Development of a Monoclonal Antibody-Based cELISA for the Analysis of Sulfadimethoxine. 2. Evaluation of Rapid Extraction Methods and Implications for the Analysis of Incurred Residues in Chicken Liver Tissue

Mark T. Muldoon,[†,‡] Sandra A. Buckley,[†] Sudhir S. Deshpande,[§] Carol K. Holtzapple,[*,†] Ross C. Beier,[†] and Larry H. Stanker[†]

Food Animal Protection Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, Texas 77845, and L.J.L. Biosystems, 404 Pasman Drive, Sunnyvale, California 94089

Several rapid extraction methods were evaluated for use with a monoclonal antibody-based competitive inhibition ELISA (cELISA) to detect sulfadimethoxine (SDM) in chicken liver tissue. These methods included extraction of the samples with (1) aqueous buffer with or without ultrafiltration, (2) acetonitrile/water, (3) methanol/water, or (4) acetone. The organic extraction methods were evaluated with or without solvent evaporation prior to dilution into assay buffer for the cELISA. The aqueous-based extraction methods were compatible with the cELISA. However, of the organic extraction methods, only the acetone liver extract with solvent evaporation prior to analysis was compatible with the cELISA. The cELISA method coupled to aqueous- or acetone-based sample extraction as well as an HPLC method was evaluated for the analysis of chicken liver tissues fortified with SDM at levels from 0.2 to 0.025 ppm. Mean SDM recoveries for the HPLC method and for the cELISA method using samples prepared by aqueous extraction, aqueous extraction and ultrafiltration, or acetone extraction, evaporation, and reconstitution were 68.9, 95.7, 60.1, and 52.5%, respectively. For the analysis of samples obtained from an SDM incurred residue study, HPLC and cELISA analysis of the same organic extract gave results that were highly correlated ($R^2 = 0.976$; $p < 0.0001$). However, results obtained from the analysis of aqueous extracts by cELISA did not correlate well with those obtained by HPLC ($R^2 = 0.61$, $p > 0.0006$). This was attributed to the coextraction of cross-reactive SDM-related residues that were not quantified by the HPLC method. The presence of these residues should be considered during data interpretation when ELISA methods coupled with rapid aqueous extraction of samples are used in SDM residue monitoring programs.

Keywords: *Sulfadimethoxine; immunoassay; extraction methods; food safety*

INTRODUCTION

In the preceding paper (Muldoon et al., 2000) we described the development and characterization of a panel of monoclonal antibodies against the sulfonamide antibiotic sulfadimethoxine (SDM). This sulfonamide is particularly important in agriculture; it is used to control and prevent bacterial and protozoan-borne diseases in food animals (Lindsay and Blagburn, 1995). Sulfonamides are monitored in edible tissues of meat and poultry at a tolerance level of 0.1 μg/g (U.S. Department of Agriculture, 1994, 21CFR 500.640).

Immunoassays designed to detect sulfonamides in animal tissues often utilize a cleanup step prior to analysis (Renson et al., 1993; Walker and Barker, 1994). The purpose of this study was to evaluate the use of rapid extraction methods coupled to a monoclonal antibody-based ELISA for the analysis of SDM in chicken liver tissue. Ideally, this approach would increase sample throughput and decrease or eliminate the use of hazardous organic solvents. For these studies, we used a highly sensitive and specific monoclonal antibody, designated SDM-18, in an indirect cELISA format to detect SDM in both fortified samples and samples derived from a SDM incurred residue study. In addition, samples were analyzed for the presence of SDM using an established HPLC method that employs an acetonitrile/water extraction step followed by extensive extract cleanup. Both the immunological and the HPLC methods gave comparable results in spike recovery studies. In addition, in the incurred residue study, we found that the cELISA and HPLC results were highly correlated when the purified organic extracts were analyzed by both methods. However, when an aqueous extraction step was used, the cELISA method overestimated the amount of SDM present compared to that observed with the HPLC method. Ultrafiltration of the aqueous extracts (to remove protein-bound conjugates) improved the correlation between the HPLC and cELISA results as did a rapid acetone extraction step coupled to cELISA analysis. These results suggest that water-soluble, cross-reactive components are present in the simple

* Author to whom correspondence should be addressed [e-mail holtzapple@ffsru.tamu.edu; fax (409) 260-9332].

[†] U.S. Department of Agriculture.

[‡] Present address: Strategic Diagnostics, Inc., 128 Sandy Dr., Newark, DE 19713.

[§] L.J.L. Biosystems.

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aqueous extract that are not present in the filtered aqueous or the organic extracts. Therefore, the cELISA coupled with a simple aqueous extraction method measures total residue composition (including SDM−protein conjugates) as opposed to the HPLC method that quantifies levels of only SDM and its free metabolite N^4-acetyl-SDM.

MATERIALS AND METHODS

Chemicals and Supplies. Sulfadimethoxine (SDM) was purchased from Fluka (Buchs, Switzerland). N^4-Acetylsulfadimethoxine was a gift from Steven A. Barker, Department of Veterinary Physiology, Louisiana State University, Baton Rouge, LA. Goat anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (GαMIgG−HRP) was purchased from Sigma (St. Louis, MO). K-Blue (enzyme substrate) was purchased from ELISA Technologies (Lexington, KY). Nonfat dry milk (NFDM) was obtained from a local grocery store. Control chicken liver tissue was obtained from the Texas A&M University Poultry Center, College Station, TX. Centriprep-3 concentrators (MW cutoff = 3000) were purchased from Amicon, Inc. (Beverly, MA).

Buffers. All buffer components were of cell culture or reagent grade. The compositions of the various buffers were previously described (Muldoon et al., 1997). Briefly, assay buffer consisted of 0.1 M Tris (pH 7.75) containing 0.001% NFDM and 0.005% Tween 20 (v/v). Coating buffer was 0.5 M carbonate−bicarbonate (pH 9.6) containing 3 mM magnesium chloride. Phosphate-buffered saline (pH 7; PBS-7) consisted of 100 mM sodium phosphate and 150 mM NaCl. Blocking buffer consisted of PBS (pH 9; PBS-9) containing 3% NFDM.

Equipment. Cell culture plasticware was purchased from Costar (Cambridge, MA). Microtiter plates used for ELISA analyses were flat-bottom Nunc Immunoplate II Maxisorp (Nunc, Roskilde, Denmark). Microtiter plate optical density (OD) measurements were made using a Bio-Rad model 3550 microplate reader (Richmond, CA). Data were collected using a Macintosh II computer with Reader Driver 1.0 and Microplate Manager 1.0 software (Bio-Rad). Other calculations utilized Excel spreadsheet software (Microsoft Corp., Redmond, WA).

The high-performance liquid chromatograph (HPLC) system was a Bio-LC from Dionex (Sunnyvale, CA) equipped with a UV−visible variable wavelength detector (set at 265 nm). The reversed-phase column was a 4.6 mm × 25 cm LC18-DB (deactivated; 5 μm) from Supelco, Inc. (Bellefonte, PA). The mobile phase was 25% acetonitrile in 0.05 M monosodium dihydrogen phosphate in water (pH 3) at a flow rate of 1.0 mL/min. Fifty-microliter injections were made.

Competitive Inhibition ELISA (cELISA). Microtiter plates were coated with BSA−SDM and blocked as previously described (Muldoon et al., 2000). One hundred microliters of sample diluted in assay buffer was added to the microtiter plate well followed by 100 μL of an optimized amount of monoclonal antibody SDM-18 diluted in assay buffer. The sample−antibody mixture was incubated at room temperature on the plate for 1 h. After the plate had been washed, 100 μL of GαMIgG−HRP diluted 1:1000 in assay buffer was added to each well, and the plates were again incubated at room temperature for 1 h. After the plate had been washed to remove unbound antibody−enzyme conjugate, 100 μL of enzyme substrate, K-Blue, was added. The plates were incubated for 20 min, and optical density (OD) measurements were made at 450 nm after the addition of 50 μL of 1 M H_2SO_4. Data were transformed to B/B_0 values, where B is the OD value obtained for the standard or sample and B_0 is the OD value obtained for the zero competitor control in the appropriate matrix. IC_{50} values (representing the concentration of the inhibitor that produces a 50% decrease in the signal compared to that of the zero competitor control) were obtained using the four-parameter curve fitting function in Microplate Manager 1.0.

Preparation of Aqueous Extracts of Chicken Liver Tissue. Chicken liver was homogenized using an Ultra-Turrax model SDT tissue homogenizer (Ika-Werk, Staufen, Germany). A 4-g sample of the homogenized tissue was weighed into a 50-mL polypropylene centrifuge tube. Assay buffer (36 mL) was added, and the sample was vortexed for 10 s to suspend the homogenate. The sample was centrifuged at 1000*g* for 10 min. The supernatant was further diluted in assay buffer prior to cELISA analysis (ELISA-1). In some cases (ELISA-2), 10 mL of the supernatant was added to a concentrator device (MW cutoff = 3000) and centrifuged for 1 h at 1000*g*, and the filtrate was diluted in assay buffer prior to cELISA analysis.

Preparation of Organic Extracts of Chicken Liver Tissue. Acetonitrile/water [90:10 (v/v)], methanol/water [80:20 (v/v)], and acetone were evaluated as rapid extraction solvents for use with the cELISA. Solvent (7.5 mL) was added to homogenized liver tissue (2.5 g) in a 50-mL polypropylene centrifuge tube. The sample was vortexed for 10 s and centrifuged at 1000*g* for 10 min. An aliquot of the supernatant was either diluted in assay buffer or evaporated to dryness and reconstituted in assay buffer prior to ELISA analysis.

Evaluation of the Various Liver Extracts on ELISA Performance. Control liver was homogenized and extracted by each of the procedures described above. Sulfadimethoxine standards ranging from 10 to 0.039 ppb were prepared in assay buffer or in various dilutions of liver extract. For the aqueous extract, liver extract dilutions of 1:10, 1:25, 1:50, and 1:100 were evaluated. For the organic extracts, liver extract dilutions of 1:20, 1:50, 1:100, and 1:200 were evaluated. Each ELISA plate (two per experiment) consisted of duplicate wells of each concentration of SDM made in assay buffer (eight concentrations and an unspiked control per set) and triplicate wells of each concentration of SDM and an unspiked control made in two of the extract dilutions.

Organic Extraction and HPLC Analysis of SDM in Chicken Liver Tissue. The HPLC extraction method was an adaptation of the method reported by Furusawa and Mukai (1994). Five grams of homogenized liver was weighed into a 50-mL polypropylene centrifuge tube. To this were added 25 mL of acetonitrile/water [90:10 (v/v)] and 20 mL of hexane. The tube was vortexed for 1 min and centrifuged at 1000*g* for 10 min. The supernatant was collected in a separatory funnel, and the pellet was extracted two additional times. The pooled supernatants were allowed to separate, and the acetonitrile layer was filtered and evaporated in vacuo to dryness. The residue was redissolved in chloroform (10 mL) and transferred to a glass column (30 cm × 15 mm i.d.) containing aluminum oxide (type I, basic) that was prewashed with chloroform (20 mL). The column was rinsed with 20 mL of chloroform, and SDM was eluted with 20 mL of acetonitrile/water [90:10 (v/v)]. The eluent was collected and evaporated to dryness in vacuo. The residue was dissolved in 1 mL of HPLC mobile phase. Aliquots (50 μL) were injected onto the HPLC column.

Analysis of SDM in Spiked Chicken Liver Homogenates by cELISA and HPLC. Aliquots of chicken liver homogenate (5 g for analysis by HPLC, $n = 2$; 4 g for analysis by ELISA-1 and -2, $n = 3$; 2.5 g for analysis by ELISA-3, $n = 4$) were fortified with SDM to give tissue levels of 0.2, 0.1, 0.05, and 0.025 ppm of SDM. These fortified samples and aliquots of unspiked tissue were analyzed by cELISA and HPLC as previously described.

Production of Incurred SDM Residue Tissues in Broiler Chickens. Albon antibacterial soluble powder (containing per packet 94.6 g of SDM as the sodium salt and disodium edetate) was used to produce incurred residue tissues in broiler chickens. Fifteen 6-week-old Peterson × Hubbard broilers that had not been previously exposed to sulfonamide antibiotics were obtained from the Texas A&M University Poultry Center, College Station, TX. They were housed in Petersime finishing units and provided with fresh clean corn/soybean feed and drinking water ad libitum throughout the study. The birds were divided into five sets of three birds each: the control group was given untreated drinking water; three groups were given water treated with SDM at the labeled (1×) dose [0.05% (w/v) SDM]; and one group was given water

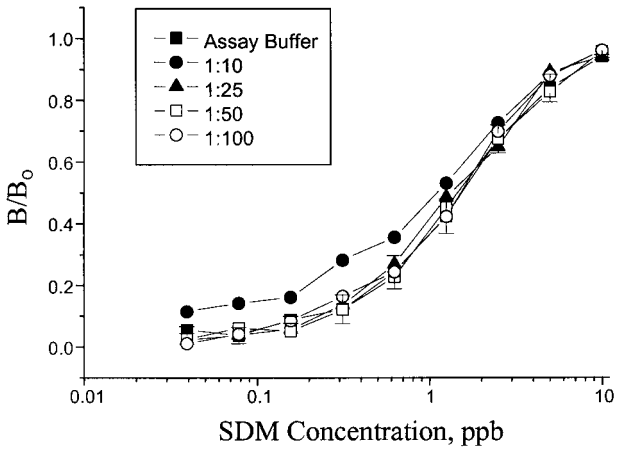

Figure 1. cELISA standard curves for SDM obtained with assay buffer or with various dilutions of aqueous buffer extracts of control liver (ELISA-1). Error bars represent standard deviations at each concentration for the cELISA performed in assay buffer ($n = 4$).

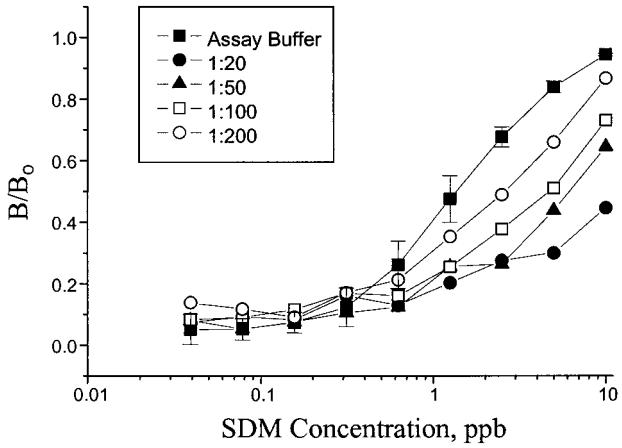

Figure 2. cELISA standard curves for SDM obtained with assay buffer or with various dilutions of acetonitrile/water extracts of control liver without solvent evaporation. Error bars represent standard deviations at each concentration for the cELISA performed in assay buffer ($n = 4$).

treated at twice the labeled (2×) dose [0.1% (w/v) SDM]. The broilers were kept on their respective drinking water treatments for five consecutive days, with their water changed daily. After 5 days, the control group, one of the 1×-dosage groups, and the 2×-dosage group were slaughtered via cervical dislocation, and tissues (liver and breast muscle) were collected. The remaining 2 groups that received the 1× dosage were given fresh untreated water and slaughtered at either 2 or 5 days after withdrawal from SDM treatment. Tissues were immediately frozen at −70 °C until analyzed by HPLC and the various cELISA methods.

Analysis of SDM Incurred Residue Chicken Liver Tissues. Individual liver samples obtained from each bird were homogenized. Aliquots from each were analyzed by HPLC and by the cELISA method using (a) HPLC organic extracts that had been evaporated and reconstituted in assay buffer (ELISA-organic), (b) aqueous extracts (ELISA-1), (c) aqueous extracts that had been subjected to ultrafiltration (ELISA-2), and (d) acetone extracts that had been evaporated and reconstituted in assay buffer (ELISA-3).

RESULTS AND DISCUSSION

Evaluation of the Effects of Various Liver Extracts on ELISA Performance. *Assay Buffer Extraction*. Sulfadimethoxine standard curves were prepared in assay buffer and in dilutions of the various extracts of control liver tissue. These were analyzed by cELISA to determine whether the extract matrix interfered with the assay and whether nonspecific interferences could be eliminated or minimized by further sample dilution. Figure 1 shows the cELISA results obtained using the assay buffer extraction procedure. The average OD values (450 nm) obtained with unspiked assay buffer or with control liver extracts diluted 1:10, 1:25, 1:50, and 1:100 in assay buffer were 1.70, 1.37, 1.52, 1.83, and 1.76 absorbance units, respectively. The IC_{50} values derived from cELISA curves generated using concentrations of SDM ranging from 10 to 0.039 ng/mL prepared in assay buffer or in control liver extracts diluted 1:10, 1:25, 1:50, and 1:100 were 1.65, 1.40, 1.51, 1.74, and 1.52 ppb of SDM, respectively. When OD measurements were normalized to B/B_0 values, using the appropriate matrix control for B_0, the standard curve obtained with the 1:10 extract was not identical to that obtained with assay buffer (Figure 1). In contrast, standard curves obtained using higher dilutions of liver extract were identical to the standard curve obtained using assay buffer alone. This result suggests that the assay buffer extract of chicken liver did not interfere with cELISA performance when the extract was used at a minimum dilution of 1:25. Similar results were obtained using extracts subjected to ultrafiltration (data not shown).

Organic Extracts. Crude extracts of liver tissue prepared in acetonitrile/water, methanol/water, or acetone were not compatible with the cELISA. Figure 2 summarizes the cELISA results obtained using different dilutions (in assay buffer) of the acetonitrile/water extracts of control chicken liver spiked with various concentrations of SDM. In these experiments, the average OD values (450 nm) obtained with unspiked assay buffer or with control liver extracts diluted 1:20, 1:50, 1:100, and 1:200 in assay buffer were 0.99, 1.75, 1.63, 2.76, and 1.91 absorbance units, respectively. The IC_{50} value for SDM obtained in assay buffer was 1.46 ppb. The IC_{50} values for SDM obtained using spiked liver extracts diluted 1:20 and 1:50 were >10 ppb, the highest SDM concentration used in the experiment. The IC_{50} values for SDM obtained using spiked liver extracts diluted 1:100 and 1:200 were 3.70 and 2.95 ppb, respectively. Equally high IC_{50} values were obtained when liver samples were extracted with methanol/water. Furthermore, no improvement was observed using either the acetonitrile/water or the methanol/water extracts when the extracts were evaporated to dryness and reconstituted in assay buffer prior to analysis (data not shown). The acetonitrile/water and methanol/water extracts appeared to increase antibody binding to the BSA−SDM coated plate, hence the increase in the OD values of the zero competitor controls in the presence of the control liver extracts. In addition, there appeared to be a decrease in the relative affinity of the antibody for free SDM as there was a concurrent increase in the IC_{50} value for free SDM.

A similar but less dramatic increase in the OD values of the zero competitor controls was observed when the cELISA was conducted in acetone extracts of liver (Figure 3). In this experiment, the average OD values (450 nm) obtained with unspiked assay buffer or with acetone extracts of control liver diluted 1:20, 1:50, 1:100, and 1:200 in assay buffer were 0.745, 1.05, 0.99, 1.27, and 1.13 absorbance units, respectively. Thus, acetone extraction appeared to have less of an effect on the maximum signal. The IC_{50} values for SDM obtained

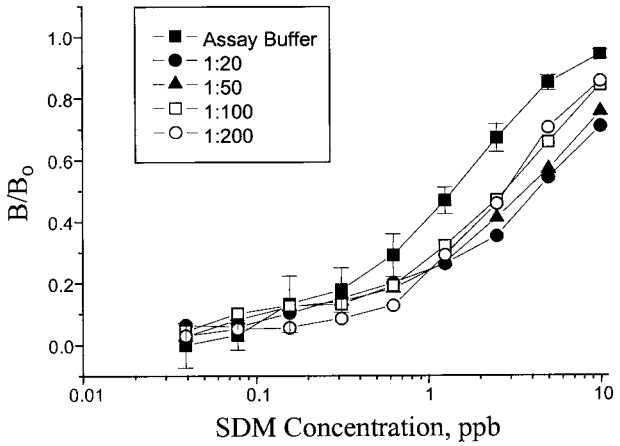

Figure 3. cELISA standard curves for SDM obtained with assay buffer or with various dilutions of acetone extracts of control liver without solvent evaporation. Error bars represent standard deviations at each concentration for the cELISA performed in assay buffer ($n = 4$).

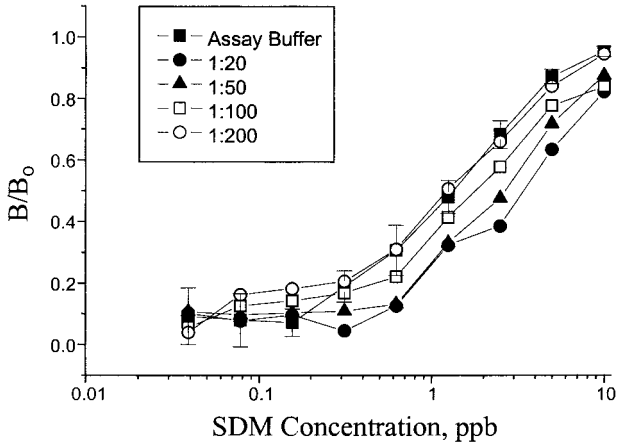

Figure 4. cELISA standard curves for SDM obtained with assay buffer or with various dilutions of acetone extracts of control liver after solvent evaporation and reconstitution in assay buffer (ELISA-3). Error bars represent standard deviations at each concentration for the cELISA performed in assay buffer ($n = 4$).

with assay buffer or with fortified control liver extracts diluted 1:20, 1:50, 1:100, and 1:200 in assay buffer were 1.35, 3.10, 2.80, 2.67, and 2.52 ppb, respectively. As was observed for the other organic solvents, there was a negative effect on antibody affinity for SDM, resulting in an increase in the IC_{50} values. However, in contrast to what was observed with the acetonitrile/water and methanol/water extracts, when the acetone extract was evaporated to dryness and reconstituted in assay buffer prior to cELISA analysis, the curves obtained with the 1:100 or 1:200 extract dilutions overlapped the standard curve prepared in assay buffer alone (Figure 4). In this experiment, the average OD values (450 nm) obtained with unspiked assay buffer or with acetone extracts of control liver reconstituted in assay buffer and then diluted 1:20, 1:50, 1:100, and 1:200 in assay buffer were 0.942, 1.19, 1.24, 1.16, and 1.11 absorbance units, respectively. The IC_{50} values for SDM obtained with assay buffer or with reconstituted fortified liver extracts diluted 1:20, 1:50, 1:100, and 1:200 in assay buffer were 1.49, 3.15, 2.66, 1.71, and 1.48 ppb, respectively. These results suggest that, for acetone, removal of the extract solvent prior to cELISA analysis improved assay performance and that for a sample dilution of at least 1:100, samples can be analyzed on the basis of a standard curve obtained using spiked assay buffer.

Analysis of SDM in Fortified Chicken Liver Tissue by cELISA and HPLC. Homogenized chicken liver tissue was fortified with SDM and analyzed by the cELISA method using aqueous buffer extracts (ELISA-1), aqueous buffer extracts subjected to ultrafiltration (ELISA-2), and acetone extracts that had been evaporated and reconstituted in assay buffer (ELISA-3) and by the HPLC method, which uses an acetonitrile/water extraction procedure followed by cleanup of the extract (as described under Materials and Methods). The results of this study are shown in Table 1. The cELISA coupled to the aqueous extraction method (ELISA-1) gave the highest recovery of the methods tested. However, the HPLC method gave the most consistent recoveries and the most precise results. Both of these methods recovered N^4-acetyl-SDM to the same extent as the parent drug (data not shown). The other two cELISA methods, using either the aqueous buffer extracts subjected to ultrafiltration (ELISA-2) or evaporated acetone extracts reconstituted in assay buffer (ELISA-3), exhibited mean SDM recoveries $\leq$60%. Consequently, these latter two methods may be used as screening methods, but they cannot be used to quantify SDM near the tolerance level of 0.1 ppm in tissue. As shown in previous experiments, a 1:100 dilution of the acetone extracts is necessary when they are used in the cELISA. This presents a problem near the tolerance level; however, at higher SDM concentrations (1.0 and 2.0 ppm), the acetone extraction method is quantitative.

Analysis of SDM Incurred Residue Tissues by HPLC and ELISA with Various Extraction Methods. Following SDM treatment in chickens, the withdrawal time recommended to ensure that residue levels in edible tissues are below the tolerance level of 0.1 ppm is 5 days (U.S. Department of Agriculture, 1994, 21CFR 500.640). This tolerance was established using conventional chromatographic analytical methodology. To evaluate the effectiveness of the various ELISA methods as residue monitoring methods, it was necessary to compare results obtained with these methods to those obtained with a conventional analytical method, such as HPLC, using tissues containing incurred SDM residues.

Six-week-old broiler chickens were treated with SDM in their drinking water for 5 days at the recommended dose or at twice the recommended dose and withdrawn from medication for various lengths of time prior to slaughter. Liver tissues from the treated birds, as well as from birds slaughtered after being given unmedicated drinking water (controls), were analyzed by HPLC and by cELISA using several extraction methods. The recovery results of this study are shown in Table 2, and regression data are presented in Table 3. The HPLC results demonstrate that tissue residue levels were high in birds given SDM at the recommended dose immediately after withdrawal and that these levels rapidly diminished after 2 days. After 5 days, residues were below the tolerance level of 0.1 ppm as measured by HPLC (Table 2). When aliquots of the same extracts were evaporated, reconstituted in assay buffer, and analyzed by cELISA (ELISA-organic) the results correlated well with the HPLC results (Table 3). Although this extraction method was not evaluated in conjunction with the cELISA in spike−recovery experiments, the extracts obtained by using this method were clearly

Table 1. Recovery of SDM from Fortified Chicken Liver Tissue As Measured by HPLC and ELISA Using Various Extraction Methods

	% recovery (%CV)[a]			
spike level, ppm	HPLC-acetonitrile/ water extraction	ELISA-aqueous extraction	ELISA-aqueous extraction/ ultrafiltration	ELISA-acetone extraction/ evaporation
2.0	—[b]	—	—	114.9 (9.1%)
1.0	—	—	—	101.0 (14.9%)
0.2	68.1 (3.0%)	81.6 (12.2%)	51.4 (2.1%)	56.6 (9.6%)
0.1	70.6 (3.4%)	102.7 (11.9%)	58.0 (10.2%)	48.4 (21.1%)
0.05	68.1 (8.8%)	91.0 (16.4%)	63.8 (10.1%)	nd[c]
0.025	68.9 (0.5%)	107.4 (9.9%)	70.5 (8.0%)	nd
mean % recovery	68.9	95.7	60.1	52.5[d]

[a] %CV, coefficient of variation, where $n = 2$ for HPLC, $n = 3$ for ELISA's with aqueous extraction, and $n = 4$ for ELISA with acetone extraction. [b] These sample concentrations were not analyzed. [c] nd, not detected. [d] Mean recovery of 0.2 and 0.1 ppm samples.

Table 2. Results from the Analysis of SDM Incurred Residue Tissues by HPLC and ELISA with Various Extraction Methods

			concentration, ppm[a]				
sample	treatment	withdrawal time, days	HPLC-organic[b]	ELISA-organic[c]	ELISA-1[d]	ELISA-2[e]	ELISA-3[f]
221	control	0	0.00	0.00	0.00	0.00	0.00
220	control	0	0.00	0.00	0.00	0.01	0.00
214	control	0	0.03	0.01	0.00	0.00	0.00
221	1×[g]	0	31.25	40.03	69.15	49.25	53.75
244	1×	0	16.36	18.05	68.40	32.57	35.19
204	1×	0	34.65	37.67	113.4	45.90	59.74
219	1×	2	0.11	0.15	18.39	1.31	0.68
222	1×	2	7.32	9.84	11.81	1.17	0.49
209	1×	2	0.20	0.29	27.57	1.67	2.01
223	1×	5	0.02	0.02	4.79	0.57	0.33
203	1×	5	0.00	0.03	6.10	0.56	0.26
284	1×	5	0.01	0.03	8.44	1.01	0.07
281	2×[h]	0	92.04	81.51	128.01	97.67	153.26
202	2×	0	41.61	38.39	224.37	79.91	131.85
205	2×	0	54.50	60.29	106.00	55.33	99.80

[a] Concentrations reported by the HPLC method are the sum of SDM and N^4-acetyl-SDM. Concentrations reported by the cELISA method are SDM equivalents that are based on the SDM standard curve analyzed on each plate. [b] HPLC-organic, acetonitrile/water extraction and cleanup. [c] ELISA-organic, acetonitrile/water extraction and cleanup. [d] ELISA-1, aqueous buffer extraction. [e] ELISA-2, aqueous buffer extraction and ultrafiltration. [f] ELISA-3, acetone extraction, evaporation, and reconstitution in assay buffer. [g] The 1× dose was 0.05% (w/v) SDM in drinking water. [h] The 2× dose was 0.1% (w/v) SDM in the drinking water.

Table 3. Linear Regression Data for the Analysis of SDM Incurred Residue Tissues by HPLC and ELISA with Various Extraction Methods

comparison vs HPLC-organic[a]	slope	R^2 ($n = 15$)	p value
ELISA-organic[b]	0.952	0.976	<0.0001
ELISA-1[c]	1.87	0.609	6.01×10^{-4}
ELISA-2[d]	1.16	0.909	<0.0001
ELISA-3[e]	1.85	0.912	<0.0001

[a] HPLC-organic, acetonitrile/water extraction and cleanup. [b] ELISA-organic, acetonitrile/water extraction and cleanup. [c] ELISA-1, aqueous buffer extraction. [d] ELISA-2, aqueous buffer extraction and ultrafiltration. [e] ELISA-3, acetone extraction, evaporation, and reconstitution in assay buffer.

compatible with the cELISA, although crude acetonitrile/water liver extracts were not (Figure 2).

When the aqueous extraction method was used for the cELISA analysis (ELISA-1), the residue levels observed immediately after withdrawal were ~2-fold greater than those observed with the HPLC method. Although the cELISA levels decreased with time, they did not fall below the tolerance level of 0.1 ppm in the 5-day postwithdrawal samples (Table 2). In these samples, in which the observed SDM and N^4-acetyl-SDM levels measured by HPLC were below the tolerance level, residue levels measured using ELISA-1 were >10-fold greater. Obviously, the cELISA results did not correlate well with the HPLC results (Table 3). Because the aqueous extract did not interfere with the cELISA (Figure 1) and because there were no false positives in the control birds (Table 2), these results suggest that when coupled to the aqueous buffer extraction method, the cELISA detects other cross-reactive compounds such as SDM−protein conjugates and free metabolites (in addition to N^4-acetyl-SDM) in the aqueous sample.

Samples were re-extracted and subjected to ultrafiltration to remove components with a molecular mass >3000 Da, such as protein or any SDM−protein conjugates. As opposed to the unfiltered supernatants, which were opaque, these filtered samples were clear. The results obtained from the analysis of these samples by cELISA (ELISA-2) demonstrated better correlation with the HPLC results (Table 3) than those obtained without filtration, but the cELISA results were still significantly higher than those obtained by HPLC. In particular, residue levels observed in birds slaughtered after the recommended 5-day withdrawal time remained above the tolerance level of 0.1 ppm. These results suggest that the cELISA probably detects water-soluble components other than protein conjugates that are not detected with the HPLC method.

The cELISA results obtained using acetone extracts that had been evaporated and reconstituted in assay buffer correlated well with the HPLC results (Table 3) ($p < 0.0001$). However, the levels detected with the cELISA were again higher than those obtained by HPLC. The acetone extraction step, like the filtration step, should eliminate protein-bound drug. However,

because acetone is miscible with water, other water-soluble, cross-reactive metabolites may be extracted with this solvent.

It is well-known that sulfonamides bind extensively to circulating proteins (Bevil, 1988) and, in some species, SDM−protein complexes may account for >95% of the circulating drug (Mengelers et al., 1995). Few data exist on the identification of metabolites of SDM other than N^4-acetyl-SDM. In one paper, a desmethyl metabolite was reported in the excrement from chickens fed SDM (Takahashi, 1985). In chicken livers stored at −20 °C for 1 year, >60% of the spiked SDM was transformed to unknown metabolite(s) other than N^4-acetyl-SDM or an N^4-glycosyl derivative (Parks, 1994). Other sulfonamides, such as sulfamethazine and sulfathiazole, can undergo N^4-acetylation and deamination or form N^4-glucosides, N^4-glucuronides, diconjugates of N^4-acetylated metabolites, and other uncharacterized metabolites (Kuiper et al., 1988; Aschbacher et al., 1995).

Our results demonstrate that the amount of SDM-related residues detected in incurred residue tissue were dependent on the particular extraction method used but independent of the detection method. When both HPLC and cELISA analyses were performed on the same extract, the results were nearly identical (Table 2). However, when aqueous-based extraction methods were used, the results obtained by using the cELISA and HPLC methods were not highly correlated. The use of [^{14}C]SDM for production of incurred residues could help to identify coextracting, antibody-reactive SDM residues (conjugated or free) and to evaluate/optimize rapid extraction methods designed to be used with immunoassays. If rapid, aqueous-based extraction methods are to be used with the cELISA, different criteria will need to be considered for establishing tissue residue tolerances, because this method is more sensitive to other SDM-related residues that are not quantified using conventional organic solvent-based extraction methods.

Conclusions. We have evaluated the use of a monoclonal antibody-based cELISA coupled to a series of rapid extraction methods for the analysis of SDM in chicken liver tissue. Aqueous-based extraction followed by cELISA analysis provided a highly sensitive method for detecting SDM in fortified liver tissue homogenates. However, in incurred residue tissues, where the parent drug may not be the predominant species, cELISA analysis coupled to an aqueous-based extraction detected SDM-related residues that were not detected using the HPLC method coupled to an organic-based extraction and extract cleanup. Because these ELISA-reactive species may not be regulated, use of the immunochemical technique may warrant the establishment of different residue tolerance criteria.

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