by the antibodies. Even the structurally similar sulfonamide, SMT, showed only a 5.4% or less cross-reactivity when compared to the same molar ratio of SMR. Other authors have found 30% (Fleeker and Lovett, 1985), 12.1% (Singh *et al.*, 1989), or 10% (McCaughey *et al.*, 1990) cross-reactivity with antisera produced against SMT when tested with samples containing SMR. Antibodies prepared against SMT may find it easier to accommodate the smaller SMR into their binding sites, whereas SMR-specific antibodies may find it more difficult to fit the extra methyl group of SMT into their binding sites.

3-Sulvit-Treated Cows. 3-Sulvit is a premix of sulfonamides, vitamins, and electrolytes for the treatment of swine, sheep, and cattle bacterial infections and shipping fever. Although the product was easily obtained from a local pharmacy without a prescription, a fine print warning on the package states that 3-Sulvit treatment should not be given to animals within 10 days of slaughter and that it should not be administered to lactating dairy animals. The obvious reason for this warning is that use of 3-Sulvit would result in high levels of sulfonamide residue in the animal's meat or milk. Milk from sulfonamide-treated cows was desirable for test evaluations, especially in view of our difficulty in spiking raw milk at very low sulfonamide levels. It also allowed for evaluation of the effect of sulfonamide metabolites. Various sulfonamide metabolic products are formed by the cow, but commonly sulfonamides are acetylated at the N^4-position (Rehm *et al.*, 1986; Medina *et al.*, 1992; Paulson *et al.*, 1992b). Paulson *et al.* (1992a) also reported that a major metabolite in milk can result from lactose conjugation at the N^4-position of the sulfonamide. Because the immunogen used in this study was produced through conjugation to the carrier protein at the N^4-position, the EIA should be able to recognize the N^4 altered metabolites of SMR. The ability of antibody-based tests to recognize N^4 sulfonamide metabolites has been reported by Sheth *et al.* (1990) and Medina *et al.* (1992).

Analysis of SMR EIA Conditions. For the evaluation of milk samples, only SMR–BSA(L) and SMR-SA–BSA-(L) were chosen for further evaluation. These two competitor conjugates had the best detection levels and similar hapten–protein molar ratios but different linking arms. The emphasis was switched from a comparison of the competitor conjugates under similar conditions to attaining the maximum performance from each conjugate under its own optimal conditions with SMR homogenized milk dilutions. Using homogenized milk rather than water for preparing the SMR standard curves resulted in an overall decrease in the maximum absorbances (fewer antibodies binding to the competitor conjugates) but improved EIA detection capabilities for all conjugates, an effect noted in other systems (Assil and Sporns, 1991). Since homogenized milk is a complex solution of fats, proteins, sugars, and salts, it was surprising that the detection capabilities of the conjugates were not reduced due to the presence of potentially interfering compounds. The improvements in detection capabilities noted in our milk EIAs may be explained by the selection of antibodies with higher binding affinities for SMR in the polyclonal antiserum. Lower affinity antibodies in the antiserum may have experienced increased interference from the milk components.

Under the newly optimized conditions, it was found that the hemisuccinate-linked competitor conjugate [SMR-SA–BSA(L)] required half the concentration for coating of the microtiter plate and polyclonal rabbit serum as did the azo-linked competitor conjugate [SMR–BSA(L)] to produce approximately the same absorbance values. Also, the SMR–BSA(L) conjugate still maintained the lower detection limit. These two findings seemed to contradict what might be logically expected. Since the polyclonal antibodies were prepared using an azo-linked immunogen, it is presumed that although a different carrier protein was used, more antibodies would have compatible binding sites for SMR–BSA(L) rather than SMR-SA–BSA(L). Therefore, if the ability of the carrier protein (BSA) to bind to the microtiter plate was equal for both competitor conjugates, the SMR–BSA(L) competitor conjugate should have required a smaller concentration for coating the microtiter plate and less antibody in solution than the SMR-SA–BSA(L) competitor conjugate. This was not the case. Also, it was assumed that changing the linking arm of the immunogen and the competitor conjugate would decrease the bias for plate-bound hapten, resulting in a more evenly balanced competition and a lower detection limit for the SMR-SA–BSA(L) conjugate. This was also not the case, as the SMR–BSA(L) conjugate had the lowest detection limit. The answer to these contradictions may be in the nature of the azo- and hemisuccinate-linked competitor conjugates.

Since Landsteiner and Van der Scheer's (1936) experimentation with azo-linked haptens, problems of using an azo-linking method for the immunogen and the competitor conjugate seem negligible. It may be the rigidity of the azo linkage that aids in reversible binding and improved competition. The flexible arm of the hemisuccinate linkage may lead to a more stable antibody–antigen interaction for the plate-bound hapten, resulting in less effective completion by free hapten. Also, more plate-bound hapten may be exposed by this flexible arm, resulting in an increased concentration of antibody bound to the plate.

Analysis of Sulfonamide-Contaminated Raw Milk Samples by SMR EIA, Charm II Assay, and CITE Sulfa Trio Test. The results of the analyses for the three test cows' milk samples are focused around the detection of sulfonamide in the milk at the 10 ppb level, since this was the level that the CITE sulfa trio test and Charm II microbial receptor assays have designated a control point. Tables 6–8 contain the results of the milk analyses for cows 8421, 8881, and 9020, respectively. Day 1/a.m. milk for all cows, as indicated by the Charm II assay, contained less than 10 ppb of sulfonamide. Within approximately 5 h of treatment (day 1/p.m.), all cows had sulfonamide in their milk above the 10 ppb level, as determined by the Charm II assay and SMR EIAs. Examination of results from day 1/p.m. until day 7/a.m. demonstrated the sulfonamide detection capabilities of each test.

For the SMR EIAs there was a measurable decrease in SMR content of the milk from day 3/p.m. to day 7/a.m. Taking into consideration only the ppb values determined from the linear regions of the standard curves, there was considerable agreement between the azo- and hemisuccinate-linked competitor conjugates as to the amount of SMR in the milk for cows 8421 and 8881 near and below the 10 ppb range. However, there was slightly less agreement as to the SMR concentration in the milk of cow 9020 determined by the two competitor conjugates.

The CITE sulfa trio test was used to determine SMT and STZ contents of the cows' milk. Although the CITE test will also determine SDX, this sulfonamide was not present in 3-Sulvit, and the test spots for SDX were consistently negative in tests with the cows' milk samples. The CITE sulfa trio test was used to determine the

Table 12. Identification of a 10 ppb Cutoff Date for Milk Samples Using Sulfonamide Assays

cow ID no.	SMR-BSA(L) EIA (sulfamerazine)[a]	SMR-SA-BSA(L) EIA (sulfamerazine)	CITE sulfa trio		Charm II assay (all sulfonamides)
			sulfamethazine	sulfathiazole	
8421	4/a.m.	4/a.m.	5/a.m.	4/p.m.	6/p.m.
8881	5/a.m.	5/a.m.	5/a.m.	4/p.m.	5/p.m.
9020	4/a.m.	4/a.m.	4/p.m.	4/p.m.	4/p.m.

[a] Sulfonamide(s) detected by assay.

sulfonamide content of the milk through the examination of a range of milk samples that were known to have a 10 ppb sulfonamide concentration by Charm II analysis. At times it was possible to distinguish between the concentration of STZ and SMT through the intensity of the spot that was formed. This provided the results at day 4/p.m. for cows 8421 and 8881, where the SMT spot could be reported as being >10 ppb while the STZ spot was <10 ppb.

The operator's manual for the Charm II assay sets for criteria for a safe level control point for determining negative samples as the control point (which was determined to be 1100 cpm) plus 15% (1265 cpm). For the purposes of our study, we were interested in quantifying the sulfonamide concentration in the milk at a 10 ppb level, so we chose to use 1100 cpm as our control point. This meant that values of <1100 cpm represented a concentration of sulfonamide in the milk of >10 ppb, and values of >1100 cpm represented a sulfonamide concentration of <10 ppb. By day 7/a.m. results of the Charm II indicated that all cows' milk was below the 10 ppb level even with the 15% safety factor.

Considering that there were three individual cows and four test situations used to examine the sulfonamide-contaminated milk samples, all test results were in relative agreement as to date when the sulfonamide concentration in the milk samples fell below 10 ppb, or a 10 ppb cutoff data (Table 12). For cow 8421, the findings by Charm II assay of day 6/p.m., being the first milk <10 ppb, are probably exaggerated. However, since day 6/a.m. milk was not available, determinations had to be made based on day 6/p.m. results. The different daily concentrations of sulfonamides in the milk samples may be attributed to three factors. First, there were the different levels of the three sulfonamides found in 3-Sulvit. Since SMR represented the smallest proportion, it was logical that the SMR EIAs would show the quickest reduction in sulfonamides, followed by the other two tests. Second, metabolic factors can influenced the rate of excretion of sulfonamides into milk. Rehm *et al.* (1986) reported that there are considerable variations in the rates of sulfonamide excretion between individual animals of the same species. Also, a sulfonamide's state of ionization has been found to influence its excretion rate (Rasmussen, 1958). The third and final factor was the variation of sulfonamide sensitivity of each analyses. For example, the operator's manual for the Charm II assay reports that the assay is 2.5 times more sensitive to SMR and STZ than to SMT, for which the 10 ppb level was established. This increased sensitivity to SMR and STZ for the Charm II assay would have prolonged the 10 ppb cutoff date.

The structural similarities between SMR and SMT and the availability of SMR in commercially produced sulfonamide preparations may require the positive identification of SMR residues in milk with screening tests such as immunoassays. This study has developed two SMR EIAs that have the detection limits and specificity required to detect SMR in milk.

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