Table III—Recovery Studies of Known Ethambutol Hydrochloride Samples and Commercially Available Tablets

Amount Weighed, mg, or Claimed, mg/Tablet	Concentration Level, μg/ml	Amount Found, mg or mg/Tablet		Percent Recovery or Percent of Claim	
		Proposed Method	USP XIX Method	Proposed Method	USP XIX Method
		Known Samples			
42.1	3.10	42.6	—	101.2	—
46.4	3.42	46.2	—	99.6	—
50.6	1.86	48.7	—	96.2	—
50.7	3.74	50.2	—	99.0	—
63.8	9.40	62.8	—	98.4	—
201.6	—	—	192.8	—	95.6
203.1	—	—	195.3	—	96.2
Overall recovery, %				98.9	95.9
SEM				0.82	
		Commercial Tablets			
100.0	3.73	99.1	99.3	99.1	99.3
100.0	3.67	98.0	97.4	98.0	97.4
400.0	1.86	376.0	388.2	94.1	97.1
400.0	1.87	385.9	391.7	96.5	97.9

and each was analyzed at different concentration levels. The overall percent recovery of the five samples was 98.9% with a standard error of the mean of 0.82. Results obtained by applying the method to the analysis of commercially available ethambutol hydrochloride tablets are in agreement with those obtained by the compendial method.

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[x] To whom inquiries should be directed.

GLC Determination of Lasalocid and Its Bromo Analog as Their Silyl Derivatives

GERALD MANIUS and V. VISWANATHAN [x]

Abstract □ A GLC method is presented for the determination of lasalocid and its bromo analog. The method is based on the quantitative trimethylsilylation of the compounds without any molecular cleavage, followed by chromatography on a nonpolar silicone column. Silylation was carried out directly without any extraction or prior cleanup, despite the complexity of the dosage forms. This procedure was used for the assay of pure substances, pellets, premixes, experimental ampul solutions, and mycelial filter cakes. The results were in good agreement with data obtained by microbiological procedures.

Keyphrases □ Lasalocid and bromo analog—GLC analysis, various preparations and dosage forms □ GLC—analysis, lasalocid and bromo analog, various preparations and dosage forms □ Coccidiostatic antibacterials—lasalocid and bromo analog, GLC analysis, various preparations and dosage forms

The isolation of lasalocid (I), an antibiotic, was reported by Berger *et al.* (1). The structural configuration and the existence of four homologs for this antibiotic were described subsequently (2–4). Recently, a microbiological cylinder-plate assay (5) and a spectrofluorometric procedure for the determination of lasalocid in premixes (6) were reported. A GLC procedure also was reported in which the antibiotic was assayed after thermal degradation to yield the retroaldol ketone (7).

The bioassay procedure is a good indicator of the mi-

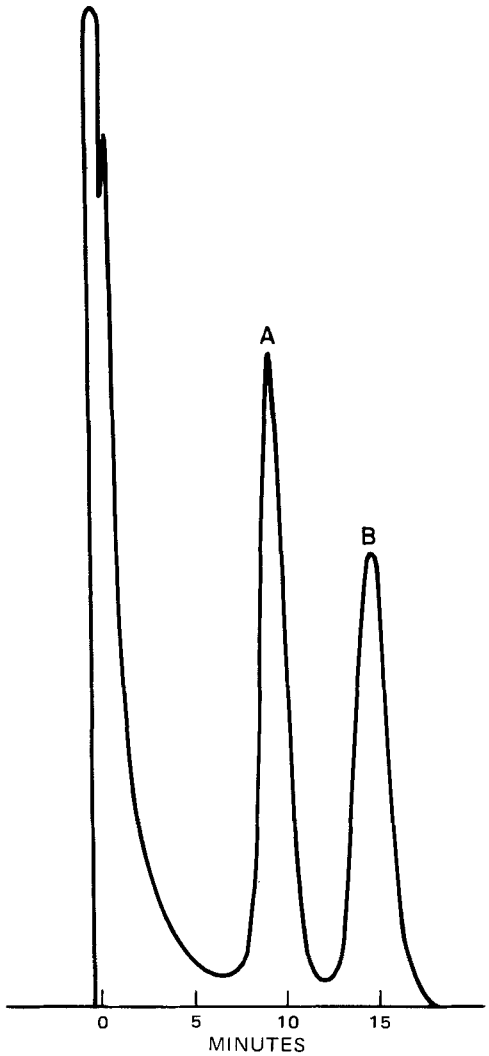


Figure 1—*Chromatogram of I (A) and II (B) trimethylsilyl derivatives.*

crobiological activity of the antibiotic. However, it is generally subject to interference from other antibiotics or biologically active degradation products and is very time consuming. Chemical methods such as the spectrofluorometric procedure require extraction and cleanup of the samples prior to analysis. GLC of the thermolysis product of lasalocid was reported to be quantitative; however, the degradation product of the antibiotic was assayed rather than the intact compound to establish sample purity. In most analytical applications, this approach is considered unsatisfactory, particularly for the assay of finished products for clinical use.

During this investigation, emphasis was placed on developing a specific assay procedure for the antibiotic in various dosage forms within a reasonably short time and with minimal sample preparation. Chromatographing the intact molecule in the form of a stable derivative appeared suitable, since this method would offer several advantages such as specificity for the antibiotic and the ability to resolve and quantitate the active ingredient in the presence of decomposition products and substances that interfere in other methods. In addition, a suitable derivative of the antibiotic would lend itself easily to confirmatory tests such as mass spectrometry when the intact antibiotic must be detected in complex media. Trimethylsilylation of the

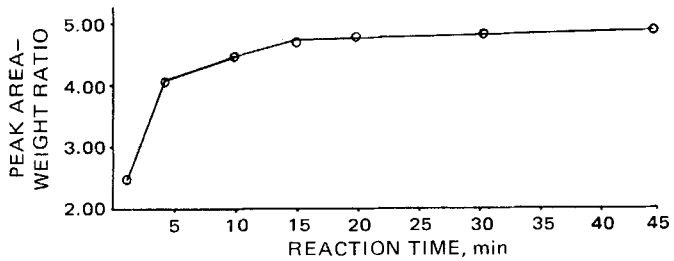


Figure 2—*Completeness of silylation reaction.*

carboxyl and secondary alcohol functions of the antibiotic was chosen, since this approach was used successfully for the assay of other antibiotics such as chloramphenicol, penicillin, erythromycin, neomycin, and lincomycin (8–14).

EXPERIMENTAL

Apparatus—A gas chromatograph equipped with a hydrogen flame-ionization detector was used. The chromatographic column was type 304 stainless steel, 61 cm in length and 3.2 mm o.d., fitted for on-column injections. The packing was 3% methyl silicone fluid on acid-washed, silane-treated diatomite support, which was sieved to specification before and after liquid phase coating. Data acquisition and quantitation were carried out using a computer system. All injections were made using a 10-μl syringe.

Reagents—*N,O*-Bis(trimethylsilyl)acetamide, *N*-trimethylsilylimidazole, trimethylchlorosilane, and tetrahydrofuran (reagent grade) were used as received. For the silylation reaction, a 3:3:2 (v/v) mixture of *N,O*-bis(trimethylsilyl)acetamide, *N*-trimethylsilylimidazole, and trimethylchlorosilane was made. To protect the stability of the reagent, mixtures were prepared fresh in small quantities and stored in tightly sealed containers under refrigeration.

Operational Parameters—For the chromatography of lasalocid and its bromo analog (II), the temperatures were: column, 260°, isothermal; injector port, 260°; and detector, 290°. The flow rate for the nitrogen carrier gas was 30 ml/min; the flow rates of hydrogen and air to the detector were set at 30 and 300 ml/min, respectively. The injection volume was 5 μl, and the attenuation was nominally set at 64× at a range setting of 10^{-11} amp/mv. Under these conditions, the trimethylsilyl derivatives of I and II eluted at retention times of 10 and 15 min, respectively.

Preparation of Solutions for Assay—*Reference Standard*—Approximately 20 mg of I or II reference material was accurately weighed into a 10-ml volumetric flask. One milliliter of the silylating reagent mixture was added, and the flask was capped and allowed to stand at ambient temperature for a minimum of 30 min. After the reaction was completed, the solution was diluted to 10 ml with tetrahydrofuran.

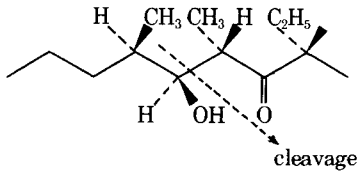
Sample Solutions—Pure substances were prepared in the same manner as the reference standard preparation. For premix pellets, final premixes, or mycelial cake materials, samples equivalent to approximately 20 mg of active ingredient were weighed into a 10-ml volumetric flask, silylated for 30 min or longer, and diluted as described. Small, accurately measured quantities of experimental ampul solutions equivalent to 2.0 mg of I were transferred into small vials, 2–3 drops of anhydrous methanol were added, and the mixture was evaporated to dryness under mild heat with a stream of nitrogen and reacted with exactly 1.0 ml of silylating reagent prior to chromatography.

Precautions were taken to exclude excessive heat, moisture, and metallic contact during the standard and sample preparations.

Table I—Statistical Evaluation of III Assay

Determination[a]	Assay, %
1	89.9
2	90.1
3	88.7
4	90.4
5	91.3
6	87.3
Mean	89.6
SD	±1.41

[a] Each determination represents individual silylation and assay.



Ia

Prior to analyzing the samples, the chromatographic column was conditioned by making several 5.0-μl injections of appropriate reference standard solution until a constant response was obtained. The calculations were carried out in a conventional manner using standard and sample peak areas.

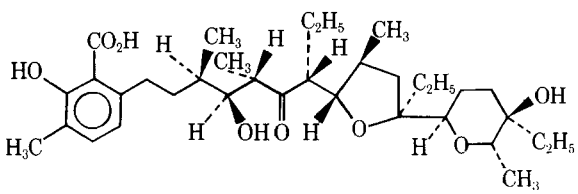
RESULTS AND DISCUSSION

Chemical Stability—High temperature causes cleavage of I at an apparent weak point (Ia) due to hydrogen bonding stress between the hydroxyl and carbonyl groups. Westley *et al.* (7) utilized this thermal degradation as the basis of a GLC assay wherein the retroaldol ketone formed during the degradation was assayed rather than the intact antibiotic.

Even though these authors utilized an injector port at 300° as an "on-site" pyrolytic chamber, experiments indicated that I in solutions would degrade with relative ease at a much lower temperature range. Due to the inherent nature of the compound to undergo cleavage at high temperatures, derivatization methods to chromatograph the intact molecule had to be carried out under ambient conditions. Attempts to silylate the compound at elevated temperatures did indeed result in multiple peaks, indicating degradation.

Silylation—*N,O*-Bis(trimethylsilyl)acetamide silylated the free acid to some extent at ambient temperatures but had no effect on the sodium salt of lasalocid (III). The addition of trimethylchlorosilane had the twofold effect of freeing I from its sodium chelate by acting as a Lewis acid and catalyzing the formation of more silylated derivative. This reagent combination, however, required 12 hr or longer to produce a quantitatively silylated product. The resulting derivative was unstable on standing (indicated by shoulder peaks), particularly after dilution with solvent, probably due to dissociation of the trimethylsilyl group from hindered sites.

The addition of the third ingredient, *N*-trimethylsilylimidazole, enhanced the reaction considerably. This reagent has the distinct advantage



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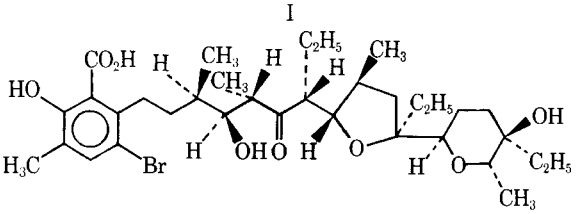

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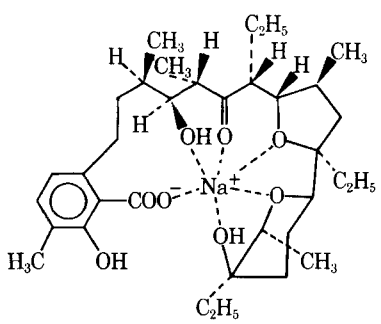

III

Table II—GLC Assay of Pure Substances

Sample	GLC Method, %	Alternative Method, %
I free acid	91.9	92.0[a]
I free acid	95.6	94.5[a]
II free acid	98.9	99.0[a]
III	89.8	88.8[b]
III	91.5	89.5[b]
III	90.8	89.5[b]

[a] Titration. [b] Microbiological.

of silylating the hindered hydroxyl groups due to its superior ability to accommodate a negative charge in the transition state (15). It also may act as a synergistic catalyst in the presence of trimethylchlorosilane to enhance the silylation of all active sites. The quantities of excess reagent present in sample solutions effectively prevented any dissociation of trimethylsilyl groups. The prepared solutions were stable during chromatography, as indicated by reproducible, well-resolved peaks for I and II derivatives (Fig. 1).

A time study was carried out on the silylation reaction at ambient temperature, and derivative formation reached equilibrium in about 30 min (Fig. 2). A time range of 30–45 min was satisfactory for the silylation of I in dosage forms. Due to the very reactive nature of the reagent mixture, contact with metallic surfaces such as aluminum cap liners was avoided throughout the study. Excess moisture in samples also was removed with mild heat and nitrogen prior to silylation to prevent an exothermic reaction with the reagent. Samples of I derivatives were subjected to GLC–mass spectrometric analysis, and the results confirmed the anticipated trimethylsilyl substitution of all carboxyl and alcohol functions, with the molecular ion observed at *m/e* 878.

Detector Response—The response of the flame-ionization detector to I and II derivatives was linear over a wide range. The II derivative was also chromatographed using scandium electron-capture detection, and the minimum detectable level was approximately 30 ng *versus* 300 ng for hydrogen flame-ionization detection.

An ideal internal standard could not be found, mainly due to solubility and elution characteristics associated with high molecular weight compounds. The external standard method used in the study yielded reproducible results.

Assay Results—A statistical analysis was carried out on the assay of III; the relative standard deviation was ±1.4%, significantly better than what is generally feasible in any microbiological assay method (Table I). Several samples of I and II pure substances also were assayed by this method, and the results were in good agreement with data obtained using other analytical techniques (Table II).

The procedure was used for the simultaneous assay of meglumine and active ingredients in I and II complexes with meglumine. The results were useful in establishing the molar concentrations of each component, depending upon the purity of materials used. However, the results could not be compared to other methods, since available chemical methodology was not reproducible for the assay of both chemical moieties of the complex (Table III). Other complex dosage forms such as ampuls (containing propylene glycol, preservative, and water), pellets, and feed premixes also were assayed with no special sample preparation. The chromatographic peak was free from interference, and the results are listed in Table IV.

All reported results are averages of duplicate determinations.

Specificity—During the assay of meglumine complexes, the chromatographic run was carried out at an initial column temperature of 160° for the elution of the meglumine trimethylsilyl derivative peak. Subse-

Table III—Assay of Experimental I and II Complexes with Meglumine

Sample	Percent Active by GLC	Percent Theory	Meglumine Percent by GLC	Meglumine Percent Theory[a]
I–monomeglumine	75.3	78	20.3	22
I–dimeglumine	52.5	62	39.8	38
I–dimeglumine	52.1	62	36.3	38
II–dimeglumine	64.8	65	35.6	35
II–dimeglumine	61.4	65	38.6	35

[a] Percent theory was calculated using molar ratios. Variations from the theoretical results were due to the presence of residual crystallization solvents, excess meglumine, and technical grade drug in the complexes.

Table IV—Assay of I in Other Matrixes

Sample	GLC Assay	Theoretical
Ampul solution 1	16.2 mg/2 ml[a]	20 mg/2 ml
Ampul solution 2	18.4 mg/2 ml[a]	20 mg/2 ml
Ampul solution 3	21.2 mg/2 ml	20 mg/2 ml
Premix pellets	2.53%	2.5%
Premix 1	14.8%	15.0%
Premix 2	16.1%	17.3%
Premix 3	16.4%	16.5%
Premix 4	16.2%	17.3%
Mycelial cake 1	6.3%	NA[b]
Mycelial cake 2	6.5%	NA
Mycelial cake 3	5.0%	NA
Mycelial cake 4[c] 1.1% excess	7.6%	7.4

[a] Decomposition of I in solution was indicated by the presence of an unknown GLC peak. [b] NA = not available. [c] Sample 1 to which had been added 1.1% of active ingredient to get an indication of recovery.

quently, the column temperature was raised to 260° to elute the I or II derivative peaks.

If any retroaldol ketone (known thermal degradation product) is present in the samples, it also would be silylated, resulting in a well-resolved peak suitable for quantitation. Samples of I and II complexes with meglumine were experimentally spiked with known amounts of retroaldol ketone and chromatographed after silylation. When the column temperature was programmed from 100 to 300°, trimethylsilyl derivatives of retroaldol ketone, meglumine, I, and II eluted at retention times of 7.5, 15, 30, and 35 min, respectively, clearly indicating the specificity and capability of the method for the assay of the antibiotic in multicomponent systems.

Furthermore, the I derivative peak area was approximately twice that of the retroaldol ketone when equal concentrations of I were subjected to the GLC–thermolysis method and the silylation procedure. This enhancement in peak response would be an added advantage in the analysis of low levels of I as a trimethylsilyl derivative.

The method presented has been applied to the assay of I in various solid and liquid preparations. It is specific for the antibiotic and does not require any extraction or pretreatment of the samples as in other reported methods.

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[x] To whom inquiries should be directed.

New Synthesis of 1,4,2-Dioxazoles and Their Pharmacological Properties

NED D. HEINDEL[*x], WILLIAM P. FIVES[*], and RICHARD A. CARRANO[‡§]

Abstract □ A series of 3-substituted 5-methoxycarbonyl-5-methoxycarbonylmethyl-1,4,2-dioxazoles was prepared by addition of hydroxamic acids to acetylene esters. Eleven of these previously unknown compounds were submitted to general pharmacological screening, and several displayed modest CNS depressant activity.

Keyphrases □ 1,4,2-Dioxazoles, substituted—synthesis, general pharmacological screening □ CNS activity—substituted 1,4,2-dioxazoles screened □ Structure-activity relationships—substituted 1,4,2-dioxazoles, general pharmacological screening

Most reported 1,4,2-dioxazoles are derived from the condensation of hydroxamic acids with phosgene (1, 2) or diethylacetals (3). Newer routes include a 1,3-dipolar addition method involving ketones and nitrile oxides (4) and a ring expansion of 2-acyloxaziridines (5). Except for some claims of antifungal activity in the 5-oxo derivatives (6) and of antibacterial potency in 5-benzenesulfonamido-substituted 1,4,2-dioxazoles (7), the possible pharmacological utility of this family of heterocyclics is still unknown.

CHEMISTRY

Reports from these laboratories noted the general utility of acetylenic esters in the synthesis of heterocyclic compounds generated by double nucleophilic additions to the same alkyne carbon (8, 9). In a similar fashion, hydroxamic acids, presumably in their enol tautomers (I) (10), reacted with dimethyl acetylenedicarboxylate (II*a*) and/or methyl propiolate (II*b*) (Schemes I and II) to produce high yields of 3-substituted 5-methoxycarbonyl-5-methoxycarbonylmethyl-1,4,2-dioxazoles (III*a*–III*j* and IV, Table I). In view of the paucity of information available on this heterocyclic family and the unique nature of this particular subclass, the general biological responses are now reported.

Available spectral evidence supported the assigned dioxazole structure.