Journal of Chromatography, 229 (1982) 167—178
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1165

DETERMINATION OF BROMO-LASALOCID IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

MARVIN A. BROOKS*, NORMAN STROJNY, MARTIN R. HACKMAN and J. ARTHUR F. de SILVA

Department of Pharmacokinetics and Biopharmaceutics, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

(Received October 28th, 1981)

SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of bromo-lasalocid in plasma. The compound was extracted into isooctane—ethyl acetate (90:10) from plasma saturated with potassium chloride and adjusted to strongly alkaline pH. The residue of this extract was dissolved in methanol—2-methoxyethanol (95:5) and analyzed by HPLC on a 10-μm C_{18} column [mobile phase of methanol—water—2-methoxyethanol—1 *M* potassium phosphate buffer, pH 3.0 (90:10:2.5:0.2)] using fluorescence detection with excitation at 215 nm and emission at wavelengths greater than 370 nm. The overall recovery of the assay was 65%, with a limit of sensitivity of 0.1 μg/ml. The method was used to obtain plasma concentration—time profiles in the dog following oral administration of bromo-lasalocid·ethanolate.

INTRODUCTION

Bromo-lasalocid [I] (Fig. 1), synthesized from the antibiotic X-537 A (lasalocid) [1], is presently under investigation as an ionophoric cardiovascular agent [2]. Investigation of the chemical transformations of [I] via pyrolysis and base-catalysis have been reported [3].

Analytical methods for lasalocid, the parent compound, include pyrolytic gas—liquid chromatographic—flame ionization detection (GLC—FID) determination in fermentation broths [4], bio-autoradiographic analysis in chicken tissues [5], spectrofluorometric assays in finished feeds and premixes [6] and in dog blood [7], and high-performance liquid chromatography (HPLC) with either UV [8] or fluorometric detection [9]. The GLC—FID analysis of intact lasalocid and bromo-lasalocid as their trimethylsilyl derivatives was also reported [10].

0378-4347/82/0000—0000/$02.75

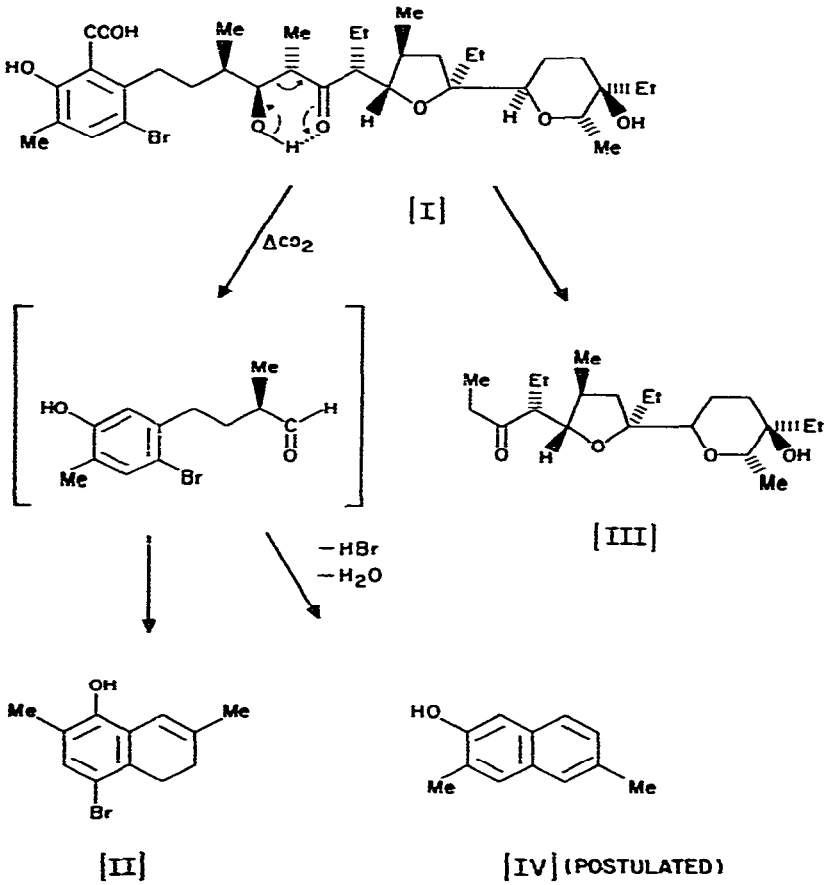


Fig. 1. GLC pyrolysis of bromo-lasalocid [4].

The present work describes a reversed-phase HPLC assay using fluorescence detection for the analysis of [I] in organic extracts of alkalinized dog plasma following intravenous and oral administration of [I]·ethanolate. The overall recovery of the assay is 65% with a limit of sensitivity of 0.1 μg/ml. In addition port. The HPLC assay is, however, preferred over the GLC—ECD assay assay is also described which utilizes the 4-bromo-5,6-dihydro-2,7-dimethyl-1-naphthol [II] (Fig. 1), produced by pyrolytic cleavage of [I] in the injection port. The HPLC assya is, however, preferred over the GLC—ECD assay due to its simplicity, higher specificity, and lower sensitivity limit.

EXPERIMENTAL

Column

The column used for reversed-phase liquid chromatography was a prepacked 30 cm × 3.9 mm I.D. stainless-steel column containing a μBondapak C-18 reversed-phase 10-μm microparticulate packing (Serial No. 115415, Waters Assoc., Milford, MA, U.S.A.).

HPLC instrumental parameters

An HPLC system (Model ALC/GPC 204/6000A, Waters Assoc.), equipped with a 400 bar (6000 p.s.i.) pump, loop injection system and UV detector with a 254-nm wavelength kit was used for chromatography. A 2-μm pre-column filter was used to improve column life. An HPLC fluorescence detector (Model FS-970, Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.),

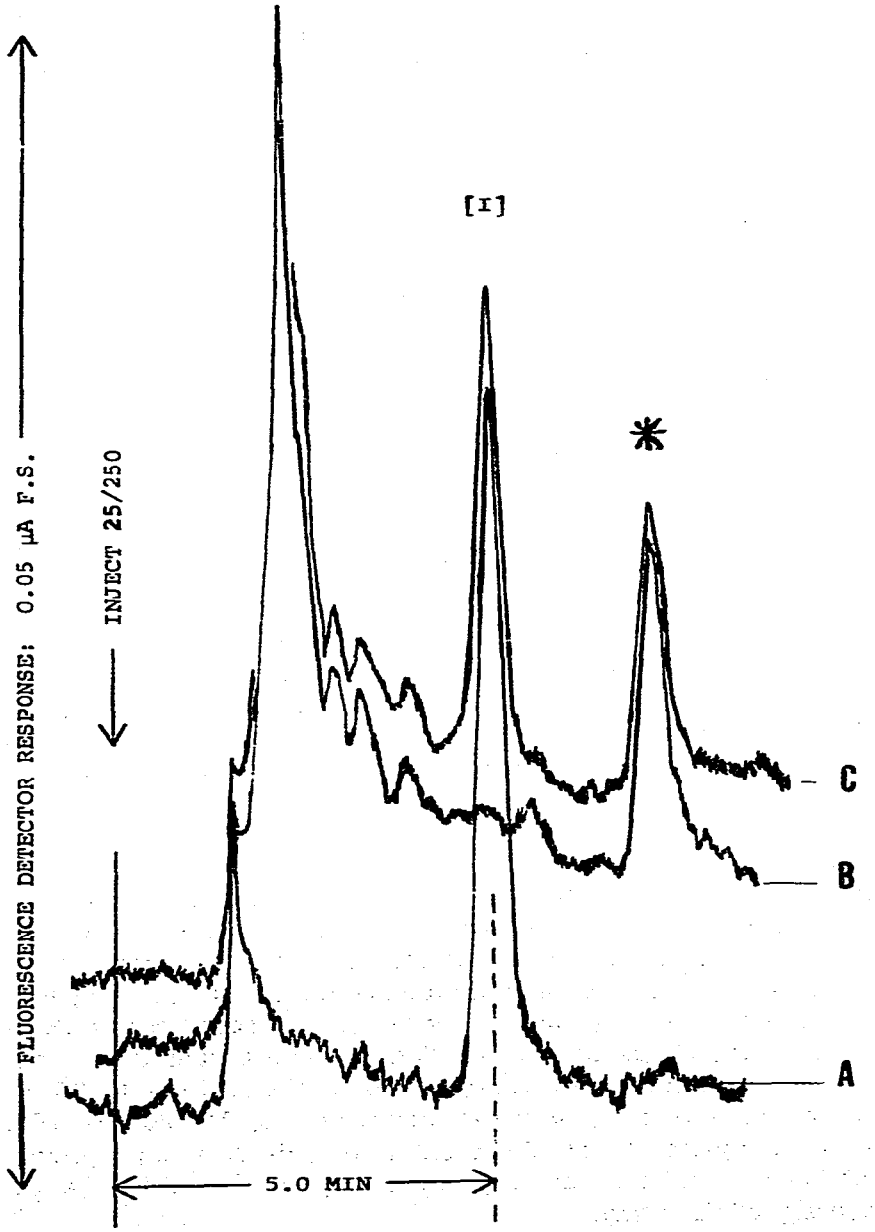


Fig. 2. HPLC separation of (A) authentic standard of 20 ng [I] injected, and extracts of 1-ml aliquots of (B) control human plasma, (C) control human plasma containing 200 ng added authentic [I] per ml. (*) Endogenous component.

operated at 215 nm for excitation with an emission filter passing wavelengths greater than 370 nm, was used for fluorometric detection. The mobile phase used for isocratic reversed-phase chromatography consisted of 900 ml of methanol, 100 ml of water, 25 ml of 2-methoxyethanol, and 2.0 ml of 1 *M* potassium phosphate buffer, pH 3.0. The solvent flow-rate was 1.5 ml/min resulting in a column head pressure of about 100 MPa. Under these conditions, the capacity factor (k') for [I] was 2.6 (retention time = 5.0 min) (Fig. 2A), and 20 ng of [I] injected gave approximately 70% full scale pen response with the detector range at 0.05 μA full scale and the photomultiplier sensitivity at 520 V. The chart speed on the dual-channel recorder (Model 7132A with option 108, Hewlett-Packard, Palo Alto, CA, U.S.A.) was 0.5 in./min.

Spectrophoto/fluorometric instrumentation

Ultraviolet absorbance spectra were recorded using a double-beam ratio-recording spectrophotometer (Coleman Model EPS-3T Hitachi Spectrometer, Coleman Instruments, Maywood, IL, U.S.A.). Corrected luminescence excitation and emission spectra (10 nm bandpass) were recorded using a spectrofluorometer equipped for direct recording of corrected excitation—emission spectra (Farrand Mark I, Farrand Optical Co., Valhalla, NY, U.S.A.). The quantum yields were determined with excitation at the absorption maxima of 310—320 nm rather than those at 209—212 nm because the diminished xenon arc lamp intensity at less than 250 nm does not yield reliable, noise-free emission spectra.

Reagents

All inorganic reagents were analytical reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.). Organic solvents suitable for spectrophotometry and liquid chromatography were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Preparation of standard solutions

A stock solution of the ethanolate salt of bromo-lasalocid, $C_{34}H_{53}BrO_8 \cdot C_2H_6O$, mol. wt. = 715.75, m.p. = 110°C, was prepared by weighing 10.7 mg (equivalent to 10.0 mg of free acid) into a 10-ml amberized volumetric flask and dissolving in 10 ml of methanol—2-methoxyethanol (95:5) to give a stock solution containing 1.0 mg [I] per ml. Five working standards containing 0.1, 0.2, 0.5, 1.0, and 2.0 μg of [I] per 250 μl were prepared by serial dilution of the stock solution with methanol—2-methoxyethanol (95:5).

Aliquots (25 μl) of each working solution, equivalent to 10, 20, 50, 100, and 200 ng of [I] were injected to obtain an external calibration curve, which was used to establish the linearity and reproducibility of the HPLC system and the overall recovery of the assay.

Procedure

A 1.0-ml aliquot of each unknown plasma sample was added to separate

13 × 100 mm disposable borosilicate culture tubes (Cat. No. 14-962-10C, Fisher Scientific, Pittsburgh, PA, U.S.A.). Specimens of control plasma containing [I] were prepared by transferring 250-μl aliquots of selected working standards into separate tubes and evaporating in a stream of clean, dry nitrogen at room temperature, and then adding a 1.0-ml aliquot of control plasma to each standard and mixing well. These specimens were processed along with the unknowns as the recovery standards for the determination of [I] in the unknowns.

A 5-ml aliquot of isooctane—ethyl acetate (90:10) was added to each tube, followed by approximately 0.5 g potassium chloride and 1.0 ml 1 *N* potassium hydroxide. The tubes were stoppered with polyethylene caps (Plugtite cat. No. 127-0019-100, Elkay Products, Shrewsbury, MA, U.S.A.) and shaken for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 50—80 strokes/min. The samples were centrifuged at 950 *g* for 10 min at 0—5°C in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC, Needham, MA, U.S.A.) and a 4.5-ml aliquot of the upper organic layer was transferred into a clean culture tube. The organic extract was evaporated to dryness at 20—25°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. The residue was dissolved in 250 μl of methanol—2-methoxyethanol (95:5) and a 25-μl aliquot was injected for HPLC analysis using reversed-phase chromatography. The Schoeffel fluorescence detector was set at 0.05 μA full scale for maximum sensitivity. Typical chromatograms are shown in Fig. 2B and C.

The concentration of [I] in each unknown was determined by interpolation from the calibration curve of the recovered standards processed with least squares regression analysis using an exponential equation.

RESULTS

Statistical validation of the method

The HPLC analysis of authentic (non-recovered) standards of [I] in the concentration range 0.01—0.20 μg injected is best described using least squares regression analysis with an exponential equation of the form $Y = mX^b$ ($Y = 2.89\ X^{0.971}$), where X = concentration injected and Y = fluorometric response (chromatographic peak height). The correlation coefficient (r) of 0.998 demonstrates the linearity and precision of the system.

The recovery of [I] from human plasma using the described assay was determined in the concentration range of 0.1—2.0 μg/ml of plasma by substitution of the fluorometric response obtained for these samples into the equation describing the calibration curve of the authentic (non-recovered) standards (see above); with the appropriate correction for aliquot injected (25/250) and for the extraction aliquot of 4.5/5.0. The mean overall recovery of bromo-lasalocid [I] ($n = 14$) was 64 ± 11% (S.D.).

The precision and accuracy of the method were determined by construction of a linear least squares regression calibration curve (exponential equation) from the responses of the recovered standards [$Y = 1.82\ X^{1.10}$ ($r = 0.994$), where Y = fluorometric response and X = concentration of [I] per ml of plasma] and substitution of the experimentally determined responses into the

TABLE I

INTRA-ASSAY STATISTICAL EVALUATION

Amount [I] added (μg/ml)	Amount [I] found ± S.D. (μg/ml)	n	Relative S.D. (± %)
0.1	0.088 ± 0.005	2	5.6
0.2	0.22 ± 0.02	3	10.5
0.5	0.55 ± 0.04	3	7.7
1.0	0.90 ± 0.06	3	6.8
2.0	2.03 ± 0.03	3	1.3
		Mean	6.2
$Y = mX^b = 1.82\ X^{1.18}$		$r = 0.994$	

where X = concentration of [I] in μg/ml and Y = fluorometric response (peak height)

theoretical equation to yield the amount [I] found (Table I).

The sensitivity limit of the assay was validated at 0.1 μg [I] per ml, using a 1.0-ml sample of human plasma per assay. The intra-assay mean relative standard deviation was 6.2% over the concentration range of 0.1—2.0 μg [I] per ml of plasma (Table I).

TABLE II

PLASMA CONCENTRATION OF COMPOUND [I]

Determination by HPLC—fluorometric analysis in the dog following a single oral administration of 28.57 mg [I]·ethanolate per kg (equivalent to 26.69 mg [I] per kg of body weight).

Sampling time (h)	Concentration μg [I] per ml of plasma
0.167	N.M.*
0.333	0.12
0.5	0.74
0.75	2.6
1.0	2.0
1.5	3.5
2	5.3
3	6.1
4	5.2
6	3.9
8	3.0
10	2.5
24	0.36
30	0.16
48	N.M.
72	N.M.

*N.M. = nonmeasurable, < 0.1 μg/ml of plasma.

Application of the method to biological specimens

The assay was applied to the determination of plasma concentrations of [I] in the dog following oral doses of [I]·ethanolate. Typical data are shown in Table II for the analysis of [I] in the dog following an oral dose of 28.57 mg/kg of [I]·ethanolate (equivalent to 26.69 mg of the free acid of [I] per kg of body weight) as a capsule formulation.

DISCUSSION

Gas—liquid chromatographic analysis of [I]

Pyrolysis of intact [I]. Initial development of an assay for [I] in biological fluids was based upon the pyrolytic properties of the molecule during GLC analysis [4]. The pyrolysis of [I] in the injection port during GLC analysis, monitored by a flame ionization detector, yielded three major peaks (Fig. 3). The peaks at 6.2 and 15.5 min were identified by GC—mass spectrometric analysis [11] as 4-bromo-5,6-dihydro-2,7-dimethyl-1-naphthol [II], and a retroaldol ketone [III] (Fig. 1), respectively (identical to that formed on the pyrolysis of lasalocid [3]). The peak at 2.5 min has not been identified but is

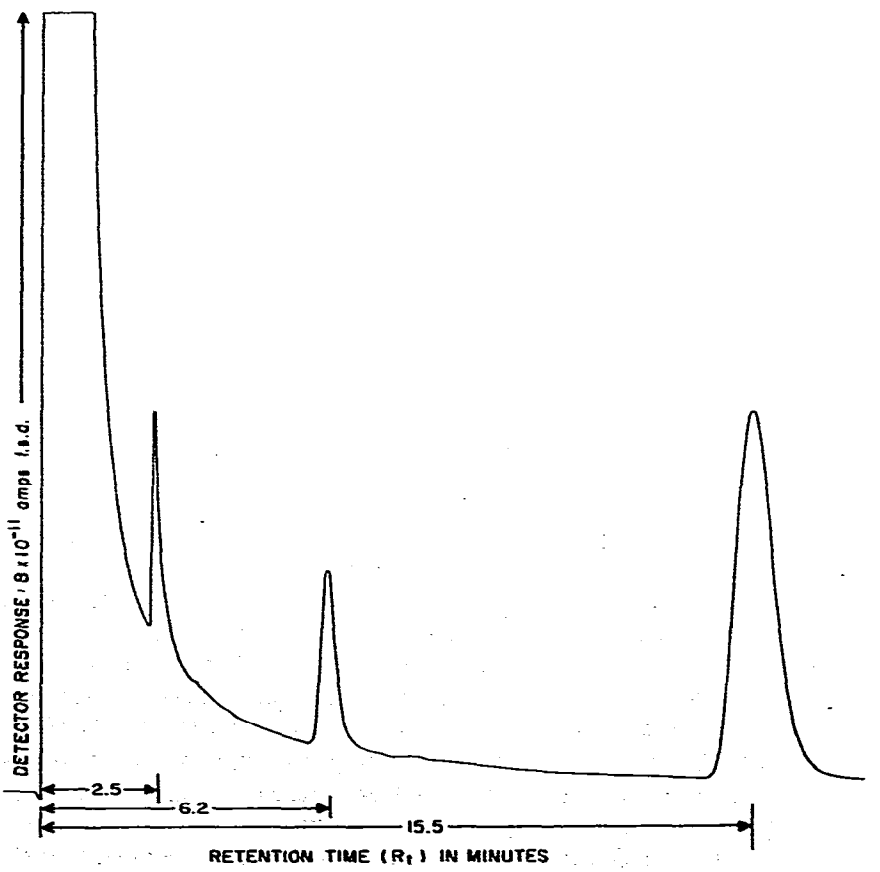


Fig. 3. GLC pyrolysis of 1 μg of [I] monitored by the flame ionization detector (GLC conditions, see Table III).

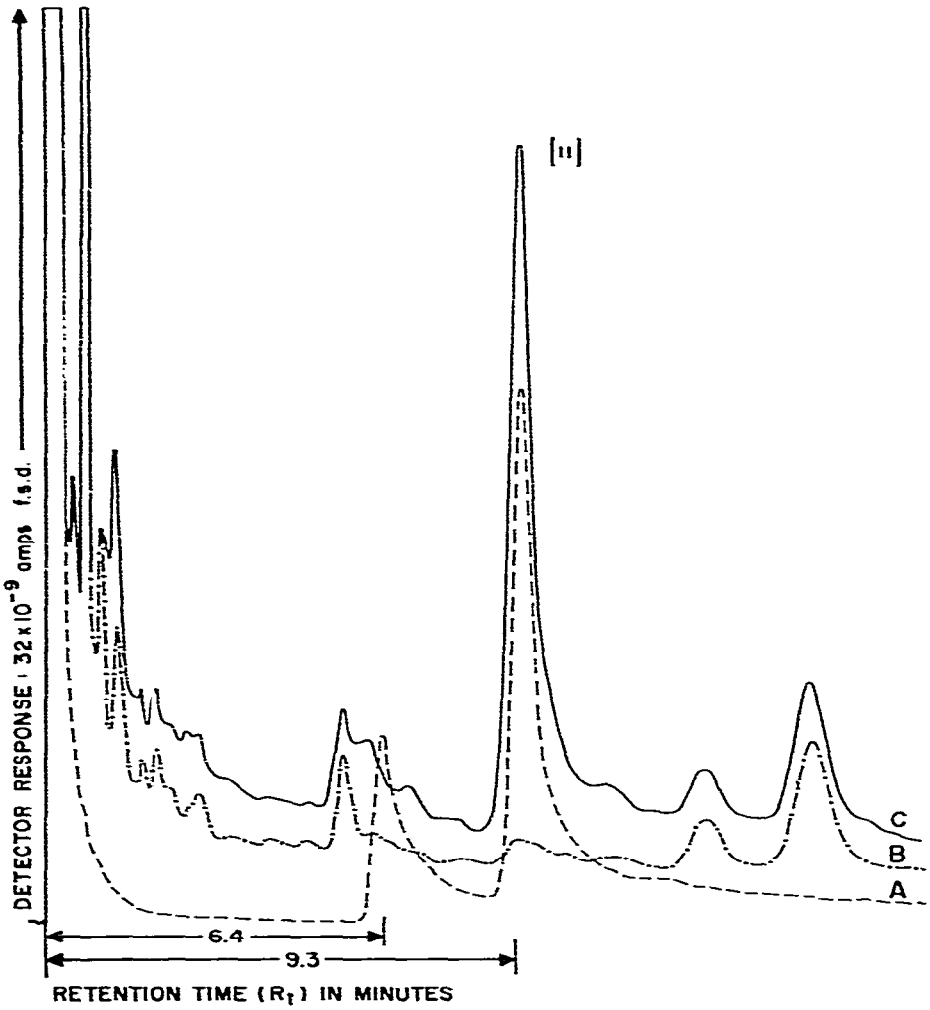


Fig. 4. Electron-capture GLC pyrolysis of (A) authentic standard, 50 ng [I] injected; (B) control blood extract; (C) extract from sample containing 750 ng of [I] per ml of blood (injection aliquot 5/50 μl) (GLC conditions, see text).

postulated to be 3,6-dimethyl-2-naphthol [IV] which is also a pyrolysis product of lasalocid (Fig. 1). The pyrolysis of [I] monitored under the same conditions with an electron-capture (EC) detector yielded two peaks (Fig. 4A), the second of which was compound [II]. This was confirmed using an authentic standard of [II] which had an identical retention time to that of the second peak monitored by both detectors. The identification was also confirmed using relative retention to 2-methylamino-5-chloro-benzophenone (MACB) (Table III) on both 3% OV-1 and 3% OV-17 column packings, using electron-capture and flame ionization detectors. The relative retention times also indicated that the first peak monitored by each detector was not the same material. Presumably, the first EC peak was a minor pyrolysis product with high sensitivity by ECD while peak 3 monitored by FID was identified as [III] by comparison to the authentic compound.

TABLE III

RELATIVE RETENTION TIMES OF PYROLYSIS PRODUCTS OF [I] TO MACB

GLC conditions: 3% phase on Gas-Chrom Q (60—80 mesh); oven temperature: 170°C; injection port: 260°C; detector temperatures: ECD, 325°C; FID, 300°C; flow-rates: ECD, 90 ml/min column flow argon—methane (90:10), 20 ml/min detector purge flow; FID, 90 ml/min nitrogen, 70 ml/min hydrogen, 570 ml/min air; samples ECD, 100 ng [I] per 10 μl injected; FID, 1 μg [I] per 10 μl injected.

Detector	OV-17 column			OV-1 column		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
FID	0.17	0.42	1.04	0.25	0.53	1.98
EC	0.30	0.43	N.D.*	0.44	0.54	N.D.

*N.D. = not detected.

The limit of sensitivity for the measurement of [II] by ECD was 10 ng injected and that for [II] and [III] by FID was 100 and 500 ng injected, respectively. The higher sensitivity of the EC detector also enabled the determination of concentrations of [I] in the dog following administration of [I]·ethanolate.

The chromatographic conditions for the analysis of [I] must be rigidly controlled to ensure reproducible pyrolysis with a linear concentration response of [II] for ECD over the concentration range of 25—100 ng injected. Variation of the injection port temperature from 225°C to 300°C demonstrated that the highest yields of [II] were obtained between 250°C and 280°C. At temperatures above 285°C larger amounts of the by-product (retention time = 6.4 min, Fig. 4A) were present than at other temperatures. The flow-rate of the argon—methane (90:10) carrier gas and the volume of sample injected are also critical for reproducible pyrolysis. Relatively slow flow-rates (⩽ 50 ml/min), and small volumes (⩽ 5 μl) gave the highest yield of [II] with small amounts of by-product. With slow flow-rates and small injection volumes, the residence time of [I] in the injection port was longer and resulted in an optimal and reproducible yield of [II].

The analysis of [I] by GLC—ECD was performed on the residue of a benzene (nanograde, Mallinckrodt, St. Louis, MO, U.S.A.) extract of blood which had been adjusted to pH 12.6 with Na_3PO_4. GLC—ECD analysis was performed on a 5-μl aliquot of the residue which was reconstituted in 50 μl of acetone. A Tracor MT-220 gas chromatograph equipped with a ^{63}Ni EC detector containing a 15 mCi ^{63}Ni β-ionization source was used. Argon—methane (9:1) (Matheson Gas Products, East Rutherford, NJ, U.S.A.) was used as the carrier gas and the column head pressure was pre-set at 40 p.s.i.g. (2.76 bar), with a column flow-rate of 50 ml/min and a detector purge of 20 ml/min. The temperature settings were as follows: oven 175°C; injection port 280°C; detector 325°C. The conditions of flow-rate and column temperature must be adjusted to obtain a retention time of 9.3 min for [II] (Fig. 4).

The solid state electrometer (Model No. 8169) input was set at 10^2, and the output attenuation was 32 giving a response of $3.2 \cdot 10^{-9}$ A for full scale deflec-

tion, the chart speed was 30 in./h, and the time constant on the 1.0-mV Honeywell recorder (Model No. 194) was 1 sec (full scale deflection). The response of the ^{63}Ni EC detector (operated in the pulsed mode) to [II] showed maximum sensitivity at 45 V d.c. at a 270-μsec pulse rate and a 4-μsec pulse width.

The column packing was a pretested phase containing 3% OV-17 on 60—80 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) packed in a U-shaped 1.22 m × 4 mm I.D. borosilicate glass column. The packed column was conditioned at 315°C under no-flow conditions for 4 h and then at 265°C for at least 18 h with a nitrogen flow-rate of 40 ml/min. The GLC—ECD assay for bromo-lasalocid has an overall recovery of 65 ± 15% (S.D.) in the concentration range of 0.5—10 μg/ml of dog blood. This relatively high standard deviation is directly related to the degree of cleanliness of the extracts used for analysis. The assay of these extracts, which are slightly colored and contain material which is sometimes insoluble in acetone, often yields responses (area [II]/ng [I] injected) which are lower than those obtained for the pure material. This is probably due either to the saturation of the EC detector or to the inhibition of pyrolysis. In order to obtain meaningful recovery data, it is essential that analysis of the recovery standards and the external standards be in an alternating sequence. Attempts to clean-up the samples by either formation of protein free filtrates and/or back-extraction resulted in significant losses of compound [I] with overall recovery of approximately 25%.

Derivatization reactions. Assay of intact [I] as its silyl derivative [10] was attempted; however, interferences originating from the silylation reagent made quantitation impractical. In addition, the sensitivity obtained by pyrolysis GLC—ECD was higher than that reported for the silyl derivative (300 ng). GLC—ECD examination of the pentafluorobenzyl ester and/or phenolic ether of [I] formed with pentafluorobenzyl bromide (PFBB) demonstrated that the single product formed (confirmed by thin-layer chromatography) underwent pyrolytic cleavage to yield two electron-capturing derivatives. These products were identified as having retention times of 7 and 11 min, respectively, on a 1.22 m × 2 mm glass column containing 3% OV-17 coated on 80—100 mesh Gas-Chrom Q at oven and injector temperatures of 200°C and 290°C, respectively. This approach was abandoned because reproducible pyrolysis of the pentafluorobenzyl ester/ether derivative of [I] yielding a single GLC component could not be obtained.

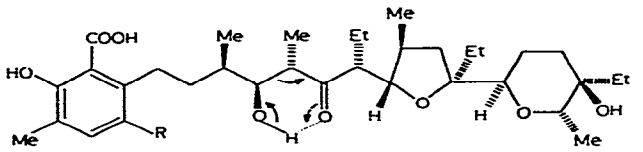
High-performance liquid chromatography

The major limitation of pyrolysis GLC—ECD analysis of [I] was the undefined specificity of the determination. The pyrolysis product, 4-bromo-5,6-dihydro-2,7-dimethyl-1-naphthol [II] constitutes only a small portion of the total bromo-lasalocid molecule [I], and as such metabolic transformation in the larger portion of the molecule would be undetected. The HPLC analysis of intact [I] was undertaken due to the undefined specificity of GLC—ECD.

Ultraviolet detection. The UV spectrum of [I] in methanol shows absorbance maxima at 212 and 315 nm, the former being approximately eightfold more intense (Table IV). Reversed-phase HPLC examination of the residue

TABLE IV

UV ABSORPTION AND FLUORESCENCE EMISSION CHARACTERISTICS OF BROMO-LASALOCID AND ITS ANALOGUES DETERMINED IN THE HPLC MOBILE PHASE



Compound (free acid)	R	UV absorption		Fluorescence	
		$\lambda_{abs.\ max}$ (nm)	Absorptivity (L/g-cm)	λ_{em} (nm)	Quantum efficiency* (Φ F)
Bromo-lasalocid	Br	212	43		
		315	5.5	425	0.014
Lasalocid	H	209	59		
		245	8.3		
		310	6.4	418	0.16
Chloro-lasalocid	Cl	210	58		
		315	6.4	423	0.22
Iodo-lasalocid	I	212	47		
		318	4.7	425	0.025

*The quantum efficiencies were determined from emission spectra obtained while exciting at the 310—320 nm maxima.

of benzene extracts of alkalinized plasma (pH 12.8, 0.7 *M* Na_3PO_4) on a μBondapak C_{18} column using a mobile phase of methanol—water—glacial acetic acid (90:10:1) showed that endogenous constituents in the control sample precluded measurement of [I] at 212 nm (variable-wavelength UV detector, Tracor Model 970A, Austin, TX, U.S.A.). However, [I] in this extract could be quantitated at 254 or 313 nm (fixed wavelength mercury-lamp detectors, Model 440, Waters Assoc.) with no interference from endogenous materials. Detection at 254 nm is preferred since the signal is 1.36 times greater with a signal-to-noise ratio two- to four-fold higher than that at 313 nm.

The HPLC—UV assay described above for [I] has an overall recovery of 65—70% in the concentration range of 1.0—5.0 μg of [I] added per ml of plasma. The data are best described by a linear regression equation of the form $Y = aX + b$ ($Y = 1.755\ X$—0.19) with a correlation coefficient (r) of 0.985 and an average deviation of 8.47%.

Fluorescence detection. The use of conventional spectrophotofluorometry [7] or fluorescence detection following HPLC separation [9] with excitation at 315 nm (as described for the high-sensitivity determination of lasalocid) is not feasible for [I] due to its ten-fold lower quantum efficiency yield (Table IV). This phenomenon is probably directly attributable to the bromine atom in the salicylic acid moiety which causes delocalization of the π electrons of the molecule.

The use of fluorescence detection with excitation at 315 nm following

HPLC did not increase the sensitivity of the analysis above that obtained using absorptiometric detection at 254 nm or 313 nm. However, the use of fluorescence detection with excitation at short UV wavelengths (215 nm) in conjunction with the use of solvents transparent at short UV wavelengths provided a five- to ten-fold increase in sensitivity beyond that obtained with UV absorption. In addition, the inherent specificity of fluorescence detection provided a much cleaner chromatographic baseline for plasma extracts than did absorptiometric detection at 215 nm.

A search for an analogue with fluorescence and HPLC characteristics similar to [I] suitable for use as an internal standard did not yield a useable compound. Although lasalocid, chloro-lasalocid, and iodo-lasalocid (Table IV) possessed UV absorbance and fluorescence emission characteristics suitable for either form of detection, they were not resolved from bromo-lasalocid under the conditions of the HPLC assay.

Compound [I] exhibits adsorption onto plasma constituents and onto glassware and stainless-steel surfaces at low concentrations. The extraction solvent was therefore changed from benzene (GLC—ECD analysis) to the more polar isooctane—ethyl acetate (90:10) which yielded more reproducible recovery at low concentrations, while 2-methoxyethanol was added to both the solvent and the mobile phase to improve the desorption and solvation of [I]. Solvent evaporation was performed at room temperature since elevated temperatures resulted in lower and more variable recovery of [I].

ACKNOWLEDGEMENTS

The authors are indebted to Mr. V. Mattaliano, Jr. for supplying the dog biological specimens, and to Mrs. W. Morley for the preparation of this manuscript.

REFERENCES

1 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 (1973) 397.
2 M. Osborne, J. Wenger, M. Zeuko, F. Kovselove and M. Cohan, in J.W. Westley (Editor), Polyether Antibiotics: Carboxylic Ionophores, Marcel Dekker, New York, 1982, Ch. 10.
3 J.W. Westley, R.H. Evans, Jr., T. Williams, Jr. and A. Stempel, J. Org. Chem., 38 (1973) 3431.
4 J.W. Westley, R.H. Evans, Jr. and A. Stempel, Anal. Biochem., 59 (1974) 574.
5 A. MacDonald, G. Chen, M.J. Duke and A. Popick, Poultry Sci., 52 (1973) 2055.
6 M. Osadca and M. Araujo, J. Ass. Offic. Anal. Chem., 57 (1974) 636.
7 M.A. Brooks, L. D'Arconte, J.A.F. de Silva, G. Chen and G. Crowley, J. Pharm. Sci., 64 (1975) 1874.
8 R.B. Hagel, J. Ass. Offic. Anal. Chem., 61 (1978) 1070.
9 M. Osadca and M. Araujo, J. Ass. Offic. Anal. Chem., 61 (1978) 1074.
10 G. Manius and V. Viswanathan, J. Pharm. Sci., 66 (1977) 769.
11 F. Vane and J. Pao, Hoffmann-La Roche, Nutley, NJ, 1975, unpublished data.