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LITERATURE CITED

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Lasalocid Determination in Animal Blood by High-Performance Liquid Chromatography Fluorescence Detection

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A simple and rapid procedure for the determination of lasalocid (Ro 2-2985) in bovine, chicken, dog, rat, and mouse blood has been developed and validated within the 5-ppb–5-ppm range with better than an 84% average recovery for each species (c.v. range = 4–8%). Lasalocid is extracted from blood with ethyl acetate, and an aliquot is evaporated to dryness at 60 °C under nitrogen. The residue is reconstituted with a basic HPLC mobile phase and injected onto a Partisil PXS 5/25 column. The effluent is monitored by a fluoresence detector (λ_{ex} = 310 nm and λ_{em} = 430 nm) and the lasalocid peak area is measured. Standards are linear over a wide range (3.0–500 ng/mL).

Lasalocid has been proven to be effective against coccidiosis in poultry and in increasing feed efficiency in confined feedlot cattle (Mitrovic and Schildknecht, 1973; Bartley et al., 1979). Presently the dog, rat, and mouse are the toxicological models used to gather data on lasalocid in order to establish a safe dosage level in cattle which will not yield edible tissue concentrations high enough to be toxic to humans. In order to compare pharmacokinetic profiles of these toxicological models and relate them to the target species, namely, the bovine and chicken, it became necessary to develop a sensitive procedure for determining lasalocid concentrations in the blood of the five species.

Previously, a sensitive HPLC method with fluoresence detection was developed to quantitate lasalocid in bovine liver as low as 25 ppb with a 10-g sample (Weiss et al., 1983). A basic HPLC mobile phase, previously used for separation of lasalocid in feed (Osadca and Araujo, 1978) and in bovine liver, was modified by addition of triethylamine in order to obtain base-line separation of lasalocid from blood background peaks. The fluoresence response of lasalocid is maximized in a basic mobile phase.

MATERIALS AND METHODS

Solvents. Distilled in glass (UV) hexane, tetrahydrofuran, ethyl acetate, and methanol were from Burdick & Jackson; triethylamine was from Eastman Kodak Co.; the water was distilled and deionized.

Standard Solutions. Lasalocid sodium working standard solutions of 3, 5, 10, 25, 100, 250, and 500 ng/mL were prepared by diluting with mobile phase aliquots of a 1 mg/mL stock standard in tetrahydrofuran. Sample fortification standards of 0.1, 1.0, 10, 100, and 500 μg/mL in methanol were prepared by diluting a methanolic 500 μg/mL stock solution.

Decontamination of Apparatus. All clean glassware (pipets, centrifuge tubes, stoppers, culture tubes, etc.) which comes in contact with the samples and standard solutions or in contact with solutions which are transferred to samples should be placed in a 225–250 °C oven for a minimum of 2 h to destroy any lasalocid contamination (Weiss et al., 1983). The stainless steel cannulas used on the evaporation apparatus should also be decontaminated after each use.

Preparation of Fortified Samples. The animal control blood sample (1 mL for mouse or rat, 10 mL for bovine, dog, or chicken) was transferred by using a sterile serological pipet into a 50-mL graduated centrifuge tube. Sample fortification standard solution (50–100 μL) was added with a Hamilton syringe, and the sample was swirled. The procedure was continued as indicated in the sample preparation procedure. The concentration of the fortified sample was approximately the same concentration as was expected to be present in the sample. Also, one control and one reagent blank were processed with each set of samples.

Preparation of Sample. *Bovine and Dog.* Ten milliliters of blood was transferred, by using a sterile serological pipet, into a 50-mL graduated centrifuge tube. One milliliter of 1 N NaOH was added, and the tube was stoppered and shaken vigorously for 10–15 s. For bovine blood, the sample was allowed to stand at room temperature for 5 min and for dog blood, 20 min. The sample was extracted with 20 mL of ethyl acetate by vigorous shaking for 50–60 s and centrifuged with a stopper on at 1500 rpm (RCF = 350*g*) for 10–15 min at room temperature. An aliquot (1–10 mL) of ethyl acetate solution was transferred by using a volumetric pipet into a 25-mL pear-shaped centrifuge tube. The tube was placed in a water bath set at 60 ± 2 °C, and the solution was evaporated to dryness under nitrogen. Sufficient volume of the mobile phase was added to the sample residue to bring the lasalocid concentration into the calibration curve range, preferably the midrange of the curve. It is best to start with 1 mL of the mobile phase and make further dilutions with the mobile phase, if necessary. The sample was vortexed vigorously for 1 min.

Chicken. Ten milliliters of blood was transferred, by using a sterile serological pipet, into a 50-mL graduated

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centrifuge tube. The sample was extracted with 20 mL of ethyl acetate by shaking vigorously for 10–15 s by hand. The tube was placed on a reciprocal shaker for 5 min at high speed followed by centrifugation, with a stopper on, at 1500 rpm (RCF = 350*g*) for 10–15 min at room temperature. The procedure was continued as indicated in the procedure for bovine and dog blood, "An aliquot (1–10 mL) of ethyl acetate solution..."

Rat and Mouse. One milliliter of blood was transferred, by using a sterile serological pipet, into a 50-mL graduated centrifuge tube. The sample was extracted with 10 mL of ethyl acetate by shaking vigorously for 30–40 s by hand. The tube was placed on a reciprocal shaker for 5 min at high speed. The tube was removed from the shaker, shaken vigorously by hand for 10–15 s to loosen blood adhering to the wall, and centrifuged with a stopper on at 1500 rpm (RCF = 350*g*) for 10–15 min at room temperature. A 9.0-mL aliquot of ethyl acetate solution was transferred into a 25-mL pear-shaped centrifuge tube. The procedure was continued as indicated in procedure for bovine and dog blood. "The tube was placed in a water bath..."

High-Pressure Liquid Chromatography. The components of the instrumentation used consisted of a Spectra Physics 3500B pump, a Valco CV-6-VHPa-M60 loop injector (42- and 100-μL loops), a Whatman Partisil PXS 10/25 (10-μm microparticulate silica, 25 cm) preinjector column, a Whatman Partisil PXS 5/25 (5-μm microparticulate silica, 25 cm) analytical column, and a Perkin-Elmer 650-10LC fluorescence spectrophotometer equipped with an 18-μL flow cell. The excitation wavelength was set at 310 nm (8-mm slit) and the emission wavelength at 430 nm (8-mm slit). The sensitivity was set at 10, 3, or 1 as needed to keep the lasalocid peak on scale. A Hewlett-Packard 3380A recording integrator was used.

The mobile phase was prepared by mixing hexane/tetrahydrofuran/methanol/triethylamine/ammonium hydroxide (810/140/20/20/10). This mixture was shaken for 30–40 s vigorously and allowed 25–30 min for phase separation. The lower aqueous phase was discarded, and an additional 10 mL of tetrahydrofuran was added to remove turbidity in the solvent mixture. New mobile phase was prepared every 3 days and was not degassed because of the presence of ammonium hydroxide. Mobile phase and samples were not filtered. The mobile phase was pumped through the column at a rate of 0.9 mL/min, and the entire system was allowed to equilibrate until a stable base line was obtained (30–45 min). An external standard solution was injected several times until a constant fluorescent response was obtained. A new column required overnight equilibration. The retention time of lasalocid can be between 6 and 8 min, depending on the condition of the column, without affecting base-line separation and peak area. When retention time decreased below 6 min, the column was flushed with methanol for 2–3 h at 2 mL/min, followed by overnight equilibration with mobile phase at 0.9 mL/min before reuse. When the HPLC system was not used for 8 h or longer, the entire system was flushed with hexane for 30 min at a rate of 2 mL/min.

Three different concentrations of lasalocid standards were injected before and after each set of samples, and a calibration curve was generated by linear regression analysis. The concentration of lasalocid standards injected was above, below, and approximately the same concentration as was expected to be present in the final dilution of the samples which was assayed.

RESULTS AND DISCUSSION

The extractability of lasalocid is due to its ionophoric property. Lasalocid forms lipid-soluble complexes with cations as shown in Figure 1. The molecule assumes a conformation which focuses the various oxygen groups toward a central location where a cation can complex via ion–dipole interaction, thereby making the outer portion of the molecule nonpolar (Pressman, 1976). The sodium form of lasalocid can, therefore, be extracted into organic solvents from an aqueous phase.

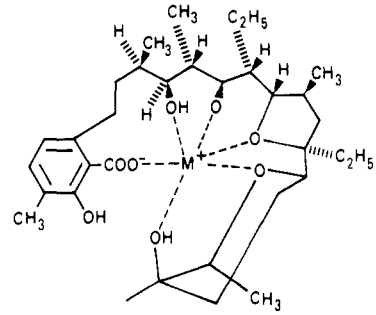

Figure 1. Structure of the lasalocid–cation complex.

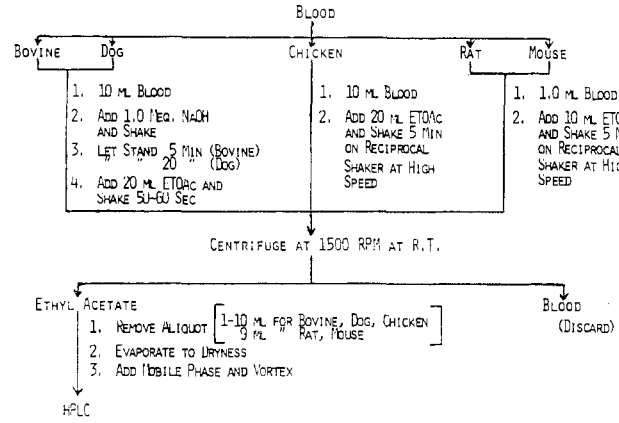

Figure 2. Schematic for determination of lasalocid in five animal species.

Table I. Recovery of Lasalocid from 10 mL of Bovine Blood as a Function of Concentration

spiked level of lasalocid, ppb	recovery, %
5.0	96
10.0	82
50.0	71
100.0	33

Ethyl acetate was the solvent of choice for extraction of lasalocid due to its low miscibility with blood, its high extraction efficiency for lasalocid, and its low extraction efficiency for blood background. This simple procedure, outlined in Figure 2, yielded good recoveries of lasaloacid from chicken, rat, and mouse blood but not from bovine or dog blood.

When bovine blood at fortified levels between 5 and 100 ppb was extracted with ethyl acetate, it was observed that recoveries steadily dropped off, yielding a high recovery of 96% at 5 ppb to a low recovery of 33% at 100 ppb as shown in Table I. So that this problem could be overcome, increasing amounts of sodium hydroxide were added to 10 mL of bovine and dog blood foritified at 100 ppb and allowed to stand at room temperature for 1 min before extraction with ethyl acetate. The recovery of lasalocid reached a maximum in both species at 1.0 mequiv of NaOH addition, as shown in Table II.

Increasing the contact time of 1.0 mequiv of NaOH with 10 mL of bovine and dog blood yielded the recoveries listed in Table III. Lasalocid recovery from bovine blood

Table II. Effect of NaOH Addition to 10 mL of Bovine and Dog Blood on Recovery of Lasalocid at 100 ppb

mequiv of NaOH added[a]	recovery, % bovine	dog
0	56.5	62
0.1	80.6	
0.5	91.0	
1.0	94.6	76
3.0	72.0	30
6.0	35.0	29
12.0	27.0	

[a] Contact time of NaOH before extraction was 1 min.

Table III. Effect of Contact Time of 1.0 mequiv of NaOH on Recovery of Lasalocid at 100 ppb from 10 mL of Bovine and Dog Blood

NaOH contact time, min	recovery, % bovine	dog
0	56.5	62
0.5	89.8	
1.0	91.3	76
2.0	93.9	
5.0	93.7	78
10.0	98.0	79
15.0	93.7	
20.0		83
30.0	98.0	84

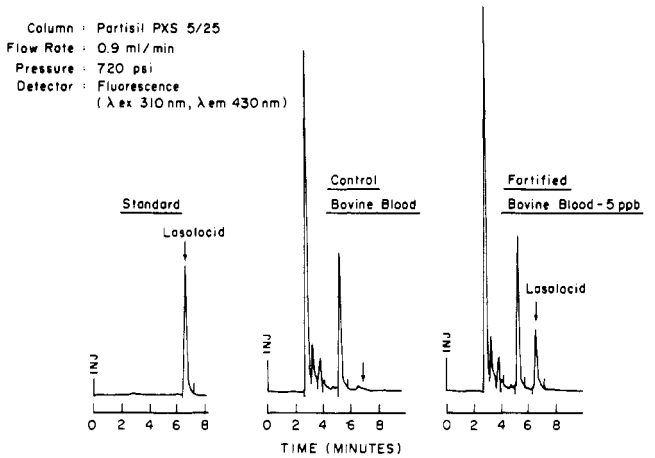

Figure 3. HPLC chromatograms of standard lasalocid, control bovine blood, and bovine blood fortified at 5 ppb.

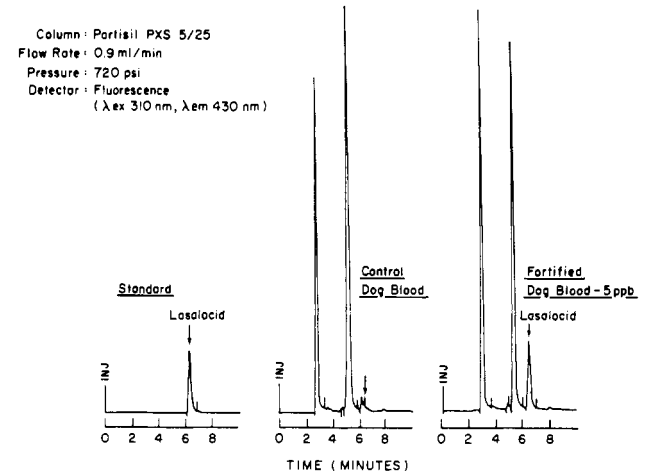

Figure 4. HPLC chromatograms of standard lasalocid, control dog blood, and dog blood fortified at 5 ppb.

reached a plateau of about 95% within 5 min, and recovery from dog blood reached a plateau of about 84% within 20 min. Therefore, the NaOH addition was incorporated into the procedure and allowed the minimal contact time to yield maximum recovery of lasalocid.

For best resolution, a single 5-μm Whatman Partisil PXS column was employed. Because of the basic mobile phase, it was necessary to add a 10-μm Partisil column before the injector to establish column equilibrium and a reproducible retention time of approximately 6.5 min for lasalocid.

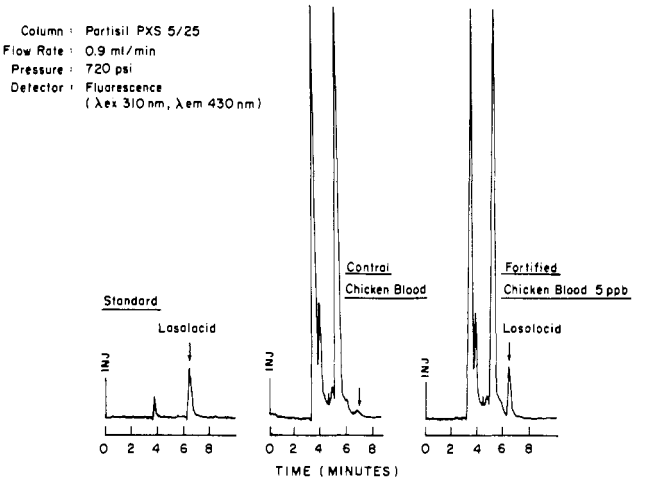

Figure 5. HPLC chromatograms of standard lasalocid, control chicken blood, and chicken blood fortified at 5 ppb.

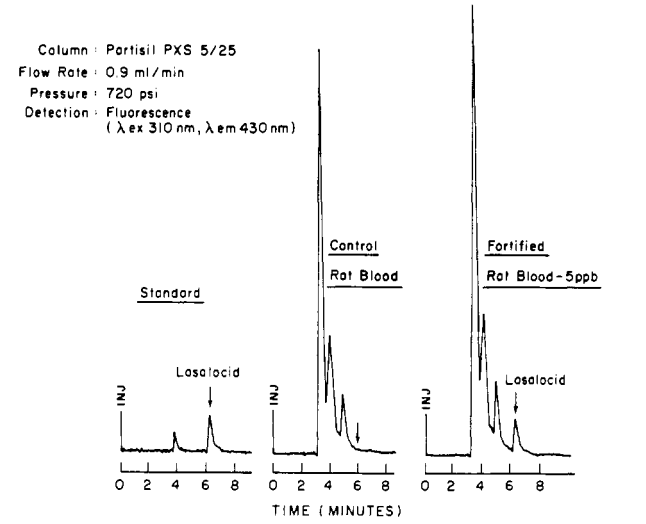

Figure 6. HPLC chromatograms of standard lasalocid, control rat blood, and rat blood fortified at 5 ppb.

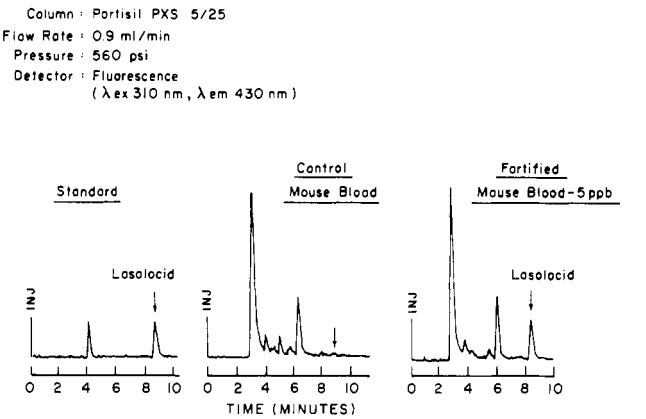

Figure 7. HPLC chromatograms of standard lasalocid, control mouse blood, and mouse blood fortified at 5 ppb.

Table IV. Lasalocid Standard Calibration Curve

concn, ng/mL	42-μL loop mean area[a]	42-μL loop c.v., %	100-μL loop mean area[a]	100-μL loop c.v., %
3.0			8 432	1.2
5.0			13 077	3.0
10.0	3 035	6.7	26 335	3.5
25.0	7 591	1.3	61 466	2.7
100.0	30 724	0.3	256 802	3.0
250.0	76 980	0.9	640 678	0.6
500.0	154 506	0.5	1 252 853	1.2

[a] Average of three injections on a given day.

Table V. Recovery of Lasalocid from Fortified Blood

animal	ppb added	mean recovery, %[a]	± SD
bovine	5.0	92.4	2.0
	10.0	88.3	2.5
	100.0	94.6	2.5
	1000.0	88.7	3.5
	5000.0	80.9	2.6
	mean:[b]	88.7	5.5
dog	5.0	91.6	4.5
	10.0	90.0	3.3
	100.0	81.1	3.0
	1000.0	81.6	1.8
	5000.0	81.0	1.1
	mean:[b]	85.0	5.6
chicken	5.0	93.9	3.8
	10.0	91.9	2.4
	100.0	93.2	1.7
	1000.0	91.4	1.6
	5000.0	86.5	4.3
	mean:[b]	91.4	3.8
rat	5.0	96.4	10.0
	10.0	97.4	4.4
	100.0	84.3	3.3
	1000.0	91.4	4.3
	5000.0	86.5	4.5
	mean:[b]	91.2	7.5
mouse	5.0	87.2	4.2
	10.0	86.4	3.6
	100.0	80.5	2.8
	1000.0	83.5	4.0
	5000.0	92.9	0.7
	mean:[b]	84.2	4.3

[a] Average of five determinations at each fortification level. [b] Average of 25 determinations.

A loop volume of 42-μL was used for analysis of bovine, dog, and chicken samples, since the amount of lasalocid extracted from 10 mL of blood at the 5-ppb concentration was sufficiently high to produce clean chromatograms with insignificant background detection. The chromatograms of standard lasalocid, control blood, and blood fortified at 5 ppb are shown in Figures 3, 4, and 5 for bovine, dog, and chicken.

The method had to be modified to accommodate 1 mL of rat and mouse blood samples, since only about 1–2 mL of blood can be obtained from a rat or mouse of average weight. Consequently, a reduction in extractable lasalocid at the 5- and 10-ppb blood concentrations required greater detection sensitivity. Therefore, the loop volume was increased to 100 μL, and the detector sensitivity range was increased from 3 to 10. The chromatograms of standard lasalocid, control blood, and blood fortified at 5 ppb are shown in Figures 6 and 7 for the rat and mouse.

A standard calibration curve of lasalocid showed good linearity between 3.0 and 500 ng/mL of mobile phase using the 100-μL injection loop and between 10 and 500 ng/mL using the 42-μL injection loop (correlation coefficient = 0.9999). The coefficient of variation for three injections on a given day were better than 4.0% in the midrange of the curve as seen in Table IV.

Recovery of lasalocid from fortified blood of each of the five species was determined in the range between 5 ppb and 5 ppm. Five replicates were assayed at each of five fortification levels, and these results are listed in Table V. Recoveries averaged better than 80% at each level and better than 84% for each species with a coefficient of variation range between 4 and 8%. The procedure described in this report for analysis of lasalocid in animal blood by HPLC fluoresence detection offers good sensitivity, precision, specificity, and speed.

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