Then:

$$\begin{matrix}\text{between-subject} \\ \text{sum of squares}\end{matrix} = \sum_{i=1}^{12} \frac{S_i^2}{2} - \frac{\left(\sum_{i=1}^{12} S_i\right)^2}{24} \quad \text{(Eq. A10)}$$

with 11 degrees of freedom, $S_i = X_{i_A} + X_{i_E}$, and:

$$\begin{matrix}\text{within-subject} \\ \text{sum of squares}\end{matrix} = \sum_{i=1}^{12} \frac{(X_{i_A} - X_{i_E})^2}{2} \quad \text{(Eq. A11)}$$

with 12 degrees of freedom.

In Eqs. A10 and A11, X_{i_A} and X_{i_E} refer to individual subject data following Treatments A and E, respectively.

An estimate of the ratio of between-subject variability to within-subject variability is given by Eq. A12:

$$B/W = \frac{MS_{\text{between}}}{MS_{\text{within}}} \times \frac{1}{k} - \frac{1}{k} \quad \text{(Eq. A12)}$$

when MS_{between} and MS_{within} represent the between-subject mean square and within-subject mean square, respectively, obtained by dividing the appropriate sum of squares by the corresponding number of degrees of freedom; and k is the number of treatments.

The 95% confidence interval for B/W is given by:

$$\text{prob}\left[\frac{MS_{\text{between}}}{kMS_{\text{within}}} \times \frac{1}{F_{0.975}(11,12)} - \frac{1}{k} \leq B/W \leq \frac{MS_{\text{between}}}{kMS_{\text{within}}} \times \frac{1}{F_{0.025}(11,12)} - \frac{1}{k}\right] = 0.95 \quad \text{(Eq. A13)}$$

If the left end-point of the constructed interval exceeds unity, between-subject variability is considered greater than within-subject variability, the magnitude of which is given by Eq. A12.

Area under Curve and Half-Life Estimation—The area under the curve (AUC) was calculated for each subject following each treatment by the following equation:

$$AUC_\infty = AUC_T + \hat{C}_T/\beta \quad \text{(Eq. A14)}$$

where AUC_∞ is the area under the curve through infinity, AUC_T is the area estimated by the trapezoidal rule up to time T, T is the last sampling time (usually 16 hr) when the observed concentration was above the sensitivity limit of the radioimmunoassay, β is the apparent elimination rate constant obtained by the method of least squares in the terminal log-linear phase, and $\hat{C}_T$ is the estimated serum concentration at time T observed by use of the exponential equation that defines the log-linear region.

The half-life ($t_{1/2}$) was estimated by the equation:

$$t_{1/2} = 0.693/\beta \quad \text{(Eq. A15)}$$

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NOTES

Separation of Penicillin G Potassium and Its Degradation Products Using High-Pressure Liquid Chromatography

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Received November 20, 1978, from the *Department of Pharmacy, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104.* Accepted for publication March 8, 1979. *Present address: Pharmaceutical Division, Ciba-Geigy Corp., Summit, NJ 07901. ‡Marion Laboratories, Kansas City, MO 64141.

Abstract □ A high-pressure liquid chromatographic technique was developed for the separation of penicillin G potassium and several of its decomposition products. The method utilized a buffered acetonitrile-phosphate mobile phase on a reversed-phase C_{18} column. Separation of penicillin G potassium and six degradation products was attained within 25 min.

Keyphrases □ Penicillin G potassium—analysis, high-pressure liquid chromatography, separation from degradation products □ High-pressure liquid chromatography—analysis, penicillin G potassium, separation from degradation products □ Antibacterial agents—penicillin G potassium, high-pressure liquid chromatographic analysis, separation from degradation products

The separation of penicillin G from mixtures of penicillin or related decomposition products was reported previously (1–7). Separation methods include TLC (1–3), GLC (4, 5), and high-pressure liquid chromatography (HPLC) (6, 7). Continuing interest in this area is due in part to ongoing efforts to identify the causative agents in penicillin allergy. Most degradation products formed during penicillin G hydrolysis can elicit an allergic response (8).

This report describes an HPLC technique which separates penicillin G potassium and six decomposition products within 25 min.

EXPERIMENTAL

Materials—Penicillin G potassium[1] and D,L-penicillamine[2] were obtained commercially and used as received. Benzylpenicilloic acid, benzylpenillic acid, benzylpenilloic acid, and benzylpenamaldic acid were prepared using standard procedures (9–11). Acetonitrile[3] was spectral

[1] Lot W732511, Wyeth Laboratories, West Chester, Pa.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Matheson, Coleman and Bell, Norwood, Ohio.

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Table I—Retention Time of Penicillin G Potassium and Various Degradation Products Separated by HPLC [a]

Compound	Retention Time, min
Buffer salts	3.6
Penicillamine	3.9
Compound F	4.9
Benzylpenillic acid	6.7
Benzylpenicilloic acid	12.7
Benzylpenilloic acid	14.5
Benzylpenamaldic acid	18.0
Penicillin G potassium	25.0

[a] μBondapak C_{18}; conditions as described.

quality; all other chemicals were reagent grade. Distilled, deionized, degassed water was used to prepare all solutions.

Apparatus—A liquid chromatograph[4] equipped with a 254-nm UV detector and a reciprocating pump[5] to deliver the mobile phase were used. A stainless steel column (4 mm i.d. × 30 cm), prepacked with a small diameter silica having a monomolecular layer of octadecyltrichlorosilane[6] chemically bonded to its surface, was obtained commercially. A precolumn (3 mm i.d. × 10.16 cm) filled with a high-capacity reversed-phase packing[7] was used to protect the microparticular packing from disturbance and contamination.

Mobile Phase—The mobile phase was prepared by dissolving 4.24 g of monobasic potassium phosphate in sufficient water to make 400 ml. One hundred milliliters of acetonitrile was added to this solution, and the pH of the final solution was adjusted to 4.15 with 1.00 *N* HCl.

Chromatographic Conditions—The chromatographic column was operated at ambient temperature, and injections were made into a stopped-flow injection port[8] using a 10-μl syringe[9]. The UV detector was set at 0.08 absorbance unit full scale (aufs) for penicillin G potassium, benzylpenicilloic acid, benzylpenamaldic acid, benzylpenilloic acid, and penicillamine. The detector range was varied for benzylpenillic acid. Chromatograms were recorded on a single-pen strip-chart recorder[10]. The flow rate and chart speed were maintained at 0.75 ml/min and 1.27 mm/min, respectively. Fifty milliliters of mobile phase was passed through the column prior to use each day.

Individual samples of penicillin G potassium and possible acid degradation products were prepared by dissolving an appropriate amount of material in sufficient water to make 50.0 ml. Samples of penicillin G potassium degraded in acidic media were prepared by dissolving penicillin G potassium in sufficient preheated pH 2.83 phosphate buffer to make 50.0 ml.

RESULTS AND DISCUSSION

In the past, the separation of penicillin G in mixtures of penicillins or related degradation products has been accomplished using TLC and GLC. However, these methods have several shortcomings. The long development time required for TLC separation does not lend itself to kinetic studies; and, while GLC provides shorter analysis time, the separations require derivatization and are limited by the labile nature of the compounds.

Several recent reports noted the use of HPLC with anionic (6) and cationic (7) exchange resins to effect separation. The reported retention times of benzylpenicilloic acid and benzylpenilloic acid using cation exchange were 156 and 768 min, respectively, making this procedure unsuitable for kinetic studies. Another criticism of ion exchange is the inherent potential of the system to serve as a possible source of degradation catalysis.

Liquid-liquid reversed-phase chromatography, with its less severe operating conditions, provides an advantage over previously reported methods.

Initial separations were obtained using a pellicular C_{18} packing[11] with various aqueous buffer solutions mixed with methanol or acetonitrile. However, a tailing problem could not be eliminated.

A reversed-phase packing with a heavier coating of stationary liquid phase[11] was used with some success. Initial separations were accomplished using a prepacked column (2 mm i.d. × 61 cm) and also a hand-packed (12) column (3.18 mm i.d. × 1 m). Improvement in the technique resulted when two of the latter columns were butted end to end.

Vastly improved separation was obtained with a microparticular reversed-phase packing[6]. The packing has a monomolecular layer of octa-

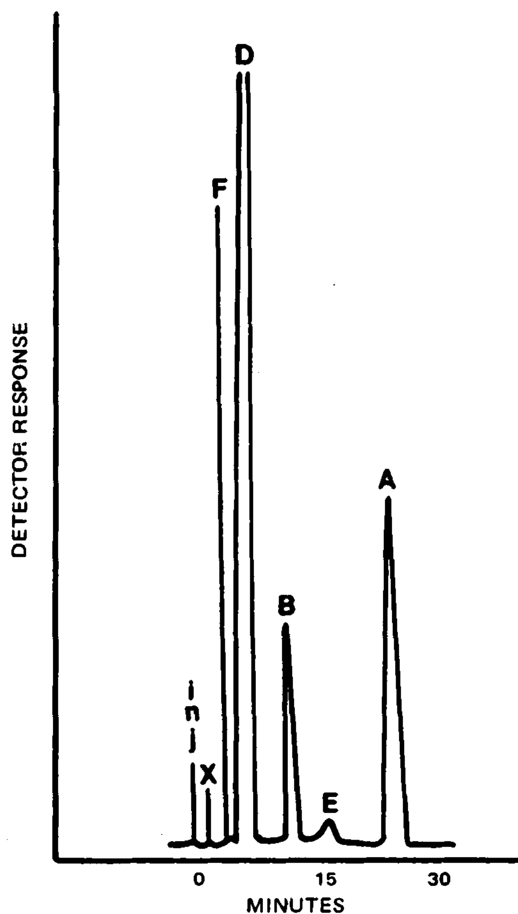

Figure 1—*Representative high-pressure liquid chromatogram of an aged penicillin potassium G sample in 0.100* M *phosphate buffer. Key: A, penicillin G potassium; B, benzylpenicilloic acid; D, benzylpenillic acid; E, benzylpenilloic acid; F, Compound F; and X, buffer salts.*

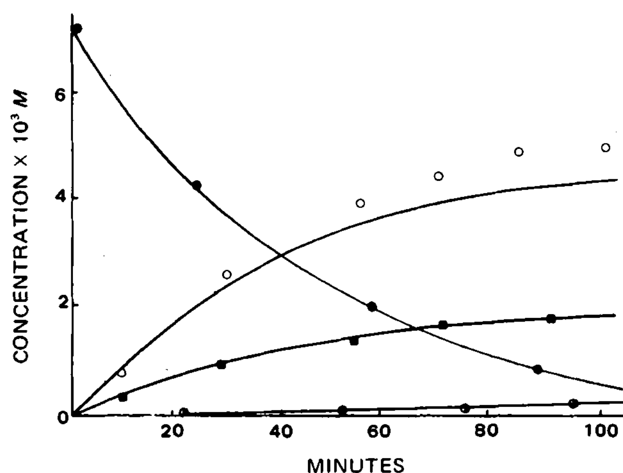

Figure 2—*Comparison of experimental data and predicted values based on proposed model. Degradation of penicillin G potassium in 0.100* M *phosphate buffer at pH 2.83, μ = 0.500, and 30.5°. Key: solid lines, predicted value; ●, penicillin G potassium; ■, benzylpenillic acid; ○, benzylpenicilloic acid, and ◑, benzylpenilloic acid.*

[4] Model 1205, Laboratory Data Control, Riviera Beach, Fla.
[5] Mini-Pump, Milton Roy Co., St. Petersburg, Fla.
[6] μBondapak C_{18}, Waters Associates, Milford, Mass.
[7] C_{18} Porasil B, Waters Associates, Milford, Mass.
[8] Model LIB, Reeve Angel, Clifton, N.J.
[9] Model LC-210, Precision Sampling Corp., Baton Rouge, La.
[10] Model 3301, Laboratory Data Control, Riviera Beach, Fla.

[11] VYDAC RP, The Separations Group, Hesperia, Calif.

decyltrichlorosilane chemically bonded to a small diameter (<10 μm) silica particle. The smaller particle size results in faster analysis time with improved resolution.

The retention times of penicillin G potassium and several degradation products formed during acid hydrolysis of penicillin G potassium at pH 2.83 are given in Table I. Identification of penicillin G potassium and related compounds was accomplished by comparing the retention time of the unknown with that of known standards. An unidentified compound with a retention time of 4.9 min was detected and designated as Compound F. A representative chromatogram for pH 2.83 is shown in Fig. 1. No penicillamine and penamaldic acid were detected at this pH.

The stability of penicillin G potassium and its degradation products in the HPLC mobile phase also was determined. Samples of penicillin G potassium and its degradation products were aged in the acetonitrile–phosphate mobile phase for a period at least equal to the retention for the particular compound. Samples were injected onto the HPLC column to observe any change in peak height or any increase in the number of peaks. Additionally, solutions containing mixtures of penicillin G potassium and its degradation products were aged in the mobile phase and subjected to the same test. No change in the number of peaks or peak height was noted.

Figure 2 illustrates the results obtained for penicillin G potassium degradation at pH 2.83 using the described HPLC process. The studies are continuing, and the complete kinetic scheme will be reported in a subsequent article.

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Sensitive High-Pressure Liquid Chromatographic Determination of Disopyramide and Mono-*N*-dealkyldisopyramide

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Received March 19, 1979, from the *College of Pharmacy, North Dakota State University, Fargo, ND 58105.* Accepted for publication April 5, 1979.

Abstract □ A high-pressure liquid chromatographic procedure for the accurate determination of disopyramide and its chief metabolite in plasma is presented. The method is suitable for monitoring patients receiving disopyramide therapy. A reversed-phase cyanopropylsilane column is utilized with a mobile phase of 50% acetonitrile and 50% 0.01 *M* sodium acetate buffer at pH 4.0. Absorption was monitored at 254 nm with a detection limit of 0.2 μg/ml of plasma. The reproducibility and precision of the procedure were demonstrated on samples containing 0.50–12 μg/ml of plasma.

Keyphrases □ Disopyramide—analysis, high-pressure liquid chromatography, plasma, metabolites, humans □ Disopyramide—metabolites, analysis, high-pressure liquid chromatography, humans □ Mono-*N*-dealkyldisopyramide—analysis, high-pressure liquid chromatography, plasma, humans □ Antiarrhythmic agents—disopyramide, high-pressure liquid chromatographic analysis, plasma, humans

Disopyramide phosphate[1] is a relatively new antiarrhythmic drug with electrophysiological properties similar to quinidine, but it is reported to be better tolerated (1). The wide range of plasma half-lives (2) of disopyramide phosphate in humans (4–18 hr) indicates that patients may require individualized dosage regimens. The side effects of the drug (3, 4) and the antiarrhythmic effects of disopyramide phosphate have been related to concentration (5, 6). Consequently, measurement of the concentration may provide a method to maximize therapeutic effect while minimizing potential side effects.

Direct spectrophotometric (7) and spectrofluorometric (8) determinations have been reported. Both these techniques are nonspecific because the major metabolite (mono-*N*-dealkyldisopyramide) has similar spectral characteristics to the parent compound (7, 8). GLC procedures also have been reported, but they either require derivatization of the compound (9) or a selective (nitrogen) detector (10). Quantitation of disopyramide and its metabolite has also been performed by high-pressure liquid chromatography (HPLC). The reported methods require the ion-pair modification of octadecylsilane reversed-phase chromatography (11) or the use of a multiwavelength detector (12, 13).

This paper describes a rapid, specific method for the determination of disopyramide and its major metabolite by HPLC using a reversed-phase cyanopropylsilane column and a 254-nm detector. The method is applicable to the direct determination of plasma levels, even in the

[1] Norpace.

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