such as sucrose or other volume indicators, from a layer of water of hydration of the phospholipid. For dimyristoyl phosphatidyl choline liposomes, this "nonsolvent water" has been shown to be 11.5 mol of water/mol of phosphatidyl choline, or approximately one-third of the enclosed liposomal water (13). The second factor is the difference between the true liposomal structure and the geometric approximations made in our model. For example, any change in the shape of the model to a nonspherical geometry will decrease the trapped volume relative to the percent exposure (r^2 *versus* r^3). This does not imply that multi-bilayers are nonspherical, but deviations from sphericity can account for this discrepancy.

In conclusion, our calculations clearly show that the estimation by Schwartz and McConnell for the number of lamellae in multilamellar vesicles is essentially correct. This, in fact, implies that the wall of the average liposome spontaneously formed by hydration of phospholipids, consists of 5–10 bilayers and has a total thickness $< \sim 0.2$ μm. Thus, "fully swollen" (spherical) liposomes of a diameter of 1.4 μm, on the average, contain an inner aqueous cavity >1.0 μm. When viewed obliquely, a nonspherical multi-bilayer could result in the observed space-filling "onion-like" structures seen in some electron micrographs and give a somewhat misleading impression of the large central cavity which we calculate to be present in multilamellar liposomal structures. Finally, we conclude that therapeutic strategies using encapsulation of aqueous components into simple multilamellar lipid dispersions must take into account the fact that the vast majority of encapsulated material will reside in the central cavity of these liposomes.

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Epimerization of Benzylpenicilloic Acid in Alkaline Media

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Abstract □ 5*R*,6*R*-Benzylpenicilloic acid was found to epimerize slowly in alkaline media to 5*S*,6*R*-benzylpenicilloic acid until equilibrium was established. Epimerization proceeded *via* the imine tautomer of penamaldic acid rather than the enamine form and was found to favor the 5*S*,6*R*-epimer at equilibrium. The conversion process was monitored using both reverse-phase high-performance liquid chromatography and NMR spectroscopy.

Keyphrases □ Benzylpenicilloic acid—epimerization, alkaline media, imine tautomer of penamaldic acid as intermediate □ Epimerization—benzylpenicilloic acid in alkaline media, imine tautomer of penamaldic acid as intermediate □ Penamaldic acid—imine tautomer, intermediate in the epimerization of benzylpenicilloic acid

Penicilloic acid has been reported to be the principal hydrolysis and excretion product of penicillin (1, 2). It has also been cited as a minor antigenic determinant, although it is structurally incapable of reacting directly with proteins to form the highly reactive penicilloyl conjugate (3). This compound is believed to exert its antigenic activity by reacting with disulfide linkages of proteins (4). Despite the important role which penicilloic acid may play as an antigenic determinant and its reported existence as a mixture of isomers in alkaline media (5, 6), virtually no attempt has been made either to separate the isomers or to investigate the isomerization process.

In this paper, a combination of high-performance liquid chromatography (HPLC), NMR, polarography, and UV spectroscopy was used to separate the 5*R*,6*R*- and 5*S*,6*R*-epimers of benzylpenicilloic acid, monitor the conversion process, and examine the epimerization mechanism.

EXPERIMENTAL

Chemicals and Reagents—Penicillin G potassium[1] was obtained commercially and used without further treatment. Acetonitrile[2] was HPLC grade, while all other chemicals were either USP or reagent grade. Double-distilled water was used to prepare buffer solutions.

Preparation of Disodium Penicilloate—The disodium salts of 5*R*,6*R*- and 5*S*,6*R*-benzylpenicilloic acids were prepared by procedures similar to those used in the preparation of penicic and epipenicic acids (7).

Disodium 5R,6R-Benzylpenicilloate—An 8.64-g sample of penicillin G potassium was dissolved in 15 mL of double-distilled water and allowed to cool in an ice-salt bath. After the solution temperature reached ~0°C, 4.64 mL of cold 10 M NaOH was added in one increment. Fifteen minutes later, the pH was adjusted to 8.7 [the equivalence point of the disodium salt of benzylpenicilloic acid (7)] using 1 M HCl, and the reaction mixture was immediately lyophilized. ^{13}C-NMR: δ 26.4 and 26.7 (C-2$^{\alpha}$ and C-2$^{\beta}$), 42.5 (C-9), 58.6 (C-2), 59.9 (C-6), 66.1 (C-5), 75.4 (C-3), 127.4 (C-4′), 129.0 (C-3′), 129.4 (C-2′), 135.0 (C-1′), and 173.9, 175.4, and 175.9 (C-3$^{\alpha}$, C-6$^{\alpha}$, and C-8). ^{1}H-NMR (D_2O): δ 1.07 (s, CH_3), 1.34 (s, CH_3), 3.26 (s, H-3), 3.53

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Waters Associates, Milford, Mass.

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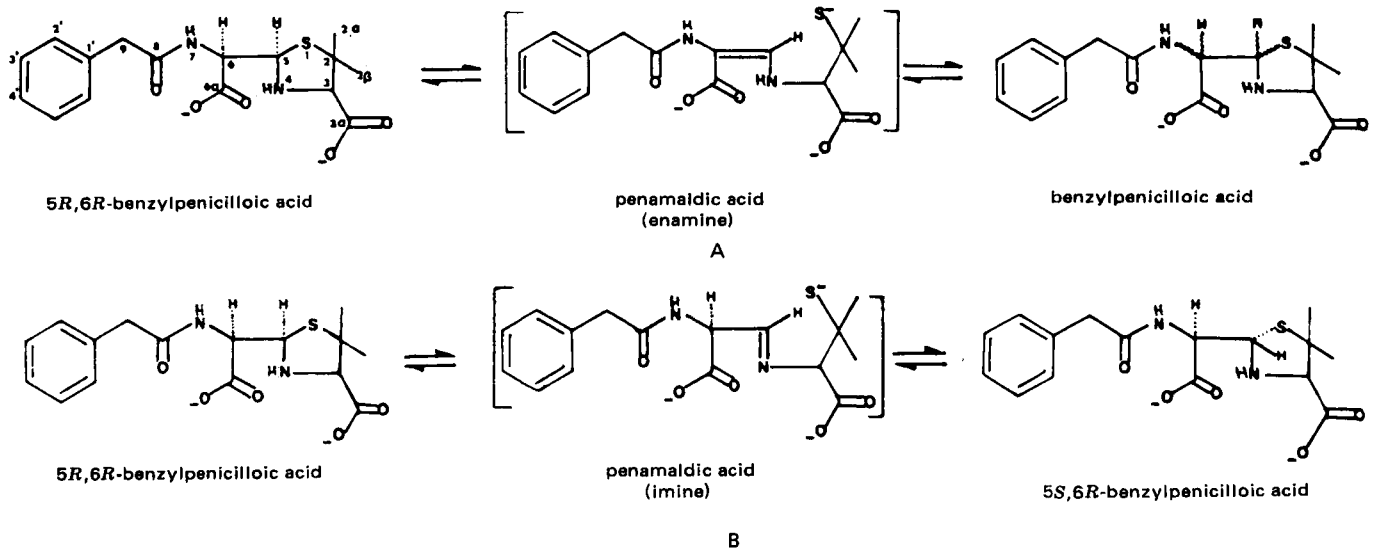


Scheme I—Proposed epimerization pathways for benzylpenicilloic acid.

(s, CH_2), 4.09 (d, J = 5.7 Hz, H-6), 4.92 (d, J = 5.7 Hz, H-5), and 7.23 (s, C_6H_5).

Disodium 5S,6R-Benzylpenicilloate—A 3.73-g sample of penicillin G potassium was dissolved in 100 mL of 0.2 M NaOH at 25°C. The mixture was stirred continuously using a magnetic stirrer, and the pH remained greater than 12 throughout the reaction time. After 30 min, the pH was adjusted to 8.7 by the addition of 1 M HCl, and the mixture was allowed to stand at 30°C for 48 h, and then was lyophilized. ^{13}C-NMR: δ 28.0 (C-2$^{\alpha}$ and C-2$^{\beta}$), 42.6 (C-9), 55.3 (C-6), 58.4 (C-2), 67.2 (C-5), 75.8 (C-3), 127.5 (C-4′), 129.2 (C-3′), 129.6 (C-2′), 134.4 (C-1′), and 174.6 and 174.8 (C-3$^{\alpha}$, C-6$^{\alpha}$, and C-8). ^{1}H-NMR (D_2O): δ 0.87 (s, CH_3), 1.41 (s, CH_3), 3.25 (s, H-3), 3.61 (s, CH_2), 4.61 (d, J = 3.1 Hz, H-6), 4.90 (d, J = 3.1 Hz, H-5), and 7.24 (s, C_6H_5).

Assignments of the structures of the 5*R*,6*R*- and 5*S*,6*R*-benzylpenicilloic acids were made after the ^{1}H- and ^{13}C-NMR spectra of the two isomers were examined. The proton-decoupled ^{13}C-NMR spectra showed clearly that these two epimers were distinctly different species. The structural difference of the two compounds was unequivocally established partly by focusing on the chemical shifts of C-5 and C-6, which are significantly different. Since one or both of these carbons are likely to participate in the isomerization of benzylpenicilloic acid, this distinction has also an additional importance in the present work. The peaks were assigned to the various carbons primarily on the basis of the proton-coupled ^{13}C-NMR spectra of the two isomers. In addition, comparison has been made with the reported assignments for penicillin G potassium (8). The ^{1}H-NMR spectra also revealed that the compounds have different coupling constants for the protons at C-5 and C-6. 5*R*,6*R*-benzylpenicilloic acid displayed a coupling constant of 5.7 Hz, while the 5*S*,6*R*-epimer exhibited a coupling constant of 3.1 Hz. This significant difference in coupling constants is in close agreement with that reported for the dimethyl esters of the two epimers (9). Moreover, examination of the signals of the protons on the methyl groups of C-2 and the chemical shifts of the proton on C-6 clearly demonstrate that the two compounds are indeed structurally different.

Table I—HPLC Data Demonstrating the Relative Peak Height Ratios of 5*R*,6*R*-Benzylpenicilloic Acid to 5*S*,6*R*-Benzylpenicilloic Acid as a Function of Time

Time, h	Peak Height Ratio (5*R*,6*R*/5*S*,6*R*)
0.50	11.4
1.00	7.8
1.50	5.9
2.00	4.9
3.00	3.6
6.00	2.1
12.00	1.0
24.00	0.5
48.00	0.3
72.00	0.2
96.00	0.2
120.00	0.2

Instrumentation—*HPLC*—A liquid chromatograph consisting of a solvent delivery system[3] and an UV absorbance detector[4] that operates at 254 nm was used. A commercially produced stainless steel column[5] (250 × 4.6-mm i.d.) prepacked with 5-μm particles that are chemically bonded to a monomolecular layer of octadecyl groups was employed. The mobile phase was composed of acetonitrile–0.05 M KH_2PO_4 (17:83) adjusted to pH 4.0 using 1 M H_3PO_4. The mixture was filtered through a 0.2-μm pore filter[6] and deaerated prior to use. Samples were introduced *via* an injection port[7] fitted with a 2.0-mL loop. The flow rate and chart speed were 1.2 mL/min and 0.25 cm/min, respectively.

^{13}C-NMR—The natural-abundance ^{13}C-NMR spectra were obtained on a spectrometer[8] operating at 25.1 MHz. The free-induction decays were recorded using a 5-KHz spectral width and 16K computer memory. This allowed a digital resolution of 0.61 Hz/data point in the transforms. The sample was irradiated with a 10-μs pulse (40° flip angle) at 4-s intervals. A noise-modulated 100-MHz frequency source was used to

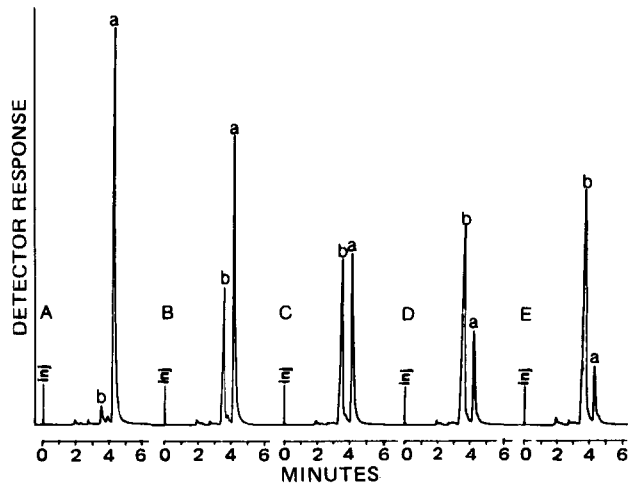


Figure 1—*Reverse-phase chromatograms of* 5R,6R-*penicilloic acid (a) and the* 5S,6R-*epimer (b) at specific time intervals during the epimerization process obtained using an ultrasphere-ODS column with a mobile phase of CH_3CN–0.05 M KH_2PO_4 (17:83) at pH 4.0 and detection at 254 nm. Key: (A) 0.03 h; (B) 6 h; (C) 12 h; (D) 24 h; (E) 48 h.*

[3] Model 6000A; Waters Associates.
[4] Model 440 Absorbance Detector; Waters Associates.
[5] Ultrasphere-ODS; Beckman Instruments, Irvine, Calif.
[6] Millipore Corp., Bedford, Mass.
[7] U6K; Waters Associates.
[8] Joel PFT-100; Joel Co., Cranford, N.J.

Table II—NMR Data Obtained During the Epimerization of 5*R*,6*R*-Benzylpenicilloic Acid to 5*S*,6*R*-Benzylpenicilloic Acid

Time, h	5*R*,6*R*/IS[a]	5*S*,6*R*/IS[a]	5*R*,6*R*/5*S*,6*R*
0.25	0.71	0.04	20
1.0	0.66	0.07	9.1
3.0	0.61	0.11	5.6
12.0	0.51	0.26	2.0
24.0	0.38	0.40	0.95
48.0	0.25	0.54	0.46
72.0	0.20	0.57	0.35
96.0	0.15	0.61	0.25
120.0	0.15	0.61	0.25

[a] IS = internal standard.

achieve broad-band decoupling of the protons. Proton-coupled ^{13}C-NMR spectra were obtained by gated decoupling (*i.e.*, decoupler off during acquisition) in order to maintain the nuclear Overhauser enhancement for the carbon signals. The carbon signals were referenced to an external standard of dioxane at 66.6 ppm.

^{1}H-NMR—The ^{1}H-NMR spectra of disodium penicilloate in deuterium oxide or sodium deuteroxide in deuterium oxide were obtained on a spectrometer[9] operating at 79.59 MHz. The free-induction decays were recorded using a 1000-Hz spectral width and 8K computer memory. This permitted a digital resolution of 0.24 Hz/data point in the transforms. The sample was irradiated with 15-μs pulse (30° flip angle) at 9-s intervals. All chemical shifts were referenced to acetone at 2.04 ppm.

Polarography—Polarographic data were obtained with a polarographic analyzer[10] which was used in conjunction with a drop timer[11]. Dropping mercury was used as the working electrode while the reference electrode was a saturated calomel electrode (SCE). A platinum wire was employed as an auxilliary electrode.

UV Spectroscopy—UV scans were made using a double-beam spectrophotometer[12] equipped with a single-pen recorder[13].

RESULTS AND DISCUSSION

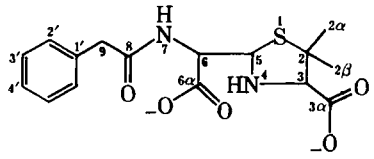
Levine (1) investigated the decomposition of benzylpenicillin at pH 7.5 and 37°C and suggested that benzylpenicilloic acid appears to exist as a mixture of isomers. Furthermore, studies[14] on benzylpenicillin degradation indicated that benzylpenicilloic acid is extremely stable in mildly alkaline solutions and, hence, remains unchanged for extended periods of time. The epimerization of benzylpenicilloic acid was, therefore, studied in alkaline media where it is the most stable.

A reverse-phase HPLC technique was developed to separate the 5*R*,6*R*- and 5*S*,6*R*-epimers of benzylpenicilloic acid. The method was specific for the two epimers and, consequently, was utilized to monitor the epimerization process. The chromatograms in Fig. 1 demonstrate that in aqueous alkaline solutions, 5*R*,6*R*-benzylpenicilloic acid gradually epimerizes at room temperature to 5*S*,6*R*-benzylpenicilloic acid until equilibrium is established. At equilibrium, 5*S*,6*R*-benzylpenicilloic acid was found to be the favored product. Table I shows the ratios of the relative concentrations of the two epimers at specific time intervals.

^{1}H-NMR was also employed to examine the epimerization process. Once again, the spectra of benzylpenicilloic acid in alkaline media revealed the progressive conversion of 5*R*,6*R*-benzylpenicilloic acid to 5*S*,6*R*-benzylpenicilloic acid with a preponderance of the latter epimer forming at equilibrium (Table II). The conversion of 5*R*,6*R*-benzylpenicilloic acid to the 5*S*,6*R*-epimer was monitored by utilizing, respectively, the methyl signals at δ 1.07 and 6.87 ppm (Fig. 2). Acetone was used both as a reference for chemical shifts and as an internal standard. Integrations are not shown for the sake of clarity.

Based on mechanistic considerations, two pathways can be proposed for the epimerization process. The first (Scheme I, pathway A) involves the enamine tautomer of penamaldic acid, a compound which exhibits strong UV absorption at 280 nm, as an intermediate, while the second (Scheme I, pathway B) involves the imine tautomer, a compound which possesses a unique polarographically active group (C=N). These analytically significant differences in the proposed intermediates were, therefore, utilized not only to distinguish between the intermediates themselves, but also to elucidate the actual epimerization pathway.

[9] FT-80; Varian Instruments, Palo Alto, Calif.
[10] Model 174A; Princeton Applied Research Corp., Princeton, N.J.
[11] Model 174/70; Princeton Applied Research Corp., Princeton, N.J.
[12] Perkin-Elmer, Norwalk, Conn.
[13] Beckman Instruments, Irvine, Calif.
[14] Unpublished observations.



It has been suggested that the enamine form of penamaldic acid exists in equilibrium with benzylpenicilloic acid in alkaline solutions (10, 11). If this were the case, alkaline solutions of benzylpenicilloic acid would be expected to exhibit a strong absorption in the 280-nm region of the UV spectrum. In addition, deuterium exchange would occur at C-6 if C-6 were, indeed, involved in any way in the isomerization process (Scheme I, pathway A). However, solutions of the disodium salt of benzylpenicilloic acid in water, phosphate buffer (pH 7.4), or borate buffer (pH 10.0) did not show strong absorption at 280 nm during the lifetime of the experiment. This phenomenon was also observed by Busson and coworkers (9). Moreover, to determine whether C-6 participates in the epimerization process as would be the case if the enamine tautomer of penamaldic acid were an intermediate, a sample of disodium 5*R*,6*R*-benzylpenicilloate was dissolved in dilute aqueous solution of sodium deuteroxide and allowed to stand at room temperature. ^{1}H-NMR spectra revealed that conversion of the 5*R*,6*R*- to the 5*S*,6*R*-epimer proceeded without deu-

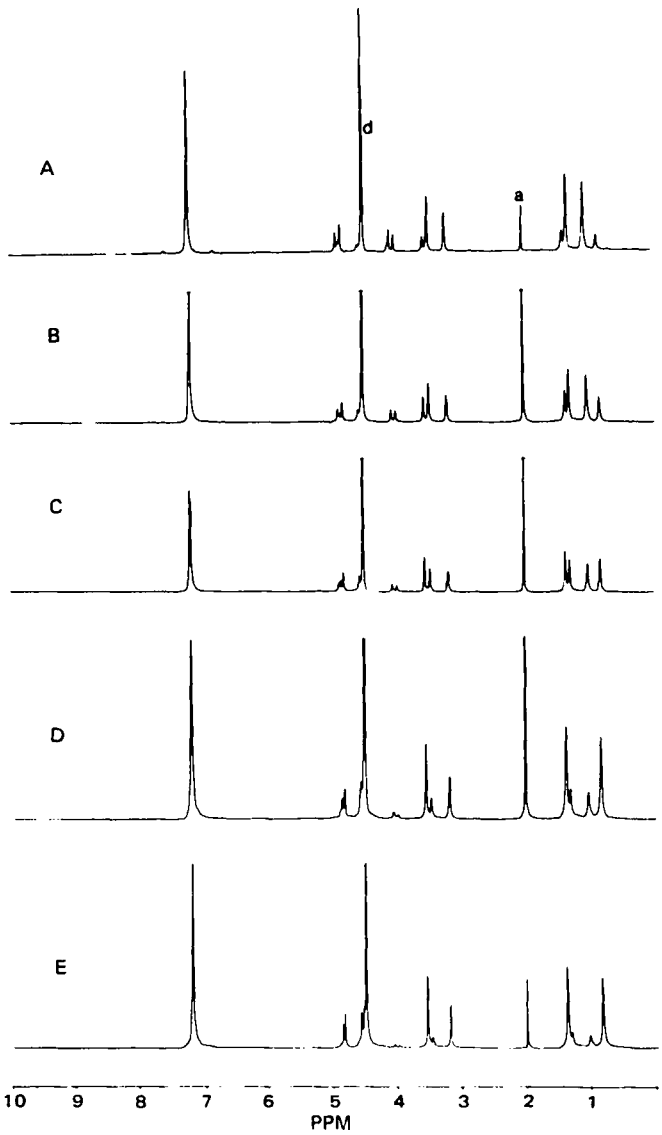


Figure 2—*^{1}H-NMR spectra showing the epimerization of* 5R,6R-*benzylpenicilloic acid (A) to* 5S,6R-*benzylpenicilloic acid (E) at specific time intervals. Key: (A) 6 h; (B) 12 h; (C) 24 h; (D) 48 h; (E) 120 h; (a) acetone; (d) water.*

terium incorporation. Though the chemical shifts of the protons at C-6 and C-5 and their coupling constants changed due to the conversion of one isomer to the other, the protons themselves remained coupled to each other, and the intensities of the peaks remained unchanged relative to the peaks of the other protons in the spectrum. These observations appear to contradict the suggestion that the enamine form of penamaldic acid exists in equilibrium with penicilloic acid in alkaline media and, consequently, exclude the enamine as an intermediate in the conversion of 5*R*,6*R*-benzylpenicilloic acid to the 5*S*,6*R*-epimer. Hence, the imine tautomer of penamaldic acid remains as the only probable intermediate involved in the epimerization process (Scheme I, pathway B).

An analytical technique employed to determine the imine in the presence of other degradation products including the enamine at very low concentrations was differential pulse polarography. Unlike the other compounds that may be present in solution, the imine tautomer contains a C=N group which is reducible at an electrode surface. Consequently, a solution of disodium 5*R*,6*R*-benzylpenicilloate was prepared and polarographed in the differential-pulse mode of operation. A reduction wave with a peak potential at −1.1 V *versus* SCE was observed. When aliquots were withdrawn from the reaction vessel after 12, 24, and 36 h and polarographed, the wave remained constant in height. An oxidation wave was also observed in the sulfhydryl region of the polarogram (~ −0.6 V *versus* SCE). However, it was not utilized for unequivocal identification since it is subject to interference from other sulfhydryl-containing compounds. Based on these results, it is concluded that in alkaline aqueous solutions 5*R*,6*R*-benzylpenicilloic acid epimerizes to 5*S*,6*R*-benzylpenicilloic acid and involves the imine tautomer rather than the enamine form of penamaldic acid as an intermediate.

The kinetic transformation of 5*R*,6*R*-benzylpenicilloic acid to its 5*S*,6*R*-epimer was determined quantitatively using the ^{1}H-NMR data presented in Table II. Assuming that the intermediate attains a steady-state concentration during the epimerization process and that the reaction rates are first order, the rate constants for both the forward and reverse reactions were computed using the following equations:

$$R \underset{k_r}{\overset{k_f}{\rightleftharpoons}} S \qquad \text{(Eq. 1)}$$

$$\log \frac{R_0 - R_{eq}}{R - R_{eq}} = \frac{(k_f + k_r)}{2.303} t \qquad \text{(Eq. 2)}$$

and

$$K = \frac{k_f}{k_r} = \frac{S_{eq}}{R_{eq}} \qquad \text{(Eq. 3)}$$

where R_0 is the initial concentration of 5*R*,6*R*-benzylpenicilloic acid, R_{eq} is the equilibrium concentration of 5*R*,6*R*-benzylpenicilloic acid, R is the concentration of 5*R*,6*R*-benzylpenicilloic acid at time t, k_f is the forward rate constant, k_r is the reverse rate constant, K is the equilibrium constant of the reaction, S is the concentration of 5*S*,6*R*-benzylpenicilloic acid at time t, and S_{eq} is the equilibrium concentration of 5*S*,6*R*-benzylpenicilloic acid; k_f was found to be 7.4×10^{-2} h^{-1}, while k_r was 1.8×10^{-2} h^{-1}. These values indicate that, at equilibrium, the ratio of the concentration of 5*R*,6*R*-benzylpenicilloic acid was ~0.2 × that of the 5*S*,6*R*-epimer, a value that is in agreement with the HPLC analysis (Table I).

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Simultaneous Determination of Methyl, Ethyl, Propyl, and Butyl 4-Hydroxybenzoates and 4-Hydroxybenzoic Acid in Liquid Antacid Formulations by Gas Chromatography

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Abstract □ An isothermal chromatographic (GC) method employing an SE-30 column and flame-ionization detection has been developed for the simultaneous assay of methyl, ethyl, propyl, and butyl 4-hydroxybenzoates and 4-hydroxybenzoic acid in liquid antacid formulations. The method, which uses a silica column chromatographic cleanup step prior to GC, is specific for the compounds with respect to possible degradation products, impurities, and excipients.

Keyphrases □ 4-Hydroxybenzoic acid—simultaneous determination with its methyl, ethyl, propyl, and butyl esters, liquid antacid formulations, gas chromatography □ Antacid formulations—liquid, simultaneous determination of 4-hydroxybenzoic acid and its methyl, ethyl, propyl, and butyl esters, gas chromatography

The methyl (I), ethyl (II), propyl (III), and butyl (IV) esters of 4-hydroxybenzoic acid (V) in various combinations are commonly used as preservatives in liquid pharmaceutical preparations. Since the antimicrobial activity of various combinations of esters are generally more than additive (1), a method for simultaneously determining the

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