Development and Validation of a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for Salinomycin in Chicken Liver Tissue

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Salinomycin is one of the most widely used coccidiostats in U.S. agriculture. A rapid and accurate analytical method for this drug should provide producers and users with an effective management tool. The current chromatographic methods are sensitive but are labor-intensive. In addition, they require large amounts of expensive organic solvents for extraction and cleanup, which requires proper (and expensive) disposal. This paper reports the development of an enzyme-linked immunosorbent assay (ELISA) coupled to a simple aqueous extraction procedure for the analysis of salinomycin in chicken liver tissue. Recovery from spiked liver homogenates was quantitative in the range from 5.0 to 0.05 ppm. Analysis of chicken livers containing incurred residue by ELISA and high-performance liquid chromatography (HPLC) showed the results to be highly correlated ($p < 0.0001$). The ELISA method described here has a limit of quantitation of 50 ppb, which is more sensitive than the HPLC method.

INTRODUCTION

Salinomycin (SAL) is a polyether monocarboxylic acid produced by a strain of *Streptomyces albus*. Other members of this class of compounds include monensin, narasin, and lasalocid, all of which possess relatively broad spectrum anticoccidial activities (McDougald and Roberson, 1988). Their mode of action is attributed to their ionophoric properties and effects on cell membrane function (Pressman and Fahim, 1982). In 1990, SAL was the most widely used coccidiostat in agriculture; its sales accounted for 55% of the U.S. market (SRI International, 1992). SAL is registered for use in the United States as a feed additive at concentrations of 44–66 ppm to control coccidiosis in broiler chickens. Toxicity can occur when broilers are fed SAL above these recommended levels and when susceptible species, such as turkeys or horses, are unintentionally exposed (McDougald and Roberson, 1988). Due to its importance in poultry production, an analytical method for SAL that is sensitive, accurate, and rapid should provide a useful management tool. Salinomycin is commonly in the form of a nonvolatile sodium salt, making it difficult to analyze by gas chromatography. Also, since SAL does not possess a chromophore, it cannot be readily detected spectrophotometrically without prior derivatization. To circumvent this limitation, a thin-layer bioautographic method was developed for the analysis of SAL in chicken liver (Dimenna et al., 1986a). The bioautographic method was sensitive, with a limit of detection of 25 ppb. However, it required over 18 h for its completion. A method for the analysis of SAL in chicken skin and fat was reported by Dimenna et al. (1986b). They extracted the analyte from tissue, oxidized it to a UV-absorbing species, and used high-performance liquid chromatography (HPLC) with UV detection. Their limit of detection was 100 ppb. Martinez and Shimoda (1986) and Asukabe et al. (1994) made fluorescent derivatives of the various polyether ionophores (including SAL) in order to detect these in beef liver tissue and animal feeds, respectively. Both of these methods required extensive sample purification prior to derivatization, and additional purification of the derivatized mixture was necessary before separation by HPLC and fluorescence detection. Their limit of detection for the analysis of beef liver tissue was 150 ppb. Vanillin has been employed for making chromophoric derivatives of the polyether ionophores following HPLC separation (Goras and LaCourse, 1984; Blanchflower et al., 1985; Lapointe and Cohen, 1988).

Our group reported the development of a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for SAL (Elissalde et al., 1993; Stanker et al., 1994). Sixteen monoclonal antibodies were produced that recognized salinomycin and the structurally similar coccidiostat narasin to the same extent. The antibodies did not recognize either monensin or lasalocid. The method could detect less than 0.3 ng of SAL in a 100 μL test sample. Buffer extracts of chicken livers which were spiked with SAL at concentrations from 1.25 to 5.0 ppm were analyzed using the method. Salinomycin recovery was quantitative in this range. The purpose of the current study was to further optimize the ELISA method for the analysis of chicken liver tissue, determine the limitations of the method, apply the method to the analysis of chicken liver tissues from an incurred residue study, and validate these results using conventional HPLC methodology.

MATERIALS AND METHODS

Chemicals and Supplies. Salinomycin (sodium salt, 90%) was purchased from Calbiochem (La Jolla, CA). The sodium salinomycin premix (Bio-Cox, Agri-Bio, Gainsville, GA) was obtained from a commercial supplier and mixed with the feed as directed. OmniSolv methanol and instrumental grade sulfuric acid were from EM Science (Gibbstown, NJ). Methylene chloride (B&J Brand) was from Baxter (Muskegon, MI). Glacial acetic acid (A-38), sodium bicarbonate (S-233), sodium carbonate (S-264), and potassium phosphate (monobasic, P-285) were from Fisher Scientific (Fair Lawn, NJ). The following were from Sigma (St. Louis, MO): vanillin (V-2375),

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Tween 20 (P-5927), sodium chloride (S-9888), potassium chloride (P-8041), sodium phosphate (dibasic, S-0876), Tris hydrochloride (T-3253), Tris base (T-8524), magnesium chloride (M-8266), and goat anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (A-5278) (GaMIgG–HRP). Bovine serum albumin (fraction V, 98%) was purchased from Hazelton Biologics (Lenexa, KS). K-Blue (enzyme substrate) was purchased from ELISA Technologies (Lexington, KY). Preparation of bovine serum albumin conjugated to SAL (BSA–SAL) and production of anti-salinomycin monoclonal antibody were previously described (Elissalde et al., 1993). Nonfat dry milk (NFDM) and broiler chicken livers (control liver) were obtained from a local grocery store. Approximately 2 kg of pooled chicken livers was diced, aliquoted into 60 g portions, and frozen at −80 °C until used.

Buffers. Assay buffer (pH 7.75) contained, per liter of water, 11.4 g of Tris-HCl, 3.32 g of Tris base, 8.7 g of sodium chloride, 0.01 g of NFDM, and 0.005% (v/v) Tween 20. Coating buffer (pH 9.6) contained, per liter of water, 1.59 g of sodium carbonate, 2.93 g of sodium bicarbonate, and 0.203 g of magnesium chloride. Blocking buffer (pH 7) contained, per liter of water, 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of sodium phosphate (dibasic), 0.2 g of potassium phosphate, and 0.05% (v/v) Tween 20.

Equipment. The high-performance liquid chromatography (HPLC) method was a modification from previously described methods (Blanchflower et al., 1985; Lapointe and Cohen, 1988). It was performed using a Dionex (Sunnyvale, CA) microbore system consisting of an advanced gradient pump and a VDM-2 variable-wavelength detector monitored at 520 nm and controlled using an AI-450 chromatography workstation. The column was a 15 cm × 2.1 mm, 5 μm, Supelcosil LC-18 from Supelco (Bellefonte, PA). The isocratic solvent system was 95% methanol/acetic acid (1% v/v, water). The flow rate was 0.25 mL/min. The gradient solvent system was 80% methanol/acetic acid (1% v/v, water), maintained for 1 min postinjection, and then a 80–100% methanol/acetic acid linear gradient was initiated and reached at 4 min; 100% methanol was maintained until 18 min. A 100–80% methanol/acetic acid gradient was initiated and reached at 20 min postinjection. The solvent flow rate was 0.25 mL/min. The postcolumn derivatization system used a RP-1 reagent pump and a PCH-2 postcolumn reaction heater that were both obtained from Dionex. The derivatization reagent consisted of 10% (w/v) vanillin in methanol containing 2% (v/v) sulfuric acid, which was delivered at a flow rate of 0.5 mL/min. Samples (25 μL) were injected onto the system using a Spectra-Physics (San Jose, CA) SP 8880 autosampler. Microtiter plates were Nunc Immunoplate II Maxisorp (Nunc no. 442404). Microtiter plate optical density (OD) measurements were made using a Bio-Rad Model 3550 microplate reader. Data were collected using a Macintosh II computer and Reader Driver 1.0 software (Bio-Rad). Some ELISA data calculations were made using SOFTmax 2.01 software (Molecular Devices Corp., Menlo Park, CA). Other calculations utilized Excel spreadsheet software (Microsoft Corp., Redmond, WA).

Competitive Inhibition ELISA. The ELISA procedure used here was a modification of the previously reported method (Elissalde et al., 1993). Anti-salinomycin monoclonal antibody was purified from ascites fluid using an immobilized protein G column (Pharmacia, Piscataway, NJ). Wells of microtiter plates were coated with 250 ng of BSA–SAL in 100 μL of coating buffer, incubated overnight (approximately 18 h) at 4 °C, and washed with distilled water containing 0.05% (v/v) Tween 20. Wells were blocked with 100 μg of BSA in 200 μL of blocking buffer, incubated for 60 min at ambient room temperature, washed, and stored at −20 °C until used. For the ELISA, 100 μL of sample diluted in assay buffer was added to the microtiter plate well. This was followed by 2 ng of anti-salinomycin monoclonal antibody in 100 μL of assay buffer. The mixture was incubated at room temperature for 60 min, and then the plate was washed. One hundred microliters of GaMIgG–HRP diluted 1:500 in assay buffer was added to each well and incubated at room temperature for 60 min, and then the plate was washed. One hundred microliters of enzyme substrate was added, and plate optical density (OD) measurements (655 nm) were taken at 30 min. In some cases, 50 μL of 3 N sulfuric acid was added and the plate OD measured at 450 nm.

Aqueous Extraction of Salinomycin from Chicken Liver Tissue. Sixty gram portions of control liver were homogenized for 1 min using an Ultra-Turrax Model SDT (IKA-Werk, Staufen, Germany) tissue homogenizer. Four grams of the homogenized liver was then weighed into a 50 mL polypropylene centrifuge tube. Assay buffer (40 mL) was added, and the sample was vortexed for 1 min to suspend the homogenate. The sample was centrifuged at 1000*g* for 10 min. The supernatant was further diluted in assay buffer prior to ELISA analysis.

Methanolic Extraction of Salinomycin from Chicken Liver Tissue. The extraction method was an adaptation of a previously described method used for the analysis of SAL in beef liver tissue (Martinez and Shimoda, 1986). Ten grams of homogenized liver was weighed into a 50 mL polypropylene centrifuge tube. Twenty-five milliliters of 80% (v/v) methanol/water was added, and the sample was vortexed for 1 min to suspend the homogenate. The sample was shaken on a wrist-action shaker for 30 min and centrifuged at 1000*g* for 10 min, and the supernatant was poured into a separatory funnel containing 100 mL of 5% (w/v) sodium chloride. Twenty-five milliliters of 80% methanol was added to the tube (containing the pellet). The tube was vortexed for 1 min to resuspend the pellet and centrifuged, and the supernatant was added to the separatory funnel. The combined methanolic liver extracts were extracted with 3 × 30 mL volumes of methylene chloride. The methylene chloride layer was evaporated to dryness *in vacuo*, and the residue was redissolved in 1 mL of methanol. This was filtered through a Millex HV 45 μm filter (Millipore, Bedford, MA) prior to HPLC analysis.

Evaluation of the Aqueous Liver Extract on ELISA. Control liver was homogenized and extracted as described above. Assay buffer (8 mL) was added to the supernatant (2 mL) (a 1:10 dilution of liver homogenate in assay buffer), resulting in a 1:50 dilution of liver tissue. Five milliliters of the 1:50 dilution was added to 5 mL of assay buffer to give a 1:100 dilution of liver tissue. SAL standards were made ranging from 10.0 to 0.078 ng/mL in assay buffer and in each of the dilutions of aqueous liver extract. These standards (eight concentrations per set), as well as assay buffer and the nonspiked extract (at each dilution), were analyzed in duplicate wells of an ELISA plate. The experiment was replicated on a second ELISA plate. Results using both OD values and B/B_0-transformed data, where B is the OD value of the sample and B_0 is the OD value of the sample without competitor (assay buffer or the appropriate dilution of unspiked liver extract), were evaluated. IC_{50} values (concentration of inhibitor which produces a 50% decrease in signal of the no competitor control) were derived from the four-parameter curve fitting function in SOFTmax. Least squares mean IC_{50} values were tested for significant differences from the standard curve made in assay buffer ($\alpha = 0.05$). Lower and upper limits of quantitation were defined as the amounts of inhibitor that produced B/B_0 values of 0.7 and 0.2, respectively (Karu et al., 1991).

Determination of Salinomycin in Spiked Chicken Liver Tissue by ELISA and HPLC. Control liver tissue was homogenized. For ELISA analysis 4 g aliquots were weighed into 50 mL polypropylene centrifuge tubes. Aliquots not spiked were used for the 0.0 ppm SAL, and other aliquots were spiked with various amounts of SAL to give tissue concentrations of 5.0, 1.0, 0.5, 0.25, 0.1, and 0.05 ppm SAL. Three sets of spiked samples were prepared and analyzed immediately.

The ELISA analysis was performed as described above. On each plate the following were analyzed: a blank (assay buffer added in place of antibody), eight SAL standards serially diluted in assay buffer ranging from 10.0 to 0.078 ng/mL, a no-competitor control, five dilutions of the 0.0 ppm SAL extract, and five dilutions from three of the six extracts of spiked liver. Each sample was analyzed in triplicate wells of an ELISA plate, except the no-competitor control in assay buffer (nine replicate wells). Two ELISA plates were used for the analysis of each set of spiked samples. Raw OD values were transformed to B/B_0 values (where B was the OD value

of the sample and B_0 was the OD value of either the no-competitor control for the standards or the appropriate dilution of the zero-dose extract for the extracts of spiked liver). Concentrations of SAL in the extracts were calculated on the basis of the standard curve within the particular plate using the four-parameter curve fitting function in SOFTmax. The lowest extract dilution that resulted in a B/B_0 value in the linear, quantitative region of the standard curve ($B/B_0 = 0.70-0.20$) was used for determining SAL in the sample.

For the HPLC analysis, 10 g aliquots of homogenized liver were weighed into 50 mL polypropylene centrifuge tubes. Aliquots not spiked were used for the 0.0 ppm SAL, and other aliquots were spiked with various amounts of SAL to give tissue concentrations of 5.0, 1.0, 0.5, 0.25, 0.1, and 0.05 ppm SAL. Three sets of spiked samples were prepared one set at a time and analyzed immediately. The samples were extracted as described above. SAL concentrations in each extract were determined on the basis of standards of SAL made in methanol ranging from 100.0 to 0.41 ppm analyzed concurrently with the extracts from the spiked samples. All samples were injected in duplicate. The isocratic HPLC solvent system was used in this study.

Salinomycin Feeding Study. One-day-old male broiler Peterson × Hubbard chicks (mean weight ± SD = 47.5 + 0.6 g) were obtained from a commercial hatchery, individually weighed, wing banded, and housed in heated growing batteries. Throughout the experiment, the birds were exposed to continuous fluorescent lighting and supplied with water and a commercial, unmedicated, corn–soybean meal-based diet that contained levels of critical nutrients recommended by the National Research Council (1984) (control diet) *ad libitum*. At 21 days of age the chicks were removed from the batteries and randomly assigned to three treatment groups of 15 birds each. Each group was placed in a 11.148 m^2 pen with a wood chip-covered concrete floor and continued to be fed the control diet. On day 43, one of the treatment groups continued to receive only the control diet (no-dose group). The other two treatment groups were fed the same control diet with the addition of either the recommended dose of sodium salinomycin (Bio-Cox) (66 mg of sodium salinomycin/kg of feed, 66 ppm group) or twice the recommended dose (132 mg/kg of feed, 132 ppm group). The birds in each group were treated for 14 days. On day 57, five chickens from each of three treatment groups were killed by cervical dislocation, and their livers were immediately removed and frozen at −70 °C until processed. The remaining 10 birds in each of the three treatment groups were immediately given control feed and sacrificed 18 and 72 h later (5 birds/group/time period). The birds were handled as described previously.

Determination of Salinomycin in Chicken Liver Tissues from the Incurred Residue Study by HPLC and ELISA. Each set of samples consisted of a liver obtained from one bird from each treatment group per withdrawal time (nine samples), a control liver sample, and a control liver sample spiked to 1.0 ppm SAL (HPLC analysis only). One complete set of samples was analyzed at a time using both methods, and five complete sets were evaluated. Livers were thawed at room temperature, homogenized, and stored at 4 °C prior to analysis (within 18 h). Livers were extracted for ELISA and HPLC as described above. The OD values of the aqueous control liver extract were used for calculating B_0 for data transformation of the samples. For each set of samples, the ELISA analysis and extraction of SAL into methylene chloride for subsequent HPLC analysis were performed on the same day. On the next day, samples were further processed and analyzed by HPLC using the gradient solvent system.

RESULTS AND DISCUSSION

ELISA Development. The assay conditions we report here were adapted and modified from the previously reported method (Elissalde et al., 1993) to improve the sensitivity and reliability of the assay and to adapt the assay to a chicken liver matrix. The anti-salinomycin monoclonal antibody (Elissalde et al., 1993) was produced as mouse ascites fluid and subsequently purified to give milligram quantities of a defined immunoreagent.

In these studies, K-Blue was substituted for 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) enzyme substrate (Elissalde et al., 1993). Two antigen plate coating methods were compared for their effect on assay signal and well to well variability. The first method for antigen coating was previously reported (Elissalde et al., 1993). Briefly, the antigen was dissolved in distilled water, and plates were coated overnight at 37 °C. The second coating method dissolved the antigen in carbonate buffer (pH 9.6), and plates were coated overnight at 4 °C. In both ELISA procedures the same amounts of coating antigen, anti-salinomycin monoclonal antibody, and anti-mouse IgG conjugated to peroxidase were used. In these experiments we observed higher OD values for the no-competitor control (1.3 OD units compared to 0.6 OD unit) when plates were coated with the antigen in carbonate buffer (pH 9.6) overnight at 4 °C versus the coating conditions previously reported. In addition, an improvement in well to well variability was observed (CV from approximately 15.0 to less than 10.0%).

Aqueous liver extracts from control samples were prepared as described under Materials and Methods. Analysis of these extracts resulted in large background absorbance values. We observed optical density values of approximately 0.2 OD unit in these buffer control samples in which no monoclonal antibody was present. To minimize this nonspecific effect, various combinations of protein blocking solutions (BSA, ovalbumin, NFDM) and assay buffers (phosphate-buffered saline, Tris) were tested for their effect on decreasing the background absorbance measured at 450 nm (data not shown). We found that the use of BSA as a blocking protein (0.5% w/v) in phosphate-buffered saline containing Tween 20 (0.05%) gave the lowest background absorbance values (approximately 0.15 OD unit). Inclusion of NFDM in the assay buffer (0.01% w/v) further decreased this effect to background absorbance values of less than 0.10 OD unit. However, these background effects were not observed when the samples were measured at 655 nm without acidification. Thus, the nonspecific backgrounds observed in the aqueous liver extract appeared to be associated with the substrate product acidification step. Other enzyme–substrate combinations were not tested. In the final assay procedure, OD measurements were made at 655 nm in the unacidified sample. Using the above modifications an improvement in assay sensitivity and variability from an IC_{50} of 3.30 ng/mL (interaasay CV = 30.3%) (Elissalde et al., 1993) to an IC_{50} of 0.52 ng/mL (interassay CV = 20.7%, intraassay CV = 8.6%) was observed.

ELISA Performance in Aqueous Liver Extract. Salinomycin standard curves were prepared in assay buffer and in various dilutions of the aqueous liver extract and analyzed by ELISA. The resulting standard curves were compared to detect any matrix effects on the assay and to determine an appropriate extract dilution for subsequent sample analysis. The goal was to balance conditions such that minimal matrix effects occurred while providing sufficient method sensitivity (≤0.1 ppm). Maximum absorbances of 1.38, 0.85, 0.72, and 0.66 OD units were observed when the ELISA was performed in assay buffer or in aqueous liver extracts diluted 1:100, 1:50, and 1:10 in assay buffer, respectively. Thus, a significant reduction in maximal absor-

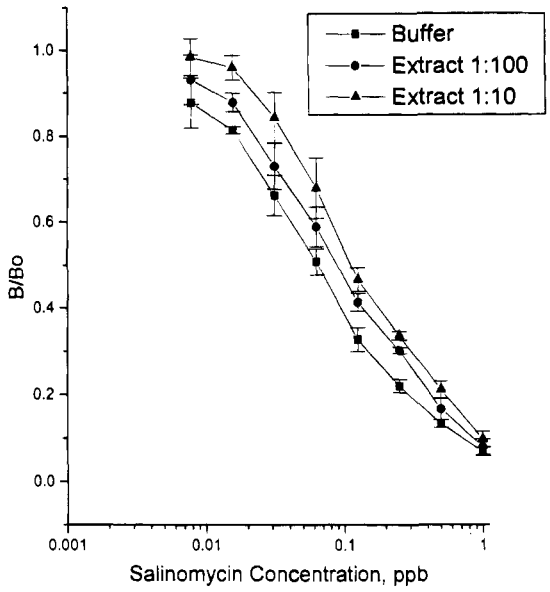

Figure 1. Salinomycin ELISA standard curves made in assay buffer and aqueous liver extract diluted in assay buffer after B/B_0 transformation of OD readings. Mean IC_{50} values derived from standard curves made in assay buffer and liver extracts diluted 1:10, 1:50, and 1:100 were 0.63, 1.25, 1.00, and 0.98 ng/mL, respectively. Mean IC_{50} values derived from standard curves made in liver extract diluted 1:10 and 1:50 were significantly different from the values obtained in assay buffer ($p > 0.05$).

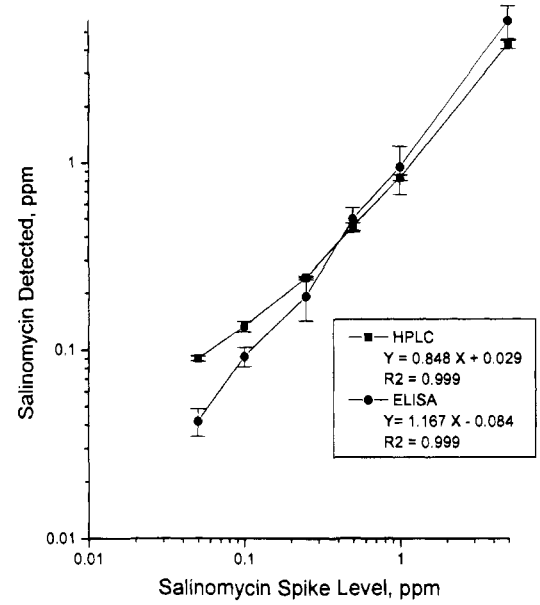

Figure 2. Results from HPLC and ELISA analysis of salinomycin in spiked chicken liver homogenates.

bance values was observed in liver extracts at every dilution tested. HPLC analysis of these control liver extracts indicated that they did not contain any salinomycin. Similar matrix effects have been reported in other ELISAs used to detect low molecular weight residues following widely differing extraction procedures: organic extraction (Stanker et al., 1989, 1993), aqueous extraction (Lehotay and Argauer, 1993), and supercritical fluid extraction (Nam and King, 1994) of animal tissue samples. Clearly interfering substances are present in these extracts which are eliminated in part by sample dilution.

Due to these interferences, use of a simple buffer control to determine B_0 is not possible. Therefore, OD measurements were transformed to B/B_0 values, where B_0 was the mean OD measurement of the no-competitor control in buffer for the standards, and the appropriate dilution of control liver extract for the samples. The results of these experiments are summarized in Figure 1. The standard curves prepared in buffer and aqueous liver extract diluted 1:100 with assay buffer overlapped. The IC_{50} value from the standard curve made in liver extract diluted 1:100 (0.98 ng/mL, CV = 7.52%) was not different ($p > 0.05$) from the value obtained in assay buffer (0.63 ng/mL, CV = 5.65%). These results demonstrate that it is possible to use a B/B_0-transformed standard curve prepared in buffer for extrapolating B/B_0-transformed sample data, with the requirement that a valid matrix control be used for the sample B_0 and the samples are diluted to a minimum of 1:100.

Determination of Salinomycin in Spiked Chicken Liver Tissue by ELISA and HPLC. Next the HPLC and ELISA methods were evaluated for estimating SAL in spiked chicken liver tissue. Figure 2 shows the results observed with the ELISA and the HPLC methods. Also included in Figure 2 are the linear regression data for the amount added and the amount detected. For both the ELISA method and the HPLC method, the amount detected was highly correlated to the amount added ($R^2 = 0.999$, $p < 0.0001$). The ELISA method was more accurate than the HPLC method at the lower tissue concentrations. Furthermore, the ELISA method gave quantitative mean recoveries over the entire range tested. We observed a higher variability between triplicate extraction and analyses by ELISA (average CV = 19.9%) than with the HPLC method (average CV = 4.11%). This difference was probably due in part to differences in extraction efficiencies for the two methods. For the ELISA, the average CV observed for individual sample determinations was 11.8% ($n = 3$), and 4.13% ($n = 2$) was the average CV for the HPLC method.

The limit of quantitation of the ELISA method was approximately 50 ppb SAL in liver tissue. Figure 1 shows that the analysis of a 1:100 dilution of aqueous liver extract spiked at a level corresponding to 50 ppb SAL in liver (0.5 ppb) gave B/B_0 values which were within the linear, quantitative range of the assay. In addition, acceptable recoveries (83%) were obtained from control liver samples spiked at this level and analyzed by ELISA (Figure 2). Statistically, the limit of quantitation for the HPLC method, defined as 10 times the variance (10σ) (Keith, 1991), is also approximately 50 ppb. However, the HPLC method showed a large deviation from linearity below 100 ppb, resulting in an overestimation of analyte. This effect limits the accuracy of the HPLC method to levels at or above 100 ppb. Therefore, we report a 100 ppb limit of quantitation for HPLC. This limit is similar to the limit of quantitation reported by others (Dimenna et al., 1986b; Martinez and Shimoda, 1986) for instrumental methods developed for the analysis of SAL in animal tissues using either pre- or postcolumn derivatization.

Determination of Salinomycin in Chicken Liver Tissues from the Incurred Residue Study. In this study, we used a gradient HPLC solvent system that resulted in a longer analyte retention time on the reversed-phase column (from 7 to 10 min) and an increased analyte signal in comparison to the isocratic solvent system. A positive control spiked liver sample (1 ppm) was analyzed in parallel with each set of incurred residue samples to verify efficient salinomycin recovery and detection. The mean recovery of SAL from

Table 1. HPLC and ELISA Analysis of Salinomycin in Chicken Liver Tissues from an Incurred Residue Study

		HPLC concn (ppb) at withdrawal time of			ELISA concn (ppb) at withdrawal time of		
treatment	bird no.	0 h	18 h	72 h	0 h	18 h	72 h
no dose	1	–[a]	–	–	–	–	–
	2	–	–	–	–	–	–
	3	–	–	–	–	–	–
	4	–	–	–	–	–	–
	5	–	–	–	–	–	–
66 ppm	1	–	–	–	101.2	–	–
	2	339.2	–	–	459.0	–	–
	3	–	–	–	153.0	–	–
	4	93.8	–	–	146.4	–	–
	5	168.1	–	–	155.3	–	–
132 ppm	1	278.0	–	–	317.9	–	–
	2	364.4	–	–	181.6	–	–
	3	157.6	–	–	688.0	–	–
	4	245.5	111.6	–	315.3	–	–
	5	238.6	–	–	251.5	–	–

[a] Below limits of quantitation: 100 ppb for HPLC; 50 ppb for ELISA. ELISA and HPLC results were highly correlated, $p < 0.0001$.

these samples was 89.1% (CV = 7.6%, $n = 4$), which indicated near quantitative recovery under these conditions. The limit of quantitation of the ELISA and HPLC methods were 50 and 100 ppb, respectively, as described in the previous section.

Liver samples were obtained from broiler chickens fed control feed or feed that was fortified with 1 or 2 times the therapeutic dose of SAL. Analysis of the control feed and feed medicated at 66 and 132 ppm SAL using the HPLC method of Blanchflower et al. (1985) gave respective concentrations of less than 5.0 (below limit of detection), 55.1, and 120.4 ppm SAL, respectively. The birds were withdrawn from medicated feed for various times prior to slaughter.

Table 1 is a summary of the results from the incurred residue study. The ELISA and HPLC results (45 samples) were tested for correlation using a t test. To include all of the data in the analysis, sample determinations that were below the method's limit of quantitation (71 of 90 sample determinations) were designated zero. This was important since we were interested in evaluating the capabilities of the methods to detect negative samples. Results from the analysis of incurred residue samples were highly correlated ($p < 0.0001$). Comparison of ELISA results to HPLC results (45 samples) showed only 1 false negative and 2 false positives by ELISA. However, these occurred near the limit of quantitation for the HPLC method (100 ppb). At this limit, these results could also be interpreted as being 1 false positive and 2 two false negatives by HPLC. Clearly, these results indicate that the ELISA method is a reliable screening tool for SAL in chicken liver tissue. In samples for which the SAL concentrations obtained by the two methods were above their limits of quantitation, there was good agreement between the two methods. The exceptions were with birds 2 and 3 in the 132 ppm, 0 h withdrawal time group for which concentration estimates by ELISA were underestimated (bird 2) and overestimated (bird 3). These inconsistencies may be due to sample matrix effects that occur with both methods.

The incurred residue data obtained by using each of the methods provided the same information concerning the disappearance of SAL from liver. Residues are present immediately following withdrawal from medicated feed and are undetectable after 18 h. Similar results were reported by Atef et al. (1993).

Conclusions. Current analytical methodologies for the analysis of SAL in animal tissues normally require organic solvent extraction followed by sample cleanup, chromatography, and pre- or postcolumn derivatization resulting in a chromophoric species for detection. These extensive procedures may limit the number of samples that can be processed in a timely manner. We have demonstrated the use of an aqueous buffer extraction followed by ELISA for the analysis of SAL in chicken liver tissue with both improved sensitivity and accuracy over those of commonly employed methods. Both HPLC and ELISA results from the analysis of incurred residue liver samples showed a rapid disappearance of SAL from tissue. The advantages of reduced organic solvent use and increased sample throughput realized by the ELISA method should save time and expense in residue monitoring for this commercially important product.

LITERATURE CITED

Asukabe, H.; Murata, H.; Harada, K.; Suzuki, M. Oka, H.; Ikai, Y. Improvement of chemical analysis of antibiotics. 21. Simultaneous determination of three polyether antibiotics in feeds using high-performance liquid chromatography with fluorescence detection. *J. Agric. Food Chem.* **1994**, *42*, 112–117.

Atef, M.; Ramadan, A.; Youssef, S. A. H.; Abo El-Sooud, K. Kinetic distribution, systemic disposition and tissue distribution of salinomycin in chickens. *Res. Vet. Sci.* **1993**, *54*, 179–183.

Blanchflower, W. J.; Rice, D. A.; Hamilton, J. T. G. Simultaneous high-performance liquid chromatographic determination of monensin, narasin, and salinomycin in feeds using post-column derivatisation. *Analyst* **1985**, *110*, 1283–1287.

Dimenna, G. P.; Walker, B. E.; Turnbull, L. B.; Wright, G. J. Thin-layer bioautographic assay for salinomycin in chicken liver. *J. Agric. Food Chem.* **1986a**, *34*, 472–474.

Dimenna, G. P.; Creegan, J. A.; Turnbull, L. B.; Wright, G. J. Determination of sodium salinomycin in chicken skin/fat by high-performance liquid chromatography utilizing column switching and UV detection. *J. Agric. Food Chem.* **1986b**, *34*, 805–810.

Elissalde, M. H.; Beier, R. C.; Rowe, L. D.; Stanker, L. H. Development of a monoclonal-based enzyme-linked immunosorbent assay for the coccidiostat salinomycin. *J. Agric. Food Chem.* **1993**, *41*, 2167–2171.

Goras, J. T.; LaCourse, W. R. Liquid chromatographic determination of sodium salinomycin in feeds, with post-column reaction. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 701–706.

Karu, A. E.; Harrison, R. O.; Schmidt, D. J.; Clarkson, C. E.; Grassman, J.; Goodrow, M. H.; Lucas, A.; Hammock, B. D.; White, R. J.; Van Emon, J. M. Monoclonal immunoassay of triazine herbicides: development and implementation. In *Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Foods, and Environment*; Vanderlaan, M., Stanker, L. H., Watkins, B. E., Roberts, D. W., Eds.; ACS Symposium Series 451; American Chemical Society: Washington, DC, 1991; pp 59–77.

Keith, L. H. In *Environmental Sampling and Analysis. A Practical Guide*; Lewis: Chelsea, MI, 1991.

Lapointe, M. R.; Cohen, H. High-speed liquid chromatographic determination of monensin, narasin, and salinomycin in feeds, using post-column derivatization. *J. Assoc. Off. Anal. Chem.* **1988**, 480–484.

Lehotay, S. J.; Argauer, R. J. Detection of aldicarb sulfone and carbofuran in fortified meat and liver with commercial ELISA kits after rapid extraction. *J. Agric. Food Chem.* **1993**, *41*, 2006–2010.

Martinez, E. E.; Shimoda, W. Liquid chromatographic determination of multiresidue fluorescent derivatives of ionophore compounds, monensin, salinomycin, narasin, and

lasalocid, in beef liver tissue. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 637–641.

McDougald, L. R.; Roberson, E. L. Antiprotozoan drugs. In *Veterinary Pharmacology and Therapeutics*; Booth, N. H., McDonald, L. E., Eds.; Iowa State Press: Ames, IA, 1988; pp 950–968.

Nam, K.; King, J. W. Supercritical fluid extraction and enzyme immunoassay for pesticide detection in meat products. *J. Agric. Food Chem.* **1994**, *42*, 1469–1474.

National Research Council. *Nutritional Requirements of poultry. 1. Nutrient requirements of domestic animals*, 8th ed.; National Academy of Science: Washington, DC, 1984.

Pressman, B. C.; Fahim, M. Pharmacology and toxicology of the monovalent carboxylic ionophores. *Annu. Rev. Pharmacol. Toxicol.* **1982**, *71*, 480–484.

SRI International. *Chemical Economics Handbook Animals: Chemical inputs for nutrition and health—overview*; Menlo Park, CA, 1992; Dialog Information Services, File 359, CEH Accession 201.8002B-2.

Stanker, L. H.; Bigbee, C.; Van Emon, J.; Watkins, B.; Jenson, R. H.; Morris, C.; Vanderlaan, M. An immunoassay for pyrethroids: Detection of permethrin in meat. *J. Agric. Food Chem.* **1989**, *37*, 834–839.

Stanker, L. H.; McKeown, C.; Watkins, B.; Vanderlaan, M.; Ellis, R.; Rajan, J. Detection of dimetrazole and other nitroimidazole residues in turkey using immunoassay. *J. Agric. Food Chem.* **1993**, *41*, 1332–1336.

Stanker, L. H.; Elissalde, M. H.; Rowe, L. D.; Beier, R. C.; Nasr, M. I. A. Detection of coccidiostats by immunoassay. *Food Agric. Immunol.* **1994**, *6*, 45–54.

Received for review January 12, 1995. Revised manuscript received April 17, 1995. Accepted April 20, 1995.[⊗]

JF950030Z

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.