variability observed in the fluorescence intensity of the different albumin solutions at each time point.

The results of linear regression analysis of the data for each type of human albumin are presented in Table I. While the r^2 value for each set of data is very low, the slope of each set of data does have a small negative value and zero is not included in the 95% confidence interval of the slope. The points at $t = 0$ and $t = 510$ min in Fig. 1 represent the linear least-squares fit of the experimental data. The actual lines were not drawn so that the experimental data could be examined without obstruction.

To determine if the small decrease in fluorescence intensity with time was due to inadequate washing of fluorescence sample cells between sample measurements, the experiment with fraction V human albumin was repeated. In this second study, fluorescence measurements were taken every hour to decrease the use of each sample cell. After each fluorescence measurement the cells were rinsed once with water, filled with sulfuric acid–dichromate cleaning solution for 15 min and rinsed using the procedure used in the previous experiments. The results are presented in Fig. 2. The fluorescence intensities of the solutions again appeared to undergo a small decrease with time. The results of linear regression analysis of the data are presented in Table I. The slope has a small negative value which is within the 95% confidence interval of the slope previously obtained for fraction V human albumin. As observed previously, zero is not within the 95% confidence interval of the slope. Thus, this different method of sample cell treatment between fluorescence measurements had no significant effect on the apparent decrease in fluorescence intensity of these protein solutions with time.

In conclusion, the intrinsic fluorescence intensity of each of the solutions of human albumin examined in this study was very stable for the first 8 hr after preparation. Based on these results, the practice of preparing solutions of human albumin several hours prior to use simply to achieve fluorescence stability is unnecessary. Since the fluorescence intensity of each of the albumin solutions examined underwent a small, consistent decrease with time, it may be advisable to use a reference solution of human albumin, as described previously (6), to correct binding data obtained from the quenching of intrinsic human albumin fluorescence by ligand.

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Separation of Penicillin and Its Major Degradation Products by Ion-Pair Reversed-Phase High-Pressure Liquid Chromatography

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Abstract □ An ion-pair reversed-phase high-pressure liquid chromatographic technique capable of separating penicillin and its major degradation products within 8 min was developed. The influence of pH, counterion concentration, buffer concentration, and organic modifier content was studied and the observed behavior of the compounds during the chromatographic process was discussed.

Keyphrases □ Penicillin—major degradation products, separation by ion-pair reversed-phase high-pressure liquid chromatography □ High-pressure liquid chromatography—ion-pair reversed-phase, separation of penicillin and its major degradation products □ Degradation products—penicillin, separation by ion-pair reversed-phase high-pressure liquid chromatography

Various analytical methods for the detection, separation, and/or quantification of penicillin in the presence of its degradation products have been reported (1–7). The most recent papers deal with anion-exchange chromatography (5), reversed-phase chromatography (6), and NMR spectroscopy (7). NMR generally lacks sensitivity while the chromatographic approaches result in relatively long analysis times. However, the availability of small-particle size packings for reversed-phase chromatography suggests that very high chromatographic efficiencies and shorter analysis times can be expected with this procedure (8, 9). The present paper describes a high-pressure liquid chromatographic (HPLC) method that utilizes an ion-pair reversed-phase technique to separate penicillin and its three major degradation products: penillic acid, penicilloic acid, and penilloic acid in <8 min.

EXPERIMENTAL

Chemicals and Reagents—Penicillin G potassium[1] and tetrabutylammonium chloride[2] were obtained commercially and used without further treatment. Penillic acid, penicilloic acid, and penilloic acid were synthesized by standard methods (10). Acetonitrile was HPLC grade[3] while all other chemicals were either USP or reagent grade. Double-distilled water was used to prepare buffer solutions.

Apparatus—A liquid chromatograph[4] equipped with a fixed-wavelength UV absorbance detector set at 254 nm was used. A commercial stainless steel column[5] (150-mm × 4.6-mm i.d.) prepacked with 5-μm particles, with the silanol groups chemically bonded to a monomolecular layer of octadecylsilane, was used. A strip-chart recorder[6] recorded the detector output.

Chromatographic Conditions—The mobile phase was composed of acetonitrile, phosphate buffer, and a counterion. The ion-pair reagent, tetrabutylammonium chloride, was dissolved in the phosphate buffer

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Waters Associates, Milford, Mass.
[4] Model ALC 202, Waters Associates, Framingham, Mass.
[5] Ultrasphere-ODS, Beckman Instruments, Irvine, Calif.
[6] Omniscribe recorder, Houston Instruments, Houston, Tex.

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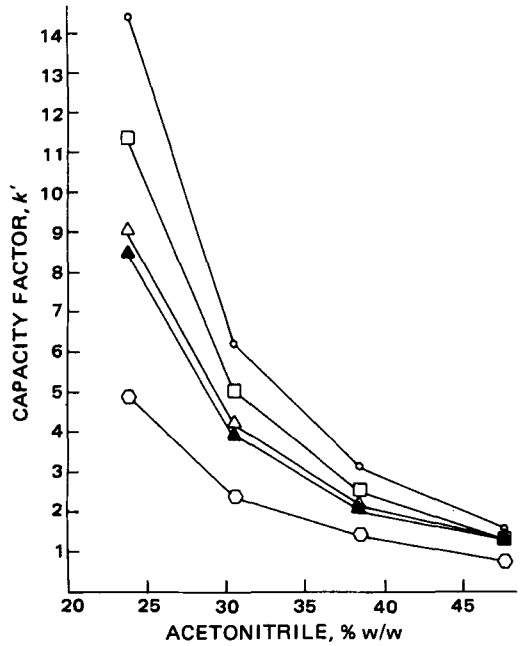

Figure 1—*Plots of the capacity factor* versus *the acetonitrile concentration in the mobile phase. Key:* ○, *penicillin;* □, *penicilloic acid;* △,▲, *penilloic acid; and* ○, *penillic acid.*

and the pH was adjusted with 1 *M* phosphoric acid before the required volume of acetonitrile was added. The mixture was filtered through a 0.45-μm filter[7] and degassed prior to use. The column was operated at ambient temperatures. Samples were introduced *via* an injection port[8] fitted with a 20-μl loop. The flow rate and the chart speed were 1.5 ml/min and 0.51 cm/min, respectively. The sensitivity range of the detector was set at 0.16 aufs.

RESULTS AND DISCUSSION

Penicillin and its major degradation products contain carboxylic acid functional groups with pH-dependent ionic states. Consequently, in the past, anion-exchange chromatography was usually used for the separation

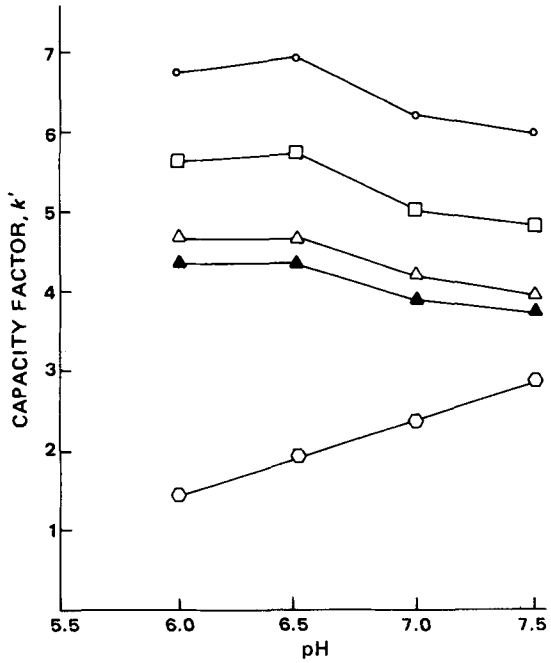

Figure 2—*Plots of the capacity factor* versus *the mobile phase pH. Key:* ○, *penicillin;* □, *penicilloic acid;* △,▲, *penilloic acid; and* ○, *penillic acid.*

[7] Millipore Corp., Bedford, Mass.
[8] Rheodyne model 70-10, Rheodyne, Berkeley, Calif.

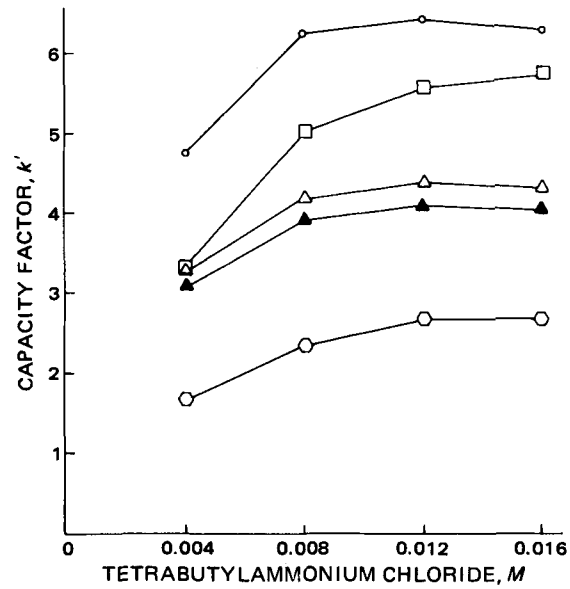

Figure 3—*Plots of the capacity factor* versus *the concentration of the ion-pair reagent in the mobile phase. Key:* ○, *penicillin;* □, *penicilloic acid;* △,▲, *penilloic acid; and* ○, *penillic acid.*

of these compounds. A reversed-phase mode utilizing an ion-suppression technique also was employed to achieve separation. However, in addition to having long analysis times, both methods use mobile phases with pH values less than optimum with respect to the stabilities of the compounds being analyzed.

The ion-pair reversed-phase technique reported here combines the advantages of both methods and also utilizes mobile phases with pH values compatible with the chemical stabilities of the solutes (10, 11). The pH range selected (6.0–7.5) satisfies the requirement that bonded-phase columns not be used at high pH while also maximizing the ionization of the solutes, despite the reported rise in pKa values as organic modifiers are added to aqueous solutions (12).

The effects of pH, counterion concentration, acetonitrile content, and phosphate buffer concentration on the capacity factors (k') and separation selectivities were studied (Figs. 1–4). The k' values of penicillin and its major decomposition products were affected substantially by a change in the percent composition of acetonitrile in the mobile phase (Fig. 1).

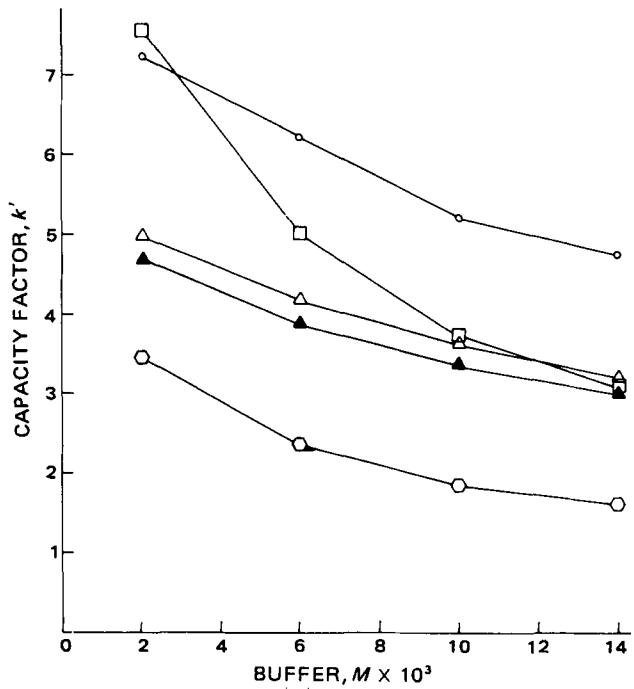

Figure 4—*Plots of the capacity factor* versus *the phosphate buffer concentration in the mobile phase. Key:* ○, *penicillin;* □, *penicilloic acid;* △,▲, *penilloic acids; and* ○, *penillic acid.*

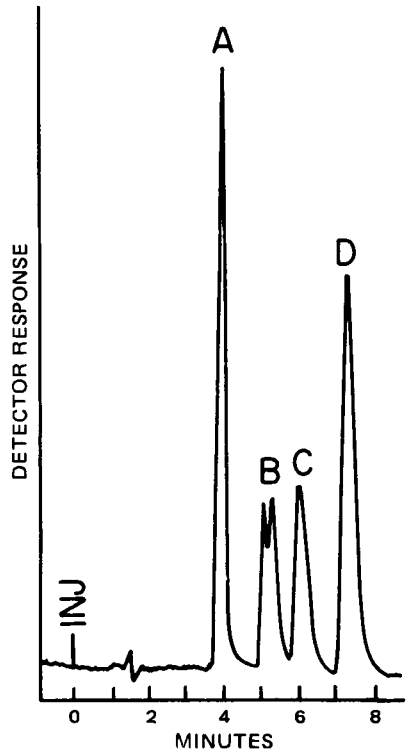

Figure 5—*Ion-pair reversed-phase high-pressure liquid chromatogram of penicillin and its major degradation products. Key: A, penillic acid; B, penilloic acid; C, penicilloic acid; and D, penicillin.*

At high acetonitrile concentrations, all four compounds gave sharp and symmetrical peaks; however, the capacity factors were so close to each other that the resolution was inadequate. Lowering the acetonitrile content improved the resolution significantly. Even the single peak of penilloic acid was partially resolved into two peaks which presumably represent the 5*R* and 5*S*, diastereoisomers of the compound (7).

Maximum k' values for penicillin, penicilloic acid, and penilloic acid were obtained between pH 6.0 and 6.5, when the compounds were completely ionized and ion-pair formation was at a maximum (Fig. 2). As the pH of the mobile phase increased, the k' values of the three anionic compounds decreased, presumably because hydroxyl ions compete with them and reduce the number of ion-pairs that would be formed (13). Although the penillic acid pK_1 and pK_2 values were similar to the ionization constants of the other three compounds (14), its capacity factor increased proportionally with an increase in pH.

Initially, the k' values of the sample molecules increased as the concentration of the ion-pair reagent was raised, but leveled off at higher concentrations (Fig. 3). This was expected because, once all sample anions form ion-pairs, the excess counterions would have no direct effect on retention. However, the ionic strength of the mobile phase increases and, in turn, varies the capacity factors. At low concentrations of ion-pair reagent, the retention times of the compounds are greatly reduced since the number of counterions available is insufficient for maximum ion-pair formation.

Furthermore, an increase in buffer concentration in the mobile phase led to a significant decrease in k' values of penicillin and its degradation products (Fig. 4). This was thought to be due mainly to the phosphate ions tying up the counterions, which would otherwise form ion-pairs with sample molecules. The simultaneous increase in ionic strength also influences the k' values of sample anions.

Logarithmic plots of the capacity factors and the four parameters studied showed linear relationships with correlation coefficients >0.99. The slopes of the curves in each graph were not always the same, clearly indicating the array of selectivities offered by the chromatographic system.

The composition of the mobile phase used to develop the chromatogram in Fig. 5 was determined by these studies. A pH of 7.5 was chosen because the peaks of the compounds were significantly removed from the solvent front and the compounds that eluted last had the lowest k' value compared to those at other pH values. Both 0.008 and 0.012 *M* tetrabutylammonium chloride yielded excellent resolution of the four compounds, but the former concentration was selected because it gave better resolution between the penicillin and penicilloic acid peaks, thus reducing the probability of overlap that might occur at high penicillin concentrations. A 0.006 *M* buffer concentration in the mobile phase offered the best selectivity and was taken as the optimum value. A 30% acetonitrile concentration was used since it provided maximum separation and reasonable analysis time.

The described chromatographic method could be extended to include other degradation products of penicillin. The four compounds studied were selected primarily for two reasons: (*a*) penicilloic acid, penillic acid, and penilloic acid are of prime importance in stability studies since they are major decomposition products of penicillin in acidic and basic media, and (*b*) since penicillin and penilloic acid are monocarboxylic acids, and penicilloic and penillic acids are dicarboxylic acids, a detailed study of their behavior in ion-pair reversed-phase chromatography could assist in the application of this analytical method for other degradation products of penicillin and similar compounds of pharmaceutical interest.

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