JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

Development of a Fluorescent Latex Immunoassay for Detection of a Spectinomycin Antibiotic

MARJORIE B. MEDINA*

Microbial Biophysics and Residue Chemistry and Core Technologies Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038

There is a need to develop a rapid and sensitive method to detect spectinomycin residues in animal tissues. A latex fluorescent immunoassay was designed using reagents developed for this assay. The spectinomycin antibody was produced in sheep, and the immunoglobulin (IgG) was purified through a Protein G affinity column and was immobilized onto latex particles. Spectinomycin was labeled with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF). The optimum assay conditions consisted of preincubating the latex−IgG with spectinomycin in buffer solutions or in bovine kidney extracts. DTAF−spectinomycin was added and was further incubated. The bound spectinomycin−DTAF/IgG−latex complex was separated by centrifugation at 4000*g* for 10 min. The fluorescence signals of the unbound spectinomycin−DTAF in the supernatant were measured at 485/535 nm excitation/emission. The measured signals were directly proportional to the concentration of spectinomycin in the samples, and spectinomycin was detected at 0−100 ppb with minimum detectability of 5 ppb. The mean regression correlation of four trials in buffer was 0.936 when the % bound complex vs spectinomycin concentration was plotted. Analysis of the kidney extract spiked with 0−100 ppb spectinomycin had a regression correlation of 0.959. This assay provides a rapid screening method for low ppb detection of spectinomycin.

KEYWORDS: Spectinomycin; fluorescent immunoassay; latex immunoassay

INTRODUCTION

Spectinomycin dihydrochloride (**Figure 1**) is used as an oral treatment to control bacterial enteritis in swine and to prevent and control losses due to chronic respiratory disease in chickens (*1*). It is subcutaneously injected into turkey poults to control bacterial infections and to prevent and control mortality (*1*). The tolerance for spectinomycin residue is 0.1 parts per million (ppm, μg/mL) in the uncooked edible tissues of chickens (*2*) and turkeys. Spectinomycin sulfate was also approved for subcutaneous treatment of bovine respiratory disease in nonlactating cattle with a 4 ppm residue tolerance of the parent compound in kidney (target tissue) and a 0.25 ppm residue tolerance of the parent compound in muscle tissues (*1*, *2*). The *Code of Federal Regulations* (*3*) describes a microturbidimetric assay with a lowest detectable level of 2.8 ppm in an analytical range of 24−37.5 ppm for the detection of this drug residue in edible tissues. A radiochemical microbial receptor assay was evaluated for quantitative analysis of spectinomycin spiked in milk with recoveries of 30 ng/mL (*4*). High-performance liquid chromatographic (HPLC) methods for spectinomycin reported 1.42 ppm detection in turkey plasma (*5*) and 50 ppb in swine, chicken, and calf plasma (*6*), in chicken eggs and edible fat, kidney liver, muscle tissues from calf, poultry, swine, and sheep (*7*), and in bovine milk (*8*). Ion-pairing HPLC coupled to an ion mass spectrometer (MS) allowed detection of 20 ng in a 20 ppm (μg/mL) spiked bovine kidney (*9*). This method was also used to confirm the presence of spectinomycin in milk at 50−100 ng/mL (*10*). Hornish and Wiest (*11*) reported an ion exchange HPLC method followed by reversed phase HPLC−atmospheric pressure chemical ionization tandem mass spectrometry for spectinomycin residue in bovine kidney, liver, muscle, and fat. The spectinomycin residue was validated in a

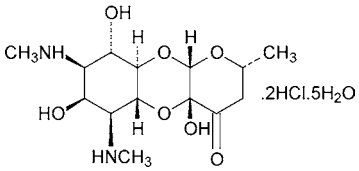

Figure 1. Chemical structure of spectinomycin dihydrochloride pentahydrate.

* To whom correspondence should be addressed. Tel: 215-233-6436. Fax: 215-233-6581. E-mail: mmedina@arserrc.gov.

10.1021/jf030542a
Published on Web 05/06/2004

range of 0.1−10 ppm. I also reported a rapid and sensitive (5−100 ng/mL) screening method utilizing a nonimmunofluorescent latex particle assay for detection of spectinomycin, using lysozyme as the aminoglycoside binding protein and fluorescein isothiocyanate (FITC) label (*12*, *13*). My current study presents a modified approach to the latex particle fluorescent assay by using anti-spectinomycin for the specific capture of spectinomycin and dichlorotriazinylaminofluorescein (DTAF)-labeled spectinomycin.

The objective of this study was to develop an immunochemical screening assay to detect low ppb levels of spectinomycin. My research goals were to produce sheep polyclonal anti-spectinomycin, purify the immunoglobulin (IgG) through a Protein G affinity column, covalently immobilize the purified IgG to latex particles, label the spectinomycin with DTAF, optimize conditions for the latex particle fluorescent immunoassay, and determine assay performance in spiked bovine tissue extracts.

MATERIALS AND METHODS

Equipment and Reagent. The keyhole limpet hemocyanin (KLH) and ImmunoPure Immobilized Protein G Plus gel were obtained from Pierce (Rockford, IL); DTAF, spectinomycin dihydrochloride, fluorescamine, Hepes (free acid), ethylenediaminetetraacetic acid (EDTA) (Na salt), sodium citrate, citric acid, $Na_3PO_4 \cdot 10H_2O$, Sigma Protein Standard, sodium azide, and Brij surfactant were from Sigma Chemicals (St. Louis, MO); the Bio-Rad Protein Assay Reagent (Coomassie Brilliant Blue G-250) and a two-way luer control stopcock lock were from Bio-Rad (Richmond, CA); Biacore 1000, N-hydroxy succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and ethanolamine were from Biacore Inc. (Piscataway, NJ); PD-10 columns were from Pharmacia (Piscataway, NJ); polystyrene carboxylated latex particles (0.86 μm, COOH/1, 10% w/v) were from Bangs Laboratories (Fisher, IN); the antibody production was contracted with Binding Site, Inc. (La Jolla, CA) and custom-produced by Immunogen International Limited (London, U.K.). Other equipment used in this study were an FCA fluorescent reader (Idexx, Inc., Westbrook, ME); Microplate Reader EL 312 (Biotek, Winooski, VT); Microcentrifuge mv 13 (Hill Scientific, Derby, CT); Tomy MTX150 refrigerated centrifuge (Peninsula Labortories, Inc., Belmont, CA); microtiter plate shaker (Lab-Line, Melrose Park, IL); Micro-fluor microtiter wells (Dynatech Laboratories, Chantilly, VA); and LS 50 B fluorescent and luminescent reader (Perkin-Elmer, Norwalk, CT).

Polyclonal Anti-spectinomycin Production. A spectinomycin immunogen was prepared by activating the carbonyls of the KLH protein prior to linking the KLH with the amino groups of the spectinomycin. The procedure used for the preparation of hygromycin−KLH immunogen was followed (*14*). The KLH (50 mg) was suspended in 1 mL of 0.3 M sodium bicarbonate buffer, pH 8.1. Sodium periodate (0.16 M) was added dropwise to the protein solution and stirred for 30 min at room temperature. The activated KLH was desalted by passing it through a PD-10 column and eluting it with 0.01 M sodium carbonate, pH 9.5. The protein-containing fractions were pooled, and 0.2 mL of aqueous spectinomycin (100 mg) was added drop by drop while stirring the solution and was further stirred for 3 h at room temperature. Sodium borohydride (0.5 mL of 0.5 M) was added to the reaction tube drop by drop and stirred overnight at 4 °C. The immunogen was desalted through a PD-10 column and eluted with 0.05 M Hepes−0.15 M NaCl, pH 7. The coupling efficiency was determined by measuring the total spectinomycin (before coupling) and the unbound spectinomycin after separation with a microfuge/filter concentrator (10 000 MW cutoff). The spectinomycin was derivatized by addition of fluorescamine at a 1:2 (spectinomycin:fluorescamine) molar concentration ratio, and the fluorescence signals were measured at 390 nm excitation and 485 nm emission.

Three sheep (nos. 1861, 1862, and 1863) were immunized with a 2 mg of protein equivalent of KLH−spectinomycin. Six booster shots (1 mg each) were given at weeks 4, 8, 12, 16, 20, and 24. The animals were bled at weeks 10, 14, 18, 22, and 26, and the sera were screened for antibody production with the BIAcore biosensor using the procedures previously described (*14*). A Protein G sensor surface was utilized to determine the total IgG production while a spectinomycin sensor surface was utilized to determine the specific antibody production against spectinomycin. Large volumes of antisera were collected at weeks 22, 26, 27, and 28.

Affinity Purification of Spectinomycin Antibody. The reagents utilized were a 2 mL bed volume of ImmunoPure Protein G Plus affinity column; spectinomycin−IgG serum; IgG binding buffer (HBS, pH 5), which contained 10 mM HEPES (free acid), 150 mM NaCl, and 3.4 mM EDTA (Na salt); elution buffer contained a 1:1 mixture of 100 mM citrate, pH 3, and 100 mM HCl, pH 1.86; neutralization buffer, 100 mM Na_3PO_4, pH 11.7; regeneration buffer, which contained a 1:1 ratio of 100 mM citric acid, pH 3, and 100 mM HCl, pH 1.8; and Sigma protein standard, 80 mg/mL. The calibration standard curve included 1, 0.5, 0.25, 0.1, 0.05, and 0 mg/mL in HBS.

The Protein G ImmunoPlus gel (2 mL) was packed in a 5 mL syringe barrel locked with a two-way stopcock attachment and plugged with a polystyrene frit with a medium porosity (70 μm). Another frit was also inserted at the top of the gel. The column was preconditioned with 5 column volumes of binding buffer. A 1 mL antiresrum was diluted with 3 mL of binding buffer and quantitatively transferred to the preconditioned Protein G purification column. The serum was allowed to flow completely into the gel. The column was locked, and the IgG was allowed to bind with Protein G for 30 min at room temperature. The column was washed with 15 mL of the binding buffer, and the IgG was eluted with a total of 10.5 mL of the elution buffer. The eluates were collected in 4 mL polypropylene tubes. Fraction 1 was eluted with 0.5 mL and followed by 10 × 1 mL fractions. Each 1 mL fraction was neutralized with 0.75−1 mL of 0.1 M Na_3PO_4 to a pH of around 6.5. The flow-through serum was again purified through the regenerated Protein G column to capture residual IgG.

Determination of IgG Purification Efficiency. Aliquots (20 μL) from each fraction were transferred to microtiter wells, and the protein content was determined by the addition of 200 μL of Bio-Rad protein assay reagent to screen for protein positive fractions indicated by a blue color change. The optical density was also measured in an enzyme-linked immunosorbent assay (ELISA) reader and quantified against Sigma protein standard containing 0, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/mL. The fractions containing proteins were pooled into a 15 mL graduated conical centrifuge tube, and the total volume was measured. The protein contents of the pooled fractions were measured by transferring 3 × 20 μL aliquots to microtiter wells, and 200 μL of Bio-Rad protein reagent was added. After a 15 min incubation at room temperature, the optical density was measured at 595 nm in the Biotek ELISA Reader and calibrated against the Sigma protein standard containing 0, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/mL to determine the protein concentration of the IgG pooled fraction.

Immobilization of Anti-spectinomycin IgG to Latex Particles. One milliliter of spectinomycin IgG containing 2.21 mg/mL was dialyzed in 10 mM sodium acetate buffer (pH 4) using a Pierce Slide-A-Lyzer with a 10 000 MW cutoff. Dialysis was carried out overnight (17 h) in 1 L of buffer at a refrigerated temperature. The protein concentration of the dialysate was again measured, and 10 mL of 0.3 mg spectinomycin−IgG/mL was prepared in sodium acetate buffer (pH 4) for latex coupling. The carboxy latex particles were sonicated with 10 pulses at 75% duty cycle (power setting no. 3), and 200 μL aliquots of latex particles were transferred into four 10 mL conical polystyrene centrifuge tubes. Equal volumes of EDC (75 mg/mL H_2O) and NHS (11.5 mg/mL H_2O) were mixed, and 20 μL aliquots of EDC/NHS were transferred to each of the four tubes containing the latex particles. The latex and EDC/NHS were gently mixed for 20 min at room temperature to activate the carboxyl groups. Anti-spectinomycin IgG (2 mL of 0.3 mg/mL) was added dropwise with continuous vortex mixing for 30 min at room temperature. The derivatized latex (latex−spectinomycin−IgG) was centrifuged at 8225*g* (10 000 rpm) for 5 min at 4 °C. The supernatant was assayed for protein content using a Bio-Rad assay and Sigma protein standard and compared with the initial protein concentration. The derivatized latex was resuspended in 2 mL of HBS. Ethanolamine, 1 M (20 μL), was added to block the unreacted active

carboxyls and further mixed gently for 30 min at room temperature. The latex−IgG was separated by centrifugation at 10 000 rpm (8225*g*) for 10 min, and the supernatant was discarded. The latex−IgG was stored in 2 mL of HBS, pH 7, 0.1% w/v, sodium azide and 0.015% Brij surfactant.

DTAF Labeling of Spectinomycin. Spectinomycin was labeled with DTAF utilizing and modifying the labeling procedures described by Wang et al. (*15*). Spectinomycin (300 μM) was dissolved in 10 mL of ultrapure water, and the pH was adjusted to 7 with 1 M sodium carbonate. DTAF (45 μmol) was dissolved in 1.5 mL of methanol. The spectinomycin (1 mL of 30 μM) solution was transferred to a silanized brown vial, and the DTAF solution was added dropwise until precipitation appeared. Triethylamine (12 μL of 90 μM) was added dropwise until the precipitate dissolved, and the reaction was allowed to proceed at room temperature in the dark for 3 h. The tracer was purified by preparative thin-layer chromatography in a 20 cm × 20 cm plate using chloroform/methanol/water (4/4/1 v:v:v). Using DTAF as R_f 1, the band in R_f 0.78 was scraped off and extracted with 2 mL of methanol for each plate and the eluates were pooled. To estimate the tracer concentration, the spectinomycin−DTAF was diluted in sodium bicarbonate (50 mM, pH 9) and the optical density was measured at 492 nm (excitation) and 510 (emission). The spectinomycin−DTAF tracer was stored in methanol solution in the dark at 20 °C or lower.

Immunoassay Procedures. Spectinomycin working standards containing 0, 5, 10, 25, 50, and 100 ppb were prepared from a 1 ppm (1 μg/mL) stock solution using the binding buffer (HBS, pH 5) containing 0.1% bovine serum albumin. Assay conditions were optimized for a single incubation (homogeneous assay). The spectinomycin working standards (50 μL) were transferred to 0.6 mL microfuge tubes. Latex−IgG with an IgG content of 44.6 μg IgG/0.2% latex particles was diluted with the assay buffer, and 50 μL of 1:5 or 100 μL of 1:2.5 dilution was added followed by the addition of 50 μL of 25 or 50 nM spectinomycin−DTAF. A blank well contained 150 μL of HBS binding buffer and 50 μL of spectinomycin−DTAF. The samples were mixed for 30 min at room temperature and centrifuged at 4000*g* (7000 rpm) for 10 min. Aliquots (2 × 50 μL) were transferred to black flat bottom microtiter wells, and the pH was adjusted to 8.2 with 5 μL of 0.1 M Na_3PO_4. The samples were mixed for 60 s, and the fluorescence was measured at 485/535 nm using an FCA fluorescent reader.

Conditions for a saturation assay with two incubation steps (**Figure 2**) were also optimized, and results were compared with the single incubation assay. The spectinomycin working standards (50 μL) were transferred to 0.6 mL microfuge tubes. Latex−IgG (100 μL of 1:2.5 dilution) was added and preincubated for 15 or 25 min to allow the spectinomycin to bind with IgG. Spectinomycin−DTAF (50 μL of 25 nM) was added and further mixed and incubated for 15 or 20 min at room temperature. A blank well contained 150 μL of HBS binding buffer and 50 μL of spectinomycin−DTAF. The unbound spectinomycin−DTAF was separated by centrifugation for 10 min at 7000 rpm. Aliquots (50 μL) of the supernatant were transferred in duplicate to microtiter wells containing 10 μL of 0.05 M Na_3PO_4 and 50 μL of ultrapure water in each well. After the mixture was mixed for 60 s, the fluorescence was measured at 485/535 nm.

Bovine Kidney Analysis. Tissue samples were prepared following the extraction procedure previously described (*16*). Kidney tissues (10 g) were cut into small pieces and transferred to a stomacher bag. Twenty milliliters of extraction buffer (73 mM phosphate buffer, pH 6) was added, and the sample was blended for 60 s. Blended samples were kept at 4 °C to allow residues to diffuse into the extraction buffer and to allow the solid material to settle. Ten milliliters of extracts was transferred to conical tubes, and the extracts were centrifuged for 10 min at 11 860*g* (12 000 rpm). The kidney extract (supernatant) was spiked with 0, 5, 10, 25, 50, and 100 ppb spectinomycin and analyzed. Aliquots (50 μL) of the spiked extracts were transferred to microfuge tubes. The saturation (two-incubation step) assay procedure as described above was followed. The latex−IgG (1:2.5) was added and incubated for 15 min. Spectinomycin−DTAF was added and incubated for another 20 min. The latex complex was separated, and aliquots of the supernatant containing the unbound spectinomycin−DTAF were transferred to microtiter wells and neutralized with 0.05 M Na_3PO_4. The fluorescence density was measured at 485/535 nm after a 60 s mixing.

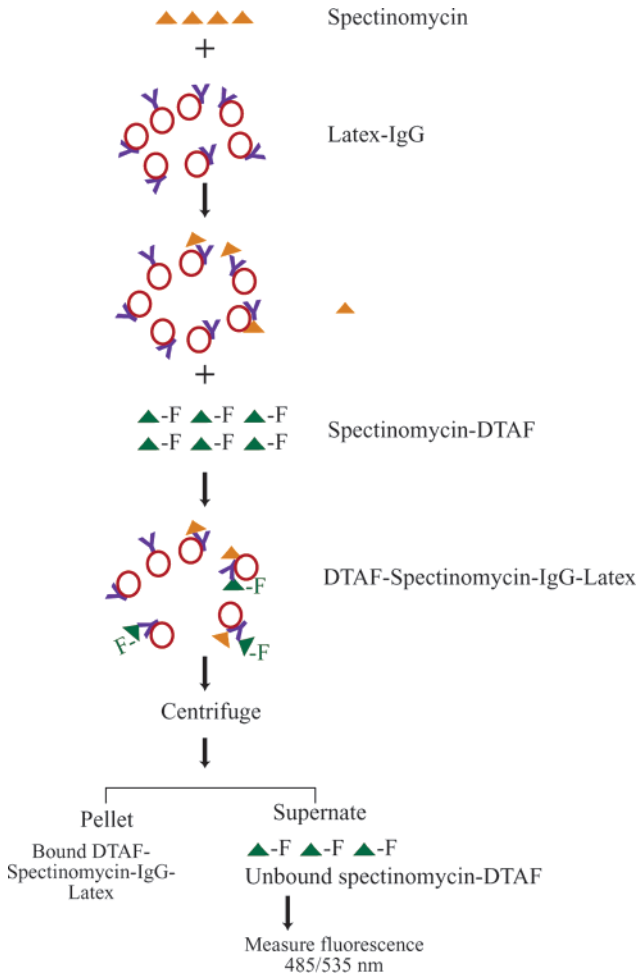

Figure 2. Schematic diagram of the principles of the fluorescent latex particle immunoassay utilizing the two step incubation assay. The first step allows the target compound to bind with the antibody, and the second incubation allows the tracer to occupy the empty binding sites of the IgG.

RESULTS AND DISCUSSION

Preparation of Immunogen and Production of Antibody. The concentration of spectinomycin−KLH immunogen was estimated at 50 mg spectinomycin per 50 mg KLH as shown by the 50% reduction of fluorescamine reaction of the initial spectinomycin concentration as compared to the final concentration after covalent coupling. The spectinomycin−KLH polyclonal antibody was produced in three sheep. The total IgG and specific spectinomycin IgG were evaluated with a Biacore biosensor utilizing a Protein G and spectinomycin sensor surfaces, respectively. The sheep no. 1862 produced a higher ratio (66−76%) of spectinomycin IgG as compared to the total IgG while the sheep nos. 1861 and 1863 yielded only 20% of specific specitinomycin IgG. The latter animals also had a low antibody production measured as total IgG. Such variability of responses suggested the physiological differences of host animals. Therefore, large volumes of serum were obtained from sheep no. 1862 prior to termination of the antisera production. The purification of spectinomycin−IgG through Protein G affinity columns yielded 99.48% recovery of the IgG in the first purification (single pass). The total protein recovered was 32.96 mg per mL antiserum when calibrated against the Sigma Protein Standard.

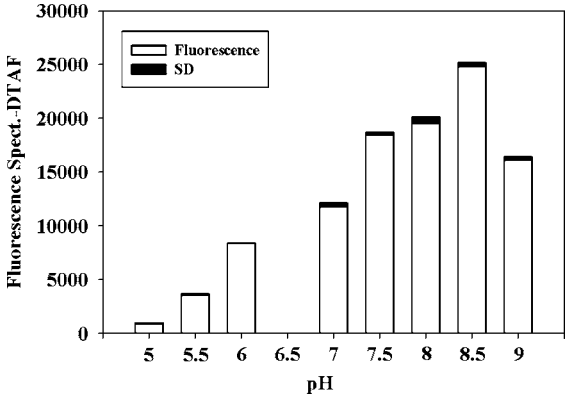

Figure 3. Optimization of the DTAF–spectinomycin fluorescent signals against the buffer pH from 5 to 9. Mean fluorescence density of triplicate samples containing 100 μL of 100 μM DTAF–spectinomycin. The optimum pH is shown at 8.5. The mean coefficient of variability (SD/mean × 100) was 2.7% in a range of 0.5–8.2%.

Tracer Preparation and Covalent Attachment of IgG to Latex Particles. The TLC separation of DTAF-labeled spectinomycin indicated three bands, with R_f values of 1.0 for DTAF, 0.9 for the intermediate product, and 0.78 for the DTAF−spectinomycin complex. A yield of 100 nM/mL DTAF−spectinomycin in methanol solution was obtained. The fluorescent tracer was scanned in a spectrophotomer to determine the optimum excitation and emission wavelengths, and the optimum pH was also determined. The maximum fluorescent signals of the DTAF−spectinomycin were observed at pH 8.5 (**Figure 3**).

Four batches of latex particles were immobilized with spectinomycin IgG. The protein load on the latex particles was 19.2, 21.8, 22.8, and 25.2 mg in each preparation with a mean of 22.3 (2.2 SD) mg protein/mg latex. The coupling efficiencies (%) were 61.1, 69.4, 72.6, and 80.3 with a mean of 70.9 (6.9 SD), respectively. The latex−IgG stock solution contained 0.223 mg/2 mL latex containing 0.5% latex particles. At 1:5 and 1:2.5 dilutions, the latex−IgG concentration was 22.3 and 44.6 μg protein/mL of 0.1% latex suspension, respectively. A 100 μL aliquot of 1:5 and 1:2.5 dilutions used in the assay contained 2.23 and 4.46 μg of IgG protein.

Fluorescent Immunoassays. The unbound DTAF−spectinomycin in the supernatant was measured, and the fluorescent signals of the free and unbound spectinomycin−DTAF were directly proportional to the concentration of the unlabeled spectinomycin in the samples. In more than a dozen experimental trials to optimize the equilibrium assay, the results suggest the following assay condition: 50 μL of latex−IgG (1:2.5 dilution), 100 μL of 25 nM spectinomycin−DTAF, and 50 μL of spectinomycin samples with 30 min incubation. Assessment of the binding capacity of the IgG and the binding of the tracer without competition and with competition of 1 ppm spectinomycin was evaluated. The % inhibition was calculated as fluorescence of 1 ppm spectinomycin − fluorescence of DTAF−spectinomycin ("0" spectinomycin)/DTAF−spectinomycin ("0" spectinomycin) × 100. Results showed that 1 ppm spectinomycin inhibited spectinomycin−DTAF binding to the IgG−latex at 24.1, 13.8, 24.1, 26.3, and 26.5%. Ideally, assay conditions should have 50% inhibition or greater.

In an attempt to obtain greater inhibition with a minimum of 40%, a saturation assay with a two step incubation was optimized with each step incubation of 15 and 20 min. The optimum preincubation time of spectinomycin binding to the IgG−latex was 15 min followed by the second incubation with the tracer for 20 min. Spectinomycin doses of 0, 5, 10, 25, 50, and 100 ppb were analyzed. A composite of eight replicate readings expressed as mean fluorescence of DTAF−spectinomycin in the supernatant vs spectinomycin concentration is shown in **Figure 4A**. The standard deviation is shown by the top bar. The % bound was calculated as total DTAF−spectinomycin in blank − sample fluorescence/total DTAF−spectinomycin fluorescence × 100. The % bound was inversely proportional to the spectinomycin concentration in samples. Plotting the % bound spectinomycin−DTAF against the spectinomycin standard concentrations in four trials, the regression correlation values (R^2) were 0.976, 0.938, 0.974, and 0.859 (mean = 0.936). The lower correlation coefficient value is perhaps due to dilution errors. Serial dilutions of standards resulted in better linearity of the calibration curve. Expressing the data as % inhibition vs spectinomycin concentration resulted in direct correlation with the spectinomycin concentration, and a typical calibration curve is shown in **Figure 4B**. The calibration standard curves had hyperbolic curves in a range of 0−100 ppb with a saturation of the IgG binding sites near 50 ppb. The limit of detection (LOD) was observed at 5 ppb, and the limit of quantitation (LOQ) was observed at 25 ppb. LOD and LOQ were determined as sample fluorescence plus 3× and 10× the standard deviations of the "0" sample, respectively. I have previously reported (*12*) that the saturation procedure with two incubation steps resulted in an assay with a more linear curve and greater sensitivity than the equilibrium assay with

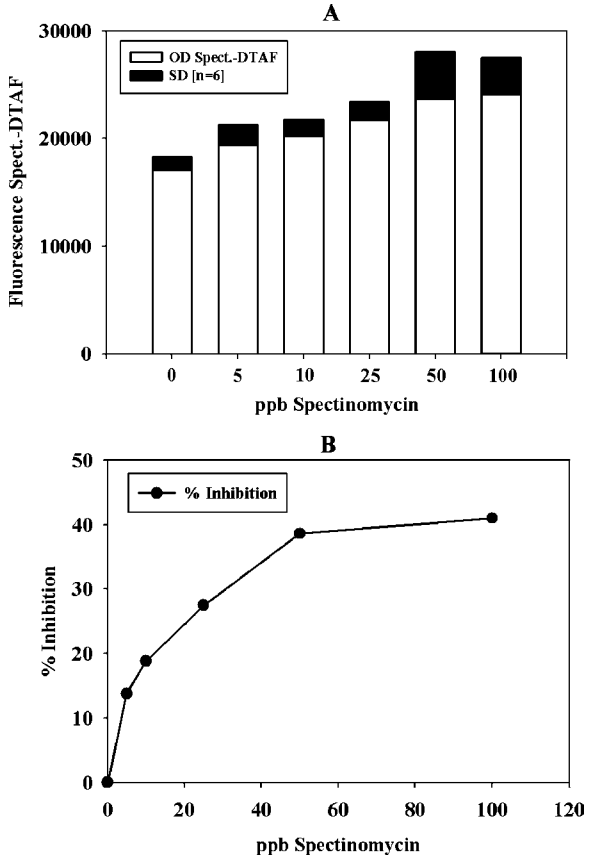

Figure 4. (**A**) Fluorescence measurement of the unbound (free) DTAF–spectinomycin in the supernate vs spectinomycin dose in a two step saturation assay. (**B**) A typical calibration curve of % inhibition of spectinomycin–DTAF binding to IgG–latex by the spectinomycin standards (5–100 ppb). Note the 50 ppb saturation point [% inhibition = DTAF–spectinomycin in sample – fluorescence in "0" sample/DTAF–spectinomycin fluorescence in "0" sample × 100].

Table 1. Data Summary of the Fluorescenct Responses (pH 8.2) of Spectinomycin in Kidney Samples and Various Ways of Analyzing the Competitive Binding Responses

spectinomycin dose (ppb)	free DTAF–spectinomycin	0	5	10	25	50	100
mean ($n = 4$) fluorescence	29 873	23 641	25 223	25 624	27 050	28 034	28 949
sample dose – 0 control[a]		0	1582	1983	3409	4983	5308
% bound/free[b]		20.9	15.6	14.2	9.5	6.2	3.1
total/bound[c]		4.4	6.4	7.0	10.6	16.2	32.3
% inhibition[d]		0	6.69	8.39	14.42	21.1	22.45

[a] Fluorescence of sample – fluorescence of "0" sample with no spectinomycin. [b] % fluorescence of DTAF–spectinomycin bound to the anti-spectinomycin–latex complex/free DTAF–spectinomycin × 100. [c] Total free DTAF–spectinomycin/DTAF–spectinomycin bound to the anti-spectinomycin–latex complex. A linear curve results by plotting total/bound vs spectinomycin dose. [d] % inhibition = sample fluorescence – "0" fluorescence/"0" fluorescence × 100.

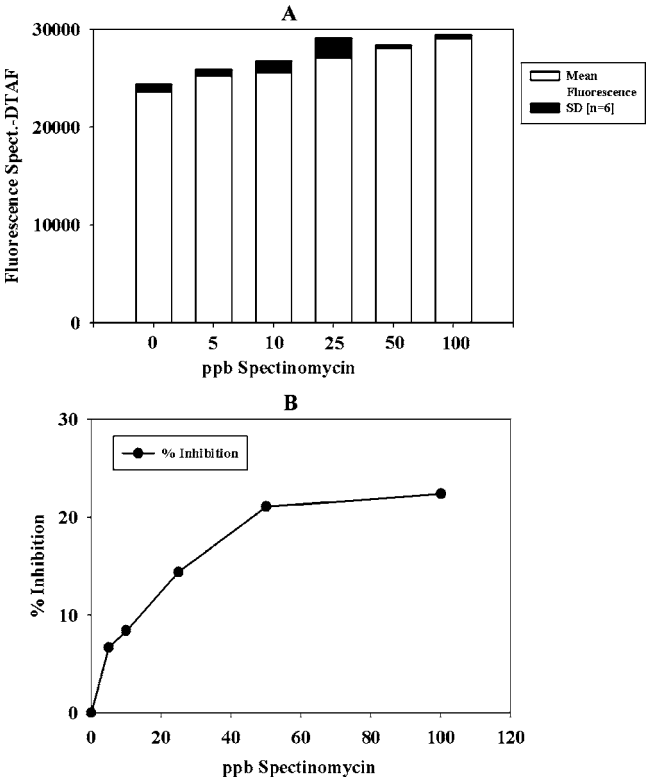

Figure 5. (**A**) Determination of fluorescence of the unbound (free) DTAF–spectinomycin in the supernatant vs spectinomycin in a bovine kidney extract. (**B**) A typical calibration curve of % inhibition of spectinomycin–DTAF binding to IgG–latex by the spectinomycin standards (5–100 ppb) in a two step saturation assay. Note the 50 ppb saturation point. Samples containing greater than 50 ppb can be diluted to obtain more accurate results.

one incubation step. However, the latter can be used if an assay can be designed with one set point for a positive or negative result. In this study, the set point for a positive or negative result is 50 ppb.

The kidney extracts were spiked with spectinomycin at 0 and 5−100 ppb and analyzed using the optimum conditions in the saturation assay with two incubation steps. The unbound DTAF−spectinomycin in the supernatant was measured. **Table 1** shows the data summary of the analysis of spectinomycin spiked in kidney extracts. The fluoresent density data were expressed as % bound/free or total free unbound DTAF spectinomycin or % inhibition of the DTAF−spectinomycin binding to the anti-spectinomycin−latex complex. These values could then be plotted against the spectinomycin dose. **Figure 5A** shows the plot of the unbound DTAF−spectinomycin in the supernatant vs the spectinomycin dose. A typical curve of the % inhibition of DTAF−spectinomycin to the IgG−latex vs spectinomycin concentration is shown in **Figure 5B** and resulted with an R^2 of 0.959. The % inhibition was directly proportional to the spectinomycin concentration in samples. The results indicated that a lower concentration of the labeled spectinomycin can be used to obtain higher sensitivity at low ppb levels. Serial dilutions of standards yielded better accuracy and lower variability of results when analyzing low ppb levels. A multiple sample assay can be completed in 1.5 h.

These studies demonstrate that the latex particle immunofluorescent assays can be used for low ppb analysis of spectinomycin, 5 (LOD) and 25 ppb (LOQ). The assay is simple and has a high throughput. The use of DTAF and anti-spectinomycin in this study shows similar results to the utilization of FITC as the tracer and lysozyme as the capturing molecule shown in my previous research. Both of these fluorescent latex particle assays have greater sensitivity than the microturbidimetric assay and most chromatographic methods with a minimum detectability of 50 ppb. The latex fluorescent assay can be used as an alternative to the radiochemical receptor assay, which is calibrated at 50 ppb sensitivity. Concentrations of spectinomycin in the original samples must be adjusted with the dilution ratio used in the sample preparation. The dilution ratio for sample preparation may vary due to different residue tolerance levels in animal tissues and fluids. My future research will modify and improve the preparation and isolation of the DTAF-labeled compound and utilize the fluorescent latex immunoassay conditions for the detection of spectinomycin in spiked tissues and in animal tissues containing "incurred" residues.

LITERATURE CITED

(1) *Code of Federal Regulations*; Title 21; Parts 520: 2122, 2123b; 522: 2120, 2121; U.S. Government Printing Office: Washington, DC, 2003; http://www.access.gpo.gov.

(2) *Code of Federal Regulations*; Title 21; Parts 556: 600; U.S. Government Printing Office: Washington, DC, 2003; http://www.access.gpo.gov.

(3) *Code of Federal Regulations*; Title 21; Parts 436: 100−200, 2000; U.S. Government Printing Office: Washington, DC, 2000.

(4) Shaikh, B.; Shemerhorn, P.; Adam, L. A.; von Bredow, J. D. Application of microbial receptor assay to quantitation of residues of spectinomycin in bovine milk and comparison with liquid chromatographic analysis. *J. AOAC Int.* **1999**, *82*, 1002−1005.

(5) Burton, S. D.; Hutchins, J. E.; Fredericksen, T. L.; Ricks, C.; Tyczkowski, J. K. *J. Chromatogr.* **1991**, *571*, 209−216.

(6) Haagsma, N.; Keegstra, J.; Scherpenisse, P. J. High performance liquid chromatographic determination of spectinomycin in swine, calf and chicken plasma. *J. Chromatogr.* **1993**, *615*, 289−295.

(7) Bergwerff, A. S. A.; Scherpenisse, P.; Haagsma, N. HPLC determination of residues of spectinomycin in various tissue types from husbandry animals. *Analyst* **1998**, *123*, 2139−2144.

(8) Shemerhorn, P.; Chu, P. S.; Kijack, P. J. Determination of spectinomycin residues in bovine milk using liquid chromatography with electrochemical detection. *J. Agric. Food Chem.* **1995**, *43*, 2122−2125.

(9) McLaughlin, L. G.; Henion, J. D. Determination of aminoglycoside antibiotics by reversed-phase ion-pairing high-performance liquid chromatography coupled with pulsed amperometry and ion-spray mass spectrometry. *J. Chromatogr.* **1992**, *591*, 195−206.

(10) Carson, M. C.; Heller, D. N. Confirmation of spectinomycin in milk using ion-pair solid-phase extraction and liquid chromatography electrospray ion trap mass spectrometry. *J. Chromatogr. B* **1998**, *718*, 95−102.

(11) Hornish, R. E.; Wiest, J. R. Quantitation of spectinomycin residues in bovine tissues by ion-exchange high performance liquid chromatography with postcolumn derivatization and confirmation by reversed-phase high performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. *J. Chromatogr. A* **1998**, *812*, 123−133.

(12) Medina, M. B. Particle concentration fluorescence assays for rapid detection of trace levels of antibiotics. In *Veterinary Drug Residues*; Medina, M., Moats, W., Eds.; American Chemical Society Symposium Series 636; American Chemical Society: Washington, DC, 1996; pp 132−143.

(13) Campbell, N. F.; Medina, M. B. Detection and separation of aminoglycosides by binding to immobilized lysozyme or alpha-lactalbumin. U.S. Patent Number 5,888,744, March 30, 1999.

(14) Medina, M. B. Hygromycin B antibody production and characterization by a surface plasmon resonance biosensor. *J. Agric. Food Chem.* **1997**, *45*, 389−394.

(15) Wang, C. H. J.; Stroups, S. D.; Jolley, M. E. Fluorescent polarization immunoassay utilizing substituted triazinylamino-fluorescein aminoglycosides. U.S. Patent Number 4,593,089, June 3, 1986.

(16) Medina, M. B.; Poole, D. J.; Anderson, M. R. A screening method for β-lactams in tissues hydrolyzed with penicillinase I and lactamase II. *J. AOAC Int.* **1998**, *81*, 963−972.

Received for review July 29, 2003. Revised manuscript received March 21, 2004. Accepted March 24, 2004. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF030542A