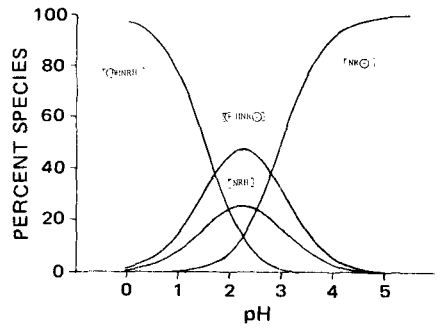

Figure 5—*Species profile for VI.*

shown. They were calculated using:

$$\%[^+HNRH] = 100 \Big/ \left\{1 + \frac{K_1}{[H^+]} + \frac{K_3}{[H^+]} + \frac{K_3K_4}{[H^+]^2}\right\} \quad \text{(Eq. 14)}$$

$$\%[NRH] = 100 \Big/ \left\{1 + \frac{[H^+]}{K_3} + \frac{K_4}{[H^+]} + \frac{K_1}{K_3}\right\} \quad \text{(Eq. 15)}$$

$$\%[^+HNR^-] = 100 \Big/ \left\{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} + \frac{K_3}{K_1}\right\} \quad \text{(Eq. 16)}$$

$$\%[NR^-] = 100 \Big/ \left\{1 + \frac{[H^+]}{K_2} + \frac{[H^+]}{K_4} + \frac{[H^+]^2}{K_3K_4}\right\} \quad \text{(Eq. 17)}$$

These equations are written in their most general forms. To simplify them, the percentages of one species can be expressed as percentage functions of the other species (7).

The maximum concentration of neutral molecules, either as zwitterions or uncharged molecules, occurs at the pH given by:

$$pH = \frac{pK_1 + pK_2}{2} = \frac{pK_3 + pK_4}{2} \quad \text{(Eq. 18)}$$

In Table IV, the pH at which the maximum zwitterion concentration occurs and the concentration ratio $[^+HNR^-]/[NRH]$ are given. Since K_1 is greater than K_3 for each compound, there are more zwitterions than uncharged molecules at any pH.

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High-Pressure Liquid Chromatographic Determination of Cycloheximide in Ointment and Suspension Formulations

LEO W. BROWN

Received June 27, 1977, from *The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication August 31, 1977.

Abstract □ A high-pressure liquid chromatographic procedure is described for the assay of the antibiotic cycloheximide in bulk drug and two experimental formulations. The method utilizes a reversed-phase C_{18} chromatographic column and refractive index detection. Possible impurities or degradation products, isocycloheximide, anhydrocycloheximide, and dimethylcyclohexanone, are well separated from cycloheximide by this procedure. Complete extraction of cycloheximide from the formulations was obtained. The assay has a relative standard deviation of approximately 1%.

Keyphrases □ Cycloheximide—high-pressure liquid chromatographic analysis in bulk drug and prepared formulations □ High-pressure liquid chromatography—analysis, cycloheximide in bulk drug and prepared formulations □ Antibiotics—cycloheximide, high-pressure liquid chromatographic analysis in bulk drug and prepared formulations

Cycloheximide[1] is used in several agricultural formulations. The compound is produced by fermentation from *Streptomyces griseus* and has been studied (1–6) for its fungicidal properties. It also has been used successfully as an abscission agent (7).

[1] Actidione, The Upjohn Co., Kalamazoo, Mich.

The chemical structure of cycloheximide (I) (mol. wt. 281; mp 120°; pK 11.2) has been characterized completely (8, 9). Degradation products of cycloheximide, 2,4-dimethylcyclohexanone, isocycloheximide (10), and anhydrocycloheximide, have been studied. Because of the β-hydroxy ketone moiety in cycloheximide, dehydration to anhydrocycloheximide is the predominant degradation pathway.

Cycloheximide has been determined microbiologically (11) and spectrophotometrically (12, 13). The latter procedures involve reactions to develop a color since cycloheximide has only end absorption in the UV. Both spectrophotometric procedures are relatively nonspecific. Even

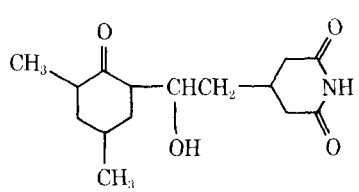

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Table I—Assay Precision of Replicate Samples of Cycloheximide Bulk Drug

Sample Weight, mg	Peak Height Ratio		Ratio/Weight
5.601	0.6141		0.1096
7.955	0.8715		0.1096
6.714	0.7309		0.1089
7.553	0.8284		0.1097
8.866	0.9833		0.1109
8.682	0.9576		0.1103
		Average	0.1098
		RSD	0.62%

though the degradation products of cycloheximide are not microbiologically active (14), making the microbiological assay specific for cycloheximide, the assay gives no indication of the amount and nature of degradation products.

A GLC method was reported for the analysis of cycloheximide and its degradation products (14). This procedure involves silylation of cycloheximide followed by treatment with isopropyl alcohol to produce a monotrimethylsilyl derivative, which chromatographs as a single peak. Recently, two new experimental formulations of cycloheximide were developed in these laboratories. Since the GLC procedure was not successful with these formulations because of interference of excipients in derivatization, a high-pressure liquid chromatographic (HPLC) procedure was developed. The method utilizes a reversed-phase C_{18} chromatographic column and refractive index detection.

EXPERIMENTAL

HPLC Conditions—A liquid chromatograph[2] was used with a differential refractometer[3] detector. The column was reversed-phase C_{18} micro silica gel[4], 30 cm × 3.9 mm. Column pressure was maintained at 900 psi (0.8 ml/min), and a 25-μl injection volume was used with a 25-μl loop injector[5]. Chart speed was 2.54 cm/5 min, and attenuation on the refractive index detector was 9.6×10^{-5} refractive index unit full scale. The mobile phase was acetonitrile–methanol–water (125:20:355).

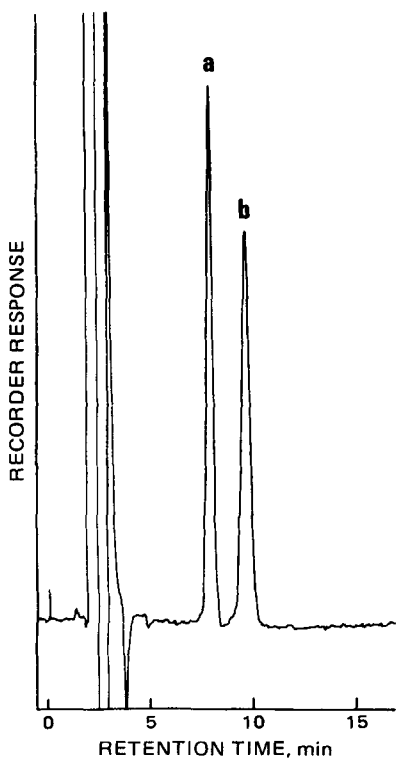

Figure 1—*Chromatogram of* p-*nitrobenzyl alcohol as internal standard (a) and cycloheximide (b).*

Table II—Assay Results of Recovery Study of Cycloheximide Added to 1.0 g of Suspension Formulation

Cycloheximide Added, mg	Peak Height Ratio		Cycloheximide Found, %
5.563	0.778		104.1
5.960	0.830		103.3
6.112	0.851		103.3
5.753	0.788		101.9
7.539	1.032		101.9
5.058	0.770		102.6
		Average	102.6
		RSD	0.9%

Internal Standard Solution—A methanol solution containing approximately 2.7 mg of *p*-nitrobenzyl alcohol/ml was prepared.

Reference Preparation—*For Cycloheximide in Ointment Formulation*—Approximately 7 mg of reference cycloheximide was accurately weighed and transferred to a 100-ml stoppered graduated cylinder. To this cylinder were added 0.7 g of ointment base, 2.0 ml of internal standard solution, 23 ml of methanol, and 75 ml of hexane. A stirring bar was added to the cylinder, which was then shaken to dissolve the ointment. After complete dissolution, the top hexane layer was removed by suction and the methanol layer was transferred to a 10-dram shell vial. The methanol was evaporated to approximately 2 ml with a nitrogen stream.

At the beginning of the evaporation, an immiscible top layer was formed and was removed by suction. Further evaporation produced a white precipitate. The mixture was transferred to a 15-ml centrifuge tube and centrifuged when approximately 2 ml of methanol remained in the

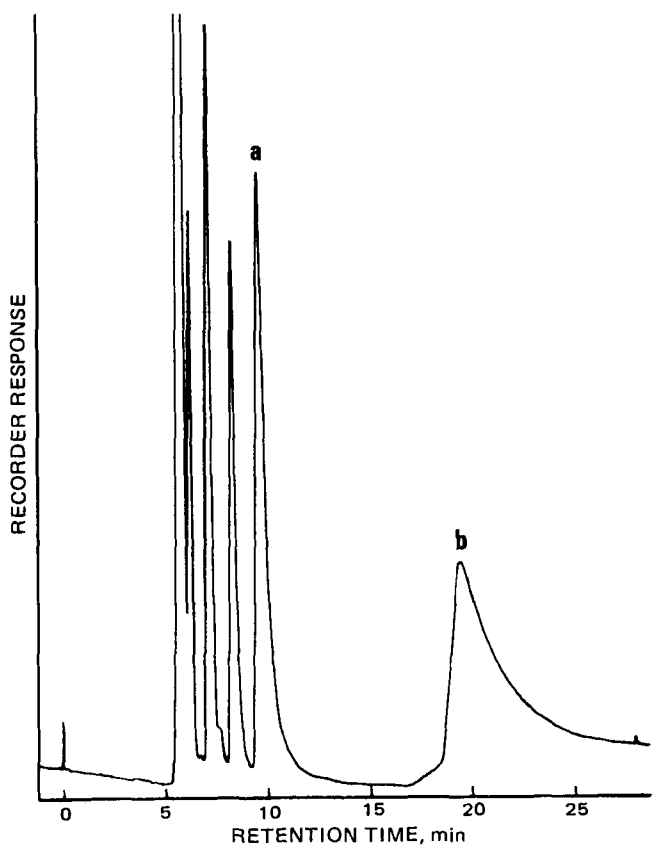

Figure 2—*Chromatogram of the suspension formulation on a silica gel column using ethyl acetate as the mobile phase. Peak a is cycloheximide, and b is propylene glycol in the formulation. All other peaks are ingredients in the formulation.*

[2] Model 830, DuPont Instrument Co., Wilmington, Del.
[3] Model R-401, Waters Associates.
[4] Bondapak, Waters Associates.
[5] Valco.

Table III—Recovery Results on Cycloheximide Added to Placebo Ointment

Weight of Cycloheximide, mg	Weight of Ointment, mg	Peak Height Ratio		Ratio/ Weight
7.019	6320	0.9231		0.1315
8.161	6530	1.0766		0.1318
7.551	7540	0.9904		0.1311
7.266	7190	0.9532		0.1311
6.717	6980	0.8815		0.1312
			Average	0.1313
			RSD	0.23%
6.657[a]	0	0.8454		0.1269
7.569[a]	0	0.9462		0.1250
			Average	0.1260
6.925[b]	0	0.8500		0.1227
6.958[b]	0	0.8564		0.1230
			Average	0.1229

[a] Carried through procedure without ointment. [b] Not extracted.

evaporation step. Approximately 1 ml of the clear supernate in the centrifuge tube was transferred to a 1-dram shell vial.

For Cycloheximide in Aqueous Suspension Formulation—Approximately 7 mg of reference cycloheximide was accurately weighed and transferred to a 3-dram shell vial. Internal standard solution, 2 ml, was added, and the vial was swirled to dissolve the cycloheximide.

Sample Preparation—*Cycloheximide in Ointment Formulation*—An ointment sample equivalent to approximately 7 mg of cycloheximide was accurately weighed and transferred to a 100-ml stoppered graduated cylinder. The sample was then treated in the same manner as the reference preparation, starting with the addition of 2.0 ml of internal standard solution.

Cycloheximide in Aqueous Suspension Formulation—The sample suspension was shaken vigorously to obtain a homogeneous mixture, and a sample equivalent to approximately 7 mg of cycloheximide was accurately weighed. The weighed sample was transferred to a 15-ml centrifuge tube. To this tube was added 10 ml of methanol and 2.0 ml of internal standard solution. Then the tube was shaken vigorously and centrifuged. Approximately 10 ml of the clear supernate was removed and evaporated with a nitrogen stream to less than 2 ml in a 3-dram shell vial. The volume was then adjusted to approximately 2 ml with methanol. The sample was mixed thoroughly and centrifuged or filtered to remove insoluble material.

Procedure—Sample and reference preparations were chromatographed using the chromatographic conditions described.

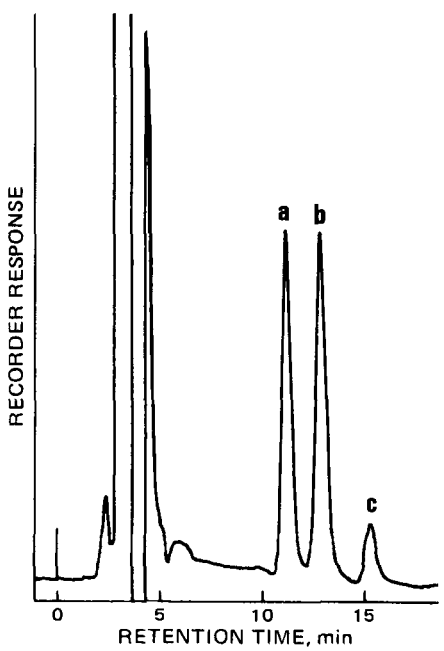

Figure 3—*Chromatogram of cycloheximide extracted from the suspension formulation. Peak a is* p*-nitrobenzyl alcohol used as the internal standard, b is cycloheximide, and c is a formulation ingredient.*

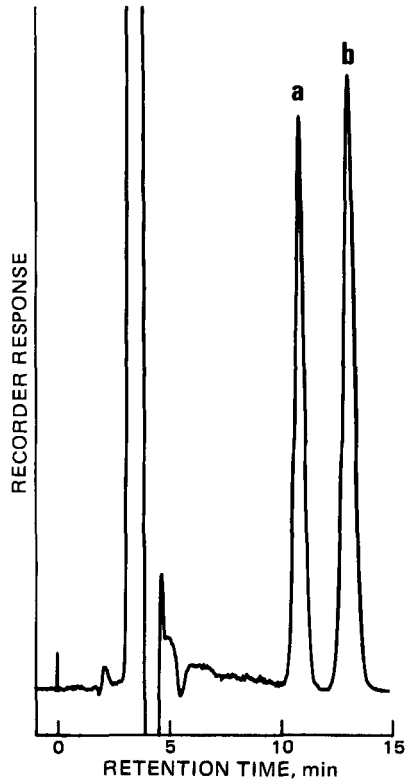

Figure 4—*Chromatogram of cycloheximide extracted from ointment. Peak a is* p*-nitrobenzyl alcohol used as an internal standard, and b is cycloheximide.*

Calculations—The following equation was used:

$$\% \text{ cycloheximide} = R_s/R_{ref} \times W_{ref}/W_s \times P \qquad \text{(Eq. 1)}$$

where R_s is the peak height ratio of the cycloheximide peak to the internal standard peak in the sample preparation; R_{ref} is the peak height ratio of the cycloheximide peak to the internal standard peak in the reference preparation; W_{ref} is the weight, in milligrams, of cycloheximide reference standard; W_s is the weight, in milligrams, of sample; and P is the purity of the cycloheximide reference expressed in percent.

RESULTS AND DISCUSSION

Samples of cycloheximide bulk drug were assayed by this HPLC procedure to check for linearity and precision (Table I). A relative standard deviation of less than 1% was obtained from six samples varying in weight from approximately 5 to 10 mg. A typical chromatogram is shown in Fig. 1.

To check recovery of cycloheximide from the aqueous suspension formulation, cycloheximide was added to a placebo formulation in varying amounts (Table II). Methanol was added to the sample preparation to increase the amount of clear supernate that could be removed after centrifugation. Addition of the internal standard solution eliminates the need to remove all of the supernate. Recovery was complete compared to a cycloheximide reference carried through the same extraction procedure. Addition of the placebo formulation to the reference preparation had no effect.

A GLC procedure (14) was tried for this aqueous suspension formulation, but the extraction procedures did not produce an extract that could be quantitatively derivatized. A silica gel column using HPLC was also tried for this formulation. A large tailing peak, identified as propylene glycol, eluted after cycloheximide, causing a longer assay time (Fig. 2). With the C_{18} reversed-phase column (Fig. 3), all formulation ingredients eluted before the cycloheximide peak except one, and it did not interfere. Only relatively polar formulation ingredients would be soluble in the methanolic solution used in the extraction. For this reason, a C_{18} column, which does not retain polar compounds, would be a better choice than a silica gel column, which retards their elution.

Development of an assay for cycloheximide in the ointment formulation was less difficult than that for the suspension formulation. A combination of hexane and methanol was used to extract the nonpolar oint-

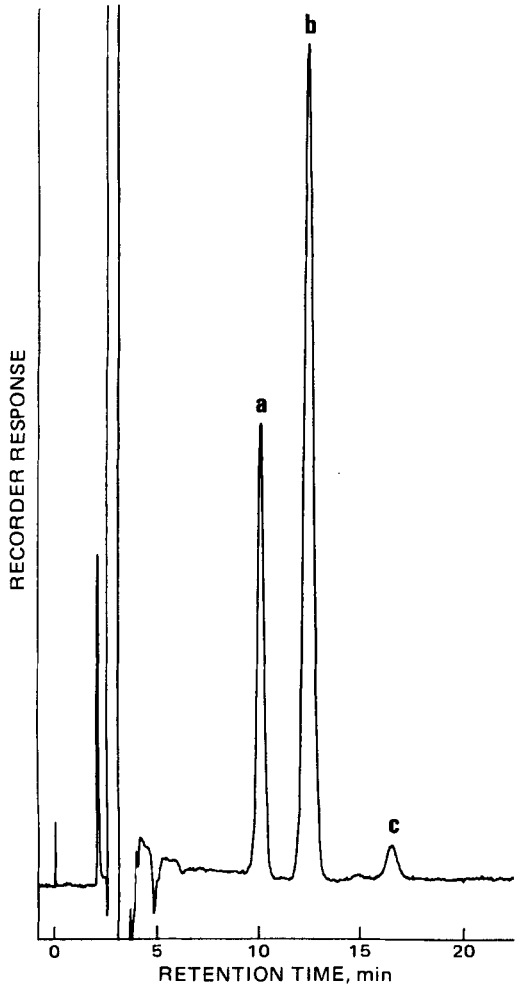

Figure 5—*Chromatogram of* p-*nitrobenzyl alcohol (a), cycloheximide (b), and isocycloheximide (c).*

ment ingredients into the upper hexane layer while the cycloheximide dissolved in the lower methanol layer. Assay results on cycloheximide added to placebo ointment, cycloheximide carried through the procedure but without placebo ointment, and cycloheximide not extracted are shown in Table III.

Results indicated only a slight difference between extracted and nonextracted cycloheximide. However, there was about a 7% difference in results comparing cycloheximide added to placebo ointment and carried through the extraction and results on nonextracted cycloheximide. This difference was due to the relative differences in solubilities of the internal standard and cycloheximide in the two solvent phases. The addition of ointment to a hexane–methanol system changes not only the relative volumes of the two layers but also their composition. For example, 50 ml each of hexane and methanol produced a system of about 14 ml in the upper layer and 84 ml in the lower layer. Addition of 1 g of ointment to the system produced an upper layer of about 20 ml and a lower layer of 79 ml.

To eliminate any bias, the reference preparation was treated in the same manner as the sample. Further work showed that the addition of 0.7 g of placebo ointment to the reference preparation was sufficient. Addition of 10 times that amount of ointment gave the same result. Complete extraction of 0.1% cycloheximide in ointment samples was obtained with a relative standard deviation of less than 0.5% by using the reference preparation with the small amount of placebo ointment added. A typical chromatogram of cycloheximide extracted from ointment is shown in Fig. 4. Placebo ointment carried through the procedure produced no interfering chromatographic peaks.

In quality control situations where many assays are performed over a short period, it is important that the chromatographic column retain its integrity. Since the column is stainless steel, it is difficult to determine whether there is buildup of formulation ingredients on the column. One way to determine this effect is to use a thin-layer plate of comparable support and to develop the plate with the mobile phase. Sample solutions of both cycloheximide formulations were applied to silica gel 60 F_{254} silanized (producing a reversed-phase plate) and developed with the mobile phase. All ointment formulation ingredients moved up the plate. However, a small amount of UV-absorbing material remained at the origin when the aqueous suspension formulation was developed on the plate. For this reason, the packing at the inlet of the HPLC column should be replaced after the assay of several suspension formulation samples.

The known impurities or degradation products, isocycloheximide, anhydrocycloheximide, and 2,4-dimethylcyclohexanone, were described previously (14) and were separated by GLC. Table IV shows the relative retention volumes for these impurities by this HPLC procedure. By GLC, the isocycloheximide peak was not baseline separated from the cycloheximide peak. By HPLC, the two peaks were well separated (Fig. 5).

Table IV—Relative Retention Volume of Possible Impurities or Degradation Products in Cycloheximide Samples

Compound	Relative Retention Volume[a]
Cycloheximide	1.00
Isocycloheximide	1.34
Anhydrocycloheximide	2.88
2,4-Dimethylcyclohexanone	0.49
Internal standard	0.77

[a] See *Experimental* for conditions.

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