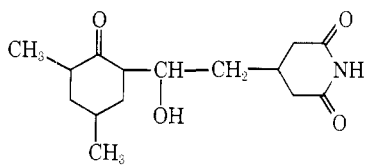
Gas Chromatographic Assay of the Antibiotic Cycloheximide

Leo W. Brown

A gas chromatographic assay procedure is described for the antibiotic cycloheximide in bulk drug and formulations. The method, which has a coefficient of variation of approximately 2%, involves silylation of cycloheximide followed by treatment with isopropyl alcohol to produce the monotrimethylsilyl derivative which chromatographs as a single peak. Isocycloheximide and the principal dehydration product (anhydrocycloheximide), two possible impurities in cycloheximide samples, can also be determined by this procedure.

Cycloheximide (marketed under the trademark Actidione by The Upjohn Company, Kalamazoo, Michigan) is obtained from *Streptomyces griseus*. Its fungicidal properties have been studied by several workers (Felber and Hammer, 1948; Gill, 1950; Leaphart and Wicker, 1968; Petersen and Cation, 1950; Tukey, 1948; Vaughn, 1951).

The structure and chemistry of cycloheximide I have been characterized (Kornfeld *et al.*, 1949; Lawes, 1962; Okuda *et al.*, 1958; Paul and Tchelitcheff, 1955). Starkovsky *et al.* (1966) have shown the β-hydroxy ketone structure to be susceptible to dehydration, forming anhydrocycloheximides. Sixteen stereoisomers of cycloheximide are possible.



I

mol wt 281; mp, 120°; pK 11.2

Whiffen (1948) assayed cycloheximide by a microbiological procedure, while Forist and Theal (1959) and Okuda *et al.* (1961) developed spectrophotometric procedures. A gas chromatographic method is presented here.

EXPERIMENTAL SECTION

Gas Chromatography Conditions. An F&M 402 gas chromatograph with flame ionization detection was used. A glass U-shaped column, 3 mm by 61 cm, was packed with 1% QF-1 on Gas Chrom Q (80 to 100 mesh). Column temperature was 200° with the detector at 225° and injection port at 200°. Helium was used as the carrier gas at a flow rate of 60 ml per min. The sample volume injected was 1 μl with the attenuation adjusted to give peaks at least 50% of full scale on the recorder. The sample was injected directly into the glass column.

Internal Standard Reagent Solution. A pyridine solution was prepared containing approximately 6 mg of cholesteryl acetate, and 0.1 ml of bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane per ml. This mixture of silylating reagents is available as Regisil TMCS (Regis Chemical Co., Chicago, Ill.).

Bulk Drug Sample Preparation. Approximately 5 mg of bulk cycloheximide was accurately weighed in a 1-dram tablet vial to which 1.0 ml of internal standard reagent solution was added. The vial was swirled gently to dissolve the solid and placed in an oil bath at 50° for 2 hr. The vial was removed from the oil bath and 1.0 ml of pyridine containing 3% isopropyl alcohol was added. After swirling the solution gently to mix, it was allowed to stand for 20 min and then chromatographed using the above conditions.

Formulations. For formulations containing materials which are soluble in organic solvents but relatively insoluble in water, a sample equivalent to 10 mg of cycloheximide was weighed in a 15-ml centrifuge tube and the cycloheximide was leached from the sample with 2 ml of water. A 1.0-ml aliquot of this solution was then extracted with chloroform and the resulting chloroform solution was evaporated to dryness with nitrogen. The sample was then treated as in the bulk drug sample preparation. For formulations containing materials such as ferrous sulfate and sodium alkyl aryl sulfonic acid, which are insoluble in organic solvents, the sample was leached directly with benzene. An aliquot of the benzene solution was evaporated to dryness, and treated as in the bulk drug sample preparation.

Reference Standard Preparation. Same as bulk drug preparation.

Calculations.

$$R_s/R_{std} \times W_{std}/W_s \times F = \text{mg of cycloheximide per mg of sample}$$

where R_s = peak area ratio of sample peak to internal standard peak, R_{std} = peak area ratio of reference standard peak to internal standard peak, W_{std} = weight of reference standard in mg, W_s = weight of sample in mg, and F = assigned potency of reference standard.

Combined Gas Chromatography–Mass Spectrometry (gc–ms). An LKB Model 9000 instrument was employed with a gas chromatographic column similar to that used in the assay work. The ion source temperature was 270°, ionizing current was 60 μA, ionizing voltage was 70 eV, and accelerating voltage was 3500 V. The scanning time was approximately 20 sec (10–500 amu).

DISCUSSION AND RESULTS

If cycloheximide is not derivatized prior to gas chromatography, it will extensively degrade on the column. Several attempts, using different reagents, columns, and temperatures, failed to yield a single peak by direct silylation. All conditions produced two peaks as shown in Figure 1. The elevated valley between the two peaks indicated one of the peaks to be labile. Gc–ms showed the peaks to be the mono and bistrimethylsilyl derivatives of cycloheximide. Since the bis derivative could not be formed quantitatively, it was considered to be very labile. Alcohol was therefore added to the reaction mixture in an attempt to convert the bis derivative quantitatively to the mono derivative. This attempt succeeded, as shown in Figure 2. As expected, addition of the same amount of alcohol (stoichiometric with silylating reagent) prior to the silylating reagents simply resulted in no silylation of the cycloheximide. Quantitative conversion of cycloheximide (I) to the monotrimethylsilyl derivative (I-TMS) can be summa-

Control Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001.

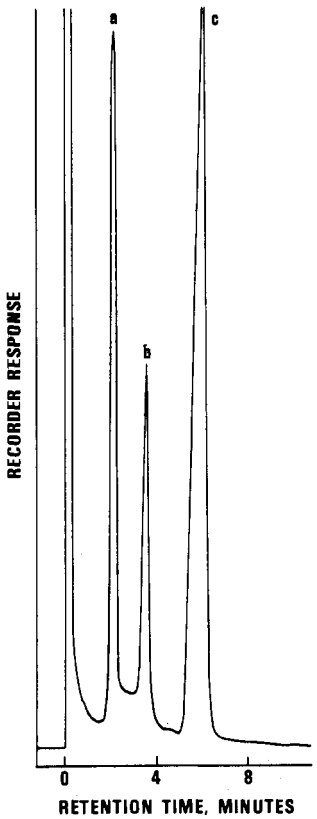


Figure 1. Chromatogram of (a) III, (b) II, and (c) cholesteryl acetate (internal standard).

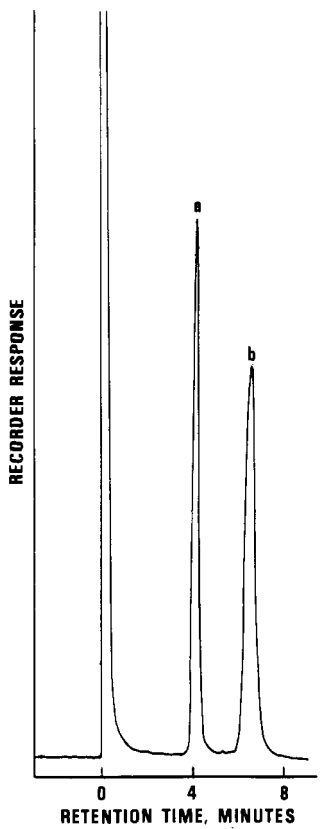


Figure 2. Chromatogram of (a) II and (b) cholesteryl acetate (internal standard).

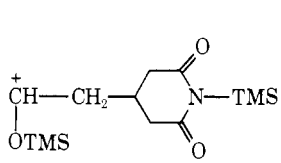
rized, therefore, by the following equations.

$$\text{I} + \text{TMS reagents} \rightarrow \text{I-TMS} + \text{I-(TMS)}_2 \quad (1)$$

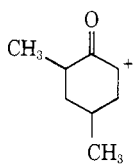
$$\text{I-(TMS)}_2 + \text{alcohol} \rightarrow \text{I-TMS} + \text{TMS} - \text{alcohol} \quad (2)$$

Several alcohols or other compounds which silylate readily can be used to convert the bis to the mono derivative. Isopropyl alcohol was arbitrarily chosen for the present assay procedure.

In order to establish the positions of the trimethylsilyl groups on the mono and bistrimethylsilyl derivatives of cycloheximide, gc–ms was employed. In the spectrum of the bistrimethylsilyl derivative a very intense *m/e* 300 was produced. A possible derivative fragment of this mass is shown in **a**.

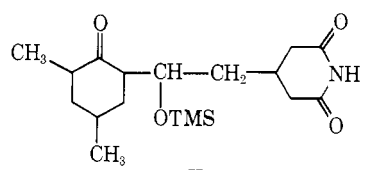


a
m/e 300

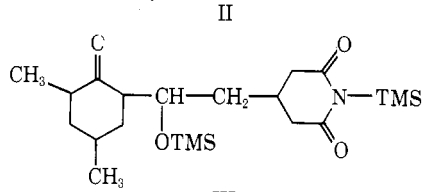


b
m/e 125

A mass of 197 would be expected if the enol form of the carbonyl in fragment **b** were silylated. Indeed, a *m/e* 197 was found in the spectrum, along with a *m/e* 125, so that no conclusions involving silylation of the enol could be drawn from these data alone. For this reason, cyclohexanone was carried through the silylation procedure as a model compound. No reaction took place, indicating that the enol form of cycloheximide does not silylate under the conditions used in this study. The two trimethylsilyl groups on the bis derivative were, therefore, considered to be on the hydroxyl oxygen and imide nitrogen as indicated by fragment **a**. To confirm this, phthalimide was also carried through the silylation procedure. Using the gc–ms technique, this compound was found to silylate to the monotrimethylsilyl derivative and then revert to the original compound upon the addition of isopropyl alcohol. In another experiment, silylation of the *N*-methyl derivative of cycloheximide produced only the monotrimethylsilyl derivative. It was concluded, therefore, that the structures of the mono and bistrimethylsilyl derivatives of cycloheximide are II and III, respectively.



II



III

With the conditions given in the Experimental Section, linearity was obtained for cycloheximide solutions ranging in concentration from 1 to 4 mg/ml. Assay results for most bulk cycloheximide and formulation samples gave a coefficient of variation of approximately 2%. Using this assay procedure, several lots of cycloheximide were assayed. Typical results for replicate samples of one lot are shown in Table I. Several products, containing 1 to 21% cycloheximide, were also assayed. The agreement of the assay value with theory was generally within 2% relative.

Nine samples of a bulk lot of cycloheximide were assayed microbiologically and by the gas chromatographic procedure. The results averaged 99.6% by the microbio-

Table I. Assay Precision and Comparison of Replicate Cycloheximide Samples *vs.* Cycloheximide Primary Standard (100%)

Sample wt, mg	Peak area ratio/weight		Percent cycloheximide in sample
	Sample	Standard	
5.020	0.3654	0.3637	100.5
5.314	0.3623	0.3606	100.5
4.884	0.3451	0.3496	98.7
4.966	0.3611	0.3567	101.2
5.104	0.3649	0.3521	103.6
5.070	0.3467	0.3543	97.9
			Average 100.4
			S.D. 2.00
			C.V. 1.99%

logical assay and 100.3% by the gc procedure (Table II). The results correlate well within the precision of the assay procedures.

Having demonstrated that the gc assay correlated satisfactorily with the microbiological assay on highly pure samples, it was of interest to determine whether this correlation would hold also for degraded samples. Since cycloheximide is known to degrade in alkaline solution, a sample was dissolved in methanol containing 0.1 *N* ammonium hydroxide. This solution was assayed at various time intervals by the gas chromatographic and microbiological assay methods. Samples of the solution were analyzed until degradation was nearly 100%. The rates of degradation of the cycloheximide sample as determined by the two methods gave a correlation coefficient of 0.983, indicating a significant correlation at the 95% probability level between the two assay methods, even on extensively degraded samples of cycloheximide. The gas chromatograms of the degraded samples showed that peaks were

Table II. Comparison of Gas Chromatographic and Microbiological Assay Results of Nine Samples of the Same Cycloheximide Bulk Drug Lot

Sample wt for gc, mg	Percent cycloheximide found	
	Gc assay	Microbiological assay
5.040	100.3	103.8
5.040	99.8	99.1
4.896	102.4	99.1
4.930	101.6	95.4
5.250	97.6	107.9
5.294	100.6	94.6
5.020	102.6	93.7
5.314	101.7	108.4
4.884	96.9	94.3
	Average 100.3	99.6
	C.V. 2.0%	5.8%

Table III. Relative Gas Chromatographic Retention Times of Cycloheximide and Related Compounds

Compound	Relative retention time
2,4-Dimethylcyclohexanone	0.18
Cycloheximide bis-TMS (III)	0.58
Isocycloheximide mono-TMS	0.79
Cycloheximide mono-TMS (II)	1.00
Anhydrocycloheximide (V)	1.29
Cholesteryl acetate (internal standard)	1.63

produced corresponding in retention times to those of isocycloheximide, an anhydrocycloheximide, and 2,4-dimethylcyclohexanone (see Table III for relative retention times).

Isocycloheximide is the isomer of cycloheximide, reported by Lemin and Ford (1960), which differs in config-

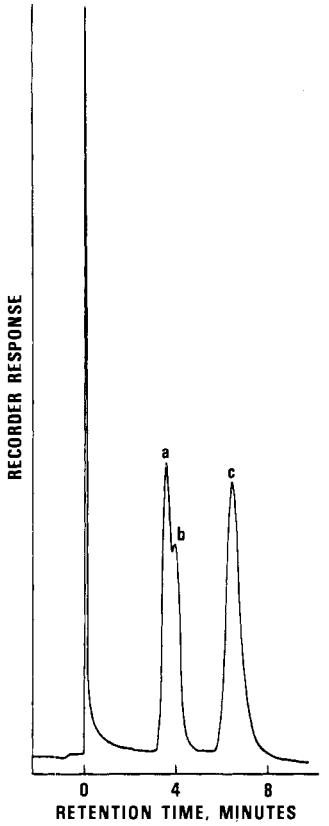


Figure 3. Chromatogram of (a) mono-TMS isocycloheximide, (b) II, and (c) cholesteryl acetate.

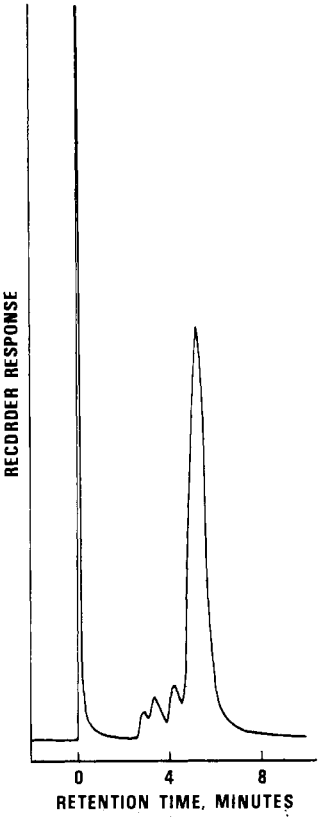
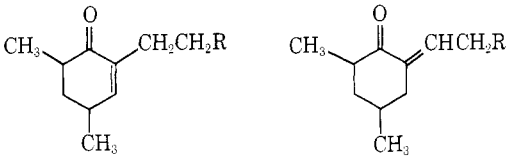


Figure 4. Chromatogram of anhydrocycloheximides.

uration at the point of attachment of the cyclohexanone ring to the other portion of the molecule. This compound was originally believed to be about one-third as active as cycloheximide by microbiological assay. As shown in Figure 3, this isomer can be detected in the presence of cycloheximide by the present gc procedure. A sample which was believed to be essentially pure isocycloheximide and which was used to determine the microbiological activity of isocycloheximide was found by the present gc procedure to contain approximately 40% cycloheximide. This indicated, therefore, that the activity in the so-called isocycloheximide sample was entirely due to the cycloheximide present and that isocycloheximide was devoid of microbiological activity. This has been confirmed by the purification of an isocycloheximide sample by column chromatography (Haak, 1972). The purified sample, which was greater than 99% pure by gas chromatography, was found to be less than 1% as active as cycloheximide by microbiological assay.

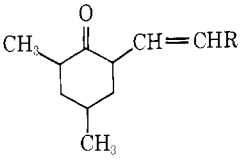
The degradation of cycloheximide in acidic solution was also investigated in order to determine whether compounds would be produced which might interfere in the gc assay. Since β-hydroxy ketone systems dehydrate very easily, cycloheximide forms anhydrocycloheximides in strongly acidic solutions. Figure 4 shows a chromatogram of cycloheximide which had been in contact with 50% sulfuric acid. At least five peaks can be produced, the relative heights of which vary with the time of contact of cycloheximide with the acid. Gc–ms of these peaks gave molecular ions of 263, suggesting that all of them are anhydrocycloheximide compounds.

Five possible structures for anhydrocycloheximides are as follows.



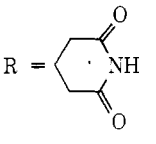
IV

Va (cis)
Vb (trans)



VIa (cis)
VIb (trans)

where



The anhydrocycloheximide (not active microbiologically) which produced the major peak in Figure 4 was prepared and confirmed to be Va or b by nuclear magnetic resonance. The structure of V was not important with regard to the assay procedure, but the fact that it is the major anhydrocycloheximide produced on degradation is important. Figure 5 shows a chromatogram of monotrimethylsilyl cycloheximide (II), V, and the internal standard, cholesteryl acetate. Complete separation of V was obtained; however, if the other anhydrocycloheximides were present in appreciable amounts, they could interfere with the silylated cycloheximide or the internal standard peaks.

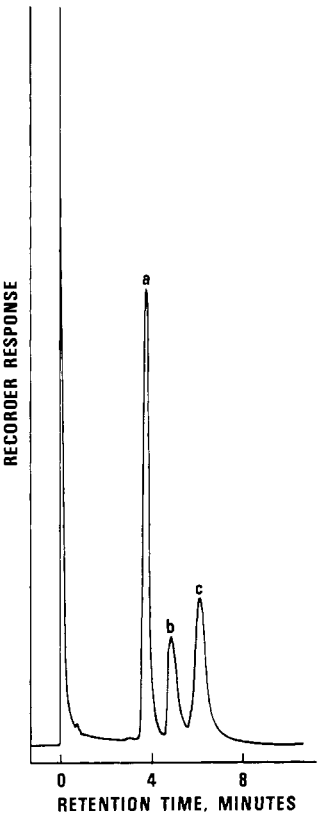


Figure 5. Chromatogram of (a) II, (b) V, and (c) cholesteryl acetate.

Several different column packings were tried for the assay of cycloheximide. A 3% OV-17 column produced better peaks than the QF-1 column finally selected but III was not separated from II, making it difficult to determine whether the isopropyl alcohol step in the procedure had removed the TMS group from the imide nitrogen. A less polar column such as OV-1 moved the principal anhydrocycloheximide peak to a shorter retention time where it interferes with the isocycloheximide peak.

ACKNOWLEDGMENT

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