Determination of Spectinomycin Residues in Bovine Milk Using Liquid Chromatography with Electrochemical Detection

Patricia G. Schermerhorn,* Pak-Sin Chu, and Philip James Kijak

Center for Veterinary Medicine, U.S. Food and Drug Administration, Beltsville, Maryland 20705

A method capable of quantifying spectinomycin in raw bovine milk was developed and validated for 100–400 ng/mL. In this procedure the milk is centrifuged at −4 °C and the top fat layer removed. The defatted milk is deproteinated by precipitation with 30% trichloroacetic acid and centrifugation. The supernatant is washed sequentially with dichloromethane, hexane, and ethyl acetate. An aliquot of the separated aqueous layer is prepared for HPLC analysis by mixing with 1-decanesulfonic acid and filtering. The analyte is separated from the matrix components using an ion-pair mobile phase and a reversed-phase column; the analyte is quantified with an electrochemical detector. Mean recoveries are 80% for milk fortified at 100 ng/mL, 76% for milk fortified at 200 ng/mL, and 77% for milk fortified at 400 ng/mL. The intralaboratory coefficients of variations are 18%, 6%, and 9%, respectively.

Keywords: *Spectinomycin; electrochemical detection; milk*

INTRODUCTION

Spectinomycin (Figure 1), an aminocyclitol antibiotic typically produced as the dihydrochloride pentahydrate salt, is widely used for veterinary purposes. It is approved for use in the United States as an injectable for 1–3-day-old turkey poults and chicks (21 CFR 522.2120) (*Code of Federal Regulations*, 1992) and as an oral solution and soluble powder for use in the drinking water of pigs under 4 weeks of age and growing chickens [21 CFR 520.2122 and 520.2123(b)] (*Code of Federal Regulations*, 1992). It is also approved and widely used in combination with lincomycin for the treatment and prevention of chronic respiratory disease associated with mycoplasmal and coliform infections for chickens up to 7 days of age [21 CFR 520.1263 (b)] (*Code of Federal Regulations*, 1992) (Haagsma et al., 1993). A tolerance of 0.1 ppm is established for residues of spectinomycin in the uncooked edible tissues of chickens (21 CFR 556.600) (*Code of Federal Regulations*, 1992).

Recently, the U.S. Food and Drug Administration (FDA) became aware of possible unapproved use of spectinomycin in lactating cattle, which has the potential of resulting in illegal residues in bovine milk. To ensure the safety of the milk supply, the FDA determined the need for an assay capable of detecting and quantifying residues at a concentration of 200 ng of spectinomycin/mL of milk.

Only a few procedures have been described for determining spectinomycin. Spectinomycin is hydrophilic (Marsh and Weiss, 1967), is lacking a UV chromophore, and is unstable in weakly alkaline or strongly acidic solutions (Brown and Bowman, 1974; Tsuji and Jenkins, 1985). Currently, the Food Safety and Inspection Service of the USDA screens for tissue residues of spectinomycin using a microbial-based method. However, this method lacks sensitivity and exhibits a minimum inhibitory concentration equivalent to 2.8 ppm of spectinomycin (U.S. Department of Agriculture, 1994).

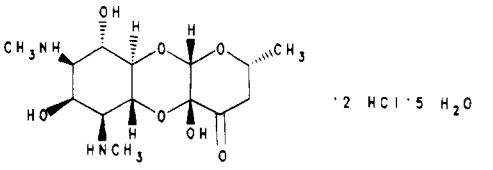

Figure 1. Structure of spectinomycin dihydrochloride pentahydrate.

Most published chromatographic techniques for spectinomycin require derivatization of the compound. Brown and Bowman (1974) developed a GC procedure for the determination of spectinomycin in bulk drugs using a trimethylsilane derivative of the compound. High-performance liquid chromatographic methods, both normal- and reversed-phase, with UV detection of spectinomycin derivatives have been developed. Tsuji and Jenkins (1985) and Haagsma et al. (1993) assayed veterinary formulations and blood plasma, respectively, by precolumn derivatization of spectinomycin with 2-naphthalene chloride, normal-phase HPLC, and detection at 254 nm. Burton et al. (1991) were able to measure therapeutic concentrations (2–100 μg/mL) in turkey plasma, after spectinomycin transformation to the respective 2,4-dinitrophenylhydrazone derivative, by employing reversed-phase HPLC and detection at 504 nm. Myers and Rindler (1979) developed a procedure for the detection of spectinomycin in process samples and fermentation beers at levels of 50–900 μg/mL using postcolumn derivatization to convert the secondary amine groups to primary amines and fluorescence detection. All of these methods either lack the required sensitivity or require the difficult formation of spectinomycin derivatives which, in some cases, yield multiple products.

Elrod et al. (1988) found spectinomycin to be electrochemically active and developed a procedure for the determination of spectinomycin using ion-pairing chromatography with electrochemical detection. However, they did not apply the method to the analysis of samples isolated from biological matrices. A similar approach was reported by McLaughlin and Henion (1992). They isolated spectinomycin from bovine kidney, but the apparent recovery was low. Both of these methods

* Author to whom correspondence should be addressed [fax (301) 504-9273; e-mail Schermerhorn@A1@CVM.FDA.GOV].

demonstrated that electrochemical detection can be used in the analysis of spectinomycin.

In this paper we describe a simple, straightforward procedure for determining spectinomycin in bovine milk at a concentration of 100–400 ng/mL using ion-pair chromatography with electrochemical detection. Samples are prepared by defatting and deproteination with trichloroacetic acid as reported previously for neomycin in milk (Shaikh and Jackson, 1989). Further cleanup prior to HPLC analysis is achieved by partitioning interfering matrix components into dichloromethane, hexane, and ethyl acetate, sequentially.

MATERIALS AND METHODS

Apparatus. Liquid chromatography was performed using a Perkin-Elmer Series 410 pump equipped with an ISS-100 autosampler (Norwalk, CT) and an ESA Coulochem II electrochemical detector (Model 5200A), equipped with a guard cell (Model 5020) and analytical cell (Model 5010) (Bedford, MA) interfaced to a Perkin-Elmer Nelson Turbochrom 3 data system. Separation was achieved using a Phenomenex Ultracarb 5 μm, ODS-2 250 × 3.2 mm column equipped with a Phenomenex Ultracarb 30 × 3.2 mm guard column (Torrance, CA), both of which were maintained at 30 °C in a column heater. A Sorvall RC5C centrifuge (Wilmington, DE) equipped with a SA-600 fixed angle rotor was used to deproteinate; a Beckman GPR refrigerated centrifuge (Palo Alto, CA) equipped with a GH-3.7 horizontal rotor was used to defat and for phase separation. (Note: a single centrifuge can be used.)

Reagents and Solutions. Liquid chromatography grade water, prepared in-house, was used in preparing all solutions. Acetonitrile, dichloromethane, hexane, and ethyl acetate were purchased as LC grade. Saturated ethyl acetate was prepared by shaking in a stoppered bottle the organic solvent with water and leaving the phases to separate; the upper, saturated organic phase was withdrawn as needed. Citric acid, sodium hydroxide solution (50% w/w), and trichloroacetic acid were of ACS reagent grade. The ion-pair reagent, 1-decanesulfonic acid sodium salt, was of chromatography grade. The ion-pair buffer was a 0.02 M citric acid and 0.002 M 1-decanesulfonic acid sodium salt solution adjusted to pH 6.1 with 50% sodium hydroxide and filtered (0.2 μm nylon). This solution is stable for 3 months if refrigerated. The mobile phase is ion-pair buffer–acetonitrile (84 + 16). The mobile phase is degassed with helium before use. As large shifts in the retention time of spectinomycin (19–30 min) occur with minute variations of both the pH and buffer/acetonitrile ratio of mobile phase, it is recommended that a sufficient quantity of mobile phase be prepared to complete any series of analyses. The ion-pair concentrate (IPC) is a 0.2 M citric acid and 0.02 M 1-decanesulfonic acid sodium salt solution adjusted to pH 6.5–7.0 with 50% sodium hydroxide. The 30% trichloroacetic acid (w/v) (TCA) is prepared with distilled, deionized water. Both solutions are stable for 1 year at room temperature. Spectinomycin (spectinomycin dihydrochloride pentahydrate) was purchased from the U.S. Pharmacopeial (USP) Convention, Inc., Rockville, MD. On the basis of the listed potency, a 100 μg of spectinomycin/mL standard solution (SS) was prepared by dissolving an amount of standard equivalent to 10 mg of the free base drug in 100 mL of distilled deionized water. Spectinomycin is light sensitive, so the use of low actinic volumetric flasks is recommended. SS was used to prepare a 10 μg of spectinomycin/mL aqueous fortification solution (SFS) for preparing fortified milk samples. SS was also used to prepare a minimum of four chromatographic standards ranging in concentration from 50 to 400 ng of spectinomycin/mL. Solutions were diluted to volume with mobile phase. All standards are stable for 1 month if stored refrigerated (Stahl et al., 1991).

Milk Samples. The control milk was obtained from the bulk tank at the USDA Agricultural Research Center in Beltsville, MD. This milk was commingled from a herd of approximately 100 animals. Incurred milk was obtained from a single cow treated by intramammary infusion with spectinomycin. A control milk sample and a control milk sample fortified at 200 ng of spectinomycin/mL should be analyzed with each set of samples to verify system performance.

Sample Preparation. Measure 5 mL of milk into polycarbonate centrifuge tubes. Cap tubes, vortex-mix for 5 s, and centrifuge at 3000 rpm (1500*g*) at −4 °C for 30 min. Carefully remove the top fat layer and discard. Add 500 μL of 30% TCA, vortex-mix for 5 s, and centrifuge at 8000 rpm (9300*g*) (temperature not critical) for 30 min. Decant the supernatant into a clean 15 mL polypropylene centrifuge tube. Add 6 mL of dichloromethane to the supernatant. Cap, vortex-mix for 30 s, and centrifuge to achieve phase separation (approximately 5 min at 3000 rpm, room temperature); transfer the top, aqueous layer to a clean 15 mL centrifuge tube. Sequentially partition contaminants from the aqueous layer with 5 mL of hexane and twice with 5 mL of saturated ethyl acetate. For each solvent, cap, vortex-mix for 30 s, centrifuge to achieve phase separation, and transfer the bottom, aqueous layer into a clean 15 mL centrifuge tube. Bring the final volume of the aqueous layer to 5 mL using water in the graduated centrifuge tube, cap, and vortex-mix for 5 s (low speed). Transfer 900 μL of aqueous sample extract into a disposable test tube, add 100 μL of IPC, and vortex-mix for 5 s (low speed). Filter the sample extract into a 1.8 mL injector vial using a 13 mm, 0.2 μm Whatman PVDF disk (Whatman, Clinton, NJ) attached to a disposable syringe. Analyze sample extracts within 24 h of preparation.

Chromatographic Conditions. The LC system was programmed to use the following parameters: flow rate, 0.75 mL/min; injection volume, 25 μL (for both standards and sample extracts); analysis time, 35 min; an additional 15 min equilibration time after analyses for sample extracts. The following detector settings were used: guard cell = 950 mV; screening electrode E_1 = 650 mV, R = 100 μA, filter = 2 s (default settings), CH_1 = 1 V, offset = 0%; working electrode E_2 = 850 mV, R = 1 μA (for older ESA detectors, 1 μAFS corresponds to a gain of 100), filter = 10 s, CH_2 = 1 V, offset = 15%. The detector should be allowed to stabilize by pumping mobile phase through the system at 0.1–0.2 mL/min for a day prior to analyzing samples. A flow of 0.1–0.2 mL/min between series of analyses should be maintained when using the system on a daily basis.

Calibration Curve. At least four spectinomycin standards spanning the range of interest should be injected prior to the injections of the sample set. A standard curve using a linear regression should be prepared. The correlation coefficient squared is generally ≥0.99. As a 900 μL aliquot of extract is diluted to 1 mL prior to injection, the concentration of the extract is multiplied by 1.111 to determine the concentration of spectinomycin in the milk sample.

System Suitability. Prior to injecting standards or sample extracts, the detector must be stable. The detector is considered stable if after blank injections of mobile phase, it does not register a negative response during the 35 min analysis time and baseline has only minimal drift. In our laboratory, the signal to noise ratio for the 200 ng/mL standard is typically 70/1. The limit of detection (LOD) for this method is approximately 30 ng/mL, and the limit of quantitation (LOQ) is approximately 50 ng/mL as defined in the *U.S. Pharmacopeia-National Formulary 1990* (1989).

RESULTS AND DISCUSSION

The purpose of this work was to develop a regulatory procedure to quantitate spectinomycin residues in bovine milk at 200 ng/mL for use in state and federal laboratories. Typically, we demonstrate method performance over a range from half to twice the level of interest. Table 1 demonstrates the performance of the method for concentrations of 100–400 ng/mL. The recoveries ranged from 76% to 80% for fortified control milk. The method precision (intralaboratory CVs) ranged from 6% to 18%. Most control milk samples did not show any peaks at the retention time of spectinomycin. One sample had a small peak which was less

Table 1. Concentration and Average Percent Recovery of Spectinomycin Measured in Milk Samples Fortified at 100, 200, and 400 ng/mL

	measd concn of spectinomycin (ng/mL)		
	100 ng/mL added	200 ng/mL added	400 ng/mL added
	100	160	360
	97	150	330
	62	160	320
	61	140	310
	71	140	300
	84	160	310
	87	150	290
	80	150	260
av	80	150	310
% recovery[a]	80	76	77
SD[b]	15	9	29
% RSD (CV)[b]	18	6	9

[a] Percent recovery = (average amount found/amount fortified) × 100. [b] Calculated from data prior to the rounding of individual values.

Table 2. Concentration of Spectinomycin Measured in Two Sets of Incurred Milk Samples from a Single Cow[a]

	measd concn of spectinomycin (ng/mL)	
	52 h postdose incurred	72 h postdose incurred
	370	100
	360	93
	450	150
	410	130
	390	140
	320	150
	320	130
	300	130
av	370	130
SD[b]	49	21
% RSD (CV)[b]	13	16

[a] These data were collected to demonstrate the suitability of the method in analyzing samples from a treated animal. [b] Calculated from data prior to the rounding of individual values.

than the LOQ of the method. For method validation, FDA policy requires the analysis of milk samples containing incurred residues of the drug being tested. Table 2 presents the amount of spectinomycin found in two sets of incurred samples when a lactating dairy cow was dosed by intramammary infusion. Milk was collected at 52 h postdose and again at 72 h postdose. Figure 2 shows chromatograms of (A) standard spectinomycin, (B) control milk, (C) fortified control milk, and (D) incurred milk. No peaks, other than the peak from spectinomycin, are seen in the incurred milk chromatogram that are not found in the control milk chromatogram.

Initially a Supelcosil LC-18-DB 5 μm, 250 × 4.6 mm column (Bellefonte, PA) was used. A flow rate of 1.5 mL/min was needed to produce a good peak shape. To conserve mobile phase and minimize the amount of hazardous waste generated, we evaluated the midbore Phenonemex Ultracarb column. We attained good peak shape at a flow rate of 0.75 mL/min, thereby reducing hazardous waste by 50%. The midbore column became the column of choice. With proper care the system is capable of running approximately 400 samples before the guard column should be changed and approximately 800 samples without column degradation.

We used applied potentials of 850 mV for the analytical cell and 650 mV for the screening electrode as reported by Elrod et al. (1988). During development, various combinations of screening and applied voltages

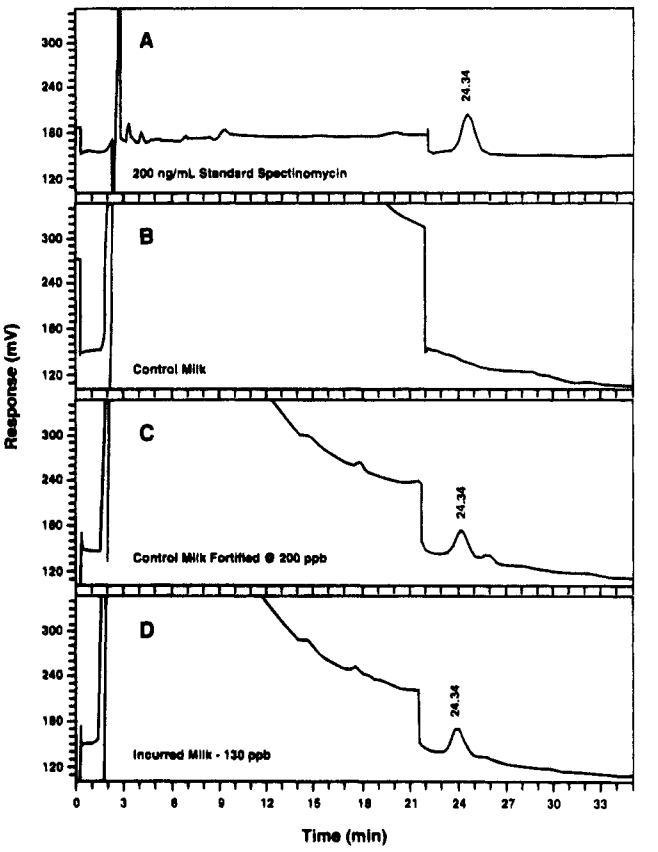

Figure 2. Chromatograms of (A) standard spectinomycin, (B) control milk from USDA bulk tank, (C) control milk fortified with spectinomycin equivalent to 200 ppb, and (D) incurred milk from a dosed cow. The detector is zeroed before spectinomycin elutes, causing the baseline shift prior to the peak.

were used in an effort to optimize the system. Our results confirm that the potentials used by Elrod et al. are well suited for the detection of spectinomycin.

To demonstrate good selectivity of the method, several common pharmaceuticals used in veterinary medicine were chromatographed to determine if they would interfere with the spectinomycin analysis. The classes of drugs tested included tetracyclines, sulfonamides, β-lactams, macrolides, and aminoglycosides; a variety of other compounds used in veterinary medicine were also tested. In addition, milk from several herds in different geographic areas was analyzed for interfering endogenous peaks. No interfering peaks were noted in these milk samples in the retention time window of spectinomycin, suggesting that the method is not susceptible to variations in regional husbandry practices.

Milk samples are sometimes analyzed after several months of storage. Since no information was available regarding the stability of spectinomycin in frozen milk, we investigated the stability of spectinomycin under these conditions. Milk was stored at −80 and at −20 °C for 6 months to determine the stability of spectinomycin in the frozen state. The milk was periodically thawed and analyzed. Over time, the milk stored at −80 °C was more stable in terms of maintaining freshness and yielded slightly higher recoveries than the milk stored at −20 °C. In contrast, milk stored at −20 °C deteriorated and curdled over the same period of time; however, the spectinomycin was still intact. During the first month of storage, there was an average loss of 15% recovery at the −80 °C storage temperature and an average loss of 24% recovery at the −20 °C storage temperature when compared to fortified fresh milk.

After this initial loss, there was no significant decrease in recovery of spectinomycin from either set of stored samples over the 6 month period. Ideally, methods developed for regulatory purposes should be easily transferable to analysts unfamiliar with the procedure. This method was given for evaluation to a second analyst in our laboratory not familiar with this work. The analyst successfully reproduced the results with similar accuracy and precision at a single level. His results showed a recovery of 68% with a CV of 7% for milk fortified at 200 ng/mL concentration.

In conclusion, this method is simple, rapid, and direct. Six to ten samples can be prepared in a normal 8 h working day by a single analyst. The method is highly practical for use in a regulatory environment.

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