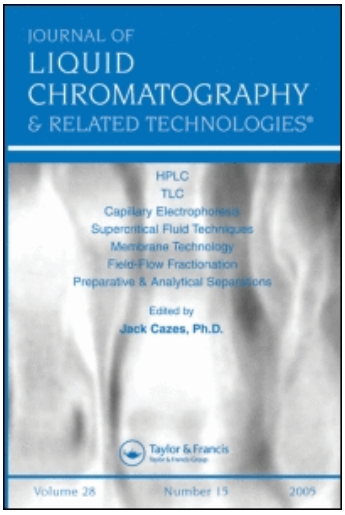

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Determination of Neomycin in Milk by Reversed Phase Ion-Pairing Liquid Chromatography

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DETERMINATION OF NEOMYCIN IN MILK BY REVERSED PHASE ION-PAIRING LIQUID CHROMATOGRAPHY

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ABSTRACT

A fluorometric high performance liquid chromatographic (HPLC) method has been developed for the determination of neomycin in milk. Whole or shelf milk was defatted by initial centrifugation at 4°C. The resulting skim milk was deproteinated with trichloroacetic acid and centrifuged again. The neomycin was determined directly in the supernate by HPLC. The HPLC conditions consisted of an ion-pairing mobile phase, a reversed-phase column, post-column derivatization with o-phthalaldehyde (OPA) reagent and fluorescence detection. The overall recovery of neomycin was 94% (coefficient of variation 6.5%), in whole milk spiked at 0.15-10 ppm and 99% (coefficient of variation 6.4%) in shelf milk spiked at 0.15-5 ppm. The method was used to detect neomycin in milk obtained from cows dosed intramuscularly with neomycin (10 mg/kg). The neomycin concentrations in milk at 8 and 24 h after dosing were 0.3 and 0.2 ug/ml, respectively.

INTRODUCTION

Neomycin, an aminoglycoside, is classified as a broad-spectrum antibiotic because it inhibits the growth of both

Gram-positive and Gram-negative bacteria (1). Like other aminoglycosides, neomycin has a narrow therapeutic range and is toxic to both the auditory branch of the eighth cranial nerve (2) and nephrons of the kidney (3) when given parenterally.

The use of antibiotics in the treatment of mastitis in cows is a potential hazard to consumers due to persistence of residues in the milk. Neomycin was detected in milk up to 24 h after intramuscular administration (4, 5) and up to 84 h after intramammary administration (4). The presence in milk of residues of other aminoglycosides such as streptomycin and dihydrostreptomycin after intramuscular (4, 6) and intrauterine (7) dosing has also been reported. Microbiological assay methods were used in those studies, but they have been characterized as lacking accuracy and specificity. Neomycin is not approved for use in lactating cows; however, in view of the possibility that its illegal use might result in significant hazards due to neomycin residues in milk, an accurate method to monitor it in milk is needed.

Liquid chromatography (LC) has been increasingly used to determine antibiotics including aminoglycosides in biological fluids (8). The majority of LC work on aminoglycosides has been done on gentamicin in serum or plasma (9-13). Recently Kubo et al. reported LC determination of streptomycin in serum by reversed phase ion-pairing liquid chromatography and fluorescence detection (14-15). Previously, Shaikh et al. reported development of an LC procedure for neomycin in animal tissues

(16). This paper describes the application of this procedure for the determination of neomycin in cow's milk.

MATERIALS AND METHODS

Apparatus

(a) Liquid chromatograph.--Waters Associated (Milford, MA) Model 6000A solvent delivery system, Model 730 systems controller, Model 721 data module, Model 712 WISP autosampler, and Perkin-Elmer (Norwalk, CT) Model LS-4 fluorescence detector set at 340 nm excitation and 455 nm emission wavelengths. Slits were set at 10 nm for both excitation and emission. Detector sensitivity setting was generally at 6 and varied when required.

(b) LC columns.--Supelcosil LC-8-DB column, 15 cm X 4.6 mm, 5 um particle size, and Supelco LC-8-DB Supelguard guard column, 2 cm X 4.6 mm, 5 um particle size (Supelco, Inc., Bellefonte, PA). Both the analytical and guard columns were placed in a column heater (Fiatron, Milwaukee, WI) set at 32.5°C.

(c) Post-column reaction system.--Model URS 051 with reaction coil at room temperature or PCR 520 with Spectroflow 400 pump and reaction coil temperature set at 33°C (Applied Biosystems, Ramsey, NJ). Temperature control should be used instead of a reaction coil where laboratory temperature is variable.

(d) Centrifuge.--IEC Model DPR-6000 (Damon/IEC Division, Needham Heights, MA) with rotor No. 269, set at 4°C, and

polypropylene centrifuge tubes with plug-type screw caps (Corning Glass Works, Corning, NY). All centrifugations were carried out at 4000 rpm (3600 x g) for 30 min.

(e) Eppendorf digital pipettes. All transfers and dilutions throughout the study were made with these pipettes.

(f) Degasser.--ERC-3510 degasser with three ports was used throughout the study (ERMA Optical Works, Ltd., Kingston, MA). This eliminated the need to degas the mobile phase by vacuum and/or ultrasonication.

Reagents

(a) Chemicals. Neomycin sulfate (U.S. Pharmacopeial Convention, Inc., Rockville, MD); sodium sulfate, granular (Mallinckrodt, Inc., St. Louis, MO); 1-pentanesulfonic acid sodium salt 1-hydrate, HPLC grade (Eastman Kodak Co., Rochester, NY, or anhydrous from Aldrich Chemical Co., Milwaukee, WI). Trichloroacetic acid and other chemicals were reagent grade.

(b) Solvents. Glass-distilled organic solvents (Burdick & Jackson Laboratories, Muskegon, MI) and distilled, deionized water were used throughout the study.

(c) Post-column derivatization reagent. A commercially available o-phthalaldehyde (OPA) reagent solution (Pierce Chemical Co., Rockford, IL) was used.

(d) Mobile phase. 0.01M 1-pentanesulfonate (0.011M for anhydrous), 0.056M sodium sulfate, 0.007M acetic acid, and 1.5% methanol, filtered through a 0.45 um Millipore filter.

(e) Standard solutions. Neomycin sulfate was dried 3 h under <5 mm Hg pressure at 60°C. The bottle was capped and placed in a desiccator to cool. Neomycin sulfate (10-20 mg) was weighed, transferred to a polypropylene tube, and dissolved in water to give a stock solution of 1000 ug/ml as free base. Aliquots of the stock solution were further transferred to polypropylene volumetric flasks and diluted with water to give standard solutions of 100, 50, and 10 ug/mL or as appropriate. All solutions were refrigerated until used.

(f) Ion-pair concentrate. A 10-fold solution of ion-pair reagent used in the mobile phase was prepared to contain 0.1M 1-pentanesulfonic acid sodium salt and 0.07M acetic acid, filtered through a 0.45 um Millipore filter, and refrigerated until used.

(g) Milk. Whole milk was obtained from a dairy (USDA/ARS, Beltsville, MD) and shelf milk (4% fat) was purchased from local grocery stores. They were used as controls and for spiking with neomycin to conduct recovery experiments.

Neomycin-incurred milk

Neomycin sulfate (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline solution to give a concentration of 350 mg/ml. A single intramuscular dose (10 mg/kg) was administered to a lactating Holstein cow. The dose was divided into two parts, and each part was injected into each hind quarter of the cow. The volume of solution injected into each quarter was kept to 10 ml or less. The milk was collected at 0

(control), 8, and 24 h and 9 days after dosing. It was portioned into 50 ml polypropylene tubes and centrifuged at 4°C and 4000 rpm for 30 min. The fat that solidified on the top layer was pushed to the side of the tube with a plastic spatula. The liquid (skim milk) under the fat layer was removed and further processed for LC analysis or stored at -20°C until analyzed.

Sample preparation and deproteination

A 1 ml portion of the skim milk obtained as above was transferred to a 15 ml polypropylene centrifuge tube, and 100 ul of 20% TCA was added. After vortex mixing, the sample was centrifuged at 4°C at 4000 rpm for 30 min. A 180 ul aliquot of supernate was transferred to a plastic insert placed in a sample vial for WISP, and 20 ul of ion-pair concentrate was added. The vial was capped, mixed by vortexing, and placed in a WISP tray for injection into the LC column.

Preparation of milk samples for recovery of neomycin

Two sets of fortification levels of neomycin in milk were prepared. Set 1: From a 10 ug/ml standard solution of neomycin, aliquots of 180, 120, 60, and 30 ul were transferred to 15 ml polypropylene tubes. Appropriate aliquots of whole milk were added to give a total volume of 2.0 ml and fortification levels of 0.9, 0.6, 0.3, and 0.15 ug/ml, respectively. The samples were mixed by vortexing and centrifuged as described in the preceding section. Set 2: From a 100 ug/ml standard solution of neomycin,

aliquots of 100 and 200 ul were transferred to 15 ml polypropylene tubes. Appropriate aliquots of milk were added to give a total volume of 2.0 ml and fortification levels of 5 and 10 ug/ml, respectively. The samples were mixed by vortexing and centrifuged as above.

Shelf milk samples were spiked with neomycin standard solution as above to give fortification levels of 0.15, 0.3, 0.6, and 5 ug/ml.

A 1 ml aliquot of skim milk of each sample was deproteinated and processed as described for Sample Preparation and Deproteination before LC analysis. Milk was fortified in 3-5 replicate samples at each fortification level.

Preparation of milk extract for use in standard curve

A large aliquot of the whole milk or shelf milk (20-50 ml) was transferred to a 50 ml polypropylene tube and centrifuged. Five ml of skim milk was transferred into a 15 ml polypropylene tube, 500 ul of 20% TCA was added, and the sample was mixed by vortexing and centrifuged. The supernate was used to make dilutions for preparation of the standard curve.

Standard Curves

Two types of standard curves were prepared, one in water and the other in milk extract.

Standard curve in water. Two curves covering lower and higher ranges of concentration were prepared. a) From the 10

ug/ml standard neomycin solution, 90, 60, 30, and 15 ul portions were transferred to 15 ml polypropylene tubes, and diluted to 1.0 ml with appropriate amounts of water to give concentration levels of 0.9, 0.6, 0.3, and 0.15 ug/ml, respectively. A 180 ul aliquot of each sample was transferred for WISP vial inserts; 20 ul of ion-pair concentrate was added to each insert and mixed by vortexing. The vials were capped and placed into the WISP tray for injection into the LC column. b) From the 50 ug/ml standard neomycin solution, 240, 160, and 80 ul portions were transferred to polypropylene tubes and diluted to 1.0 ml with appropriate amounts of water to give concentration levels of 12, 8, and 4 ug/ml, respectively. Ion-pair concentrate was added to each sample before LC analysis as described above.

Standard curve in milk extract. Both sets of standard curves covering lower and higher ranges were prepared as above except that dilutions were made with deproteinated milk extract. Before LC analysis, ion-pair concentrate was also added as described above.

RESULTS

Assay of fortified and incurred milk samples

Figures 1 and 2 show the typical LC chromatograms of spiked whole and shelf milk, respectively, along with their respective control milk. The neomycin peak is well separated from milk background peaks. Two calibration curves spanning

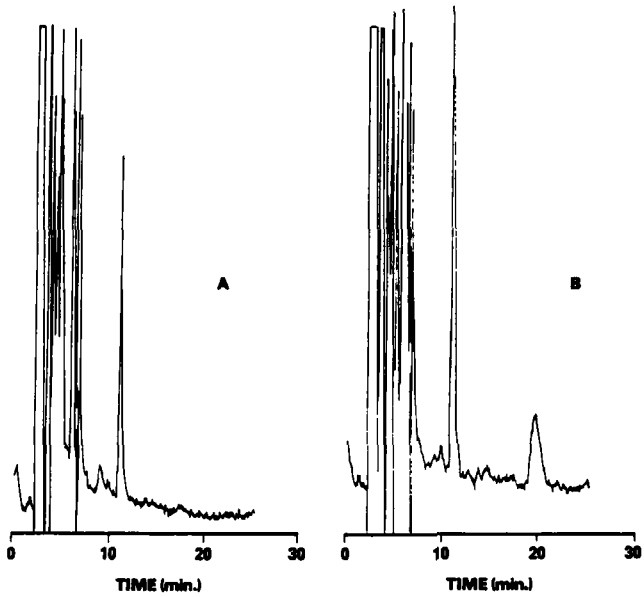

FIGURE 1

HPLC chromatogram of (A) control whole milk and (B) neomycin-spiked whole milk (0.3 ug/ml). Injection volume was 25 ul in each case.

lower (0.9, 0.6, 0.3, and 0.15 ug/ml) and higher (12, 8, and 4 ug/ml) concentration ranges were prepared to cover a wide range of detection from unknown samples. Each of these curves was prepared either in water or in milk extract, and found to be linear. Figure 3 shows an example of linear curves along with their regression analyses, covering the concentration range from 0.15 to 12 ug/ml in water, whole milk and shelf milk. The correlation coefficients for the three standard curves in water, whole milk, and shelf milk were 0.997, 0.997, and 0.998,

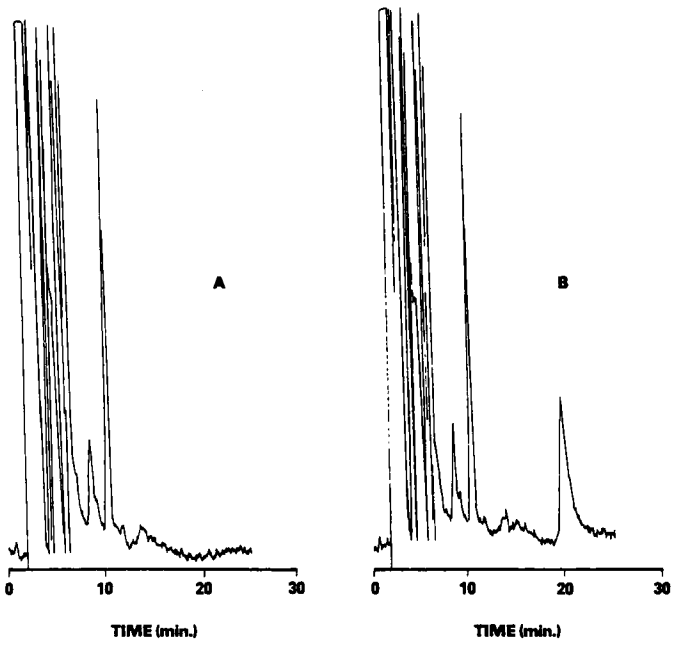

FIGURE 2

HPLC chromatogram of (A) control shelf milk and (B) neomycin-spiked shelf milk (0.6 ug/ml). Injection volume was 25 ul in each case.

respectively. The recovery of neomycin from whole milk at fortification levels of 0.15, 0.3, and 0.6 ug/ml and 0.9, 5, and 10 ug/ml is given in Table 1. Table 2 shows recoveries of neomycin at 0.15, 0.3, 0.6, and 5 ppm from shelf milk. The calibration curves covering lower ranges of concentrations were used to calculate recoveries from levels of 0.15 to 0.9 ug/ml and the curves covering higher levels of concentration were used for levels of 5 to 10 ug/ml. The overall recoveries of neomycin from whole milk using standard curves prepared in milk extract

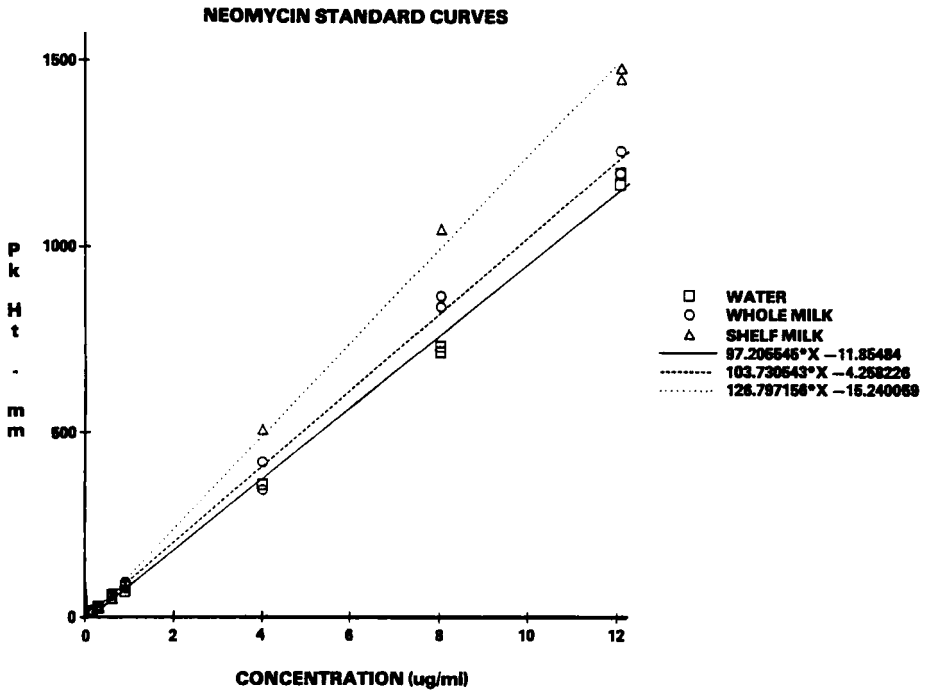

FIGURE 3

Neomycin standard curves in water, whole milk, and shelf milk (0.15, 0.3, 0.6, 0.9, 4, 8, and 12 ug/ml).

and water were 94 ± 6.8% and 122 ± 13.9%, respectively. The recovery of neomycin from shelf milk calculated by using the standard curve prepared in milk extract only was 99 ± 6.4%. For analysis of samples containing neomycin spiking levels of 5 and 10 ug/ml, the injection volume and detector sensitivity setting were decreased to 5 ul and 2, respectively (Fig 4). This kept the neomycin peak on scale and significantly reduced baseline noise and endogenous background peaks in milk. Manual

TABLE 1

Recovery of Neomycin from Fortified Whole Milk Samples

Added (ug/ml)	Recovery (%) Calculated from Standard Curve Prepared In Milk	In Water
0.15	104	142
0.15	94	132
0.15	110	152
Average ± C.V.	102 ± 8.1	142 ± 7.1
0.3	92	121
0.3	105	139
0.3	95	126
Average ± C.V.	97 ± 7.2	129 ± 7.2
0.6	99	127
0.6	85	107
0.6	100	127
Average ± C.V.	95 ± 9.2	120 ± 9.5
0.9	86	110
0.9	76	94
0.9	88	113
Average ± C.V.	83 ± 7.9	106 ± 9.5
5 PPM	97	117
5	92	116
5	93	110
Average ± C.V.	94 ± 2.8	114 ± 3.3
10	89	106
10	95	112
10	90	107
Average ± C.V.	91 ± 3.1	108 ± 3.2
Overall recovery	94 ± 6.8	122 ± 13.9

Table 2

Recovery of Neomycin from Fortified Shelf Milk Samples

Amount Added (ug/ml)	Recovery (%) Vendor 1	Vendor 2	Vendor 3
0.15	90	-	-
	93	-	-
	100	-	-
	100	-	-
	93	-	-
Average ± C.V.	95 ± 8.8	-	-
0.3	97	-	-
	97	-	-
	97	-	-
	93	-	-
	100	-	-
Average ± C.V.	97 ± 2.4	-	-
0.6	110	102	93
	108	98	100
	103	98	105
	112	100	100
	105	105	100
Average ± C.V.	108 ± 3.4	101 ± 2.9	100 ± 4.3
5	98	102	106
	102	100	100
	100	104	102
	98	100	104
Average ± C.V.	-	98	102
	99 ± 1.9	101 ± 2.3	103 ± 2.2
Overall recovery	100 ± 6.4		

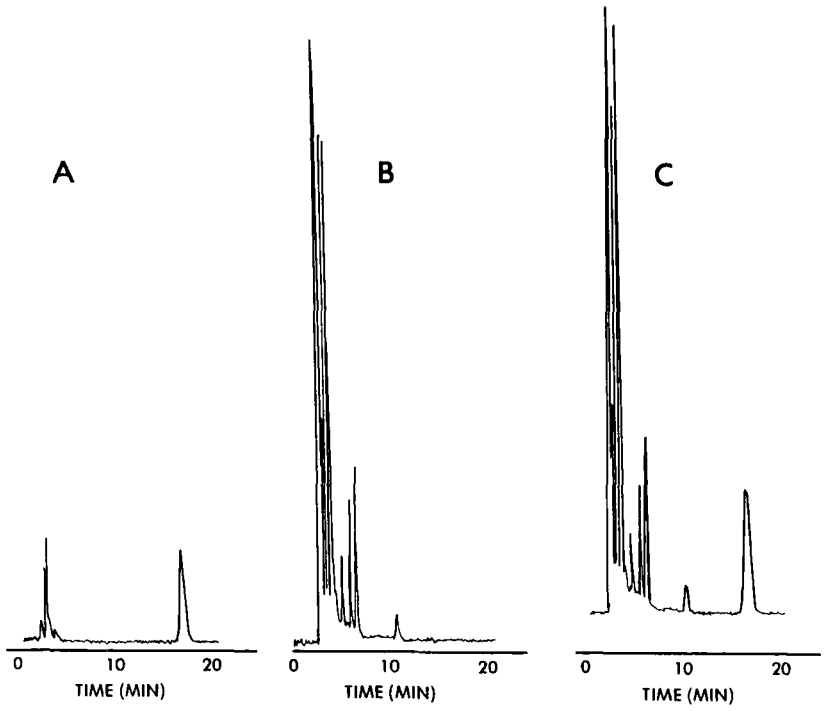

FIGURE 4

HPLC chromatogram at reduced sensitivity setting of 2 and injection volume of 5 ul: (A) 25 ng of neomycin standard (5 ug/ml), (B) control whole milk, and (C) spiked whole milk (5 ug/ml).

peak heights were used, as electronic integration area units were not reproducible because of baseline noise, particularly at lower spiking levels. The control shelf milk samples from three vendors were examined and their chromatograms showed no interfering compounds at the elution position of neomycin. The neomycin recoveries from each vendor's milk were also acceptable (Table 2).

The difference in recoveries was more pronounced at lower levels of spiking when the two standard curves were used: the lower the spiking level, the higher the recovery calculated from the standard curve prepared in water. The increased recovery was perhaps due to contributions of endogenous compounds in milk, which are, however, nullified when recoveries are calculated from the standard curve prepared in milk extract. At higher levels of spiking, detector sensitivity and injection volume were reduced; the contribution of milk endogenous compounds was therefore less pronounced, resulting in acceptable recoveries by either curve.

Figure 5 shows a chromatogram of neomycin detected in milk collected from a cow 8 h after intramuscular dosing of neomycin. The neomycin concentrations in 8 h, 24 h, and 9 day milk samples collected after dosing and calculated by using the milk standard curve were 0.3, 0.2, and 0.0 ug/ml, respectively. These data correlate well with findings that the aminoglycoside residue in milk is minimal because of the lipid barrier to polar compounds (4).

Influence of milk samples on analytical and guard columns

After about 35 injections of neomycin-contaminated or control milk samples, the neomycin peak exhibited deterioration on the analytical column; however, the peak from the standard neomycin injection was not affected. When the guard column was reversed, another 35 portions of milk samples could be injected without affecting the integrity of the neomycin peak on the

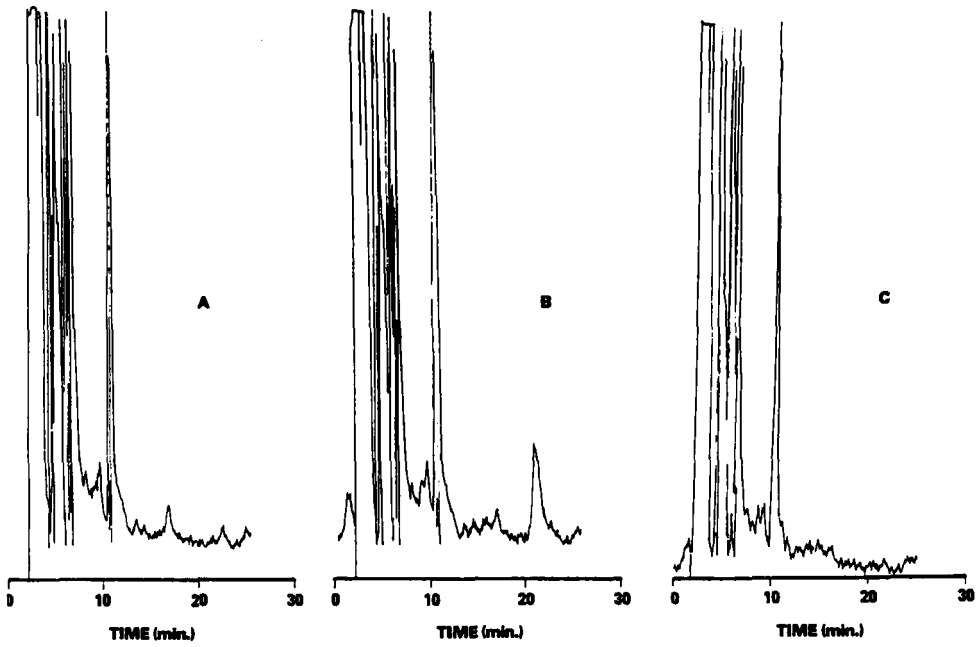

FIGURE 5

HPLC chromatogram of (A) control milk, (B) neomycin-incurred milk collected 8 h after dosing of cow, and (C) neomycin-incurred milk collected after 9 days. Injection volume was 25 ul in each case.

analytical column. Because regeneration of the guard column was unhelpful, a new guard column was installed. The routine replacement of the guard column prevented deterioration of the analytical column even after more than 200 injections of milk samples.

The retention time of neomycin was reduced as the number of injections of milk samples was increased. However, the neomycin peak was well separated from endogenous background peaks until the retention time reached approximately 10 min. When this

happened, the analytical column was regenerated by washing with about 20 column volumes each of water, 100% methanol, and 100% acetonitrile. Before the column was reconditioned with mobile phase, it was washed with 10 column volumes each of a mixure of equal parts of water and methanol and with 100% water. The above column regeneration and washing procedure is also used on a weekly basis, except that the acetonitrile is omitted.

DISCUSSION

A rapid, accurate, and sensitive method for detection of neomycin in milk has been developed. LC conditions reported for detection of neomycin in tissues were applied to detection of neomycin in milk. However, extraction and deproteination procedures for tissues could not be used to isolate neomycin from milk. Direct centrifugation of whole or shelf milk at 4°C separated lipid material from the aqueous part of the milk. Deproteination with trichloroacetic acid was selected because heat deproteination, used in tissues, did not precipitate milk proteins. No further cleanup of milk was needed since centrifugation and deproteination steps were sufficient to separate neomycin from endogenous background compounds. Whole and shelf milk samples from different sources were examined and found to contain no milk background peaks at the elution position of neomycin. Use of the guard column protected the analytical column, improved its stability, and increased its lifespan. The

use of a calibration curve when neomycin standards are prepared in milk extract is preferred, since it corrects for variations due to endogenous milk background and results in acceptable recoveries, particularly at lower levels of detection and spiking. The method is quite sensitive; it can detect neomycin in milk at 0.15 ug/ml or below and could be used to monitor illegal neomycin residues in milk.

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