Microionization Constants of Commercial Cephalosporins

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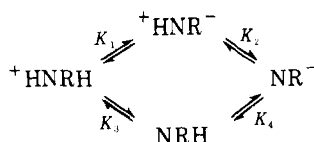
Abstract □ The equilibrium constants of five commercial cephalosporins were determined. Two are monoacidic and possess one K_a while three are amphoteric and have four microconstants. Although several assumptions were made in the calculations, good agreement was found between the compounds and with previously reported macroionization constants. By utilizing the microionization constants, the ratios of zwitterion to uncharged species were calculated to be in the 900–50,000 range and to have a maximum concentration between pH 3.5 and 5.

Keyphrases □ Cephalosporins, various—microionization equilibrium constants determined □ Microionization constants, equilibrium—determined for various cephalosporins □ Equilibrium constants, microionization—determined for various cephalosporins □ Antibacterials—various cephalosporins, microionization equilibrium constants determined

Recently, a method to determine the microionization constants of zwitterionic compounds was described (1). These amphoteric compounds, *i.e.*, those behaving as either proton acceptors or donors, undergo the equilibria given in Scheme I.



Scheme I

According to this scheme, loss of a proton from the doubly protonated molecule will form either the zwitterion, $^+HNR^-$, or the uncharged molecule, NRH. Following the loss of the first proton, a second proton can leave, with the equilibria given by the equilibrium constants K_2 and K_4. The relative amount of zwitterion and uncharged molecules is a constant given by:

$$\frac{[^+HNR^-]}{[NRH]} = \frac{K_1}{K_3} = \frac{K_4}{K_2} \quad \text{(Eq. 1)}$$

The method described for elucidating all four constants is a complex procedure. To utilize the technique without modification, the compounds must possess a pH-dependent chromophore and be soluble enough to permit potentiometric titration. It will be shown that, as an alternative, the constants can be calculated if ionization constants of a structurally similar compound are known.

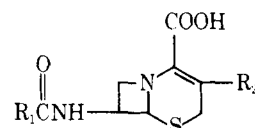
Five commercial cephalosporins were investigated: cephapirin[1] {3-[(acetyloxy)methyl]-8-oxo-7-[[(4-pyridinylthio)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monosodium salt} (I), cephaloglycin[2] {[6*R*-[6α,7β(*R**)]]-3-[(acetyloxy)methyl]-7-[(aminophenylacetyl)amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylic acid} (II), cephalexin[2] {(+)-[6*R*-[6α,7β(*R**)]]-7-[(aminophenylacetyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid} (III), cephalothin[2] {3-[(acetyloxy)methyl]-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid sodium salt} (IV), and cefazolin[2] {(6*R*-*trans*)-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)thio]-methyl]-8-oxo-7-[[(1*H*-tetrazol-1-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylic acid monosodium salt} (V). Compounds I–III have two basic groups and, therefore, will have the equilibria expressed according to Scheme I; IV and V have one basic group and, therefore, have one pKa.

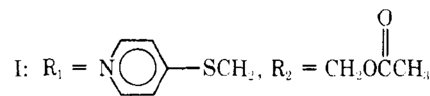
EXPERIMENTAL

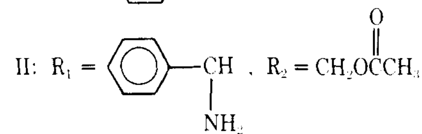
Potentiometric Measurements—Potentiometric measurements were made at 25 ± 2° using the previously described equipment (1). The appropriate amount of compound (400–700 mg) was dissolved in water, and an excess of 0.15 *N* HCl was added to doubly protonate the diprotic compounds and singly protonate the monoprotic compounds. These solutions were then titrated with 0.15 *N* KOH, and the pH was measured with a meter[3] in the expanded scale mode.

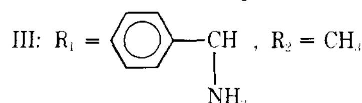
Corrections for impurities based on microbiological potency values were made on III and V; a moisture content determination of II was made, and the appropriate correction was applied. The moisture content of I and III–V was assumed to be that given by the formula according to the manufacturer. At least two titrations were made for each compound.

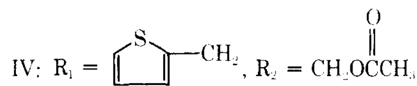
Spectrophotometric Measurements—Spectrophotometric measurements[4] were made on I. Buffers were prepared at molar ionic strengths between 0.05 and 0.1 *M* over the pH 2–7.5 range. Aliquots of 1 ml of a stock solution of I were placed into 25-ml volumetric flasks and brought to volume with the appropriate buffers (final concentration was

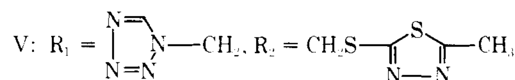


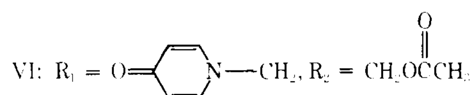












[1] Bristol Laboratories.
[2] Eli Lilly and Co.
[3] Beckman Century SS-1.
[4] Varian Cary 17.

Table I—pKa Values of Cephalosporins

Compound	pK_1	pK_2	pK_3	pK_4
I	1.83	5.48	4.78	2.53
II	1.78	7.22	6.46	2.53
III	2.48	7.59	6.46	3.60
IV	—	—	—	2.35
V	—	—	—	2.75
VI	1.70	2.80	1.97	2.53

11 μg/ml). Spectra were then obtained of these solutions. A maximum absorption at 290 nm was attributed to the protonated pyridine ring, and that at 258 nm was assigned to the nonprotonated ring.

THEORETICAL

In the following equations, { }, [ ], Y, and K represent activity, concentration, activity coefficient, and equilibrium constants, respectively, on a molar scale. The total concentrations of base added as titrant and of acid being titrated are given by [B] and [A].

Previously (1), an equation was derived having the form:

$$\alpha = K_1 \frac{Y_{+HNRH}}{Y_{+HNR^-}}\beta + K_3 \frac{Y_{+HNRH}}{Y_{NRH}}\beta + K_3K_4 \frac{Y_{+HNRH}}{Y_{NR^-}}\gamma \quad \text{(Eq. 2)}$$

where:

$$\alpha = [B]\{H^+\}^3 + \frac{\{H^+\}^4}{Y_{H^+}} - \frac{K_\omega\{H^+\}^2}{Y_{OH^-}} \quad \text{(Eq. 3)}$$

$$\beta = [A]\{H^+\}^2 - [B]\{H^+\}^2 - \frac{\{H^+\}^3}{Y_{H^+}} + \frac{K_\omega\{H^+\}}{Y_{OH^-}} \quad \text{(Eq. 4)}$$

$$\gamma = 2[A]\{H^+\} - [B]\{H^+\} - \frac{\{H^+\}^2}{Y_{H^+}} + \frac{K_\omega}{Y_{OH^-}} \quad \text{(Eq. 5)}$$

By combining two sets of experimental data (potentiometric and spectrophotometric), the four equilibrium constants could be determined by using:

$$\{H^+\}^2 \frac{Y_{NR^-}}{Y_{+HNRH}}\{[Z] - [A]\} = K_3\{H^+\}\frac{Y_{NR^-}}{Y_{NRH}}\left\{[B] + \frac{\{H^+\}}{Y_{H^+}} - \frac{K_\omega}{\{H^+\}Y_{OH^-}} - [A]\right\} + K_3K_4\left\{[B] + \frac{\{H^+\}}{Y_{H^+}} - \frac{K_\omega}{\{H^+\}Y_{OH^-}} - 2[A] + [Z]\right\} \quad \text{(Eq. 6)}$$

where [Z] is the total concentration of protonated nitrogen species obtained spectrophotometrically.

Equation 6 can be expressed in the simplified form given by:

$$\delta = K_3\epsilon + K_3K_4\xi \quad \text{(Eq. 7)}$$

from which it can be seen that values for K_3 and K_4 can be obtained by either graphical techniques or simultaneous equations.

The value of K_3 or K_4 obtained from Eq. 6 can be inserted into Eq. 2 to calculate the remaining constants. A plot of α/β *versus* γ/β should be linear with a slope of K_3K_4 and an intercept at $\alpha/\beta = 0$ equal to $K_1 + K_3$, while a plot of α/γ *versus* β/γ should be linear with a slope of $K_1 + K_3$ and an intercept at $\beta/\gamma = 0$ equal to K_3K_4. Because of the relationship between the four constants, it can be shown that $K_1K_2 = K_3K_4$. This approach to solving for the equilibrium constants leads to the conclusion that, with knowledge of one constant, the remaining constants can be determined using Eq. 2.

When acidic groups on a molecule are separated by large distances with no conjugation, there should be little interaction between the groups influencing the respective equilibria. Therefore, it should be possible to determine the microconstants for the diacidic cephalosporin compounds in Table I if a comparable equilibrium exists on a similar compound that is known. Combining the known value with either the calculated $K_1 + K_3$ or K_3K_4 will result in values for the unknown constants.

The selection of the best known value to use requires careful consideration because the sum $K_1 + K_3$ is obtained and it is better to select the smaller of these two constants as the known constant. That is, if K_3 is smaller than K_1, either K_3 or K_4 should be used as the known constant; if K_1 is smaller, then either K_1 or K_2 should be used.

Table II—Comparison of Calculated pKa Values for I

Method[a]	pK_1	pK_2	pK_3	pK_4
A	1.83	5.48	4.78	2.53
B	1.85	5.44	4.47	2.81

[a] Method A used K_4 of VI; Method B combined spectrophotometric and potentiometric data.

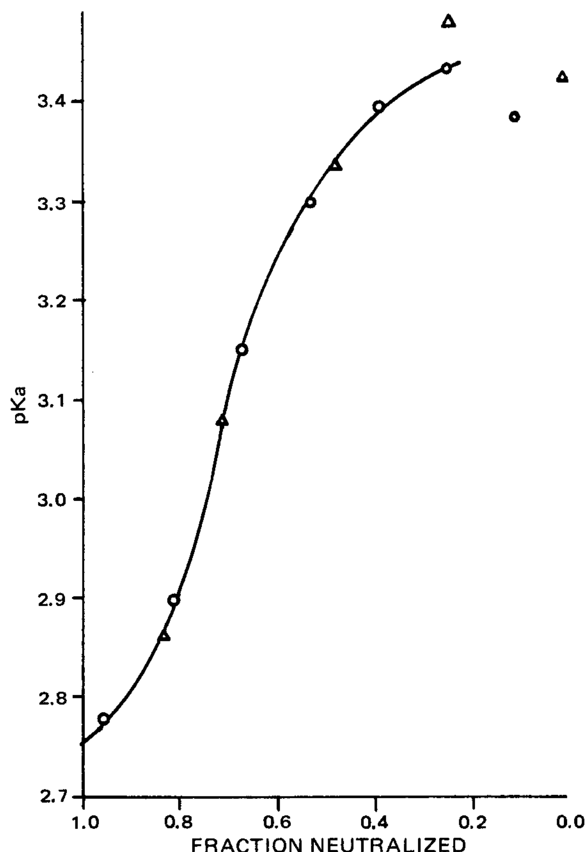


Figure 1—*Calculated pKa for V.*

The equilibrium constants for the monoprotic compounds were calculated using a standard equation in the form:

$$\eta = \theta K \quad \text{(Eq. 8)}$$

where:

$$\eta = \frac{Y_{A^-}}{Y_{HA}}\{H^+\}\left\{[B]\{H^+\} + \frac{\{H^+\}^2}{Y_{H^+}} - \frac{K_\omega}{Y_{OH^-}}\right\} \quad \text{(Eq. 9)}$$

$$\theta = [A]\{H^+\} - [B]\{H^+\} - \frac{\{H^+\}^2}{Y_{H^+}} + \frac{K_\omega}{Y_{OH^-}} \quad \text{(Eq. 10)}$$

RESULTS AND DISCUSSION

The equilibrium constants of I and II were determined using the value for pK_4 of 3-[(acetyloxy)methyl]-8-oxo-7-[[4-oxo-1(4*H*)-pyridinyl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (VI) previously reported (1). The equilibrium associated with pK_4 is the loss of the proton on the carboxylate group when the molecule is uncharged and satisfies the requirements for the selection of acceptable equilibria. On all three compounds, R_2 is the same and R_1 is different. Since R_1 is

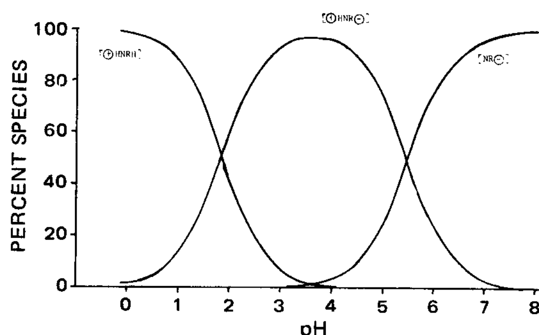


Figure 2—*Species profile for I.*

Table III—Correlation of Equilibrium Constants

Compound	—COOH pKa
Cephalosporin C[a]	3.1
D-Cephaloglycin[b]	4.4
L-Cephaloglycin[b]	4.6
7-Aminocephalosporanic acid[c]	1.75

[a] Reference 8. [b] Reference 3. [c] Reference 4.

separated from the carboxylic acid group by a large distance with no conjugation, K_4 would be expected to be the same. Table I lists the calculated pKa values for these compounds.

The equilibrium constant K_3 can be considered the same for II and III. Both compounds have the same R_1 group, with the NH_2 group acting as a proton acceptor. Since the NH_2 is separated by a large distance with no conjugation from the carboxylic acid group and R_1, K_3 should be the same. This equilibrium is the loss of a proton from the protonated NH_2 (NH_3^+) to form the uncharged molecule. The values thus obtained for III are given in Table I.

Also included in Table I are the values calculated for the monoprotic compounds IV and V. These constants were obtained from the experimental data using Eq. 8.

To check the validity of the assumption used to calculate the constants for I–III, the constants for I were determined by combining spectrophotometric with potentiometric data as explained previously (1). In Table II, the results of the calculations by both methods are given for comparison and show good correlation between the two methods for pK_1 and pK_2 and fair correlation for pK_3 and pK_4. The correlation between pK_3 and pK_4 is not unreasonable when considering the type of compound and the fact that different methods were used.

The pKa values for the carboxy group on cephalosporins having the R_2 group CH_2OCOCH_3 are listed in Table III. It would not be expected that these values should differ by more than ±0.5 unit, but a spread of 2.85 units exists. Of particular interest are the values for D- and L-cephaloglycins, which give an indication of the reliability to be expected in the pKa values for these compounds. The same investigators used the same method to obtain both values and show a difference of 0.2 pKa unit. Although they are optical isomers, the pKa values for the carboxy groups should be the same. Any effect on the pKa values due to the different isomers would manifest itself in the side-chain pKa's. On the contrary, the same values for the side-chain pKa's were found. From this result, it can be concluded that a spread of 0.2 pKa unit would not be unexpected when using the same method. When using different methods, the expected correlation would be less and a difference of 0.3 pKa unit would not be unreasonable. It was not possible to measure the microionization constants of II and III by this method since they do not possess pH-dependent chromophores.

The pKa values for I and II and compounds related to III were reported (2–4); only two K_a values (K_{13} and K_{24}) were given. It can be shown that the two K_a values are related to those reported here according to:

$$K_{13} = K_1 + K_3 \qquad \text{(Eq. 11)}$$

and:

$$\frac{1}{K_{24}} = \frac{1}{K_2} + \frac{1}{K_4} \qquad \text{(Eq. 12)}$$

The pKa values reported by Prasad *et al.* (2) for I are in reasonable agreement (2.03 and 5.35) with those given here (1.83 and 5.48). Spencer *et al.* (3) reported values for II of 4.4 and 7.22, the first of which is significantly different from that found here (1.78 and 7.22). The study by Spencer *et al.* (3), however, used a 66% dimethylformamide–water mixture for the solvent, which could cause this large a difference in the pKa values. Also, the value of 4.4 for pK_{13} is not consistent with the finding of Prasad *et al.* (2) or the value in Ref. 1, which indicate the carboxylic acid to have a pKa of about 2 with this R_2 group. Albert and Serjeant (5) discussed the effect of mixed solvents upon the pKa and indicated that it is extremely difficult to correlate equilibrium constants in mixed solvents with those in pure water.

Table IV—pH of Maximum Zwitterion Concentration and the Ratio [$^+$HNR$^-$]/[NRH]

Compound	pH	[$^+$HNR$^-$]/[NRH]
I	3.66	8.91×10^2
II	4.50	4.79×10^4
III	5.04	9.55×10^3
VI	2.25	1.86

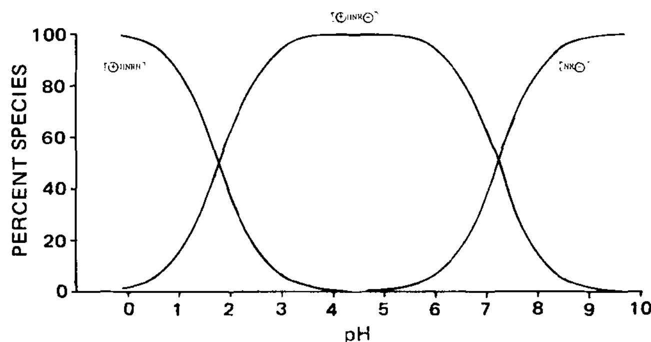


Figure 3—*Species profile for II.*

A direct comparison cannot be made with literature values for III. An estimate of the effect of the CH_3 group on carboxylic acid dissociation can be obtained from Stedman *et al.* (4). They found that the carboxy group has a pKa between 2.38 and 2.74 when the CH_3 group is present. This result is in good agreement with the 2.48 found here.

Neither pKa of the monoprotic compounds was reported. Stedman *et al.* (4) reported that changing the CH_3 group to $CH_2OC({=}O)CH_3$ lowers the pKa of the carboxy group in compounds similar to IV. The pKa of V (2.75) appears reasonable when compared to IV.

The method used to determine the pKa values of I–III introduces a slight error since the assumption is made that the largest influence on the acidic groups is made by groups close to them. The fact that pK_1 for I, II, and VI and pK_2 for II and III are nearly the same indicates that there is little or no long-range influence in the uncharged molecules because the charged molecules have little effect on the equilibria. Any influence on the equilibria due to different groups would be expected to be greater when the groups are charged.

Compounds IV and V were not completely soluble in their acid forms at the concentrations required for the potentiometric titrations. Even though precipitation occurred, calculations were made using the data obtained. The pKa values calculated were not constant but did change in a systematic manner. A plot of the pKa values *versus* the fraction of acid neutralized could be extrapolated to a value of 1. Since the compounds were soluble when completely neutralized, the pKa value at this point should be correct. A plot of this type is given in Fig. 1.

In performing the calculations, an attempt was made to correct for activity effects by utilizing Davies equation (6) to approximate the single ion activities:

$$-\log Y_i = AZ_i^2 \left\{ \frac{\sqrt{I}}{1 + \sqrt{I}} - 0.2I \right\} \qquad \text{(Eq. 13)}$$

where Y_i is the single ion activity coefficient, A is a parameter whose value at 25° is 0.5092 on a molar scale, Z_i is the charge of ion i, and I is the molar ionic strength. The advantage of this equation over the Debye–Hückel law is that it does not require the use of any ion size parameters.

An approximation was not made for the activity of the zwitterionic species because there is no expression comparable to Eq. 13. It was not expected that Y_{+-}[5] would deviate greatly from the ideal value of 1. It also was assumed that the uncharged species had an activity coefficient of 1, a reasonable assumption within the limits of the experiment.

In Figs. 2–5, the species profiles for I–III and VI, respectively, are

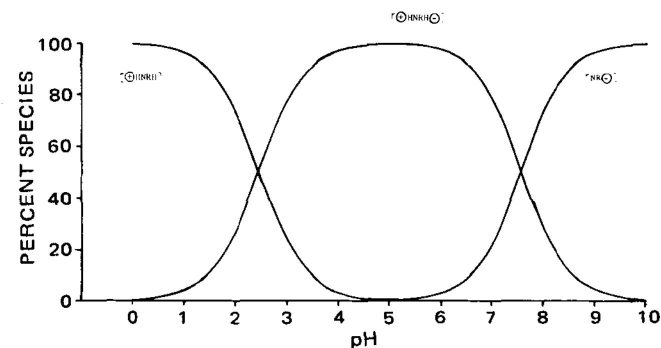


Figure 4—*Species profile for III.*

[5] The activity coefficient for the zwitterionic species.

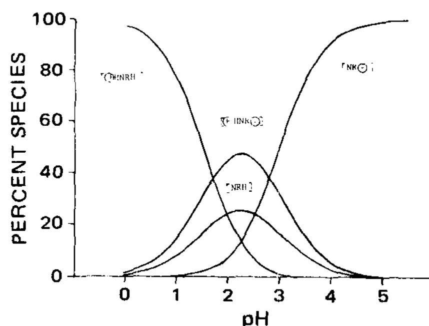


Figure 5—*Species profile for VI.*

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.

REFERENCES

(1) W. H. Streng, H. E. Huber, J. L. DeYoung, and M. A. Zoglio, *J. Pharm. Sci.*, **65**, 1034 (1976). W. H. Streng, *ibid.*, **66**, 1357 (1977).
(2) V. K. Prasad, W. H. Johns, M. W. Wingate, M. M. Mihotic, B. J. Southard, M. A. Kaplan, and A. P. Granatek, *Curr. Ther. Res.*, **16**, 1214 (1974).
(3) J. L. Spencer, E. H. Flynn, R. W. Roeske, F. Y. Siu, and R. R. Charwette, *J. Med. Chem.*, **9**, 746 (1966).
(4) R. J. Stedman, K. Swered, and J. R. E. Hoover, *ibid.*, **7**, 117 (1964).
(5) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N.Y., 1962, pp. 66–68.
(6) J. N. Butler, "Ionic Equilibrium. A Mathematical Approach," Addison-Wesley, Reading, Mass., 1964, p. 437.
(7) *Ibid.*, pp. 210–212.
(8) E. P. Abraham, *Q. Rev. Chem. Soc.*, **21**, 231 (1967).

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

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

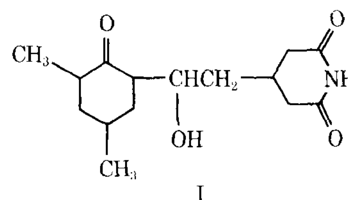
Abstract ◻ A high-pressure liquid chromatographic procedure is described for the assay of the antibiotic cycloheximide in bulk drug and two experimental formulations. The method utilizes a reversed-phase C_{18} chromatographic column and refractive index detection. Possible impurities or degradation 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).

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