assistance, and to the Delta Branch Experiment Station in Stoneville, MS, for the cotton plants.

Registry No. LC, 41653-72-9; LCME, 56362-72-2.

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Tissue Residue Regulatory Method for the Determination of Lasalocid Sodium in Cattle Liver Using High-Performance Liquid Chromatography with Fluorometric Detection

George Weiss,* Nancy R. Felicito, Maurice Kaykaty, Gloria Chen, Ann Caruso, Edythe Hargroves, Connie Crowley, and Alex MacDonald

A method has been developed for the assay of lasalocid sodium in cattle liver at the 25-ppb level. Ten grams of liver tissue was extracted with acetonitrile, which was then washed with hexane, and an aliquot was blown down to dryness with nitrogen. The residue was brought up in an aliquot of water saturated with the mobile phase, which was then extracted with the mobile phase. This extract was analyzed by HPLC using two 25-cm Whatman Partisil 10 columns in series using a basic mobile phase. Detection was by fluorescence (excitation at 310 nm, emission at 430 nm). Peak heights were used for quantitation.

Lasalocid, or antibiotic X-537A (Figure 1), is a carboxylic acid ionophore. Its discovery (Berger et al., 1951) and structure and chemistry (Westley et al., 1970, 1973; Johnson et al., 1970) have been described. The ability of ionophores to mitigate the transport of mono- and divalent ions across lipid membranes has aroused considerable interest and has led to the discovery of interesting biological activities. It has also led to attempts by synthetic chemists

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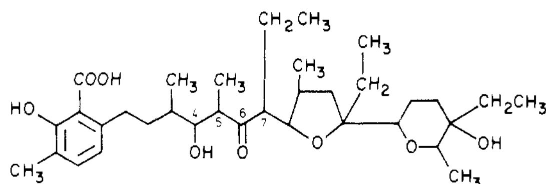

Figure 1. Structure of lasalocid.

to modify the natural ionophores and to design synthetic ones to transport specific ions (Pressman, 1676).

Our interest in lasalocid stems from its demonstrated activity as a coccidiostat in poultry (Mitrovic and Schildknecht, 1973) and its ability to increase feed efficiency and weight gain in ruminant animals, especially cattle (Bartley et al., 1979). The latter effect is thought to be due to its ability to alter the spectrum of rumen bacteria in such a way that the production of propionates is increased at the expense of the production of acetates and butyrates (Raun et al., 1974).

In experiments with ^{14}C-labeled lasalocid in cattle, it has been shown that the levels of radioactivity in all tissues except liver is extremely low. In previous experiments, we were able to show that the most abundant component of the liver residues is intact lasalocid (Weiss et al., 1981).

As part of the application for approval to market lasalocid for use in cattle, it became necessary to develop a reliable analytical method for lasalocid in cattle liver. The only previous method for the quantitation of lasalocid in animal tissues is based on TLC bioautography (MacDonald et al., 1979). This method, however, is time consuming and lacks the desired precision. Our desire was to employ HPLC with fluorescence detection to acquire the desired speed, specificity, sensitivity, and precision.

Lasalocid exhibits fluorescence with excitation λ_{max} at 308–315 nm and emission λ_{max} at 400–430 nm, depending upon the solvent. The fluorescence is most intense when the carboxylic acid moiety is in the ionized form. The intensity drops 2 orders of magnitude in going from pH 8.3 to 3.2 (Toome and Manhart, 1979). Thus, it was necessary to use a basic mobile phase to ensure optimum sensitivity.

An HPLC procedure using a microparticulate silica column and a basic mobile phase has been published for the determination of lasalocid in feed premixes (Osadca and Araujo, 1978). This system was modified and incorporated into the procedure described in this paper.

MATERIALS AND METHODS

Apparatus Decontamination. In the process of developing this method, we experienced contamination problems, and it became necessary to take special precautions to decontaminate all the apparatus. The Hobart meat grinder used to grind the liver samples to obtain uniformity was thoroughly cleaned with soapy water and rinsed with water and ethyl acetate. The Polytron 45 homogenizer used for tissue extraction was decontaminated before processing samples and between samples expected to contain different levels of lasalocid. The shaft was removed, washed thoroughly with soapy water, and rinsed with water. It was returned to the Polytron, and with the motor set at medium speed, the shaft was cleaned successively with acetone, ethyl acetate, and acetonitrile. In addition, between samples the shaft was cleaned by first wiping any remaining tissue from it and rinsing it with acetonitrile for 15–20 s with the motor set at medium speed. All glassware and steel apparatus were cleaned thoroughly and then heated at 225 °C for at least 2 h. This effectively pyrolyzes any lasalocid present and ensures that the samples will not be contaminated.

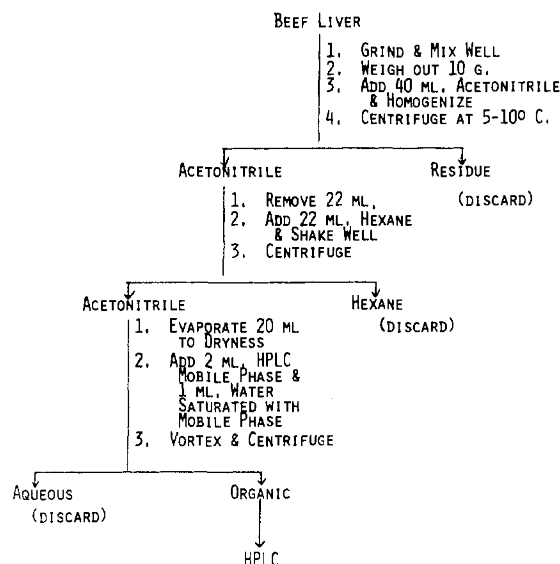

Figure 2. Scheme used for extraction and cleanup of lasalocid from beef liver.

Solvents. All solvents were Burdick & Jackson HPLC UV grade with the following exceptions: the ammonium hydroxide was Baker analyzed reagent grade; the water was distilled and deionized.

Tissue Sample Preparation. The liver samples were frozen as soon as possible after the animals were killed. So that uniformity could be obtained, they were partially defrosted, cut into appropriate pieces, ground in a Hobart meat grinder, and thoroughly mixed in a large plastic bag. Approximately 100-g samples were removed, placed in 18-oz Whirl-pak bags, and refrozen for use as needed.

Preparation of Standard Solutions. Exactly 100.0 mg of a lasalocid sodium standard was weighed out, transferred into a 100-mL volumetric flask, dissolved in tetrahydrofuran, and made up to the 100-mL mark (yields 1.0 mg/mL solution). This solution was diluted successively as needed with the HPLC mobile phase to yield a solution ranging from 100 μg/mL to 50 ng/mL for use in sample fortifications and HPLC external standards. Standard stock solutions were stored in parafilm-sealed, stoppered volumetric flasks at 4 °C and are stable for at least 5 months. Working solutions were obtained by decanting aliquots of the stock solutions into Teflon-lined screw-capped culture tubes. These were sealed with parafilm and can be stored at room temperature for 1 week.

Preparation of Water Saturated with Mobile Phase. Water saturated with the mobile phase was prepared fresh daily. Fifty milliliters of water was mixed with 100 mL of the HPLC mobile phase (prepared as described below) in a 250-mL separatory funnel. The lower aqueous layer was kept in contact with the upper layer until needed.

Sample Extraction and Cleanup. The procedure followed is outlined in Figure 2. The samples in the 18-oz Whirl-pak bags were partially defrosted and 10-g aliquots placed in 250-mL centrifuge bottles. Forty milliliters of acetonitrile was added, and the sample was homogenized for 15–30 s with a Polytron 45 homogenizer set at medium speed. Any tissue adhering to the side of the bottle above the solvent was scraped down into the solvent by using the Polytron shaft. The sample was rehomogenized as before for an additional 15–30 s. The sides of the bottle above the solvent were again scraped down by using the shaft, and any tissue remaining on the shaft was scraped into the bottle by using a stainless steel spatula.

After homogenization, the sample was centrifuged at

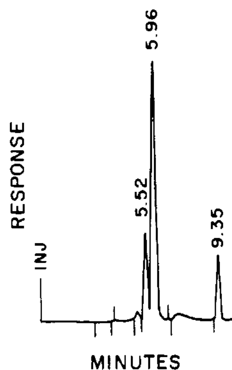

Figure 3. Typical HPLC chromatogram of beef liver extract. Response is fluorescence emission at 430 nm with excitation set at 310 nm. Lasalocid elutes at 5.96 min.

5–10 °C and 2500 rpm (1000*g*) for 15–20 min. Twenty-three milliliters of the supernatant was then transferred to a graduated, glass-stoppered, 50-mL centrifuge tube. The acetonitrile extract was washed once with an equal volume of hexane. Because the acetonitrile picks up varying amounts of the hexane, the volume change was recorded and used as a correction factor in calculations of recovery and concentration. The upper hexane layer was removed by aspiration, and 20 mL of the acetonitrile layer was transferred to a 30-mL conical-shaped centrifuge tube. The sample was evaporated to dryness under nitrogen in a water bath set at 60 °C. The sample was redissolved in 1 mL of water presaturated with the mobile phase and extracted with 2 mL of the HPLC mobile phase. An aliquot of the organic phase was transferred to a 13 × 100 mm Teflon-lined, screw-capped culture tube for HPLC analysis.

HPLC Analysis. The mobile phase was prepared by mixing the top layer of mixture A with mixture B in a 3/1 ratio. *Mixture A*: tetrahydrofuran/methanol/ammonium hydroxide/hexane, 150/30/10/810. This solution is shaken well in a separatory funnel and allowed to stand until the upper phase is clear (ca. 0.5 h). *Mixture B*: tetrahydrofuran/methanol/hexane, 150/30/820. The mobile phase was not filtered or degassed. It was stored in an amber, Teflon-lined, screw-capped bottle and was found to be usable for at least 1 week.

A component HPLC system consisting of a Spectra Physics 3500B pump, Valco manual loop injector, and Perkin-Elmer 650-10LC fluorescence spectrophotometer equipped with an 18-μL flow cell was used. Chromatography was performed on two Whatman Partisil 10 PXS 10/25 columns connected in series. In addition, an otherwise unusable silica column was placed between the pump and injector to act as a precolumn saturator.

The flow rate was set at 2 mL/min and checked frequently. The spectrophotometer excitation wavelength was set at 310 nm and the emission at 430 nm (with an 8-mm slit width for both). Under these conditions, lasalocid elutes at about 6 min. Peak heights were used for quantitation.

RESULTS AND DISCUSSION

A linear response was obtained for lasalocid from 0.3 to 625 ng (correlation coefficient = 0.9999). A typical chromatogram for a steer liver sample is shown in Figure 3, with lasalocid eluting at 5.96 min. The size of the peak that elutes just before lasalocid was found to vary greatly in size. It seemed highest for samples obtained from local stores and lowest for those from field trials. Control samples did show a small peak at the retention time of lasalocid. On average, this peak was equivalent to ca. 3 ppb of lasalocid, or 12% of the lowest level desired for this assay.

Table I. Recovery Values Obtained for Beef Liver Fortified with Lasalocid

	Fortification Level (ppm)				
	0.025	0.050	0.0100	0.500	1.000
Recovery (%)	72.3	72.7	82.7	77.8	88.2
	80.9	75.4	68.0	69.6	72.0
	68.6	63.3	85.5	76.0	74.9
	80.9	67.3	71.1	64.7	73.0
	72.3	75.6	66.5	68.4	74.3
	62.8	71.1	69.2	62.4	62.1
	77.6	81.3	61.3	65.3	67.7
	55.4	81.9	75.1	64.9	65.2
	54.2	76.6	56.2	66.5	70.2
	63.4	77.0	73.2	60.7	66.6
	84.5	77.1	78.8	70.1	70.8
	78.0		76.6	72.2	74.6
	61.1		81.9	73.1	71.7
			72.7	62.4	75.5
			79.8	65.8	69.5
				72.3	70.4
				79.2	72.2
				72.3	71.3
				61.0	80.7
					73.3
Average	70.2	74.5	73.2	68.7	72.2
S.D.	10.08	5.59	8.15	5.60	5.52
C.V. (%)	14.36	7.50	11.14	8.15	7.64
TOTAL AVERAGE RECOVERY	71.5%				
S.D.	7.15				
C.V. (%)	9.998				

Table II. Recovery Values Obtained for Acetonitrile Homogenate of Beef Liver Fortified with Lasalocid

	Fortification Level (ppm)							
	0.025	0.050	0.100	0.201	0.403	0.604	0.907	1.209
Recovery (%)	88.0	82.0	86.0	86.1	84.1	85.9	82.0	81.1
	88.0	92.0	81.0	86.6	85.1	80.3	86.8	79.7
	88.0	84.0	84.0	88.6	83.1	87.7	81.4	81.1
	88.0	88.0	84.0	86.6	85.1	85.9	85.6	78.4
	84.0	90.0	83.0	88.6	84.1	106.3[a]	82.0	78.4
						87.6		
						85.8		
						85.8		
						85.8		
						84.8		
Average	87.2	87.2	83.6	87.3	84.3	85.5	83.6	79.7
S.D.	1.8	4.1	1.8	1.2	0.8	2.2	2.5	1.4
C.V. (%)	2.1	4.7	2.1	1.4	0.9	2.6	3.0	1.8
TOTAL MEAN RECOVERY	84.9							
S.D.	3.1							
C.V. (%)	3.6							

[a] Not included in average (Invalid Measurements, "A Statistical Manual for Chemists," Bauer, E., pp. 22, 1971)

Recovery values for fortified liver were obtained by two methods. In the initial method, the standard was placed directly onto the semifrozen tissue. The sample was stirred briefly and extracted with acetonitrile. Recovery values obtained by this method from 25 to 1000 ppb over a 4-week period by three analysts, given in Table I, averaged 71.5% with a relative standard deviation of 10.0%. The recoveries were linear throughout the range.

The somewhat low recoveries obtained by using this procedure were probably due to the instability of the added lasalocid in contact with liver tissue. We carried out some stability experiments to test this hypothesis. It was found that storage of fortified samples between 0.5 and 3 h at room temperature resulted in a drop in recovery of 5.5–29.8%. However, assay values from livers of cattle treated with lasalocid actually increased by about 20%

Table III. Recovery Values Obtained for Lasalocid from Beef Liver in the Absence and Presence of an Additional Drug

Control Spiked with Drug + Lasalocid[1]				Control Spiked with Lasalocid
Drug	Liver Fortification Level (ppm)	% Lasalocid Recovery		% Lasalocid Recovery
Monensin Sodium	0.11	96.0		81.1
Sulfaethoxypyridazine	0.23	77.5		83.7
Amprolium	2.10	79.1		83.7
Sulfadimethoxine	0.25	83.0		93.4
Decoquinate	4.20	83.5		89.8
Thiobendazole	0.21	83.5		84.0
Tylosin	0.40	85.0		
Bacitracin MD	0.98	88.2	Mean	86.0
Zeranol	0.16	86.2	± S.D.	4.6
Phenothiazine	0.13	73.2		
Procaine Penicillin G	0.13	81.4		
Sulfamethazine	0.22	75.4		
Erythromycin	0.12	78.8		
Neomycin Sulfate	0.13	85.0		
Zinc Bacitracin	1.00	90.6		
Famphur	0.24	85.2		
Levamisole HCl	0.23	89.6		
Melengestrol Acetate	0.11	80.1		
Chlortetracycline HCl	0.25	91.5		
Lincomycin HCl	0.26	92.2		
Streptomycin Sulfate	0.13	90.6		
Oxytetracycline HCl	0.20	90.6		
Coumaphos	2.00	76.4		
Bacitracin	0.96	81.3		
Rabon	3.06	84.3		
Ronnel	8.34	84.0		
	Mean	84.3		
	± S.D.	5.7		

[1]Fortification level for lasalocid was 0.1 ppm

upon storage at room temperature for 3 h and about 9% upon storage at 4 °C for 6 h.

Because of this instability of added lasalocid in contact with bovine liver, recovery values were also obtained for lasalocid added to the acetonitrile homogenate. These values, given in Table II, were much higher, averaging 84.9% with a 3.6% relative standard deviation from 0.025 to 1.209 ppm.

This method has been employed in our laboratories for over 1 year. We have found that new columns need a conditioning period of about 24 h before use. Even after this period, the retention time of lasalocid tends to decrease slowly, presumably due to adsorption of components of the liver extract onto the silica. However, column life has been very good. Columns have only had to be replaced when they developed high back pressures. In normal use, one can expect the columns to last several months.

We have found that the mobile phase, though it contains a dissolved gas, remains usable for at least 1 week after preparation if stored in a Teflon-lined, screw-capped amber bottle. However, the water saturated with the mobile phase used in the final partition step was prepared daily and kept in contact with the mobile phase until use.

This method has been tested for possible interference by 26 approved drugs commonly used in cattle feed. Each drug was spiked with and without lasalocid at 0.1 ppm into control liver samples, and the recovery results were compared. Table III lists the 26 drugs tested and the levels added to the liver. It was found that none of the drugs tested affected the recovery values.

Using this method, we have analyzed 125 liver samples from treated animals, mostly in duplicate. The average standard deviation for replicate analysis of the same sample was 10.5%. The stability of lasalocid in frozen liver tissue (−20 °C) is very good. Assay values obtained over a 1-year period were essentially constant.

Registry No. Lasalocid sodium, 25999-20-6.

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A Pyrolysis Gas Chromatographic–Mass Spectrometric Confirmatory Method for Lasalocid Sodium in Bovine Liver

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A pyrolysis GC–selected ion MS method has been developed to confirm the HPLC regulatory assay for intact lasalocid in liver from cattle treated with lasalocid sodium. The effluent from a Whatman semipreparative Partisil 10 M-9 column is trimethylsilylated with Regisil (BSTFA plus 1% TCMS, Regis Chemical Co.) and analyzed by pyrolysis gas chromatography–positive chemical ionization mass spectrometry. Four ions, two from each of the two pyrolysis fragments, are monitored.

Assay methodology suitable for the regulatory monitoring of lasalocid sodium in bovine liver is reported in the preceding paper (Weiss et al., 1983). The method is based on HPLC using fluorometric detection. A highly specific procedure is still required, however, to ensure that the peak being monitored in the HPLC chromatogram is in fact lasalocid. In the method reported here, the peak corresponding to lasalocid is collected from a semipreparative

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