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Short Communication

Determination of spiramycin and neospiramycin in plasma and milk of lactating cows by reversed-phase high-performance liquid chromatography

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Abstract

After chloroform extraction, the rapid and sensitive determination of spiramycin and neospiramycin can be performed with AASP-diol clean-up cartridges prior to reversed-phase C_{18} high-performance liquid chromatography. The limits of quantification of spiramycin in plasma and milk are 0.023 and 0.013 μg/ml, respectively, and those of neospiramycin, are 0.058 and 0.006 μg/ml, respectively. Application of the method to the analysis of plasma and milk samples obtained from pharmacokinetic studies is described. Spiramycin has a terminal half-life of 14.27 h in plasma and 34.59 h in milk, while neospiramycin has a half-life of 25.62 h in plasma and 105.85 h in milk.

1. Introduction

Spiramycin, a macrolide antibiotic, is used for the treatment of Gram-positive bacterial infections in veterinary medicine [1], such as mastitis [2]. Neospiramycin, a demycarosil residue of spiramycin [3], shows similar antibiotic activity. Spiramycin and neospiramycin, which have the same spectrum of activity, cannot be distinguished by microbiological titration.

The determination of spiramycin in plasma is usually performed by microbiological techniques [4,5]. A reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the identification of spiramycin was first described by Mourot *et al.* [6]. The direct measurement of spiramycin in plasma using a column-switching technique has been described with a limit of detection of 50 ng/ml [7]. This method has been adapted for use with a Varian advanced automated sample processor (AASP) as a simple means of sample preparation, followed by analysis by C_{18} RP-HPLC [8]. This method cannot determine neospiramycin, however. We therefore modified it to determine spiramycin and neospiramycin in plasma and milk. No such HPLC method for milk has been published previously.

This paper describes an RP-HPLC method

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that can be applied to pharmacokinetic studies in lactating cows.

2. Experimental

2.1. Reagents

All reagents were of analytical-reagent grade. Methanol, chloroform, acetonitrile, sulphuric acid, tetrahydrofuran, dipotassium hydrogenphosphate and potassium hydrogenphosphate were purchased from Merck (Darmstadt, Germany). Free base spiramycin containing 4555 IU/mg was provided from Rhône-Mérieux (Toulouse, France) and neospiramycin was obtained by acid hydrolysis of spiramycin standard. A Millipore filter and reagent-grade water system was used to obtain ultrapure water (Millipore, Molsheim, France).

2.2. Apparatus

The chromatographic system consisted of a Model 5020 liquid chromatograph, a Model 2050 variable-wavelength UV detector set at 231 nm, a Rheodyne 100-μl injection loop and an AASP to prepare and inject the samples (Varian, Walnut Creek, CA, USA). Integration was carried out with a Star informatic system (Varian, Sunnyvale, CA, USA). The analytical column (12.5 × 4 mm I.D.) and guard column (4 × 4 mm I.D.) were packed with LiChrospher 100 RP-18 (Merck). The samples were extracted using AASP-diol cartridges.

2.3. Standard solutions

A stock standard solution was obtained by dissolving 25 mg of spiramycin and 25 mg of neospiramycin in methanol (5 ml) and diluting with water to 25 ml. Working standard solutions were prepared by serial dilution with water. All standard solutions were stored at 4°C.

2.4. Spiked plasma and milk

Antibiotic-free plasma and milk samples were obtained from six cows. These samples were spiked with neospiramycin and spiramycin standard solutions of 20, 5, 1, 0.2 and 0.05 μg/ml. The spiked solutions were stored at −20°C.

2.5. Extraction procedure

Liquid–liquid extraction

Spiked plasma samples were left to thaw at room temperature, then 1 ml was pipetted into a 16-ml glass vial and diluted with 0.5 ml of phosphate buffer (dipotassium hydrogenphosphate and potassium hydrogenphosphate, 50 m*M*, pH 7). After homogenization, 2 ml of chloroform were added.

Milk samples were centrifuged at 4000 *g* for 5 min at 10°C, the fat layer was separated from the aqueous phase and 1 ml was pipetted into a 16-ml glass vial followed by 2 ml of chloroform.

The samples were mixed with a Heidolf rotary stirrer for 15 min at 50 rpm. Decantation was accelerated by centrifugation at 4000 *g* for 15 min at 10°C.

Solid–liquid extraction

A 1-ml volume of the chloroform phase was removed by pipette and applied to a conditioned AASP-diol cartridge loaded on a Prepstation. Samples were driven through the sorbent with a pressure manifold (nitrogen, 6 p.s.i.). The cartridges were washed with 0.5 ml of chloroform, then 0.5 ml of water–acetonitrile (19:1, v/v) were applied.

The separation of the analytical column was carried out with a mobile phase consisting of sulphuric acid (0.25%)–acetonitrile (78:22, v/v) at a flow-rate of 1 ml/min (UV detector wavelength = 231 nm).

The amounts of spiramycin and neospiramycin in plasma samples (μg/ml) and in milk were calculated from the ratio of their peak areas to the average area for two spiked plasma samples (1 μg/ml).

2.6. Cassette preparation

Before use, each cartridge cassette was washed successively with 1 ml of acetonitrile–0.25%

phosphoric acid (1:2, v/v), 1 ml of acetonitrile–water (19:1, v/v), 1 ml of tetrahydrofuran, 1 ml of acetonitrile and 1 ml of chloroform.

2.7. Calibration

The procedure employed to validate the method follows the recommendations of Shah *et al.* [9]. At each concentration, two analyses were performed and repeated on five days. A standard was injected directly on to the analytical column via Rheodyne loop. The peak areas of spiked plasma or milk samples were compared with those of the standards to determine the recovery. A linear regression equation was obtained by plotting the peak areas (corrected by the recovery) against concentrations. The limit of quantification was calculated at ten times the baseline noise.

2.8. Drug disposition study

A solution of spiramycin (Suanovil 20; Rhône-Merieux, Toulouse, France) was administered intraveously to six cows at a dose of 30 000 IU/kg. Blood was withdrawn via the jugular vein at 0, 5, 10, 20, 30 min and 1, 2, 4, 6, 8, 24, 30, 48 h and transferred to tubes containing 10 units of heparin. The plasma was separated immediately after centrifugation and stored at −20°C. Milk samples from lactating cows were collected 2 and 4 h after the intraveous injection of spiramycin and during each milking (two per day) for seven days following the injection. Samples were stored at −20°C until use.

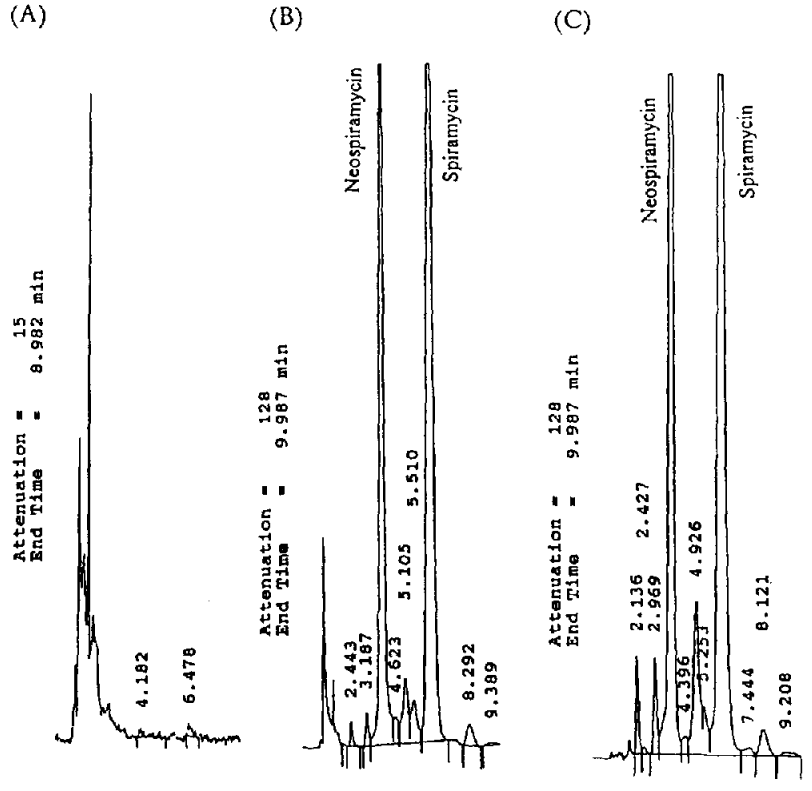

Fig. 1. (A) Chromatogram of blank cow's milk; (B) chromatogram of a standard of spiramycin and neospiramycin at 25 μg/ml; (C) chromatogram of milk spiked with 5 μg/ml of spiramycin and neospiramycin.

2.9. Determination of pharmacokinetic parameters

Pharmacokinetic analysis was carried out by a non-linear regression program [10]. The first estimations were obtained by the residual method described by Gibaldi and Perrier [11]. An adequate model equation was selected according to the minimum Akaike's information criterion [12].

The half-life was calculated from the slope of the terminal exponential phase of the curve. The volumes of distribution and the areas under the curve (AUC) were obtained from classical equations [11].

Table 1
Recoveries of spiramycin and neospiramycin in milk by the HPLC method

Dose (μg/ml)	Recovery (mean ± S.D.) (n = 10) (%)	
	Spiramycin	Neospiramycin
0.05	86.53 ± 2.76	79.99 ± 3.60
0.2	87.44 ± 1.61	78.34 ± 3.98
1	90.71 ± 2.10	85.82 ± 2.43
Mean	88.22 ± 2.81	81.38 ± 4.63
1	90.71 ± 2.10	85.82 ± 2.43
5	93.83 ± 0.83	92.86 ± 3.68
20	92.24 ± 4.20	89.29 ± 3.35
Mean	92.49 ± 2.97	89.32 ± 4.25

3. Results and discussion

3.1. Milk

Previous work in our laboratory showed that the extraction of spiramycin from milk with a plasma method (AASP-diol cartridge) gave a poor recovery [6]. We therefore tried chloroform extraction, followed by clean-up on AASP-diol, a sorbent typically used for polar extraction from non-polar solvents. Spiramycin and neospiramycin were eluted from the cartridges with 5% aqueous acetonitrile solution before analysis by RP-HPLC.

Fig. 1 shows the chromatograms of (A) blank cow milk, (B) a standard of spiramycin and neospiramycin at 25 μg/ml and (C) a spiked milk containing spiramycin and neospiramycin at 5 μg/ml. No interference from endogenous compounds was observed (Fig. 1A) and good separation between spiramycin and neospiramycin (Fig. 1B) was obtained. Recoveries from spiked milk were not linear over the five standard points reported in Table 1. We distinguish two ranges of values in which the relationship

Table 2
Least-squares regression statistics and limit of quantification of spiramycin and neospiramycin in milk (n = 50)

	Slope (mean ± S.D.)	Intercept (mean ± S.D.) (mV/s)	Correlation coefficient	Limit of quantification (μg/ml)
Spiramycin				
High range	327 474.70 ±3586.80	23 828.18 ±42 741.61	0.998	–
Low range	339 602.59 ±2124.70	−3097.78 ±1252.49	0.999	0.0131
Neospiramycin				
High range	256 325.91 ±2724.61	35 996.81 ±32 467.47	0.998	–
Low range	257 181.64 ±1891.15	−1705.45 ±1114.81	0.999	0.0201

Table 3
Inter-assay precision of the HPLC method applied to milk

Dose (μg/ml)	Spiramycin		Neospiramycin	
	Repeatability C.V. (%)	Reproducibility C.V. (%)	Repeatability C.V. (%)	Reproducibility C.V. (%)
0.05	3.60	3.60	4.25	4.53
0.2	1.96	1.96	2.47	5.32
1	2.18	2.34	2.15	2.90
5	0.90	0.90	1.01	1.30
20	5.61	5.61	4.78	4.78

between AUC and concentration is linear (Table 2).

The repeatability and reproducibility [13] coefficients of variation (C.V.) were less than 15% (Table 3). The method can be considered as reliable for the intended application [9]. The quantification limits in milk of 0.013 μg/ml for spiramycin and 0.02 μg/l for neospiramycin are lower than those achieved by a microbiological method [8].

Table 4
Recoveries of spiramycin and neospiramycin in plasma by the HPLC method

Dose (μg/ml)	Recovery (mean ± S.D., $n = 10$) (%)	
	Spiramycin	Neospiramycin
0.05	79.92 ± 5.85	87.08 ± 8.33
0.2	75.00 ± 6.96	78.39 ± 5.67
1	77.89 ± 3.90	84.20 ± 5.11
5	81.58 ± 3.29	79.80 ± 7.47
20	80.64 ± 5.54	80.31 ± 7.23
Mean	79.00 ± 5.58	81.96 ± 7.33

3.2. Plasma

The previous method employed to dose spiramycin in plasma [6–8] cannot be used to determine neospiramycin. Peaks are present at the retention time of neospiramycin. The method applied to milk can extract spiramycin and neospiramycin from bovine plasma, but the recoveries are not constant. By diluting the sample with phosphate buffer we obtained good recoveries (Table 4). The repeatability and reproducibility were in agreement with the thresholds (Table 5). Fig. 2A shows no endogenous

Table 5
Inter-assay precision of the HPLC method applied to plasma

Dose (μg/ml)	Spiramycin		Neospiramycin	
	Repeatability C.V. (%)	Reproducibility C.V. (%)	Repeatability C.V. (%)	Reproducibility C.V. (%)
0.05	4.66	7.59	5.28	9.97
0.2	5.46	5.46	4.85	7.48
1	2.69	5.23	2.31	6.38
5	2.95	2.95	2.04	9.90
20	4.77	7.09	2.10	2.56

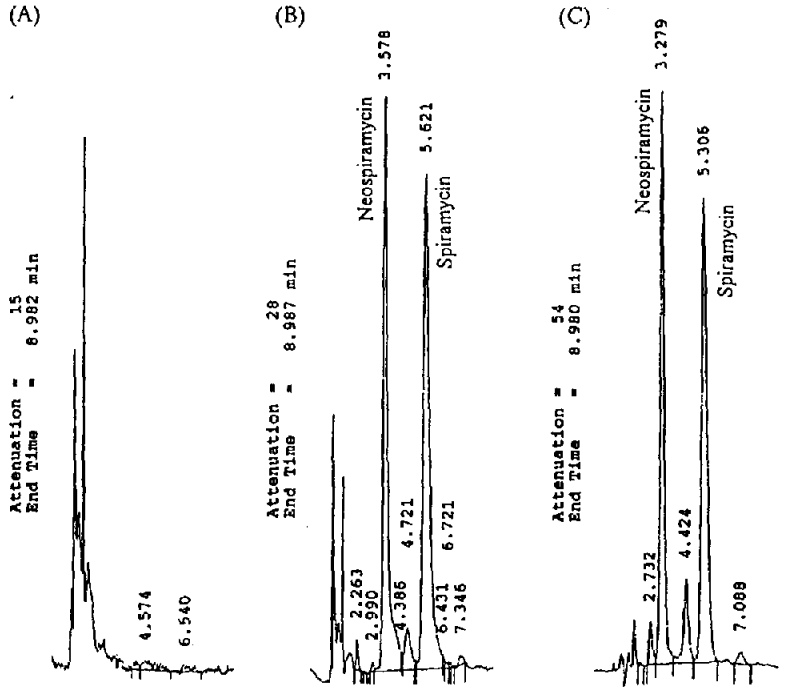

Fig. 2. (A) Chromatogram of blank cow's plasma; (B) chromatogram of spiramycin and neospiramycin standard at 5 μg/ml; (C) chromatogram of plasma spiked with 1 μg/ml of spiramycin and neospiramycin.

peaks from antibiotic-free plasma, while Fig. 2B shows a better separation of spiramycin and neospiramycin than our previous process of plasma extraction. Least-squares regression analysis was used to determine the slope, intercept and correlation coefficient for each compound over two concentration ranges for spiramycin and neospiramycin (Table 6).

The limits of quantification for spiramycin and neospiramycin in plasma were half those in milk (Tables 2 and 6). This may be explained by the lower fluidity of plasma than milk. The detection limit for spiramycin was lower than the limit reached by a recently published pyrolysis–gas chromatographic method [14].

3.3. Pharmacokinetic study

The plasma concentration–time profiles of spiramycin and neospiramycin are presented in

Table 6
Least-squares regression statistics and limit of quantification of spiramycin and neospiramycin in plasma ($n = 50$)

Slope (mean ± S.D.)	Intercept (mean ± S.D.) (mV/s)	Correlation coefficient	Limit of quantification (μg/ml)
Spiramycin			
306 520.00 ±3248.45	13 007.92 ±29 985.94	0.997	0.0228
Neospiramycin			
243 273.09 ±3074.91	26 044.42 ±28 384.02	0.996	0.0581

Fig. 3A. The plot of plasma concentrations of spiramycin was fitted to a tri-exponential equation as described by Wilson [15]. The kinetics obtained for neospiramycin were also described by a tri-exponential equation. The main parameters were half-lifes of 14.27 ± 4.08 h for spiramycin and 25.62 ± 10.39 h for neospiramycin. The volume of distribution was

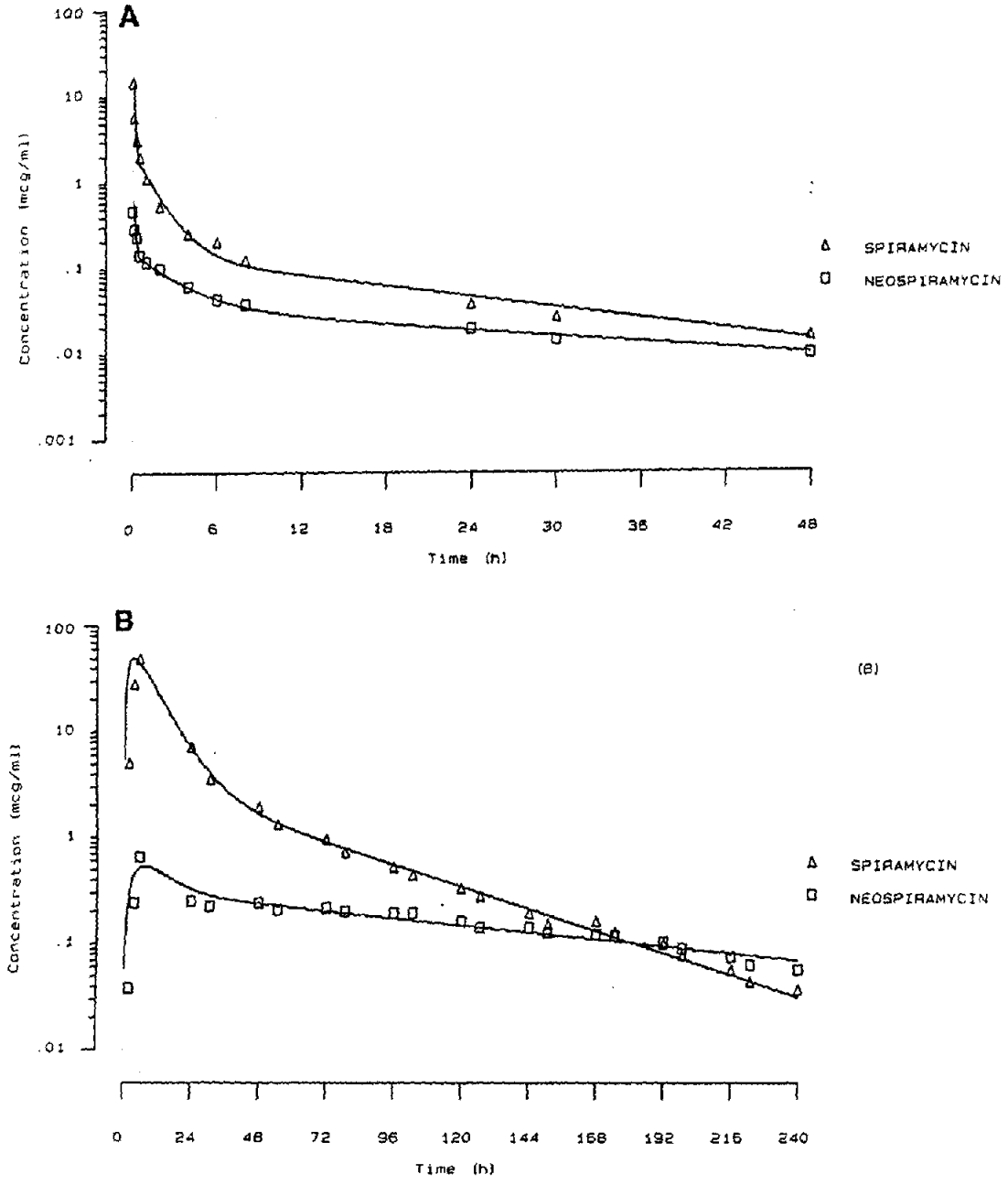

Fig. 3. (A) Plasma and (B) milk concentration–time curves for a representative cow given a 30 000 IU/kg intravenous dose of spiramycin. △ = Spiramycin; □ = neospiramycin.

16.88 ± 9.94 l/kg for spiramycin. These results are in agreement with those determined by Sanders [16], but lower than those found by Friis *et al.* [17].

The milk kinetic concentration was an exponential equation characteristic of a bicompartmental extravascular model that gave a half-life of 34.59 ± 3.84 h. The maximum concentration of 29.59 ± 24.58 μg/ml was reached at 5.04 ± 3.12 h after intravenous injection. For neospiramycin, the maximum concentration was 0.56 ± 0.10 μg/ml at 8.64 ± 3.40 h. The concentration in milk was 80 times higher than the plasma concentration and the dose excreted was 10%. These higher results compared with those found by Sanders [16] or Ziv and Sulman [18] may be explained by the lower quantification limit of the method. For neospiramycin, the maximum concentration was 0.56 ± 0.10 μg/ml at 8.64 ± 3.40 h. The concentration of neospiramycin represents 1/15th of that of spiramycin.

4. Conclusion

Quantification by RP-HPLC can distinguish the kinetics of spiramycin from those of neospiramycin. The chloroform extraction, which permits the determination of the free and bound forms of antibiotics in the biological fluids, might explain why we found a higher concentration in milk, whereas with the microbiological method only free spiramycin is able to act on bacteria.

The maximum residues limit (MRL) of spiramycin in bovine milk is 150 μg/kg and the limit of spiramycin quantification is ten times more sensitive. Our method is therefore of interest for controlling the presence of spiramycin residues in milk. Provided that the amount of neospiramycin is negligible, the detection of residues may follow only the spiramycin quantification. The present method, which is satisfactory for the quantification of these macrolides, may be used in routine tests such as in pharmacokinetic studies.

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