Determination of Lincomycin in Milk and Tissues by Reversed-Phase Liquid Chromatography

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Lincomycin cannot be distinguished from other antibiotics by microbiological tests. A procedure was developed for determination of lincomycin residues in milk and tissues using high-performance liquid chromatography. For extraction/deproteinization, samples were mixed/blended with 3 volumes of 0.1 M $NH_4H_2PO_4$. An equal volume of methanol was added, and the mixture was filtered. Then 1.5 volumes of acetonitrile was added, and the mixture was refiltered. The filtrate was concentrated to <4 mL by evaporation. An automated HPLC system was used for cleanup. An aliquot of sample extract (2 mL) was loaded onto a reversed-phase C_{18} column in the HPLC system with the mobile phase 0.01 M $NH_4H_2PO_4$–0.005 M Me_4NCl. Lincomycin was eluted with an acetonitrile gradient. A narrow fraction containing the lincomycin was collected and rechromatographed by reversed-phase HPLC at pH 7.5. Mean recoveries were 89–99% with detection limits of 20 (muscle, milk) and 50 ppb (liver, kidney).

Lincomycin is a moderately broad spectrum antibiotic that has found considerable use both in human and in veterinary medicine. It is a member of a small, chemically distinct group which includes lincomycin B and clindamycin. Commercial lincomycin is lincomycin A (Figure 1). It is reported to be a weak base with a pK_a of 7.6. It has a weak UV absorption below 220 nm (Eble, 1978). Treatment of farm animals either orally or intramuscularly resulted in significant levels in blood and tissues which were generally depleted below levels detectable by microbial assay in 48–72 h (Chaleva and Duc Luis, 1987). Intrauterine treatment of lactating diary cows gave detectable residues in milk for up to 24 h (Kaneene et al., 1986). Hornish and co-workers (Hornish et al., 1987) found that lincomycin was metabolized to a number of unidentified substances, none of which were present at sufficient levels to serve as marker residues. Residue analysis must therefore be based on the parent compound. As with other antibiotics, failure to adhere to prescribed withdrawal times can result in appreciable residues in milk and tissues.

Determination of lincomycin residues in milk and tissues has traditionally been done by microbiological assay. A procedure sensitive to 0.1 ppm has been described for residues in tissues (Barbiers and Neff, 1976). However, lincomycin cannot be distinguished from other microbial inhibitors. Methods employing high-performance liquid chromatography (HPLC) (Asmus et al., 1983), gas–liquid chromatography (GLC) (McMurray et al., 1984), and thin-layer chromatography (TLC) (Wagman and Weinstein, 1983) have been described for determination of lincomycin. These are generally not directly applicable to residue analysis. Farrington et al. (1988) recently described a method for the determination of lincomycin in kidney using gas chromatography. This procedure, while sensitive, gave only 45% recoveries. An LC cleanup was used. The present paper describes a method using an improved extraction/deproteinization procedure and liquid chromatography for both cleanup and analysis. The procedure is simpler than the GLC method and gave essentially quantitative recoveries. It is suitable for detection and quantitation of lincomycin residues at or below the U.S. tolerance limit of 0.1 ppm in edible tissues (CFR21.556.360) (*Code of Federal Regulations*, 1989).

MATERIALS AND METHODS

Materials. Acetonitrile and methanol were EM Omnisolv or equivalent (E. Merck, Gibbstown, NJ). Sodium heptanesulfonate (98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Tetramethylammonium chloride was purchased from Kodak (Rochester, NY). Ammonium dihydrogen phosphate, potassium dihydrogen phosphate, and disodium phosphate were reagent grade chemicals acquired from several sources. Lincomycin hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO) and was reported to have 1 water of hydration/mol and an activity of 850 units/mg. A stock solution of 1 mg/mL as received was prepared in water and diluted as required. The stock solutions were stable for several months when refrigerated and for a week or more at room temperature.

Animal tissues used were obtained from animals slaughtered in the Beltsville abattoir with no known history of treatment with lincomycin. Whole milk was purchased from a local dairy store.

Sample Preparation. *Tissues.* Tissue (15 g) was weighed and transferred to a 500-mL blender jar, and 45 mL of 0.1 M $NH_4H_2PO_4$ was added. The mixture was blended for 60 s, briefly at low speed and then at moderate to high speed; a variable resistance transformer was used to control speed. Then 60 mL of methanol was added, and the mixture was stirred 10 s at the lowest possible speed at which the blades would turn with the transformer. After standing for 5 min, the mixture was filtered through a coarse pleated filter (S&S 588 or equivalent), and 50 mL of filtrate was collected. Then 75 mL of acetonitrile was added to the filtrate, and the mixture was filtered as before; 100 mL of filtrate was collected. This was equivalent to 5.25 g or 5.25 mL of the original sample, allowing for loss of volume when water and methanol are mixed.

Milk. Fifteen milliliters was mixed with 45 mL of 0.1 M $NH_4H_2PO_4$ and 60 mL of methanol. After standing 5 min, the mixture was filtered through a coarse pleated filter (S&S 588 or equivalent), and 50 mL of filtrate was collected. The remainder of the procedure was the same as for tissue.

The filtrate was transferred to a 250-mL glass-stoppered side-arm flask with a rinse of 20 mL of *tert*-butyl alcohol (to suppress foaming during evaporation). The filtrate was evaporated in the side-arm flasks under reduced (water-pump) pressure. The vacuum was applied first without heating. After the flasks became cold and bubbling ceased, they were placed in a shallow (1–2 cm) water bath at 40–50 °C and weighted with plastic-coated lead rings. If foaming occurred during evaporation, a few milliliters of acetonitrile was added and evaporation under reduced pressure

was resumed without heating until bubbling stopped. The contents were evaporated to about 1–2 mL.

The liquid in the flasks was carefully transferred to 4-mL graduates or graduated 15-mL conical centrifuge tubes calibrated to the 1- and 4-mL marks. The flasks were rinsed with several small volumes of water to give a final volume of 4 mL in the graduates. This was filtered through a small plug of glass wool in the stem of a funnel to remove coarse particles (slight turbidity was not harmful). The liquid was transferred to 4-mL autosampler vials.

Automated HPLC Cleanup. The automated cleanup system consisted of a Varian (Sugarland, TX) Model 9010 pump, a Waters (Milford, MA) WISP Model 712 autosampler, a Supelco (Bellefonte, PA) LC-18 or LC-18-DB column, 4.6 × 150 mm, 5-μm particle size with matching guard cartridge, a Waters 740 data system or 990 diode array detector, and an ISCO (Lincoln, NE) FOXY fraction collector. The mobile phase was 0.01 M $NH_4H_2PO_4$–0.005 M tetramethylammonium chloride (A)–acetonitrile (B). The autosampler was used to start the gradient program, fraction collector, and data system. The autosampler had a 1500-μL syringe and 2000-μL sample loop for rapid loading. The autosampler vials were left open or closed with a nonsealing Teflon septum. A 2000-μL aliquot of the sample was loaded with the mobile phase 100A:0B, flow rate of 1 mL/min. After a delay of 3 min, a gradient was started to 50A:50B after 20 min. The column was flushed for 10 min and returned to starting condition at 30 min. The overall program was 100A:0B (0–3 min)–50A:50B (20–30 min)–100A:0B (31 min). Injection of another sample was started at 40 min. The HPLC systems were operated at ambient temperature. The retention time of lincomycin was determined, and the autosampler was set to collect a 1-mL fraction centered on the lincomycin peak with a 0.1-min delay. Fractions were collected in 15-mL calibrated conical centrifuge tubes. Acetonitrile was removed by evaporation on a Buchler (Ft. Lee, NJ) Rotary Evapomix under reduced pressure, and the final volume was adjusted to 1.0 mL. This was transferred to 1.8-mL autosampler vials. After completion of the automated cleanup sequence, the HPLC column was flushed with water and 50:50 water–acetonitrile for storage.

HPLC Analysis. The HPLC system used for analysis consisted of a Varian Model 5000 liquid chromatograph and a Varian Model 9090 autosampler with a 200-μL loop. Several detector systems were used including a Varian UV-50 detector, a Waters 481 UV–visible detector with a Waters 740 data system, and a Hewlett-Packard (Rockville, MD) Model 1050 UV–visible detector and Varian Model 650 data system. Detection was at 200–210 nm. The columns used were a Polymer Laboratories (Amherst, MA) PLRP-S column, 4.6 × 150 mm, 5-μm particle size, or a Supelco LC-18 or LC-18-DB column, 4.6 × 150 mm, 5-μm particle size, all with matching guard columns. The mobile phases consisted of 0.01 M (pH 7.5 or 8.0) phosphate buffers (A)–acetonitrile (B) with composition 80A:20B or 78A:22B, respectively, on the PLRP-S column and 76A:24B or 74A:26B on the LC-18 or LC-18-DB packings. The buffers consisted of, respectively, 0.54 g of KH_2PO_4 and 3.66 g of Na_2HPO_4 (pH 7.5) and 0.31 g of KH_2PO_4 and 3.9 g of Na_2HPO_4 (pH 8.0) in 3 L of water. Analysis was isocratic with a flow rate of 1 mL/min. Quantitation was based on either area or height of peaks, either of which was linear to at least 5 μg injected onto the column.

Sample Fortification. After tissue was weighed into the blender jars, a measured amount of lincomycin in 150–300 μL of water was applied to the solid tissue and equilibrated for 30 min before proceeding with the method as described. A similar procedure was used with milk.

RESULTS AND DISCUSSION

The structure of lincomycin (lincomycin A) is shown in Figure 1. Since there is no obvious way to derivatize the compound, detection was based on the weak UV absorption below 220 nm. This required efficient separation from interferences.

Previous studies in our laboratory have demonstrated that extraction/deproteinization with water-miscible organic solvents was a simple and effective procedure for recovery of antibiotic residues from tissues and milk (Moats, 1990). Extraction/deproteinization with 1:1 methanol gave fairly efficient precipitation of proteins when samples were buffered with 0.1 M (pH 4.6) $NH_4H_2PO_4$. Recoveries of lincomycin were excellent, but some residual protein was present which interfered with subsequent steps. Extraction/deproteinization with 2–4 volumes of acetonitrile gave lower and erratic recoveries and coextracted considerable interfering lipid material. A two-stage procedure, using first 1:1 methanol and then 3 additional volumes of acetonitrile, combined the advantages of each extraction and gave essentially quantitative recoveries.

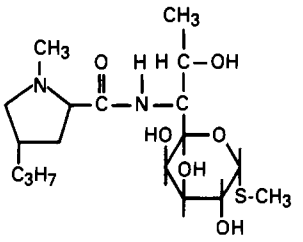

Figure 1. Structure of lincomycin (lincomycin A).

Table I. Effect of pH and Counterions on the Reversed-Phase Chromatographic Behavior of Lincomycin in Buffer–Acetonitrile Mixtures[a]

	0.01 M (pH 8.0) phosphate buffer (A)–MeCN (B)		0.01 M (pH 4.6) $NH_4H_2PO_4$ (A)–MeCN (B)	
counterion	retention time, min	peak height,[b] % FS	retention time, min	peak height, % FS
none	20.1	46	14.2	45
0.005 M Me_4NCl	19.3	48	13.8	57
0.005 M buffer, 0.001 M sodium heptanesulfonate	20.0	62	16.1	55

[a] Supelco LC-18-DB column; gradient elution: 100A:0B (0–1 min); 50A:50B (20 min). [b] 2 μg std; detection UV–200 nm, 0.1 AUFS, 1 mL/min.

Water-immiscible organic solvents were added to the filtrate to form two layers in the hope that lincomycin would partititon into either the organic or water layer formed. However, lincomycin was always distributed between the two layers. The organic solvent was therefore removed by evaporation under reduced pressure, and the water layer was concentrated to 4 mL for subsequent cleanup. Evaporation in a side-arm flask was complete in about 2 h and usually proceeded quietly without bubbling. This is a simple and efficient evaporation procedure that does not require costly and bulky apparatus. The concentrated filtrate was filtered through a plug of glass wool to remove any coarse precipitate. The concentrates were sometimes slightly turbid, but this did not adversely affect the cleanup column. Further filtration through disposable filter cartridges is possible.

The strategy adopted for cleanup and analysis was based on the fact that the retentions of the salt form and the free base were substantially different on reversed-phase packings. A liquid chromatograph was used for cleanup to achieve the rigorous separations required. Table I shows retentions at two different pHs on a Supelco LC-18-DB column. Gradient elution was used with the same solvent gradient in all cases to simplify comparison. Lincomycin has a pK_a of 7.6 so that it will be in the salt form in 0.01 M (pH 4.6) $NH_4H_2PO_4$ and largely in the free base form at pH 8.0. As expected, there was a substantial difference in retention between the two pHs. The effect of coun-

Table II. Effect of Counterions on Reversed-Phase Retention of Lincomycin in 0.01 M (pH 8.0) Buffer under Isocratic Conditions[a]

	buffer–MeCN			
	72:28		73:27	
	retention time, min	peak height, % FS	retention time, min	peak height, % FS
none	8.1	46	9.2	44
0.005 M Me_4NCl	7.7	52	8.7	44
0.005 M (pH 8.0) buffer, 0.001 M sodium heptanesulfonate	7.9	49	9.0	42

[a] Conditions as in Table I, 0.05 AUFS.

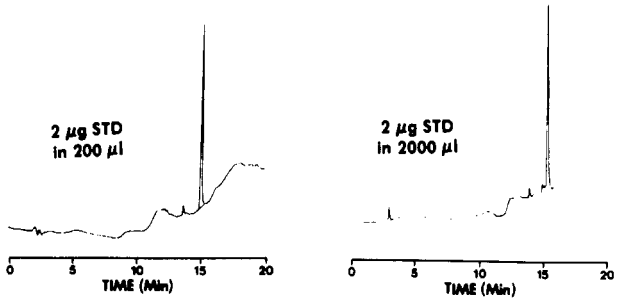

Figure 2. Gradient elution of 2 μg of lincomycin injected in 200 and 2000 μL. A Waters UV-481 detector and Model 740 data system were used, 200 nm. Conditions: 0.01 M $NH_4H_2PO_4$–MeCN; 100:0 (0–3 min)–50:50 (20 min); Supelco LC-18-DB column; WISP autosampler; 2000 μL loop.

terions is also shown. Tetramethylammonium ion also acts as a silanol blocking agent as well as an ion pair and therefore decreases retention of basic compounds on bonded packings. This was observed. It also improved column efficiency as shown by the increase in peak height at pH 4.6. Sodium heptanesulfonate was added as an ion pair. It considerably increased retention of the ionized form but had little effect at pH 8.0. Results were difficult to interpret because sodium heptanesulfonate gave system peaks with gradient elution. Table II shows isocratic elution at pH 8.0. Again, the retention and column efficiency were only slightly affected by counterions at this pH. Since separation was based on getting as large a difference as possible in retentions at the different pHs, ammonium dihydrogen phosphate–tetramethylammonium chloride was used in the cleanup step.

Any system that can be controlled by a start signal from the autosampler should be satisfactory for the automated cleanup. The requirements are a pump capable of generating a ternary gradient, an autosampler capable of injecting 1.0–2 mL, a UV detector, a fraction collector capable of collecting adjustable time windows, and a data system. The Waters WISP autosampler will inject a larger volume than others available. It would be preferable to inject a larger volume of more dilute sample extract if suitable autosamplers were available.

The sample was loaded with a solvent composition of 100% buffer. The injection cycle was then started. After a delay of 3 min for injection of the sample, an acetonitrile gradient was started to 50:50 buffer–acetonitrile in 20 min. The system was then programmed to flush the column with 50:50 acetonitrile–buffer for 10 min and to return the system to starting conditions after 30 min. A 1-mL fraction containing lincomycin was collected, 0.5 min on either side of the retention time of lincomycin with a 0.1-min delay to ensure complete recovery. Figure 2 shows standards injected in 200 and 2000 μL of buffer. The peaks are identical with respect to shape and height, demonstrating

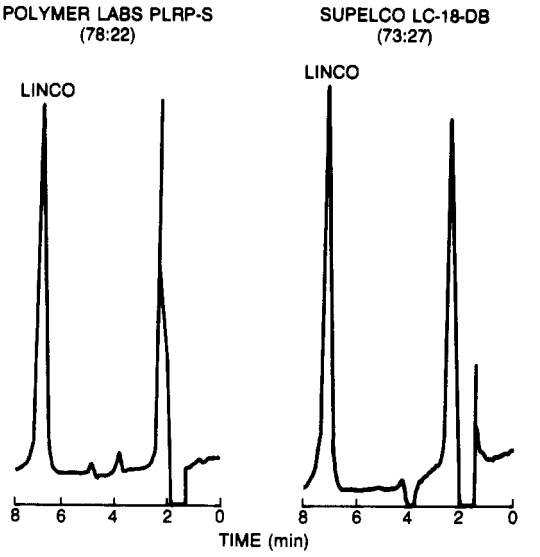

Figure 3. Comparison of a polymeric and a bonded C_{18} reversed-phase column for isocratic analysis of lincomycin. A Varian UV-50 detector was used. Conditions: 2 μg of standard; 0.1 AUFS; 200 nm; 0.01 (pH 8) MeCN.

that peak shape was unaffected by the volume of solution in which the analyte was injected under the conditions used. This is essential to the success of this cleanup approach. Lincomycin eluted as a very sharp band in less than 0.5 mL. However, a 1-mL fraction was collected to ensure complete recoveries. In sample extracts, lincomycin was completely masked by other substances.

For analysis, a Varian Model 5000 liquid chromatograph was used with detection at 200–210 nm with several detector systems. The increased sensitivity at 200 nm was offset by increased base-line noise with some detectors. The bonded packings deteriorated fairly rapidly at pH 7–8 with loss of column efficiency. It was necessary to replace guard columns after 50–100 injections. A polymeric column was more stable in this pH range. For determination, isocratic elution was used. The acetonitrile concentration in the mobile phase was adjusted to give a retention of 6–10 min depending on the type of sample. The retentivity of various brands of reversed-packings varied somewhat. Buffer of pH 7.5 or 8.0 was generally satisfactory. For analysis, acetonitrile was removed from the fractions collected by evaporation under reduced pressure by using a Buchler Rotary Evapomix. The volume was adjusted to 0.5–1.0 mL, and 200-μL aliquots were injected for analysis.

Although there is a widespread impression that polymeric columns are less efficient than bonded reversed-phase columns, our results do not support this conclusion. Figure 3 compares results with a lincomycin standard on a Polymer Laboratories PLRP-S column and a Supelco LC-18-DB column. The PLRP-S column was somewhat less retentive, so the acetonitrile concentration was reduced to give comparable retention times. Both columns were 15 cm long and used 5-μm particle size packings. The similar shapes and heights of the peaks demonstrate that column efficiencies were very similar. Peak shape was a little better on the PLRP-S packing since slight tailing was present on the LC-18-DB packing. It is claimed by the manufacturers that polymer-based packings are stable up to pH 13. However, under our conditions, the polymeric columns were unstable above pH 8. The columns developed excessive back pressures and soon became unusable. Function could be partially or completely restored by flushing with buffer of lower pH. In the pH range 7–8, the

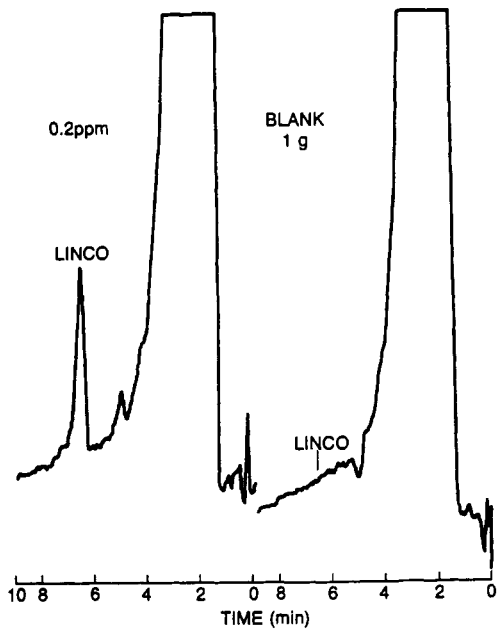

Figure 4. Lincomycin in pork muscle. A Varian UV-50 detector was used. Conditions: PLRP-S column; 0.01 M (pH 7.5) MeCN (78:22); 200 nm.

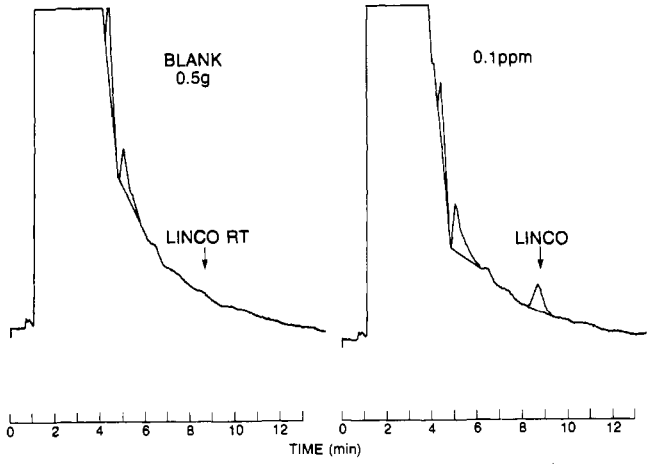

Figure 5. Lincomycin in beef muscle. A Hewlett-Packard 1050 detector and Varian 650 data system were used. Conditions: PLRP-S column; 0.01 M (pH 8) MeCN (78:22); 210 nm.

polymeric columns worked well and were more stable than bonded packings.

Figure 4 shows pork muscle spiked at 0.2 ppm and a blank. A large sharp peak was obtained free of interference. It should be possible to detect as little as 20 ppb in milk and muscle on the basis of the amount which gave a clear response above base-line noise. Figure 5 shows beef muscle spiked at 0.1 ppm with detection at 210 nm.

Liver and kidney samples contained considerably more interference after cleanup. A somewhat longer program was used to improve separation from interferences. This reduced sensitivity somewhat. A beef kidney blank and 0.2 ppm are shown in Figure 6. The retention time was increased by reducing the acetonitrile concentration. The limit of reliable sensitivity was estimated to be about 50 ppb from liver to kidney.

Recoveries from a variety of substrates are summarized in Table III. Mean recoveries were in the range 89–99% except from pork liver. The results (<10% at 1 ppm) suggest that lincomycin may be broken down rapidly when added to pork liver. The procedure was not run with

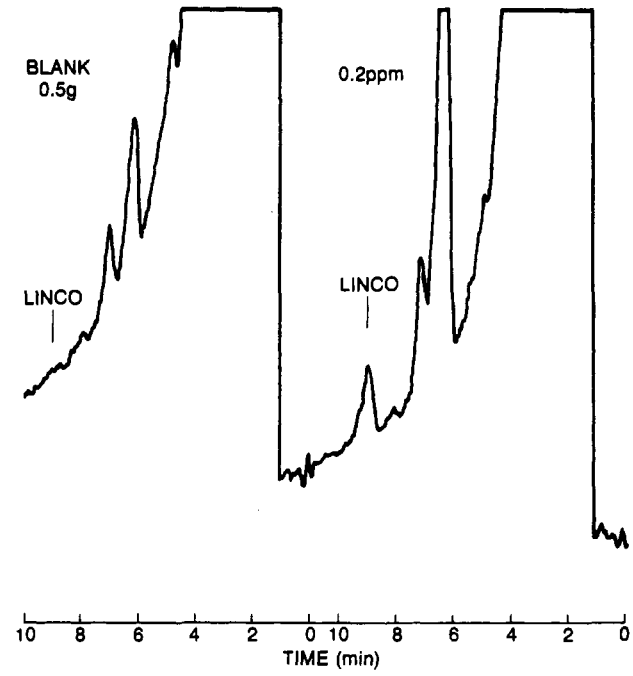

Figure 6. Lincomycin in beef kidney. A Varian UV-50 detector was used. Conditions: PLRP-S column; 0.01 M (pH 7.5) MeCN (80:20); 200 nm.

Table III. Recoveries of Lincomycin from Spiked Samples

	% recovery for added amt of				mean recovery ± SD
	1 ppm	0.2 ppm	0.1 ppm	N	
beef kidney	99, 85, 101, 102	99	99	8	98 ± 6
		106	96		
muscle	106		95, 95	3	99 ± 5
liver	103, 95, 91, 95		100, 96, 62	7	92 ± 13
milk	95, 104, 100		95, 88	9	98 ± 5
	95, 104		101, 98		
pork muscle	95	95	96, 90	4	94 ± 3
kidney	95, 77	89	ND[a]	6	89 ± 9
	103	78	93		
liver	<10		ND	2	<10

[a] ND, not detected.

incurred residues in tissues. However, when the spike is allowed to equilibrate with the tissue, there is some opportunity for interactions to occur which would be expected with incurred residues in treated animals. The results with pork liver suggest that this is the case. With incurred residues, recoveries cannot be determined since there is no way of knowing how much residue is present. Even with radiolabeled compounds, the residue may be present as a metabolite.

In summary, a simple and efficient method was developed for the determination of lincomycin residues in milk and tissues. Key steps are the use of a methanol–acetonitrile extraction/deproteinization procedure and the use of an automated LC system for cleanup. Mean recoveries were 89–99% with excellent precision. Sensitivity limits were about 20 ppb in milk and muscle and 50 ppb in liver and kidney. The extraction and cleanup procedure described herein might also be used in conjunction with a GLC analysis procedure as described by Farrington et al. (1988). We have not attempted this in our laboratory, however.

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