Immunoassays (ELISA) for Rapid, Quantitative Analysis in the Food-Processing Industry

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An indirect, competitive-type enzyme-linked immunosorbent assay (ELISA) is shown to have sufficient accuracy, precision, and sensitivity for application to total residue determinations for spectinomycin-related residues in food animal tissues. Assay optimization established the linear dynamic range, sensitivity, and method detection limits. Control tissue extracts were assayed for the presence of either positive or negative interferences. Interferences arising from cross-reactivity with other antibiotics or therapeutic agents likely to be present were evaluated. Recovery and precision from fortified tissue samples as well as measurement of operator bias from the analysis of samples with biologically incurred residues were determined. Equivalency of the ELISA method with standard radiotracer methods of analysis is demonstrated herein. Delivered in the microtiter format and using fully automated sample processing systems, immunoassays, as described, can be used for "real-time" quality control analysis in the food-processing industry.

Keywords: *Spectinomycin; dihydrospectinomycin, immunoassay; quantitative; analysis*

INTRODUCTION

Current methods and practices for the analysis of food-borne toxins, antibiotics, pathogens, and adulterants are entirely too slow, costly, and labor intensive for the on-line, real-time quality control analysis in the food-processing industry. Ever increasing public and regulatory pressure for increased monitoring of the nation's food supply to assure safety and wholesomeness is creating a demand to increase analytical laboratory capacity in the food-processing industry. Immunoassay technology delivered as rigorous analytical methods in a microtiter format compatible with automated instrumentation represents the lowest cost and most rapid means for significantly increasing analytical laboratory capacity for these and other applications.

Spectinomycin (decahydro-4a,7,9-trihydroxy-2-methyl-6,8-bis(methylamino)-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one) is an aminocyclitol antibiotic isolated from the fermentation broth of *Streptomyces spectabilis* as described by Mason *et al*. (1961). Spectinomycin is labeled for use as a feed additive in the poultry industry; however, significant "off-label" use occurs in the treatment of cattle for bovine respiratory disease (shipping fever) and consequently spectinomycin enters the food supply in an unregulated manner. In this study, spectinomycin is selected to represent the broader class of food contaminants and adulterants for which there are no methods of analysis suitable for regulatory monitoring or process quality control in the food-processing industry.

The current standard for analysis of spectinomycin is a microbiological assay first published by Marsh *et al*. (1967) and Neff *et al*. (1973). Although cumbersome, labor intensive, and slow, these methods have served as the benchmark for nearly 30 years and are the basis for the *U. S. Pharmacopeia* guidelines as well as the World Health Organization's analytical standards. Recently this methodology was modified by Stahl *et al*. (1995) to incorporate a trifluoroacetic acid extraction step.

Spectinomycin represents one of the more challenging analytical problems arising from the absence of a chromophore and its virtual insolubility in organic solvents. To improve the detectability of any analyte the analytical paradigm suggests that an analyte can be modified to enhance its detectability via chemical derivatization to introduce a chromophore into the analyte. A detection device that is more sensitive to the analyte, *e.g*. fluorometric vs colorimetric analysis, may also be utilized to improve the detectability of the analyte, or in the alternative, removal of interfering substances through the use of chromatographic separations may also enhance the detectability of the analyte.

To improve the detectability of spectinomycin several approaches to the problem have been published over the years. For instance Elrod *et al*. (1988) employed an electrochemical detector after separation of the spectinomycin and spectinomycin-related residues by HPLC, and Phillips and Simmonds (1994) used pulsed amperometric detection after cation exchange chromatography. Abbay *et al*. (1991) utilized dynamic headspace gas chromatographic analysis of bulk spectinomycin hydrochloride and Medina *et al*. (1995) used thin-layer chromatography for the semiquantitative analysis of derivatized aminocyclitols including spectinomycin. McLaughlin and Henion (1992) demonstrated reversed-phase ion-pair HPLC to separate aminoglycoside antibiotics in animal tissue matrices. Other derivatization strategies followed by chromatographic analysis have been employed by us and others. For instance amine-derivatizing agents such as 1-fluoro-2,4-nitrophenyl-5-alanine amide (FDAA or Marfey's Reagent) or 9-fluorenylmethyl chloroformate (Fmoc-chloride) have been used to derivatize spectinomycin prior to HPLC analysis with either UV or fluorescence detection, respectively. Similarly spectinomycin has been derivatized as the trifluoromethyl ethers, trifluoroacetyl esters, and trimethylsilyl ethers followed by gas chromatographic

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analysis and electron capture detection (unpublished experimental results).

Immunochemical methods of analysis address the analytical paradigm in two ways. First, the antibodies themselves are highly specific for the antigen (analyte), and second, either the antigen, the antibody, or an antiglobulin may be conjugated to an enzyme that produces an intensely colored or fluorescent product in the presence of the enzyme substrate to enhance the detectability of the analyte in an amplification step. Various formats for the immunochemical assays have been employed in either competitive or noncompetitive assays with the detector response being either directly or indirectly proportional to the analyte concentration and are thoroughly discussed and described by Clausen (1991) and the references therein.

Low molecular weight compounds such as spectinomycin are not typically antigenic, however, and are therefore not capable of illiciting an immune response by themselves. The chemistry of methods utilized to conjugate small molecules to proteins and other immunogenic substances has been extensively described by Clausen (1991) as well as by Wong (1993) and the references therein. Heterocyclic compounds, such as aminocyclitols like spectinomycin, require additional chemical modification prior to conjugation to a protein or other immunogenic substance. The chemistry for the addition of a four-carbon carboxyl spacer arm via condensation with succinic anhydride to form succinyl esters was first described for estrogenic steroids by Tulchinsky and Abraham (1971), Wu and Lundy (1971), and Emment *et al*. (1972). These reactions required several hours of incubation or reflux conditions that would degrade a thermally labile compound such as spectinomycin or would result in multiple products.

Subsequent coupling of a carboxyl containing hapten to a protein utilizes the carbodiimide condensation described by Sheehan and Hess (1955) and Khorana (1955). The reaction is a condensation of the hapten carboxyl with a substituted carbodiimide to form an active *O*-acylisourea intermediate. Nucleophilic substitution with an amine such as the ϵ-amine of lysine forms the stable amide with elimination of the substituted urea. The poor coupling efficiency of the carbodiimide reaction is substantially improved when *N*-hydroxysuccinimide is introduced to form the intermediate active *N*-hydroxysuccinyl ester as described by Anderson *et al*. (1964).

ELISA methods, such as described herein and delivered in the microtiter format, meet the criteria to be described as analytically rigorous and will ultimately become an important addition to standard methods of analysis.

MATERIALS AND METHODS

Part A. Antibody Production and Purification. *Preparation of Succinylated Spectinomycin and Conjugation to Carrier Protein*. The keto form of spectinomycin was prepared by dissolving 2 g of spectinomycin dihydrochloride pentahydrate (Sigma Chemical Co.; St. Louis, MO) in 30 mL of cold, deionized water and adding this solution to a cold slurry of 6 mL of Amberlite IRA-400 OH (Sigma Chemical Co.; St. Louis, MO) in 30 mL of deionized water. After 30 min of stirring in an ice bath, the resin was poured into a column (1 × 10 cm) with a fritted glass support and washed with ~30 mL of deionized water. The final effluent (90 mL) was transferred to a round-bottom flask and evaporated *in vacuo* in a rotary evaporator with the water bath temperature at 55 °C. The resulting glassy solid was recrystallized from water and acetone. The resulting crystalline product was dried in a vacuum oven at 60−70 °C overnight. The keto form of spectinomycin was confirmed by the ^{13}C-NMR and the ^{1}H-NMR spectra obtained using a 250 MHz NMR (Brücker Instruments; Switzerland) located at the U.S. Naval Research Laboratories in Washington, DC. The carbonyl absorbance was located at 201 ppm in the ^{13}C-NMR and vinylic absorbances arising from the enol form of spectinomycin were conspicuously absent in both the ^{13}C-NMR and the ^{1}H-NMR spectra.

In a 1-mL ReactiVial (Pierce Chemical Co.; Rockford, IL) equal molar amounts (21 μmol), of spectinomycin and [1,4-^{14}C]succinic anhydride (Sigma Chemical Co.; St. Louis, MO) were added to 9.1 mg of *N*-hydroxysuccinamide (NHS, Sigma Chemical Co.; St. Louis, MO) and dissolved in 400 μL of dimethylformamide (DMF, EM Science; Cherry Hill, NJ). The reaction mixture was stirred at room temperature for 4 h. In a separate tube 16 mg of dicyclohexylcarbodiimide (DCC, EM Science; Cherry Hill, NJ) was dissolved in 200 μL of DMF and transferred to the succinylate reaction mixture. This reaction mixture was stirred for 1 h at room temperature and transferred to a refrigerator to continue stirring overnight at 4 °C.

The carrier protein, KLH, (Imject, Pierce Chemical Co.; Rockford, IL, 20 mg of protein in phosphate buffered saline, PBS, pH 7.2 when reconstituted) was reconstituted in 5.4 mL of deionized water. The activated hapten was transferred to a microcentrifuge tube and centrifuged for 5 min to remove the substituted urea precipitate. The supernatant was transferred to the reconstituted carrier protein solution, and stirring was continued for 4 h at room temperature. The protein reaction mixture was centrifuged to remove any precipitated protein and the supernatant was applied to a desalting column (Swift, Pierce Chemical Co.; Rockford, IL) and eluted with 0.01 M PBS, pH 7.2 in 3-mL fractions. Fractions were monitored at 280 nm using a UV-1201S spectrophotometer (Shimadzu Corporation; Kyoto, Japan). The immunogen typically eluted in fractions 2−4. The fractions containing the immunogen were pooled and lyophilized. The protein conjugation procedure was repeated with bovine serum albumin (BSA, Imject, Pierce Chemical Co.; Rockford, IL, 20 mg of protein in PBS, pH 7.2 when reconstituted) to prepare the coating antigen.

The final protein concentration was determined by the BCA method (Pierce Chemical Co.; Rockford, IL), and the hapten concentration was determined by liquid scintillation analysis. A 0.1-mL aliquot of the final immunogen preparation was dissolved in 15 mL of ScintiVerse II (Fisher Scientific, Inc.; Fair Lawn, NJ) and analyzed by liquid scintillation spectrometry (RSO, Inc.; Gaithersburg, MD using a Beckman LS6000 (Beckmann Instruments, Inc.; Fullerton, CA). The gross CPMs were converted to DPMs with an internal quench curve and were background subtracted.

Immunization and Antibody Purification. Five, disease-free, female New Zealand White rabbits weighing on average 4.5 kg were immunized to produce antibodies. Prior to immunization, individual rabbits were identified by a sequential identifying number tattooed on the ear. Four days prior to the first immunization the rabbits were inoculated against *Bordetella pertusis* and a preimmunization bleed was collected to provide a background titer.

A primary inoculum was prepared from 2.5 mL of the immunogen (1 mg/mL in 0.01 M PBS) and 25 mg *M. tuberculosis* suspension in 2.5 mL of Freund's adjuvant. This suspension was homogenized until thick and creamy. The dose per rabbit was 1.0 mL by subcutaneous injection.

Booster inocula were prepared from 1.25 mL of the immunogen (1 mg/mL in 0.01 M PBS) and 1.25 mL of Freund's incomplete adjuvant. This suspension was homogenized until thick and creamy. The dose per rabbit was 0.5 mL by subcutaneous injection every 21 days. Eight days after the first and each subsequent booster injection a test bleed was assayed for antibody titers.

Antisera was assayed in a checkerboard fashion after Voller *et al*. (1976), and the titer was taken as the highest dilution of antisera at the highest dilution of coating antigen that gave a response three times the background determined from preimmunization serum.

When antibody titers reached an apparent steady state, a production bleed of approximately 20 mL was taken per rabbit. The antisera was pooled, assayed for protein content, and stored frozen (−85 °C) until used. Antibody purification was accomplished by diluting the antisera to 4.6 mg/mL and applying 2-mL aliquots to Protein A affinity columns (AffinityPak, Pierce Chemical Co.; Rockford, IL). The column was washed with 15 mL of ImmunoPure IgG Binding Buffer (Pierce Chemical Co.; Rockford, IL) and eluted with 5 mL of ImmunoPure IgG Elution Buffer (Pierce Chemical Co.; Rockford, IL) in 1-mL fractions. The absorbance of each fraction at 280 nm was monitored using a UV-1201S spectrophotometer (Shimadzu Corporation; Kyoto, Japan) and the fraction with significant absorbance (usually fraction 2) was desalted with a 5-mL Excellulose column (Pierce Chemical Co.; Rockford, IL) which had previously been conditioned with 10 mL of 0.02 M PBS (100 mM NaCl, pH 7.4). Elution was in 1-mL fractions and the absorbance was again monitored at 280 nm. Fractions containing significant absorbance (usually fractions 3−4) were pooled and assayed for total protein.

Part B. Methods of Data Analysis. *Determination of IC_{50} and Percent Inhibition*. The IC_{50} was defined as the concentration of the inhibitor required to reduce the absorbance to one-half the absorbance measured when no inhibitor was present. This was accomplished by plotting the % activity remaining vs the concentration of the inhibitor where the percent activity remaining is given in eq 1

$$\% \text{ activity} = \frac{A_0 - A_i}{A_0} 100$$

where A_0 is the measured absorbance at 410 nm when no inhibitor is present and A_i is the measured absorbance at the ith concentration of inhibitor. The percent inhibition is then given by the relation 100 − % activity.

The plot of percent activity remaining vs inhibitor concentration was analyzed by the exponential curve fitting method. To calculate the IC_{50}, the exponential equation of the form $y = bm^nx$ was then solved for x when y was set equal to 50, *i.e.* 50% activity remaining or 50% inhibition. Graphics were performed using Microsoft Excel, Ver 5.0 Graphics (Microsoft Corporation, Copyright 1993; Redmond, WA) and exponential curve fitting was performed using Microsoft Excel, Ver 5.0 Analysis ToolPak (Microsoft Corporation, Copyright 1993; Redmond, WA).

Determination of Linear Range, Sensitivity, and Limit of Detection. A plot of the average absorbance of the seven replicate analyses obtained from the seven concentrations of the inhibitors was made using Microsoft Excel, Ver 5.0 Graphics (Microsoft Corporation, Copyright 1993; Redmond, WA). The standard deviations, least-squares method of linear regression, y-intercepts, and slopes were calculated using Microsoft Excel, Ver 5.0 Analysis ToolPak (Microsoft Corporation, Copyright 1993; Redmond, WA).

The linear dynamic range was visualized as the linear portion of the curve obtained from a plot of absorbances at 410 nm from the various standard concentrations analyzed in seven replicates and having a % CV less than 20%.

The limit of quantitation (LOQ) was defined as 3 times the method detection limit (MDL). To measure the MDL the standard deviations of the replicate analyses at each concentration of inhibitor were calculated and the least squares method of regression analysis was performed. The y-intercept of the regression line was taken as the background absorbance arising from noise. The absolute value of the x-intercept was taken as the sensitivity. This background absorbance was converted to the concentration of spectinomycin from the standard curve over the linear range. The resulting concentration was defined as the estimated detection limit (EDL). A standard in the concentration range of 1−5 times the EDL is analyzed in seven replicates, and the mean and standard deviation is calculated. Three times the standard deviation in concentration units thus calculated is taken as the MDL.

Cross-Reactivity. Dihydrospectinomycin, the major metabolite of spectinomycin, is also about 20% of the bulk drug formulation. The IC_{50} for dihydrospectinomycin was calculated from the % inhibition when dihydrospectinomycin was added as the inhibitor in the concentration range of 0.1 to 100 ng/mL.

To evaluate the potential for positive interference arising from the presence of other antibiotics or therapeutic agents, a broad spectrum of commonly encountered antibiotics was used as inhibitors in the ELISA. The antibiotics and class (in parentheses) evaluated include neomycin and gentamicin (aminoglycoside), pennicillin G (β-lactam), erythromycin and tylosin (macrocyclic), sulfamethazine (sulfonamide), and tetracycline (tetracycline). The therapeutic agents evaluated include β-estradiol and progesterone (growth hormones) and thiabendazole (antihelmenthic). The concentration range evaluated for cross-reactivity was generally 1−100 ppm except for β-estradiol which was 1−25 ppm; all in a control liver matrix.

Biologically Incurred Residue Analyses. To assess the accuracy of the method and show equivalency of the ELISA method with standard methods for total residue analysis, liver and kidney tissue samples from six calves with biologically incurred [^{3}H]spectinomycin residues were obtained from Analytical Development Corporation; Colorado Springs, CO. Nominally 200-mg portions of homogenized tissues were combusted in triplicate using a Harvey Biological Oxidizer (Model OX-100, R. J. Harvey Instrument Corporation; Hillsdale, NJ) and the combustion products were trapped in 5 mL of Harvey Tritium Cocktail (R. J. Harvey Instrument Corporation; Hillsdale, NJ) and 10 mL of BetaBlend (ICN Biomedicals, Inc.; Costa Mesa, CA). Liquid scintillation analysis was performed using a Beckman LS 9800 (Beckman Instruments, Inc.; Fullerton, CA). Counting was typically for 10 min with a count terminator of 40 000 (2σ = 1%). Values were corrected for background and combustion efficiency and triplicate measurements were averaged.

Spectinomycin related residues were also extracted from triplicate 5-g portions of tissue homogenate according to the method described (see Test Protocol below). Total extractable residues were measured from 1-mL portions of each replicate added to 15 mL of BetaBlend (ICN Biomedicals, Inc.; Costa Mesa, CA) and liquid scintillation counting was performed using a Beckman LS 9800 (Beckman Instruments, Inc.; Fullerton, CA). Counting was typically for 10 min with a count terminator of 40 000 (2σ = 1%). Values were corrected for background and triplicate measurements were averaged. The unextracted [^{3}H]spectinomycin residues were measured from the solid pellet remaining from the extraction process. Nominally 200-mg portions of tissue pellet was combusted in triplicate using a Harvey Biological Oxidizer (Model OX-100, R. J. Harvey Instrument Corporation; Hillsdale, NJ) and the combustion products were trapped in 5 mL of Harvey Tritium Cocktail (R. J. Harvey Instrument Corporation; Hillsdale, NJ) and 10 mL of BetaBlend (ICN Biomedicals, Inc.; Costa Mesa, CA). Liquid scintillation analysis was performed using a Beckman LS 9800 (Beckman Instruments, Inc.; Fullerton, CA). Counting was typically for 10 min with a count terminator of 40 000 (2σ = 1%). Values were corrected for background and combustion efficiency and triplicate measurements were averaged.

Residue levels in samples were analyzed by ELISA (see Test Protocol below) using the external standard calibration curve method of quantitation. A standard curve was established in a control sample matrix for quantitation. Linear regression analyses were performed using Microsoft Excel, Ver. 5.0 Analysis ToolPak (Microsoft Corporation, Copyright 1993; Redmond, WA). The sample's nonspecific binding subtracted absorbance was matrix background subtracted using the average absorbance of the control sample with no added coating antigen. The resulting matrix background subtracted absorbance was converted to concentration of spectinomycin from the external standard curve.

Statistical Evaluation. Student's t-test was employed to determine whether or not a particular data point within a population was statistically different from the mean value for that population. Absorbances outside the critical t-value at the 95% confidence interval were rejected.

The F-statistic was used to evaluate the difference between two sample variances and the Z-test was used to compare the means of two independent data sets when the population variance was known or was approximated by the sample variance i.e., greater than 30 data points. Precision was measured as the percent coefficient of variation (% CV) of replicate measurements.

Part C. Description of the Test Protocol. The following method is a competitive enzyme-linked immunosorbant assay (ELISA) for spectinomycin residues in various animal tissues and fluids.

Tissue Extraction Procedure. A 5-g portion of homogenized tissue is weighed into a 250-mL centrifuge bottle. After the addition of 50 mL of PBST extraction buffer (0.1% Tween-20 in 0.01 M PBS, pH 7.2) the sample is homogenized using a tissue homogenizer. The homogenized sample is centrifuged at 5000*g*-av at 10 °C for 30 min to sediment the solids. The supernatant is decanted into a 250-mL graduated cylinder. The process is repeated twice more, and the combined supernatant volume is adjusted to 200 mL with PBST.

To the combined supernatants, 1 mL of 4% sodium azide is added (final concentration 0.02%) to prevent microbial growth. The entire sample is further clarified by centrifugation, and the supernatant is carefully decanted. A 4-mL aliquot of extract processed in this manner corresponds to 0.1 g of tissue.

Microtiter Plate Preparation. Microtiter plates used in the course of this study are 96-well Easy Wash, modified flat-bottom, polystyrene ELISA plates (Corning Glass Works; Corning, NY).

(1) Plate Plan. A plate plan is laid out for each microtiter plate prior to the start of the assay. The plate plan designates wells for standard curves, antigen negative wells, primary antibody negative wells, a positive control well, and samples with replicates.

(2) Coating Antigen. The coating antigen (100 μL) is added to each well according to the plate plan. The wells selected as antigen negative are coated with 0.01 M PBS only. A positive control well is designated and contains antibody instead of coating antigen. The plate is covered with sealing tape and lid, and the sealed plate is incubated overnight at 4 °C.

(3) Wash Procedure. The plates coated with coating antigen are removed from the refrigerator and are washed in three consecutive addition/aspiration steps with washing buffer (0.01% Tween-20 in 0.01 M PBS). A washer/aspirator designed for washing 96-well microtiter plates (Handiwash, Dynatech Laboratories, Inc.; Chantilly, VA) is utilized.

After the third addition of washing buffer, the wells are incubated with washing buffer at room temperature for ~3−5 min. The plate is then washed in two additional consecutive addition/aspiration steps. Excess washing buffer is aspirated from the wells, and the plate is inverted and tapped dry on a paper towel or other absorbent surface.

(4) Blocking Procedure. All wells of the microtiter plate have 300 μL of blocking solution (1% BSA in 0.01 M PBS) added to each well. The plates are incubated at room temperature for 1 h or overnight at 4 °C.

(5) Wash. The washing procedure described above is repeated to remove excess BSA. The coated, blocked, and washed plates may be prepared in advance and stored frozen (−20 °C).

Sample Spiking Procedure. Ninety-six low protein binding microtubes are arranged in a tube rack with an 8 × 12 array to correspond to the microtiter plate plan. The duplicate external standard curves are established in the first two columns of tubes. The remaining tubes are to contain up to seven replicates of the diluted tissue extracts to which is added 190 μL of diluted sample and 10 μL of the protein A purified antibody (pAIgG). The positive control tube contains 200 μL of PBST. To the antibody negative controls are added 10 μL of 0.01 M PBS buffer instead of pAIgG. All tubes are thoroughly mixed on a vortex mixer after the antibody addition. Tubes are incubated ~1 h, and 100 μL from each tube are transferred to the corresponding well in the microtiter plate according to the plate plan. The plates are incubated 1 h at 37 °C or overnight at 4 °C.

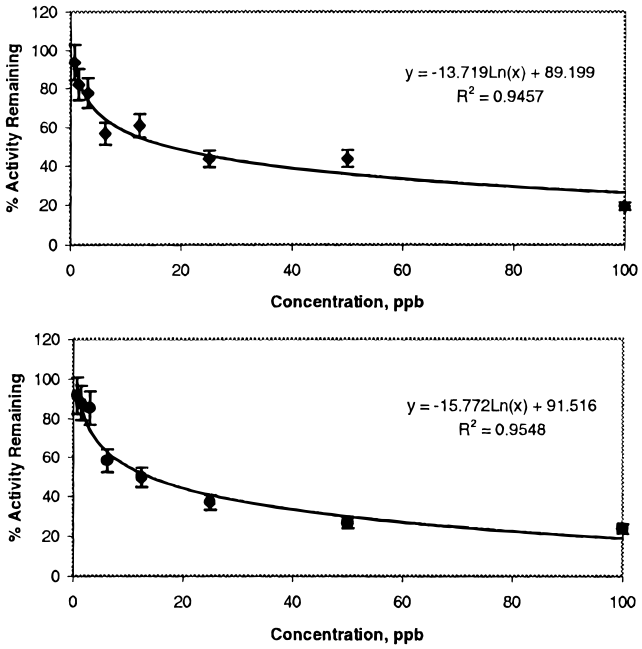

Figure 1. Inhibition curve for spectinomycin in PBST extraction buffer (top) and a liver tissue homogenate (bottom). Each data point is the average of seven replicate analyses.

Microtiter Plate Assay Procedure. (1) Wash. After incubation the plates are washed with the washing buffer as described earlier.

(2) Addition of Secondary Antibody. To each well 100 μL of the 1:5000 dilution of secondary antibody (goat, anti-rabbit IgG conjugated to alkaline phosphatase, Pierce Chemical Co.; Rockford, IL) is added, and the plate is covered with a lid and incubated for 1 h.

(3) Wash. After the incubation the unbound secondary antibody is removed by the washing procedure as described earlier.

(4) Addition of Enzyme Substrate and Absorbance Measurements. To each well 100 μL of the substrate (*p*-nitrophenyl phosphate, PNPP, Pierce Chemical Co.; Rockford, IL) is added. The plate is covered with a lid and incubated at room temperature. Color development is typically noted in the positive control well within 15 min. The incubation is carried out long enough to give absorbance in the standard curve wells that is in the range of 0.3−0.5 absorbance units when no antigen is present. Initial readings are taken after 1 h, 90 min, and 2 h incubation periods if necessary. Readings are taken using a microtiter plate reader (MR 5000, Dynatech Instruments, Inc.; Chantilly, VA) at 410 nm. Antibody negative control wells are background subtracted from the remaining wells.

RESULTS AND DISCUSSION

Antibody (IgG) and coating antigen concentrations had been previously optimized by a checkerboard assay according to Voller (1976) to be ~0.5 ug/mL (50 ng per microwell) and 658 ng/mL (66 ng per microwell) respectively. When converted to a molar basis using the calculated hapten/protein molar binding ratio of 12, the antigen is in a 36-fold molar excess of the IgG added. The inhibition curves for spectinomycin in buffer and liver homogenate matrices are presented in Figure 1. The IC_{50} was measured to be 17 ng/mL in buffer and 14 ng/mL in a control liver matrix, suggesting that liver matrix offered no significant interference to the ELISA. The linear range of the assay was observed to be 0.1 to 100 ng/mL with a calculated MDL of 0.1 ng/mL and a LOQ of 0.5 ng/mL. Figure 2 demonstrates a typical external calibration curve over the linear range of the assay.

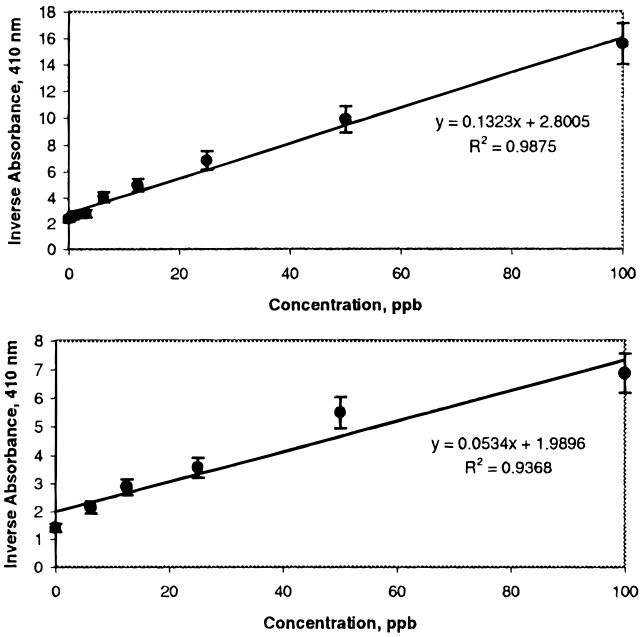

Figure 2. Calibration curves for spectinomycin-related residues in a liver tissue matrix (top) and a kidney tissue matrix (bottom). Each data point is the average of seven replicate analyses.

Table 1. Evaluation of Possible Interfering Substances in Control Liver Tissues

compound	maximum spike concentration, μg/mL	measured IC_{50}, ng/mL
dihydrospectinomycin	0.1	15
neomycin	100	>100 000
gentamicin	100	>100 000
pennicilin G	100	>100 000
erythromycin	100	>100 000
tylosin	100	>100 000
estradiol	25	>25 000
progesterone	100	>100 000
thiabendazole	100	>100 000

Table 2. Total Spectinomycin-Related Residue Extraction Efficiency

withdrawal day	10^6 DPM_{actual}	10^6 $DPM_{extracted}$	% Extracted	10^3 DPM_{after}	% remaining
		Liver Tissue			
0	6.2	5.9	95	6	0.1
3	2.8	2.8	>99	3.8	0.1
7	1.2	1.2	>99	2.1	0.1
21	0.14	0.14	>99	0.9	0.6
		Kidney Tissue			
0	15.3	14.4	94	89	0.6
3	6	5.8	97	66	0.9
7	0.6	0.5	83	5.1	0.8
21	0.03	0.03	>99	2.3	6.7

To evaluate the potential for interference arising from other therapeutic or antibiotic agents, the IC_{50} was measured for each agent in a control liver tissue matrix. No inhibition for the tested agents could be measured. These results are tabulated in Table 1.

The extraction efficiency with PBST for biologically incurred residues in liver and kidney tissue ranged from nominally 83% to >99% with an average 93% recovery from kidney and an average 98% recovery from liver. The percent of residues remaining in the postextraction pellet ranged from 0.1% to 6.7% with an average 2.3% residue remaining in kidney and an average 0.2% residue remaining in liver tissue. These results are tabulated in Table 2.

Spectinomycin residues from fortified liver tissue at concentrations of 0.5, 1.0, and 2.0 ppm were measured on three nonconsecutive days by a single analyst. The percent recovery (accuracy) was calculated from the average of five replicate analyses at each fortification level on each analysis day and ranged from 85% to 98% while the precision (% CV) within the assays ranged from 2% to 22%, and the precision between analysis days ranged from 6% to 11% with an overall precision of 7%. These results are tabulated in Table 3.

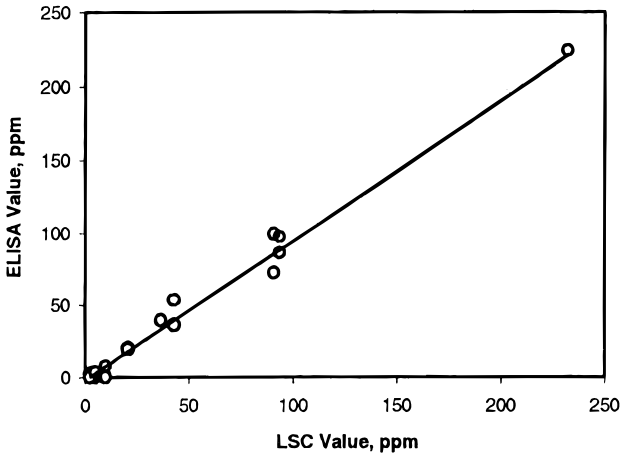

Figure 3. Correlation ($R^2 = 0.995$) between total spectinomycin-related residues from bovine liver and kidney tissues measured by ELISA and radiotracer (LSC) analysis. Results are from data collected on different analysis days by an experienced analyst ($n = 21$) and a newly trained analyst ($n = 18$).

Table 3. Precision and Accuracy Measurement from Fortified Tissue

sample	tissue concentration, μg/mL 0.5	1	2
day 1	0.47 ± 0.01	0.95 ± 0.07	1.44 ± 0.32
day 3	0.46 ± 0.01	0.78 ± 0.09	1.82 ± 0.09
day 13	0.53 ± 0.02	0.92 ± 0.05	1.83 ± 0.09
N	15	15	15
av conc (μg/mL)	0.49	0.88	1.7
% av precision (within assay)	2	8	11
% precision (between assays)	6	8	11
% accuracy	98	88	85

The accuracy of the ELISA method compared to standard isotopic tracer methods ranged from 76% to 105% with an average ($n = 21$) of 95% when the analyses were performed by an experienced analyst. The accuracy obtained by a newly trained analyst ranged from 71% to 122% with an average ($n = 18$) of 93%. Equivalency of the ELISA method compared to the standard isotopic tracer methods for total residue analysis demonstrated in Figure 3.

Some of the previously published methods of analysis have attempted to increase the detectability of spectinomycin by chemically introducing a chromaphore. For instance, N-derivatizing agents, although successful in enhancing the detectability of spectinomycin, suffer from poor overall yield primarily because the extraction from tissue matrices is poor. Other strategies have attempted to increase the detectability of spectinomycin by chromatographically removing the interferences and employing a specific electrochemical method of detection after the separation. These methods seem to work well for relatively clean sample matrices; however, in complex matrices, such liver or kidney tissue, the matrix interference simply overwhelms the detector and the

analyte is either indistinguishable from the background or extensive column washing is required between analyses. For small scale applications, e.g. research analyses, this limitation is acceptable. For industrial-scale quality control applications, however, sample throughput is a major consideration.

ELISA methods, such as described herein and delivered in the microtiter format, meet the criteria necessary to be considered analytically rigorous. Moreover, in terms of increasing laboratory sample throughput, the ELISA assay can be employed with automated assay systems to analyze over a thousand samples per day. Thus, the ELISA assay is capable of meeting the "real-time" requirements for quality control analysis in the food-processing industry as well as the regulatory requirements contemplated by the USDA Hazard Analysis and Critical Control Point (HACCP) program.

CONCLUSIONS

The *in situ* derivatization and conjugation procedure for preparation of haptens and immunogens described in this report appears to be generally applicable to most natural products and is sufficiently mild to be free of untoward side reactions and degradations with the resulting immunogen produced in acceptable yields.

The one-step liver tissue extraction procedure developed in the course of this study is simpler than methods used previously, is quantitative (>98% overall extraction efficiency) for incurred residues in liver and kidney tissues, and appears to be free of matrix interference that introduces either positive or negative bias to the assay.

The assay was shown to have acceptable accuracy and precision for incorporation into a process quality-control program or incorporation as a surveillance method for regulatory compliance monitoring. Moreover, the ELISA method was shown to have >99% equivalency to standard radiotracer methods of analysis.

Ever increasing public pressure as well as regulatory pressure for increased monitoring of the nation's food supply to assure safety and wholesomeness is creating a demand for increased analytical laboratory capacity in the food processing industry and the commercial testing laboratory industry. The incorporation of ELISA into the battery of rigorously quantitative methods of analysis is the fastest and lowest cost means to significantly increase laboratory capacity and meet the public's expectation for assurance of food safety.

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