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

| Drug | Control Spiked with Drug + Lasalocid[1] Liver Fortification Level (ppm) | % Lasalocid Recovery | Control Spiked with Lasalocid % 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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Received for review May 28, 1982. Accepted September 15, 1982.

# 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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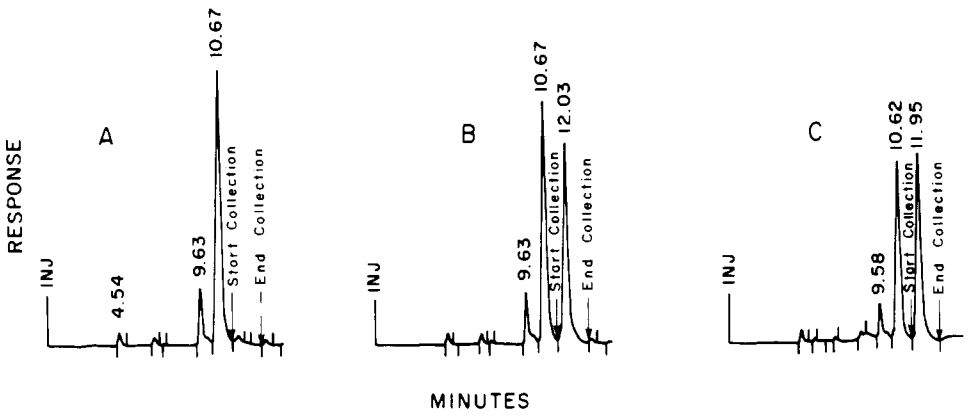


**Figure 1.** Semipreparative HPLC chromatograms of extracts from (A) control bovine liver, (B) control bovine liver fortified with lasalocid sodium at a concentration of 52.5 ppb, and (C) bovine liver from an animal treated with lasalocid sodium at a concentration of 11 ppm. An analysis of the last sample by HPLC suggested that it contained 51 ppb of intact lasalocid.

HPLC column, and the residue remaining following removal of the HPLC solvent is trimethylsilylated and analyzed by using pyrolysis GC–MS (Vane and Pao, 1975). Four ions, two from each of the two pyrolysis fragments, are monitored to confirm the presence of lasalocid.

## MATERIALS AND METHODS

**Instrumentation.** All apparatus for the preparation of tissue samples, their extraction, sample workup, and HPLC chromatography have been described (Weiss et al., 1983). The one modification was the substitution of a Whatman Partisil M9 10/25 column (9.4-mm i.d., 25 or 50 cm) for the two Partisil PXS 10/25 columns (4.6-mm i.d., 25 cm).

Mass spectra and selected ion monitoring measurements were obtained by using a Finnigan Model 1015 quadrupole mass spectrometer and a Finnigan Model 6000 data system. The mass spectrometer was coupled to a Finnigan Model 9500 gas chromatograph.

The glass GC column, 5 ft × 2 mm id, was packed with 3% SE-30 on 120–140-mesh Gas-Chrom Q (Applied Science Laboratories) and contained a 2-in. silanized glass wool plug at the inlet. Prior to use, the column was conditioned overnight at 295 °C with a nitrogen flow of 10 mL $min^{-1}$. For the assay, isobutane was used both as the GC carrier gas (1 $lb/in^2$) and CI reagent gas (ion source pressure 0.6 torr). The injector, column oven, separator oven, and transfer line were operated at 280, 237, 250, and 250 °C, respectively.

The mass spectrometer was tuned and calibrated in the positive chemical ionization mode by using perfluorotributylamine introduced through the reference inlet. Methane, 0.7 torr, was introduced into the ion source through the direct insertion probe inlet. At this time, the GC divert valve was left on to eliminate the presence of isobutane in the ion source. The ion source voltages were set to give the maximum signal intensity, consistent with optimum peak shape and unit mass resolution at $m/z$ 219 and $m/z$ 414. The filament emission and electron energy were set to their lowest value, consistent with the maximum intensity of the $m/z$ 219 ion. The conversion dynode was set at −2.5 kV and the electron multiplier was set at −1.8 kV. The ion source was operated without any external heating.

With the data system operating in the diagnose mode, the $m/z$ 219 ion from perfluorotributylamine was set to $m/z$ 218.8 by using the mass offset potentiometer. The mass spectrometer was then set to monitor $m/z$ 211, 291, 337, and 381. The threshold was set at 1 bit and the scan time set at 1 s. For recording mass spectra, the data system was used to set the mass spectrometer to scan every 1.5 s from $m/z$ 200 to $m/z$ 500.

**Procedure.** The following modifications to the procedure of Weiss et al. (1983) were made to ensure the acquisition of the maximum quantity of the sample for the GC–MS analysis. (a) The initial acetonitrile extract is not divided. Instead, the entire 40 mL is washed with hexane and subsequently blown to dryness under nitrogen at 60 °C. (b) The residue is dissolved in 2 mL of water saturated with the mobile phase and subsequently extracted with 4 mL of the HPLC mobile phase. (c) Three milliliters of the HPLC mobile phase solution from the above is removed, blown to dryness under nitrogen at 60 °C and reconstituted in 0.5 mL of the HPLC mobile phase. (d) Most (0.46 mL) of the above solution is chromatographed on a Whatman Partisil M9 column (9.4 mm i.d., 25 cm) at a 5 mL/min flow rate. The HPLC peak corresponding to lasalocid (or for control samples the area where lasalocid should have eluted) is collected in a 25-mL conical stoppered centrifuge tube.

The HPLC-purified samples are blown to dryness under nitrogen at 60 °C. The samples are trimethylsilylated by adding 50 μL of Regisil (BSTFA plus 1% TCMS, Regis Chemical Co.) to the residue and heating the solution for 1 h at 60 °C. The derivatized samples are analyzed by GC–MS on the same day that they are derivatized. Prior to analysis, the GC column is conditioned with five 1-μL injections of Silyl-8 (Pierce Chemical Co.) followed by three 5-μL injections of the final solution obtained from 10 g of lasalocid-free tissue.

Five microliters of the final 50-μL solution is injected into the GC–MS with the GC divert valve open. Thirty seconds later the divert valve is closed to allow the GC effluent to enter the ion source. The ion source filament and data system are activated 45 and 50 s, respectively, after injection.

## RESULTS AND DISCUSSION

Figure 1 shows typical HPLC chromatograms from the analysis of a control liver sample, a control liver sample fortified with lasalocid sodium to give a concentration of 52.5 ppb, and a dosed liver sample previously assayed by HPLC and found to contain 51 ppb. Also indicated are the start and end points for sample collection.

Pyrolysis GC of silylated lasalocid results in a retroaldol cleavage yielding two GC peaks (Figure 2). Under the conditions of the assay, the retention times of the first and second peaks are approximately 90 and 120 s, respectively. Positive chemical ionization mas spectra of the two peaks are shown in Figure 3. The $MH^+$ ion for the retroaldol

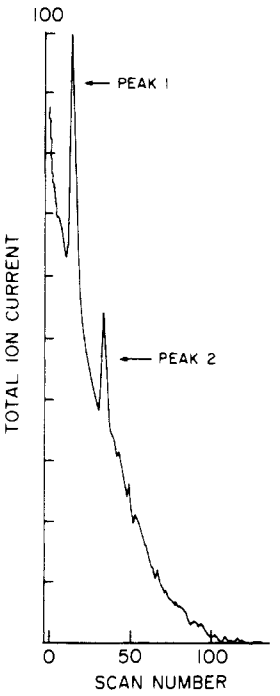


**Figure 2.** A reconstructed ion chromatogram showing the total ion current for $m/z$ 200 to $m/z$ 500 from the injection of a 21 $\mu$g/50 $\mu$L Regisil solution of lasalocid.

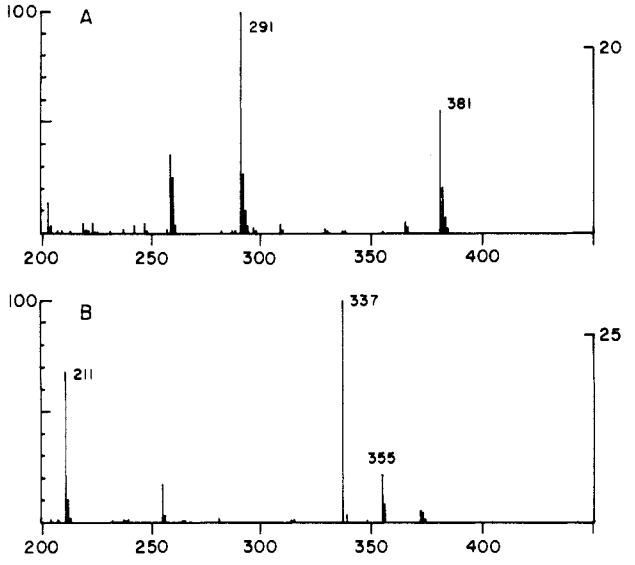


**Figure 3.** Positive chemical ionization mass spectra of the two GC pyrolysis products of trimethylsilylated lasalocid.

aldehyde and retroaldol ketone are $m/z$ 381 and $m/z$ 355, respectively, confirming the identity of GC peak 1 (scan A) and GC peak 2 (scan B). The mass spectral data indicate that under the silylating conditions used, only the carboxylic acid and phenolic moieties are derivatized.

Two ions from each cleavage product were chosen for selected ion monitoring: $m/z$ 291 and $m/z$ 381 from the aldehyde fragment and $m/z$ 211 and $m/z$ 337 from the ketone fragment. Due to background interference, $m/z$ 355 from the ketone fragment was not monitored. The retroaldol cleavage of trimethylsilylated lasalocid and the proposed fragmentation pathways for the pyrolysis products are shown in Figure 4.

Selected ion chromatograms from the samples whose HPLC chromatograms are given in Figure 1 are shown in Figure 5. Selected ion chromatograms B and C show the presence of the four ions monitored at the expected retention times, confirming that the peak in the HPLC

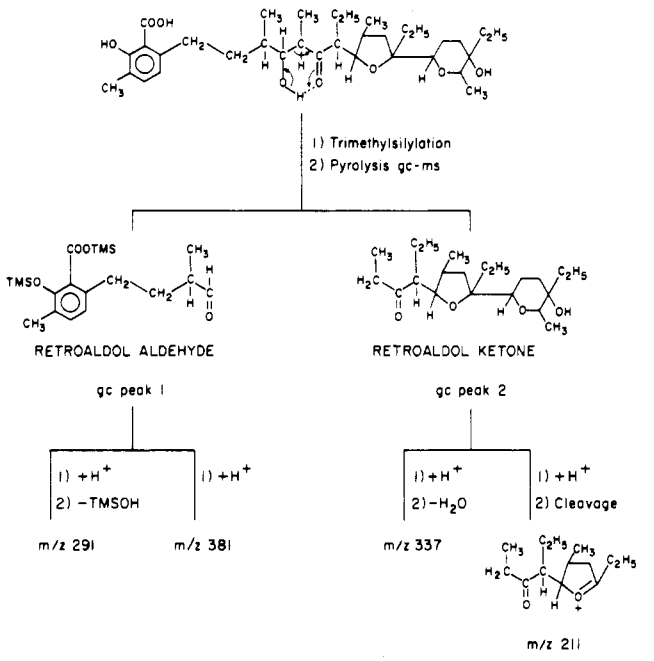


**Figure 4.** Proposed scheme for the GC pyrolysis and mass spectral fragmentation of trimethylsilylated lasalocid.

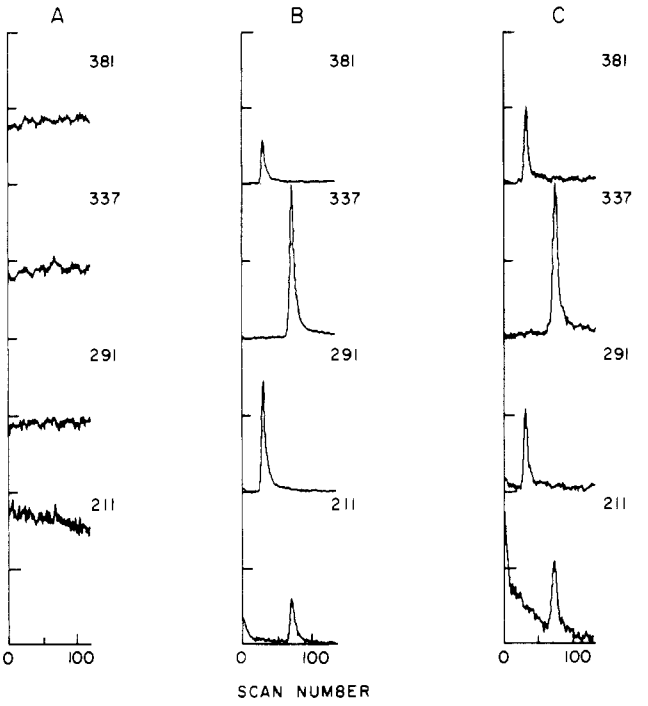


**Figure 5.** Ion chromatograms from the analysis of (A) control liver, (B) control liver fortified to give a lasalocid concentration of 52.5 ppb, and (C) a dosed liver containing 51 ppb of lasalocid.

chromatogram from dosed liver tissue assigned to lasalocid is indeed due to the presence of lasalocid in the sample.

## SUMMARY

A pyrolysis GC–CIMS method to confirm the presence of lasalocid in livers from animals dosed with lasalocid sodium has been developed. The presence of lasalocid is confirmed by (1) collection of a specific fraction of the HPLC effluent by monitoring the fluorescence at 430 nm when excited at 310 nm, (2) pyrolysis of the derivatized HPLC fraction to give two fragments, (3) separation of the two pyrolysis fragments on a gas chromatographic column, and (4) selected ion monitoring of two ions from each of the two fragments at a specific retention time.

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

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Received for review May 28, 1982. Accepted September 15, 1982.

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

Maurice Kaykaty* and George Weiss

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 ($\lambda_{ex}$ = 310 nm and $\lambda_{em}$ = 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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