Spectrofluorometric Determination of the Antibiotic Lasalocid in Blood

M. A. BROOKS *x, L. D'ARCONTE *, J. A. F. de SILVA *, G. CHEN ‡, and C. CROWLEY ‡

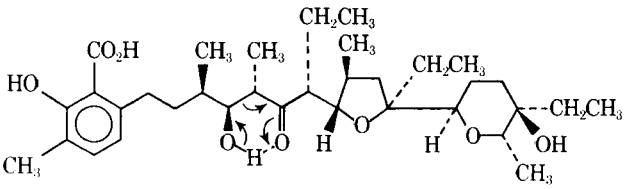
Abstract □ A spectrofluorometric assay was developed for the determination of the antibiotic lasalocid in dog blood, based on the intrinsic fluorescence of the compound in ethyl acetate. The assay can measure "total" levels of drug and any metabolites present. The specificity of the assay was verified by TLC separation of the dog blood extract, which indicated the presence of only intact drug. The overall recovery (±*SD*) of lasalocid was 62.0 ± 3.6% in the concentration range of 1.0–10 μg of compound/ml of dog blood. The sensitivity of the assay is 0.5 μg/ml. The assay was applied to the determination of blood levels of lasalocid in the dog following the intravenous administration of a 5-mg/kg dose.

Keyphrases □ Lasalocid—spectrophotofluorometric analysis in blood after intravenous administration, dogs □ Spectrophotofluorometry—analysis, lasalocid in dog blood

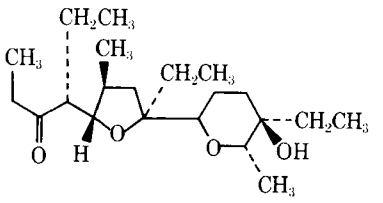
The isolation of the antibiotic lasalocid[1] (I) was first reported in 1951 (1), and it is presently under investigation as an anticoccidial agent in chicks (2). Studies pertaining to the structure elucidation (3–5) and the biosynthesis (6–8) of the compound were reported. Other investigations have dealt with chemical transformations and their effects on antibacterial activity (9), nitration (10), and pyrolytic cleavage (11).

Analytical investigations of lasalocid include GLC determination of the compound in fermentation broths (12), in which the compound is pyrolyzed in the injection port to two naphthols (III[2] and IV[3]) and a retroaldol ketone (II[4]). The ketone is used for quantitation employing a flame-ionization detector. Bioautographical procedures also were reported for the measurement of the antibiotic in tissues of chickens following administration at the 0.0075% level (13).

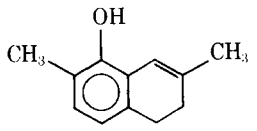
The compound shows strong optical activity in the presence of several amines and monovalent and divalent metals (14, 15). This activity was reported in terms of circular dichroic absorption (14, 15) and fluorescence emission (15) of the complexed compound. A spectrofluorometric assay to measure lasalocid in finished feeds and premixes also was reported (16). The assay involves extraction of the drug into ethyl acetate from the premixes and finished feeds, followed by a differential spectrofluorometric measurement of lasalocid.



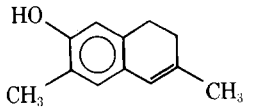
I



II



III



IV

The present work employs several modifications of the previously reported spectrofluorometric assay (16) in order to measure lasalocid in blood. The assay is based on the intrinsic fluorescence of the compound in ethyl acetate and has a sensitivity limit of 500 ng/ml. The assay measures "total" levels of the drug and any metabolites present. The specificity of the assay was verified by TLC separation of the dog blood extract, which indicated the presence of only intact drug. The method was applied to the determination of blood levels of the drug in the dog following intravenous administration of a 5-mg/kg dose.

EXPERIMENTAL

Instrument—A spectrofluorophotometer[5] equipped with a selected high energy output dc xenon arc energy source and a high sensitivity (>10 lumen rating) photomultiplier[6] was used. The 10-nm slit arrangement was used in all four positions, and the instrument was adjusted for maximum energy output using a Pyrex reference rod. Spectra were obtained on an *X–Y* recorder[7].

Standard Solutions—Prepare a stock solution (Solution A) of lasalocid (I) ($C_{34}H_{53}NaO_8$, mol. wt. = 612.78, pKa 5.13), by dissolving 10 mg of I in 100 ml of methanol. Make a 1:10 dilution of this

[1] 6-[7(*R*)-[5(*S*)- Ethyl-5-[5(*R*)-ethyltetrahydro-5-hydroxy-6(*S*)- methyl-2*H*- pyran-2(*R*)-yl]tetrahydro -3(*S*)-methyl- 2(*S*)- furanyl]- 4(*S*)-hydroxy-3(*R*),5(*S*)-dimethyl-6-oxononyl]-2-hydroxy-3-methylbenzoic acid. Lasalocid is also designated as X-537A in the literature.

[2] 5,6-Dihydro-2,7-dimethyl-1-naphthol.

[3] 7,8-Dihydro-3,6-dimethyl-2-naphthol.

[4] 4-[5-Ethyl-3-methyl-5-(ethyl-5-hydroxy-6-methyl-2-tetrahydropyranyl)-2-tetrahydrofuryl]-3-hexanone.

[5] Model Mark I, Farrand Optical Co., Mount Vernon, N.Y.

[6] RCA 1P 28 or equivalent photomultiplier tube.

[7] Model 2200-3-3, Houston Instruments, Bellaire, Tex.

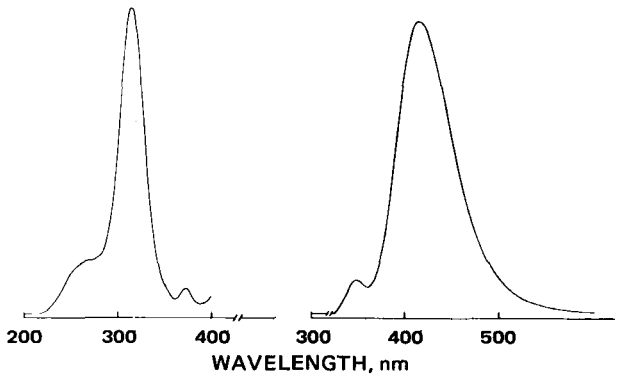


Figure 1—*Excitation–emission spectra of 1 μg of lasalocid in 5 ml of ethyl acetate. Left: excitation (λ_{max} 310 nm). Right: emission (λ_{max} 415 nm). Attenuation = 3×.*

stock solution in methanol to yield a working solution (Solution B) containing 10 μg/ml; suitable aliquots of Solution B are added to blood as internal standards.

Reagents—The reagents used were of analytical reagent grade (ACS) purity and included saturated tribasic sodium phosphate (Na_3PO_4) (pH 12.3), ethanol–0.1 *N* NaOH (80:20, spray reagent), anhydrous ether[8], benzene[9], methanol[9], and ethyl acetate[10].

Analysis in Blood—Into a 50-ml centrifuge tube, add 1 ml of sample and then mix[11] for about 5 sec. Add 1 ml of ethanol, stopper, and mix well again for about 5 sec, making sure that all of the sample has been in contact with the alcohol; then place in a water bath at about 70° for 5 min for protein precipitation.

Allow the samples to cool to room temperature. Add 5 ml of saturated tribasic sodium phosphate solution (pH 12.3), and mix[11] well to ensure that the coagulated sample is uniformly suspended in the mixture. Add 20 ml of ether, mix again for 5 sec, and place in a reciprocating shaker for 20 min. Centrifuge at 2400–2600 rpm for about 5 min.

Transfer an 18-ml aliquot of the ether extract into a clean 50-ml tube and evaporate to dryness. Reconstitute by the addition of 200 μl of ether, stopper, and mix[11] well. To the ether solution, add 10 ml of ethyl acetate (spectral grade); then mix well and measure the fluorescence of the solution in a 1-cm path quartz cell at 415 nm with excitation at 310 nm. Along with the unknowns, run internal standards of 0.5, 1.0, 2.0, 5.0, and 10.0 μg of I (0.05, 0.10, 0.20, 0.50, and 1.0 ml of Solution B, respectively, evaporated to dryness under nitrogen) in blood taken preferably from the same dog prior to medication or from a pooled control source.

TLC Analysis of Blood Extracts—The specificity of the fluorometric assay in blood can be verified as follows. Following fluorometric analysis, evaporate the ethyl acetate solution to dryness and dissolve the residue in 100 μl of ether. Transfer the extract quantitatively onto a 20 × 20-cm 60-μm silica gel chromatoplate[12]. Rinse each tube with an additional 50 μl of ether and transfer to the TLC plate to effect a quantitative transfer.

Develop the plate for 15 cm ascending in a vapor-saturated tank using benzene–methanol (90:10). Air dry the plate, spray it lightly with a mixture of ethanol–0.1 *N* sodium hydroxide (80:20), and heat in an oven at 100° for 15 min. The parent drug (I, $R_f \simeq 0.32$) is identified by reference to the R_f of 10 μg of authentic standard run alongside the sample extract as a blue fluorescent spot seen under short- and longwave UV irradiation characteristic of salicylate-like fluorescence. These zones turn yellow upon standing and exposure to the atmosphere.

Calculations—All fluorescence readings [(*TM*) = transmittance (*T*) × meter multiplier factor (*M*)] are corrected for blank readings and for any dilutions made. Unknown concentrations are interpolated from the internal standard curve or from direct comparison with one of the internal standards. The percent recovery of the assay is calculated based on the comparison of (*TM*/μg/ml) values of the internal and external standards (corrected for aliquots taken).

The percentage recovery should be calculated routinely as a check on analytical precision and reproducibility.

Recovery and Sensitivity Limits—The assay has an overall recovery (±*SD*) of 62.0 ± 3.6% in the range of 1.0–10 μg of I/ml of dog blood. The sensitivity limit of the assay is 0.5 μg of I/ml of blood. At the 0.5-μg/ml level, the recovery is approximately 15% lower than that in the 1.0–10-μg/ml range (Table I).

Table I—Recovery of Lasalocid from Dog Blood

Amount of Lasalocid Added to 1.0 ml of Control Blood, μg	Amount Recovered, μg	Recovery[a], %
0		
0		
0.5	0.20	44.4 } 44.4
0.5	0.20	44.4 }
1.0	0.56	62.2 } 62.2
1.0	0.56	62.2 }
2.0	1.18	65.4 } 65.5
2.0	1.18	65.4 }
5.0	3.12	69.3 } 69.5
5.0	3.14	69.7 }
10.0	5.82	64.6 } 68.4
10.0	6.50	72.2 }
		62.0 ± 3.6% (*SD*)[b]

[a] Corrected for dilution factor of 1.11. [b] Excluding the 0.5-μg samples.

RESULTS AND DISCUSSION

The UV absorption spectrum of lasalocid shows a major band at 305 nm with an absorbance (*A*/μg/ml) ≃0.008 in ethyl acetate, indicating a potential sensitivity limit of about 10–20 μg/ml of blood. Because of this poor sensitivity, a spectrophotometric assay was not attempted and the development of a spectrofluorometric assay based on a reported method (16) was undertaken.

The compound exhibits moderate fluorescence emission at 415 nm (*TM*/μg/ml = 498) in ethyl acetate with an excitation maximum at 310 nm (Fig. 1). The fluorescence of the compound is linear with concentration over the range of 0.04–20 μg of I/ml of ethyl

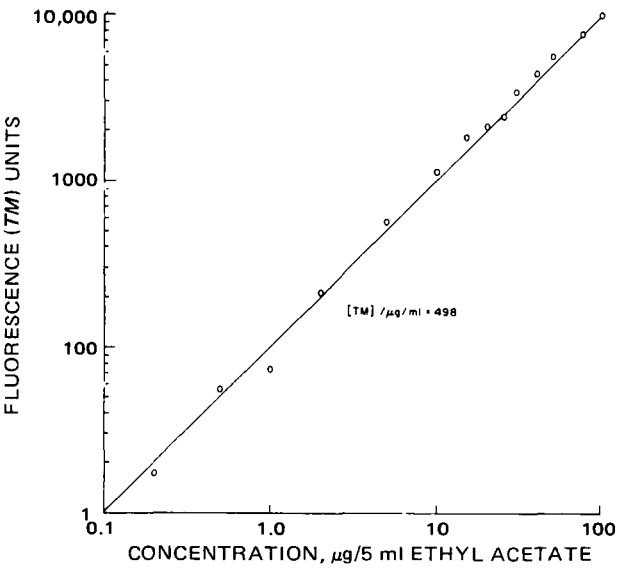


Figure 2—*Linear dynamic range of fluorescence* versus *concentration of lasalocid in ethyl acetate.*

[8] Mallinckrodt, St. Louis, MO 63160
[9] Fisher, Springfield, NJ 07081
[10] Spectroquality grade, Matheson, Coleman and Bell, East Rutherford, NJ 07073
[11] Vortex action supermixer, Catalog No. 1290, Lab Line Instruments, Melrose Park, Ill.
[12] Chromatoplates were manufactured by E. Merck (F_{254}), Darmstadt, Germany, and marketed by Brinkmann & Co., Westbury, NY.

Table II—Blood Levels of Lasalocid in a Dog following a 5-mg/kg iv Dose

Hours	Micrograms per Milliliter of Blood	
	Spectrofluorometric	Bioautography
0.0083	15.8	14.1
0.25	8.8	11.8
0.50	7.8	7.3
1	5.8	5.1
3	3.9	4.9
5	2.7	3.8
24	0.4	0.4

acetate (Fig. 2). The overall sensitivity limit of the assay, 0.5 μg of I/ml of sample, is restricted by interferences from the sample extract of the dog blood.

The ether residues of the sample extracts contain a yellow-colored coextracted biological impurity, which necessitates the addition of 200 μl of ether to effect its solution in ethyl acetate. This impurity imparts a distinct yellow color to the ethyl acetate solution used for spectrofluorometric measurement and produces fluorescence quenching to the extent of about 10%. Therefore, the samples and internal standards must be measured in the same volume of ethyl acetate.

Back-extraction of the samples, using a variety of acidic and basic aqueous solutions to effect better cleanup, was unsuccessful. Contact with solutions below pH 11 resulted in a shift of the excitation and emission maxima to 320 and 370 nm, respectively, with a corresponding decrease in overall fluorescence yield. Washing with strongly alkaline solutions also decreased the overall sensitivity.

Application of the pyrolysis flame-ionization GLC (12) was not successful due to the presence of many coextracted impurities that interfered with the measurement of the analytical retroaldol ketone (II) peak.

The spectrofluorometric assay presented is more rapid than the bioautographical assay (13), but it is not as sensitive. In the bioautographical assay, larger volumes of samples can be extracted. This same assay procedure is applicable to urine samples with recoveries ($\pm SD$) of 80.5 ± 4.2% in the concentration range of 1.0–10.0 μg/ml.

APPLICATIONS

Blood levels were determined in a male dog by the spectrofluorometric assay following intravenous administration of 5 mg of lasalocid/kg (Table II). A peak blood level of 15.8 μg of I/ml was obtained at 5 min and declined to 0.4 μg of I/ml at 24 hr.

These same samples were also analyzed by the bioautographical assay (13), and satisfactory correlation was found between the two assays. Examination of the ethyl acetate solutions by TLC following fluorometric analysis did not reveal the presence of other fluorescent zones due to possible metabolites (after spraying with ethanol–sodium hydroxide) that may interfere with the specificity or accuracy of the fluorometric assay. This finding does not, however, preclude the presence of nonfluorescent UV-absorbing metabolites that are extractable under the same conditions.

REFERENCES

(1) J. Berger, A. I. Rachlin, W. E. Scott, L. H. Sternbach, and M. W. Goldberg, *J. Amer. Chem. Soc.*, **73**, 5295(1951).

(2) M. Mitrovic and E. C. Schildknecht, *Poult. Sci.*, **53**, 1448(1974).

(3) J. W. Westley, R. H. Evans, Jr., T. Williams, and A. Stempel, *J. Chem. Soc., Chem. Commun.*, **1970**, 71.

(4) S. M. Johnson, J. Herrin, S. J. Liu, and I. C. Paul, *ibid.*, **1970**, 72.

(5) S. M. Johnson, J. Herrin, S. J. Liu, and I. C. Paul, *J. Amer. Chem. Soc.*, **92**, 4428(1970).

(6) J. W. Westley, R. H. Evans, Jr., D. L. Pruess, and A. Stempel, *J. Chem. Soc., Chem. Commun.*, **1970**, 1467.

(7) J. W. Westley, D. L. Pruess, R. G. Pitcher, *ibid.*, **1972**, 161.

(8) J. W. Westley, R. H. Evans, Jr., G. Harvey, R. G. Pitcher, D. L. Pruess, A. Stempel, and J. Berger, *J. Antibiot.*, **27**, 288(1974).

(9) J. W. Westley, E. P. Oliveto, J. Berger, R. H. Evans, Jr., R. Glass, A. Stempel, V. Toome, and T. Williams, *J. Med. Chem.*, **16**, 397(1973).

(10) J. W. Westley, J. Schneider, R. H. Evans, Jr., T. Williams, A. D. Batcho, and A. Stempel, *J. Org. Chem.*, **36**, 3621(1971).

(11) J. W. Westley, R. H. Evans, Jr., T. Williams, and A. Stempel, *ibid.*, **38**, 3431(1973).

(12) J. W. Westley, R. H. Evans, Jr., and A. Stempel, *Anal. Biochem.*, **59**, 574(1974).

(13) A. MacDonald, G. Chen, M. J. Duke, and A. Popick, *Poult. Sci.*, **52**, 2055(1973).

(14) S. R. Alpha and A. H. Brody, *J. Amer. Chem. Soc.*, **95**, 7043(1973).

(15) H. Degani, H. L. Friedman, G. Nawon, and E. M. Kosower, *J. Chem. Soc., Chem. Commun.*, **1973**, 431.

(16) M. Osadca and M. Araujo, *J. Ass. Offic. Anal. Chem.*, **57**, 636(1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 9, 1974, from the *Departments of *Biochemistry and Drug Metabolism and ‡Agricultural Research, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication February 28, 1975.

ˣ To whom inquiries should be directed.