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Determination of neomycin in plasma and urine by high-performance liquid chromatography

Application to a preliminary pharmacokinetic study

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the determination of neomycin in plasma and urine. The plasma was deproteinated with trichloroacetic acid and centrifuged. The supernatant was mixed with ion-pair concentrate and centrifuged again. The resultant supernatant was analyzed by HPLC. Urine was centrifuged to remove debris, if any, mixed with ion-pair concentrate and analyzed directly 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 average recovery of neomycin was 97 and 113% from plasma spiked at 0.25–1.0 μg/ml, using standard curves prepared in plasma extract and in water, respectively, and 94% for urine spiked at 1–10 μg/ml using a standard curve prepared in water. The method was used to detect neomycin in plasma and urine obtained from animals injected intramuscularly with neomycin. Various pharmacokinetic parameters of neomycin were also determined from its profile of plasma concentration *versus* time.

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 eight cranial nerve [2] and nephrons of the kidney [3] when given parenterally.

Ziv and Sulman [4] studied comparative pharmacokinetics of neomycin in lactating animals and determined neomycin concentrations in blood and milk. Distribution and pharmacokinetic studies of neomycin in calves have also been

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reported [5,6]. Microbiological assay methods were used in these studies; however, they have been characterized as lacking accuracy and specificity. In view of neomycin's narrow therapeutic range and toxicity, an accurate method for monitoring it in blood is necessary to achieve best therapy.

Liquid chromatography (LC) has been increasingly used to determine antibiotics, including aminoglycosides, in biological fluids [7]. The majority of LC work on aminoglycosides has been concerned with gentamicin in serum or plasma [8–12]. Recently Kubo *et al.* [13,14] reported the determination of streptomycin in serum by reversed-phase ion-pairing LC and fluorescence detection. Previously, Shaikh and co-workers [15,16] reported the development of an LC procedure for neomycin in animal tissues and milk. This paper describes an extension of this procedure for the determination of neomycin concentrations in plasma and urine.

EXPERIMENTAL

Apparatus

The LC system consisted of a Waters Assoc. (Milford, MA, USA) Model 6000A solvent delivery system, Model 730 system controller, Model 721 data module and Model 712 WISP autosampler, and a Perkin-Elmer (Norwalk, CT, USA) Model LS-4 fluorescence detector set at 340 nm (excitation) and 455 nm (emission). Slits were set at 10 nm for both excitation and emission. Detector sensitivity was generally set at 8 and varied when required.

The LC and guard columns used were as follows: a 15 cm × 4.6 mm I.D. Supelcosil LC-8-DB column, 5 μm particle size, and a 2 cm × 4.6 mm I.D. LC-8-DB Supelguard guard column, 5 μm particle size (Supelco, Bellefonte, PA, USA). Both the analytical and guard columns were placed in a column heater (Fiatron, Milwaukee, WI, USA) set at 32.5°C.

The post-column reaction system was a Model URS 051 with reaction coil at room temperature or PCR 520 with a Spectroflow 400 pump and reaction coil temperature set at 33°C (Applied Biosystems, Ramsey, NJ, USA). Use of temperature control with a reaction coil is preferred, particularly where laboratory temperature is variable.

All centrifugations were carried out at 3600 *g* for 30 min, except where noted, in an IEC Model DPR-6000 (Damon/IEC Division, Needham Heights, MA, USA) with rotor No. 269, set at 4°C, and polypropylene centrifuge tubes with plug-type screw caps (Corning Glass Works, Corning, NY, USA). All transfers and dilutions throughout the study were made with Eppendorf digital pipettes.

Use of an ERC-3510 degasser with three ports (ERMA Optical Works, Kingston, MA, USA) throughout the study eliminated the need to degas the mobile phase by vacuum and/or ultrasonication.

Reagents

Glass-distilled organic solvents (Burdick & Jackson Labs., Muskegon, MI, USA) and distilled, deionized water were used throughout the study. Except where noted, chemicals were reagent grade.

The post-column derivatization reagent was a commercially available *o*-phthalaldehyde (OPA) reagent solution (Pierce, Rockford, IL, USA).

The mobile phase consisted of 0.011 *M* 1-pentanesulfonate prepared from the anhydrous sodium salt, HPLC grade (Aldrich, Milwaukee, WI, USA), 0.056 *M* sodium sulfate, prepared from granular Na_2SO_4, reagent grade (Mallinckrodt, St. Louis, MO, USA), 0.007 *M* acetic acid and 1.5% methanol. The mobile phase was filtered through a 0.45-μm Millipore filter.

Standard solutions were prepared by drying neomycin sulfate (U.S. Pharmacopeial Convention, Rockville, MD, USA) 3 h at about 5 mmHg pressure (0.69 KPa) at 60°C, capping the bottle and placing it in a desiccator to cool; then 10–20 mg were weighed, transferred to a polypropylene tube and dissolved in water to give a stock solution of 1000 μg/ml as neomycin free base. Portions of the stock solution were further transferred to polypropylene volumetric flasks and diluted with water to give standard solutions of 100, 50 and 10 μg/ml or as appropriate. All solutions were refrigerated until used. Neomycin referred in this paper denotes to neomycin B.

A ten times concentrated solution of ion-pair reagent used in the mobile phase (IPC) was prepared to contain 0.11 *M* 1-pentanesulfonic acid sodium salt and 0.07 *M* acetic acid, filtered through a 0.45-μm Millipore filter and refrigerated until used.

A fresh 20% solution of trichloroacetic acid (TCA) was prepared every three months. Recently we have switched to a 30% solution, since it provided clear samples over a long period before LC analysis.

Animal experiments

Five Hereford Angus or Angus steers, about ten to eleven months old, were used in the experiment. On arrival, animals were maintained on pasture and grain and were proviced water *ad libitum*. During dosing and bleeding periods, they were housed in pens and fed a diet of hay and grain.

Neomycin sulfate powder was reconstituted with sterile saline solution to a concentration of 300 or 350 mg/ml.

Two animals were given intramuscular injections at a dose level of 22 mg/kg (concentration 300 mg/ml) deep into the gluteal muscles of both the right and left hind legs (9.5 ml each). Three animals were similarly injected with 11 mg/kg (concentration 350 mg/ml) into the left or right hind leg. Blood samples were collected into 50-ml polypropylene tubes containing 7.5 ml of anticoagulant (citrate dextrose solution) at the following times: 0 (control, just prior to treatment), 10, 20, 30, 60, 120, 240, 360, 480 and 1440 min after dosing. Each of the tubes was capped, mixed by vortexing and centrifuged at 4°C. The plasma was removed and stored at −20°C until it was assayed for neomycin.

Blood samples collected from animals dosed at 10 mg/kg were centrifuged at 2000 *g* for 15 min and those from animals dosed at 5 mg/kg were centrifuged at 3600 *g* for 20 min. The centrifugation at both speeds was satisfactory. After the slaughter of the animals, urine was also drawn from the bladder and kept frozen at −20°C until analyzed.

Sample preparation and deproteination

A 1-ml portion of plasma was transferred to a 15-ml polypropylene centrifuge tube, and 100 μl of 20% TCA (currently 30% TCA is being used) were added. After mixing by vortexing, the sample was centrifuged at 4°C at 3600 *g* for 30 min. A 450-μl portion of supernatant was transferred to another tube, and 50 μl of IPC were added. After mixing by vortexing, the sample was centrifuged again as above. A portion of the supernatant was carefully transferred to a plastic insert placed in a vial for WISP. The vial was capped and placed in a WISP tray for injection into the LC column. If the sample was stored for several hours or overnight before LC analysis, it became cloudy and required additional centrifugation. This problem was eliminated by using 30% TCA for deproteination.

A portion of urine was centrifuged at 4°C and 3600 *g* for 30 min. A 180-μl portion of supernatant was transferred to a plastic insert and placed in a sample vial for WISP, and 20 μl of IPC were added. The vial was capped, mixed by vortexing and placed in a WISP tray for injection into the LC column.

Preparation of plasma samples for use in standard curve

A 5-ml portion of plasma was transferred to a 15-ml polypropylene tube, and 0.5 ml of 20% TCA (currently replaced by 30%) was added. The sample was mixed by vortexing and then centrifuged at 3600 *g* for 30 min. Supernatant was used to make dilutions for preparation of the standard curve.

Standard curves

(a) From the 10 μg/ml standard neomycin solution, portions of 120, 90, 60 and 30 μl were transferred to 15-ml polypropylene tubes and diluted to 1.0 ml with appropriate amounts of water to give concentration levels of 1.2, 0.9, 0.6 and 0.3 μg/ml, respectively. (b) From the 100 μg/ml standard neomycin solution, portions of 200, 100 and 50 μl were transferred to 15-ml polypropylene tubes and diluted to 1.0 ml with water to give concentration levels of 20, 10 and 5 μg/ml, respectively. (c) A standard curve of neomycin in plasma was prepared as in (a) above except that the dilutions were made with deproteinated plasma. Other standard solutions were prepared as appropriate.

A 180-μl portion of each of the standard solutions from (a) and (b) above was transferred to WISP vial inserts, and 20 μl of IPC were added to each. The vials were capped, mixed by vortexing and placed into the WISP tray for injection into the LC column. A 450-μl portion of each of the solutions from (c) above was transferred to a 15-ml tube, and 50 μl of IPC were added to each tube. After

mixing by vortexing, the samples were centrifuged. A portion of the supernatant was transferred to a WISP insert for injection into the LC column.

Preparation of samples for recovery of neomycin

From the 10 μg/ml standard solution of neomycin, portions of 100, 50 and 25 μl were transferred to 15-ml polypropylene tubes. Appropriate portions of plasma were added to give a total volume of 1.0 ml and fortification levels of 1, 0.5 and 0.25 μg/ml, respectively. The samples were mixed by vortexing, 100 μl of 20% TCA were added and sample preparation and deproteination were carried out as described above.

From the 100 μg/ml standard solution of neomycin, portions of 10, 50 and 100 μl were transferred to 15-ml polypropylene tubes containing 1.0 ml of urine each to give fortification levels of 1, 5 and 10 μg/ml. The samples were mixed by vortexing and prepared for high-performance liquid chromatographic (HPLC) analysis as described above.

RESULTS

Figs. 1 and 2 show typical liquid chromatograms of neomycin standard,

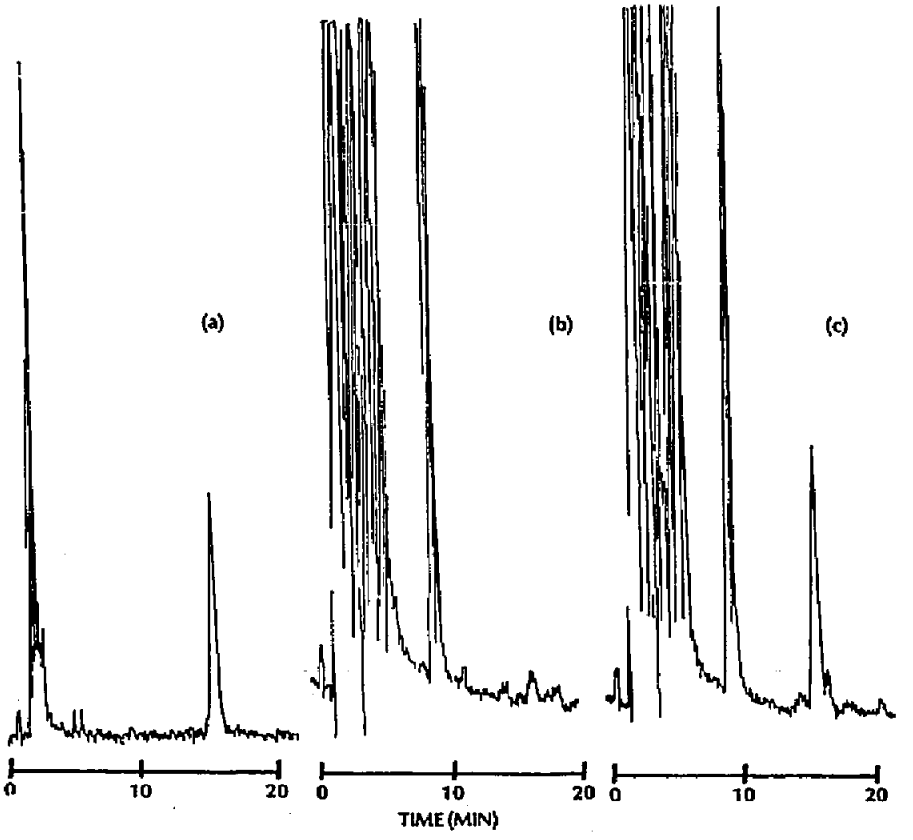

Fig. 1. HPLC of (a) neomycin standard (0.6 μg/ml), (b) control plasma and (c) neomycin-spiked plasma (0.6 μg/ml). Injection volume, 25 μl; detector sensitivity setting, 8.

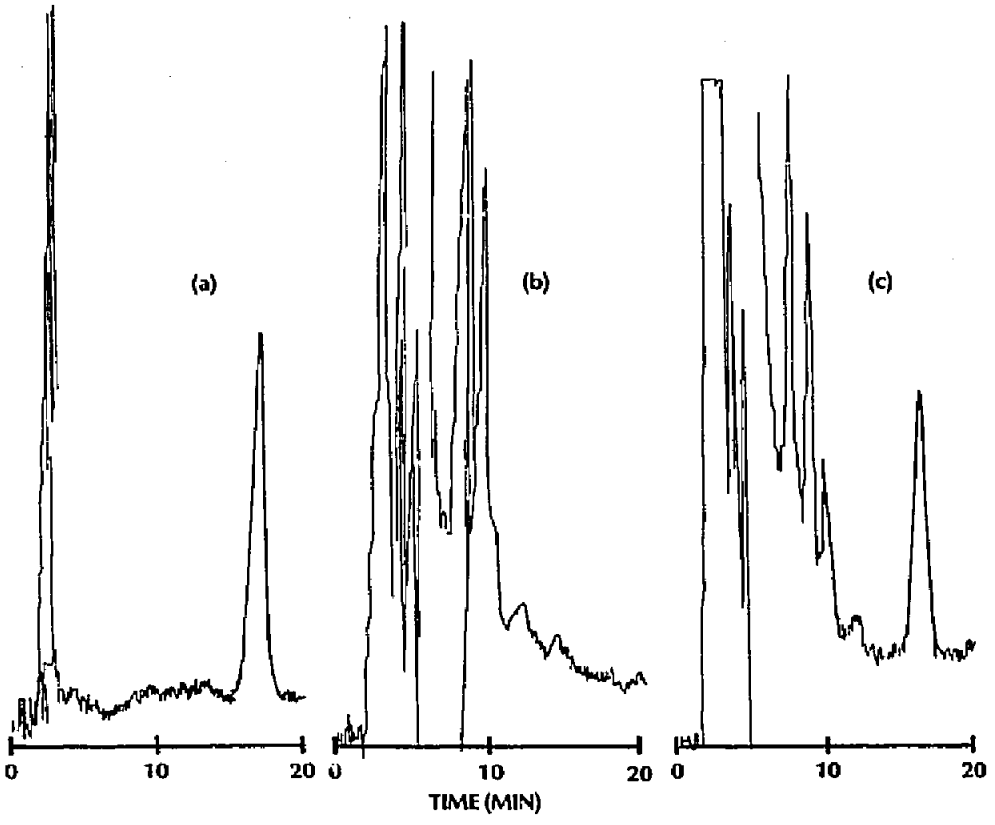

Fig. 2. HPLC of (a) neomycin standard (1.0 μg/ml), (b) control urine and (c) neomycin-spiked urine (1 μg/ml). Injection volume, 30 μl; detector sensitivity setting, 6.

spiked plasma and urine, respectively, together with their respective controls. The neomycin peak is well separated from plasma or urine background peaks. Calibration curves for plasma samples covering concentration levels of 0.3, 0.6, 0.9 and 1.2 μg/ml were prepared in both plasma extracts and water. They were found to be linear (correlation coefficients, 0.981 and 0.999, respectively). A calibration curve covering higher concentration levels of 5, 10 and 20 μg/ml were prepared in water and found to be linear (correlation coefficient, 0.999). The samples were

TABLE I

RECOVERY OF NEOMYCIN FROM FORTIFIED PLASMA

Added (μg/ml)	Recovery (mean ± C.V., $n=3$) (%) calculated from standard curve	
	Prepared in plasma	Prepared in water
0.25	105 ± 11.5	133 ± 9.1
0.5	96 ± 0.0	109 ± 0.3
1.0	91 ± 4	98 ± 3.9

TABLE II

RECOVERIES OF NEOMYCIN FROM URINE

Added (μg/ml)	Recovery (mean ± C.V., $n=3$) (%)
1	75 ± 4.2
5	104 ± 13.2
10	104 ± 10.7
Incurred urine (nine days after dosing)	1.7 ± 6.8 μg/ml

analyzed by using either standard curves or standards interspersed with samples. The recoveries of neomycin from plasma at fortification levels of 0.25, 0.5 and 1.0 μg/ml using standard curves prepared with plasma extract and water are given in Table I. The recoveries of neomycin using both standard curves appears to be reasonable at all levels except the 0.25 μg/ml spike; higher recovery is obtained by using a standard curve prepared in water. The increased recovery was perhaps

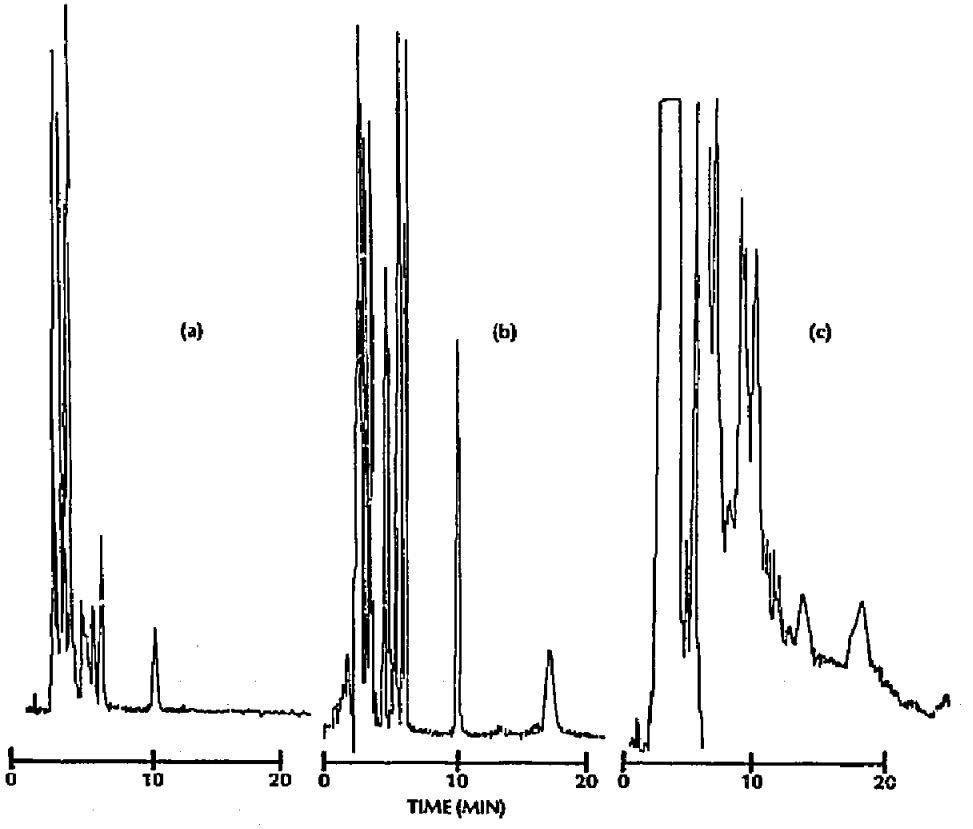

Fig. 3. HPLC of (a) control plasma, (b) neomycin-incurred plasma (8-h withdrawal) and (c) neomycin-incurred urine. Injection volume, 5 μl for both plasma and urine samples. Detector sensitivity setting 2 and 6 for plasma and urine samples, respectively.

due to contributions of endogenous compounds in plasma, which are, however, nullified when recoveries are calculated from the standard curve prepared in plasma extract. Therefore, neomycin determinations from incurred plasma samples may be slightly elevated at or below 0.25 μg/ml levels, when calculated from the standard curve prepared in water. The correlation coefficient of recovered neomycin (using the standard curve prepared in water) from plasma samples supplemented before TCA deproteination was calculated to be 0.922, an indication of linear relationship, even though the recovery at 0.25-ppm level was high.

Table II shows recoveries of neomycin from urine at 1, 5 and 10 μg/ml using the standard curve prepared in water. No attempts were made to determine recoveries at concentrations lower than 1 μg/ml. Table II also shows the concentration of neomycin (1.7 μg/ml) in urine from the bladder of a steer killed nine days after neomycin administration (11 mg/kg). No neomycin was detected in the urine of steers killed 14 or 23 days after neomycin administration. No urine was collected from steers killed one and five days after neomycin administration.

Fig. 3 shows typical LC profiles of incurred urine and plasma samples. Detector sensitivity and injection volumes were reduced for analysis of plasma samples containing higher concentrations of neomycin. Standard curves covering concentration levels of 5, 10 and 20 μg/ml or other appropriate levels were prepared in water and used to calculate incurred neomycin in all plasma samples.

Analysis of four standard curves, concentration range 5–20 μg/ml, prepared over eight days to quantitate incurred plasma samples indicated that the average

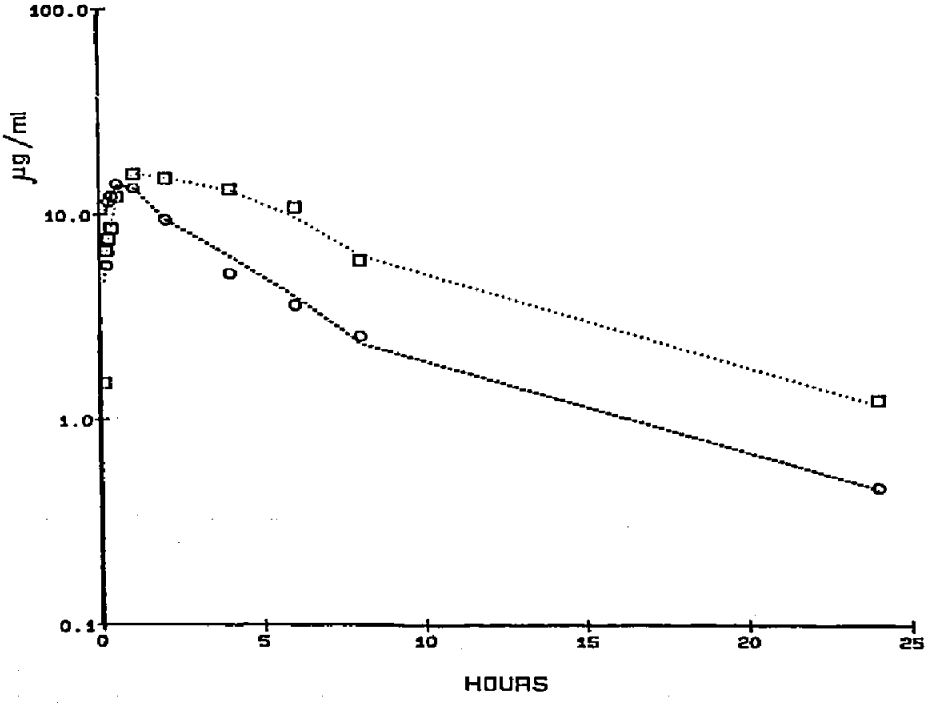

Fig. 4. Neomycin plasma concentration following intramuscular administration to calves. Dose level: (□) 22 mg/kg ($n=2$); (○) 11 mg/kg ($n=3$).

TABLE III

PHARMACOKINETIC PARAMETERS OF NEOMYCIN FOLLOWING INTRAMUSCULAR ADMINISTRATION TO CALVES

Parameter[a]	Dose (mg/kg)				
	22	22	11	11	11
C_{max} (μg/ml)	15.6	15.9	7.8	17.2	22.4
t_p (h)	1	1	2	0.50	0.50
AUC (μg/h/ml)	159.17	150.94	90.81	59.88	76.66
AUC/dose (h/kg/l)	7.24	6.86	8.26	5.44	6.97
$t_{1/2}$ (h)	8.84	4.39	7.27	5.70	5.05
MRT (h)	11.23	7.54	11.18	4.97	4.68
Cl/F (l/h/kg)	0.1382	0.1458	0.1211	0.1837	0.1435
Vd_{area}/F (l/kg)	1.76	0.92	1.27	1.51	1.04

[a] C_{max} = maximum plasma concentration; t_p = time at peak plasma concentration; AUC = area under the curve; $t_{1/2}$ = elimination half-life; MRT = mean residence time; Cl = total body clearance; Vd = volume of distribution; F = bioavailability.

inter-assay coefficient of variation (C.V.) was 9.8%. The average intra-assay C.V. of recovery samples, concentration range 0.25–1.0 μg/ml (Table I), was 4.4%.

The concentrations of neomycin in plasma samples collected at various time intervals from animals dosed at 22 and 11 mg/kg body weight were measured. The plots of the log of neomycin plasma concentration *versus* collection time at both dose levels are given in Fig. 4. The pharmacokinetic parameters were determined by using a non-compartmental model [17] and are listed in Table III. The maximum plasma concentration occurred at 1 h for the two animals dosed at 22 mg/kg, but at 30 min for two animals dosed at 11 mg/kg. A third animal (No. 3) dosed at 11 mg/mkg gave a maximum plasma concentration at 2 h, which was less than half of the maximum concentration of the other two dosed similarly. This difference could be due to improper and/or partial administration of the dose and/or variations in the rate of absorption among the animals. Elimination half-life ($t_{1/2}$) ranged from 4.39 to 8.84 h, although it was similar for the two animals (Nos. 4 and 5) dosed at 11 mg/kg (5.70 and 5.05 h).

DISCUSSION

A rapid, accurate and sensitive method for the detection of neomycin in plasma and urine has been developed. LC conditions reported for detection of neomycin in tissues [15] or milk [16] were used for its detection in plasma and urine. TCA was selected for deproteination, since other techniques were not satisfactory. Heat treatment used to deproteinate tissue extracts [15] caused the plasma to

solidify. Deproteination with acetonitrile, although satisfactory, caused organic phase to separate after IPC was added and was therefore abandoned. Urine samples required no pretreatment, except centrifugation to remove any debris, and were analyzed directly by LC after IPC was added. The differences in the values of pharmacokinetic parameters among animals is probably due to the variability in absorption from the intramuscular injection sites. More detailed and systematic studies of neomycin by intravenous, intramuscular and oral administration to calves are in progress and will be reported in the future.

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REFERENCES

1 S. A. Waksman, E. Katz and H. Lechevalier, *J. Lab. Clin. Med.*, 36 (1950) 93.
2 B. A. Waisbren and W. W. Spink, *Ann. Intern. Med.*, 33 (1950) 1099.
3 A. A. Nelson, J. L. Radomski and E. C. Hagen, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 10 (1951) 366.
4 G. Ziv and F. G. Sulman, *Res. Vet. Sci.*, 17 (1974) 68.
5 W. D. Black and M. J. Claxton, *Can. Vet. J.*, 23 (1982) 276.
6 W. D. Black, J. D. Holt and R. D. Gentry, *Can. J. Comp. Med.*, 47 (1983) 433.
7 I. Nilsson-Ehle, *J. Liq. Chromatogr.*, 6 (Suppl. 2) (1983) 251.
8 G. W. Peng, M. A. F. Godall, A. Peng, V. Smith and W. L. Chiou, *Clin. Chem.*, 23 (1977) 1838.
9 J. P. Anhalt and S. D. Brown, *Clin. Chem.*, 24 (1978) 1940.
10 D. M. Barends, J. S. Blauw, M. H. Smith and A. Hulshoff, *J. Chromatogr.*, 276 (1983) 385.
11 H. Kubo, T. Kinoshita, Y. Kobayashi and K. Tokunaga, *J. Chromatogr.*, 227 (1982) 244.
12 T. A. Getek, A.C. Hanekes and G. B. Selzer, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 172.
13 H. Kubo, Y. Kobayashi and T. Kinoshita, *Anal. Chem.*, 58 (1986) 2653.
14 H. Kubo, Y. Kobayashi and T. Kinoshita, *Anal. Biochem.*, 162 (1987) 219.
15 B. Shaikh, E. H. Allen and J. C. Gridley, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 29.
16 B. Shaikh and J. Jackson, *J. Liq. Chromatogr.*, 12 (1989) 1497.
17 M. Gibaldi, *Biopharmaceutics and Clinical Pharmacokinetics*, Lea & Febiger, Philadelphia, PA, 3rd ed., 1984, pp. 17–28.