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ANALYSIS OF ANTIBIOTICS BY GAS CHROMATOGRAPHY

I. LINCOMYCIN

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SUMMARY

The analysis of lincomycin and lincomycin B by an improved gas–liquid chromatographic method is described. The trimethylsilyl ether is formed and extracted before chromatography. The method has been applied successfully to bulk material and has been extended to determine the lincomycin content of capsules, syrups, injectables, and powders. The results compare favorably with those obtained by microbiological assay.

INTRODUCTION

Lincomycin is an antibiotic, produced by *Streptomyces lincolnensis* fermentation, which inhibits the growth primarily of Gram-positive bacteria. Its discovery and biological properties were reported by MASON *et al.*[1] and its chemical structure was elucidated by HERR AND BERGY[2] and HOEKSEMA *et al.*[3]. A congener, lincomycin B*, is produced concomitantly with lincomycin at a level of about 3% during biosynthesis[4]. The only structural difference between the two analogs is that lincomycin has a propyl substituent at position 4 on the pyrrolidine ring and lincomycin B has an ethyl group[5,6].

Lincomycin may be assayed microbiologically, either directly[7] or after isolation by thin-layer chromatography[8]. The official method used by the Food and Drug Administration in the certification of lincomycin hydrochloride monohydrate bulk and dosage forms is the microbiological assay[9]. An automated chemical assay reported recently[10] is based upon acid cleavage of the antibiotic, followed by determination of the liberated methanethiol with a disulfide color reagent. However, these methods lack specificity and lincomycin cannot be differentiated from lincomycin B or from a number of other antibiotics. Lincomycin B possesses the same antibacterial spectrum as lincomycin, but has only about 25% as much activity[11].

* Also known by the code number U-21699.

HOUTMAN *et al.*[4] developed a gas–liquid chromatographic method for the determination of both lincomycin and lincomycin B. The method employs silylation in pyridine and direct chromatographic analysis. Many determinations were performed in this laboratory using their method but several major difficulties were encountered.

One difficulty was that, since the silylating reagent reacts with practically all compounds with labile protons, lincomycin must be separated from significant amounts of substances containing groups such as hydroxyls, primary or secondary amines, and sulfhydryls. These compounds occur in dosage forms as solvents (water, glycols), saccharides (sucrose, lactose), and various additives such as flavors and preservatives. Individual adjustments were described[4] to compensate for extensive recognized interferences; for example, freeze-drying was used to eliminate the interference from water. However, this procedure does not remove sugars.

Because of the excessive silylating reagent, the electrode in the detector system of the gas chromatograph rapidly became contaminated, and the efficiency of the entire analysis was reduced considerably. The coating effect, also previously reported by other workers[12–16], could not be eliminated by injecting Freon 12 into the column as recommended by the manufacturer. After every four to five sample injections, the electrode had to be removed and cleaned to avoid gross impairment of electrode sensitivity and distortion of instrument response. This frequent cleaning resulted in undue damage to the electrode because of excessive handling, in the necessity of restandardization after each cleaning operation, and in premature replacement of the electrode.

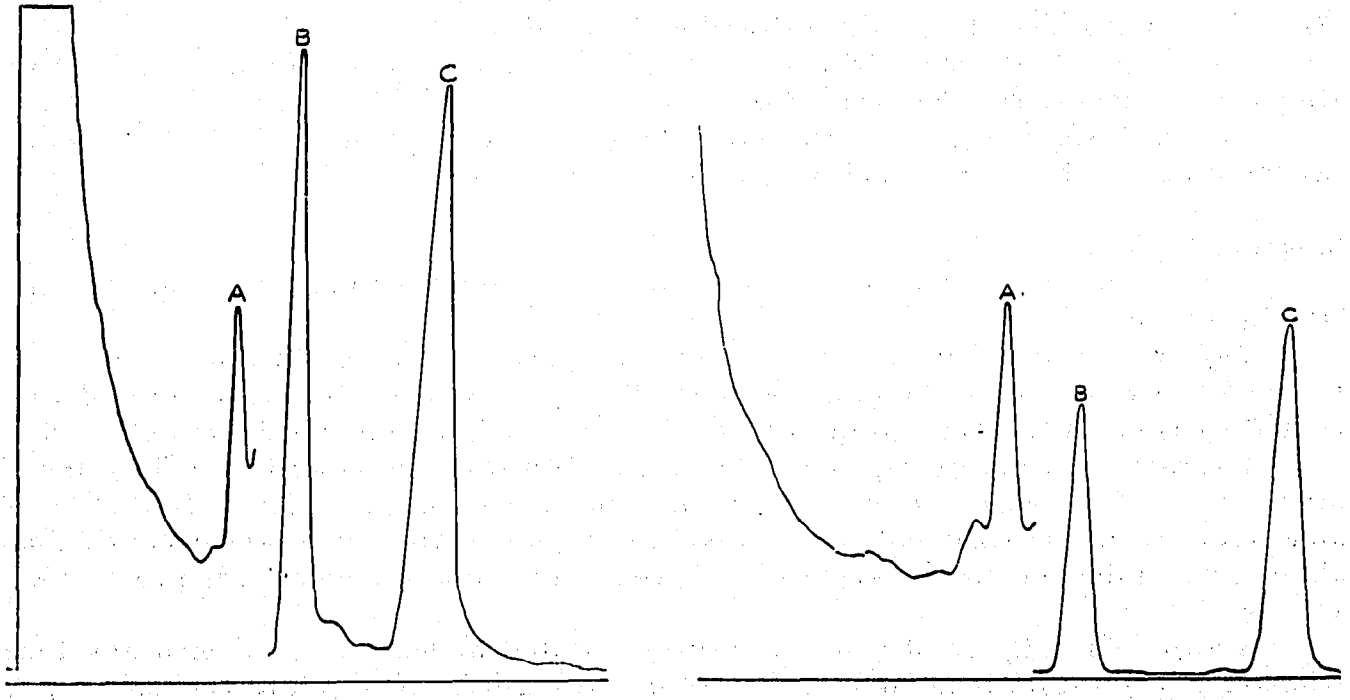


Fig. 1. Direct injection of silylated compounds in pyridine solvent. Glass column, 4 ft., 3 mm I.D., packed with 3% SE-30 on Diatoport S; column temperature, 240°; carrier, nitrogen at 51 ml/min. Key: A = lincomycin B; B = lincomycin; C = internal standard.

Fig. 2. Direct injection of silylated compounds in pyridine solvent. Glass column, 6 ft., 3 mm I.D., packed with 9.1% DC-200 on 80/100 mesh Gas Chrom Q: column temperature, 262°; carrier, nitrogen at 30 ml/min. Key: A = lincomycin B; B = lincomycin; C = internal standard.

Another difficulty was that the pyridine used as a solvent during preparation of the silyl derivative caused serious tailing in the chromatograms in the direct injection method (Figs. 1 and 2).

The method was evaluated in an attempt to minimize these difficulties, and was modified by extracting the silyl ether derivative into cyclohexane. A new method of preparing samples of dosage forms was developed and the applicability of the modified methods was studied.

EXPERIMENTAL

Apparatus

Gas chromatograph. A Barber-Colman Model 5000 gas chromatograph with a flame ionization detector was used; recorder, 5 mV range and $^1/_3$ in./min chart speed. Glass column: U-shaped, 6 ft. by 3 mm I.D., packed with 5% SE-30 on Gas-Chrom Q (80/100 mesh). Operating conditions: column temperature, 257°; detector temperature, 280°; injector temperature, 280°; carrier gas, nitrogen at 20 p.s.i., 150 ml/min; pressure of hydrogen, 32 p.s.i.; air, 40 p.s.i. (adjusted for maximum response). Current 2×10^{-8} A F.S.D.; sensitivity, 100, and attenuation, 2.

Reagents

Solvents. Anhydrous methanol, absolute ethanol, and cyclohexane, all reagent grade. Pyridine, also reagent grade, kept over potassium hydroxide.

Silylating reagent. Nine parts of hexamethyldisilazane mixed with one part of trimethylchlorosilane. The mixture is cleared by filtration.

Lincomycin standard. About 4 mg of lincomycin reference standard, accurately weighed and transferred to a centrifuge tube.

Internal standard. A saturated solution of tetraphenylcyclopentadienone* prepared in cyclohexane, and cleared by filtration.

Sample preparation

Bulk materials. Samples are prepared in the same manner as the lincomycin standard above.

Injections and sensitivity powders. The samples are diluted with or dissolved in methanol. An aliquot containing approximately 4 mg lincomycin is transferred to a centrifuge tube and is evaporated to dryness on a steam bath under a current of dry air.

Capsules. The empty capsule and its contents are added to methanol. The lincomycin is dissolved with gentle heat; the mixture is cooled and further diluted with methanol. After the sediment has settled, an aliquot of the supernatant containing about 4 mg of lincomycin is transferred to a centrifuge tube and evaporated to dryness as above.

Syrups. An aliquot of 5 ml of syrup is diluted at least 20-fold with absolute ethanol. The solution is heated, then cooled overnight to precipitate the sucrose. A portion of the supernatant is further diluted with methanol. An aliquot of the final dilution containing about 4 mg of lincomycin is transferred to a centrifuge tube and evaporated to dryness as above.

* J. T. Baker Chemical Co., Phillipsburg, N.J.

Derivatization

A lincomycin standard is treated in the same manner as the samples. Each dry or dried sample is dissolved in 1 ml of pyridine, and 0.2 ml of the silylating reagent is added. The reaction mixtures are allowed to stand not less than 30 min, 1.00 ml of the internal standard solution and 2 ml of water are added, and the mixture is shaken vigorously. The phases are separated by gravity or centrifugation.

Chromatography and calculations

Five microliters of the cyclohexane phase are injected into the gas chromatograph. The areas of each peak are measured by planimetry or by disc integration.

The lincomycin content is determined by direct comparison of the ratio of the peak areas (lincomycin:internal standard) with that of the lincomycin reference standard treated in an identical manner. Lincomycin B content in bulk material is determined as a fraction of the combined lincomycin + lincomycin B.

RESULTS AND DISCUSSION

The retention times of lincomycin, lincomycin B, the internal standards, and the residual sugars are shown in Table I. Under specified experimental conditions, the retention time of a particular entity is a good qualitative characteristic. Occasionally, identification of a substance may become complicated by the appearance of more than one major peak due to the formation of multiple derivatives, such as occurs with salicylic acid[17,18] or by the presence of such anomers as α- or β-lactose[19]. Various parabens[12,20], carbohydrates[19], and the possible interference of water[21] have been studied by silylation and gas chromatography. Under the conditions of this study, no qualitative interference has been found from any substance presently used in compounding lincomycin preparations.

TABLE I

RETENTION TIMES OF LINCOMYCIN AND OTHER COMPOUNDS

	Retention time (min)	*Relative retention time*
Lincomycin	7.65	1.00
Lincomycin B	6.12	0.78
Lactose	4.11, 6.00	0.54, 0.78
Sucrose	4.35	0.57
Internal standard	11.10	1.45

The improved method was used for single determinations of lincomycin samples, and the results were compared with the average of several microbiological assays (Tables II and III). The peak areas were obtained by a disc integrator and were normalized by the internal standard technique. The results of analyses of bulk samples of the various dosage forms show good correlation between the chromatographic and microbiological assays.

By using methanol as a solvent, most of the lactose present can easily be separated

TABLE II

ANALYSIS OF LINCOMYCIN HYDROCHLORIDE MONOHYDRATE BY IMPROVED METHOD

Sample No.	*GLC*		*Microbiological assay (μg lincomycin/mg)*	
	μg lincomycin/mg	*Lincomycin B (%)*	*Lab 1*	*Lab 2*
1	847	3.7	882	887
2	847	3.5	898	905
3	828	4.7	908	888
4	903	2.6	897	874
5	843	3.0	886	883
6	824	4.0	829	874
7	868	4.7	892	874
8	868	4.5	871	874
9	862	2.2	880	888
10	856	2.4	881	873
11	846	1.8	886	870
12	849	1.5	911	876
13	849	3.5	885	853
14	834	2.0	902	866
15	817	3.3	904	867
16	846	2.8	873	868
17	846	3.7	890	859
18	845	3.4	872	860
19	871	4.2	860	853
20	839	3.7	873	854
21	869	3.4	889	854
22	862	4.4	898	856
23	865	4.0	900	862
24	849	3.8	899	851
25	845	3.8	906	867
26	843	4.4	858	863
27	854	3.1	906	864
28	858	2.7	880	861
29	862	3.4	887	856
30	840	4.3	864	867
31	851	4.1	865	874
32	859	4.4	848	852

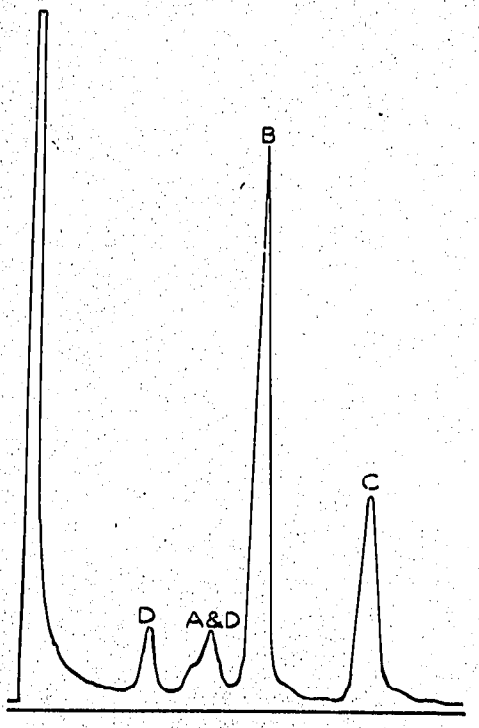
from lincomycin. As shown in Fig. 3, small amounts of residual lactose do not interfere with the gas chromatographic resolution of lincomycin.

Although sucrose in lincomycin syrup causes interference, diluting the syrup with absolute ethanol precipitates most of the sucrose, in time. Precipitation is improved and accelerated by subjecting the alcoholic solution to ultrasonic vibrations. Separation of the lincomycin from most of the sucrose can be achieved also by extracting an alkaline solution of the sample with chloroform (Fig. 4).

Water in lincomycin hydrochloride injection reacts with the silylating reagent to produce primarily trimethylsilanol. Water was eliminated simply by diluting the sample with methanol and evaporating an aliquot to dryness.

Small amounts of residual water, sugars, and other additives do not interfere, if sufficient silylating reagent remains to react quantitatively with the antibiotic (see Fig. 5). Fig. 6 shows that insufficient separation from sucrose yielded a recovery of lincomycin as low as 73%.

The silylation of compounds with labile protons is a reaction which is useful in



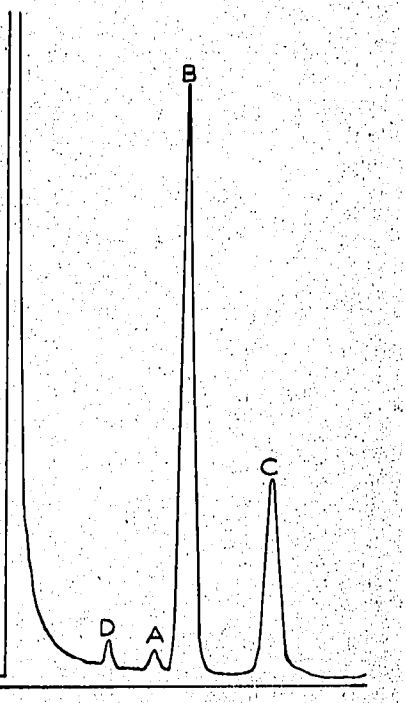
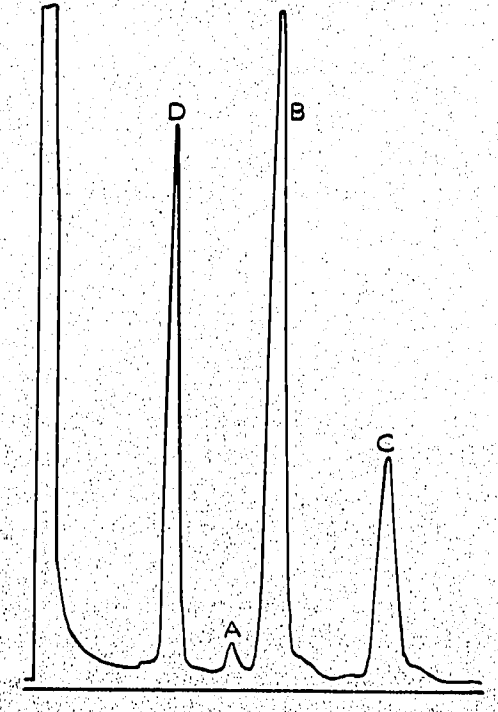


Fig. 3. Chromatogram of silylated compounds from capsules by the improved method. Key: A — lincomycin B; B = lincomycin; C — internal standard; D = lactose.

Fig. 4. Chromatogram of syrup sample extracted with chloroform. Column, 5% SE-30 on Gas Chrom Q; column temperature, 255°; carrier, nitrogen at 150 ml/min. Key: A = lincomycin B; B = lincomycin; C = internal standard; D = sucrose.



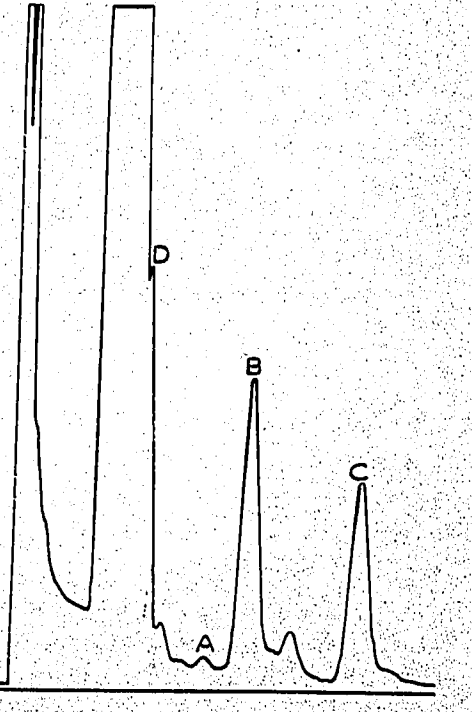


Fig. 5. Syrup with most of the sucrose removed before silylation; improved method, conditions as in Fig. 3. Key: A = lincomycin B; B — lincomycin; C = internal standard; D = sucrose.

Fig. 6. Syrup with excess sucrose in reaction medium showing incomplete recovery of lincomycin by the improved method. Key: A — lincomycin B; B = lincomycin; C = internal standard; D = sucrose.

TABLE III

ANALYSIS OF LINCOMYCIN IN DOSAGE FORMS BY IMPROVED METHOD

Sample No.	*GLC (mg)*	*Microbiological assay (mg)*	*(mg)*
		Lab. 1	*Lab 2*
	Syrup (5 ml)		
1	234	250	252
2	268	238	243
3	255	238	243
4	254	250	245
5	271	251	245
	Injection, syringe		
1	642	629	
2	627	629	
3	699	632	
4	694	632	
5	655	622	
6	655	630	
	Injection, vial (ml)		
1	323	304	327
2	328	308	319
3	330	306	320
4	307	313	314
5	299	311	330
6	334	316	323
7	323	306	341
	Capsule		
1	250	250	251
2	514	515	507
3	498	496	496
4	505	502	488
5	513	505	513
6	265	245	250
7	265	254	251
8	538	505	500
	Powder, vial		
1	20.7	21.4	20.3

organic analysis, and its use in gas chromatography, thin-layer chromatography, and infrared and mass spectroscopy is well established[22]. Silylation of organic compounds tends to increase volatility, thermal stability, and solubility in aprotic solvents. The original compound is easily converted to and recovered from the trimethylsilyl ether derivative[23,24].

Various solvents for the silylation reaction were studied. Recoveries indicated that silylation of lincomycin was only about 10% complete in dimethyl sulfoxide and from 75 to 100% complete in ethyl acetate, acetone, and acetonitrile. Based on overall qualitative and quantitative performance, acetonitrile was comparable to pyridine. Pyridine is preferred over other solvents because of its solubilizing power.

A mixed silylating reagent was employed for convenience. CARTER AND GAVER[25] have previously shown that using a silylating reagent mixture for long-chain bases

resulted in more reproducible and reliable analyses than when the reagents were added individually.

The silyl ether derivative of lincomycin was extracted into cyclohexane in an attempt to seperate it from any possible interfering substances present in antibiotic preparations. This extraction vastly reduces solvent (pyridine) tailing and chromatographic peaks are symmetrical. The water added during the extraction provided an immiscible phase which scavenges excess silylating reagent, resulting in a cleaner electrode, and dissolves the ammonium chloride formed during the silylation, eliminating the occasional sticking of solids in the microsyringe.

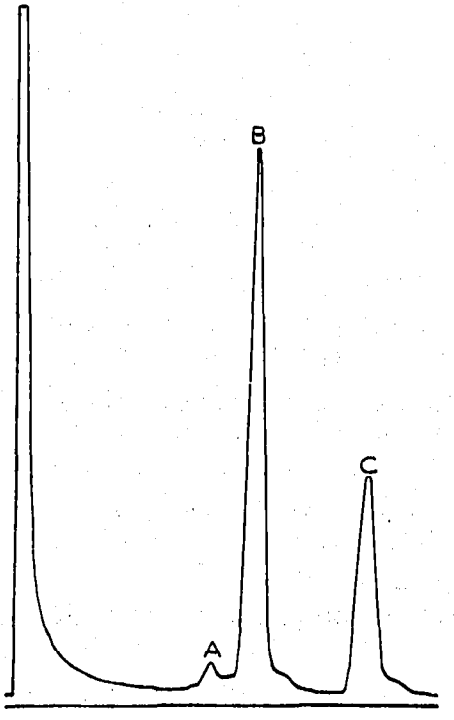


Fig. 7. Chromatogram of lincomycin derivative by the improved method; conditions as in Fig. 3. Key: A = lincomycin B; B = lincomycin; C = internal standard.

Two stationary phases were investigated. Although DC-200 (Fig. 2) was superior to SE-30 (Fig. 1), the latter was used for routine work because of its reportedly greater thermal stability. Other combinations of inert phases and supports were not studied.

A saturated solution may not be the best choice for an internal standard, but it is dictated in this case because of the relatively low solubility of tetraphenylcyclopentadienone in cyclohexane. When the samples and standard are prepared at the same time, the actual concentration of the internal standard is inconsequential, as long as the peak areas can be normalized. This procedure has worked well in practice.

During the initial phase of this study, seven bulk lots and two samples of lincomycin injection were analyzed by the direct injection technique[4]. Five methods of quantitation were used with and without the internal standard.

All these calculation methods give comparable values and the injection volume seemed to be replicated well enough to indicate that the internal standard may not always be necessary. However, in routine work it is recommended that an internal

standard be used as a built-in check of the instrument, the operator, and the procedure. The use of an integrator, mechanical or electronic, is desirable.

Initially, because changes in the attenuation of the recorder precluded the use of the disc integrator in our system, the lincomycin B content was calculated as a fraction of the total area by planimetry (Figs. 1 and 2). The response factors of each lincomycin were assumed to be essentially equal because of the close similarity of the structures and the amounts of carbon in the molecules. In the improved method, the attenuation remains fixed and an accurate disc integrator value can be obtained (Fig. 7).

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