High-Pressure Liquid Chromatographic Analysis of Penicillin G Potassium and Its Degradation Products

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Abstract □ An anion-exchange high-pressure liquid chromatographic system capable of separating penicillin G potassium from five of its degradation products was developed. The retention times were: penicillin G potassium, 17.5 min; DL-penicillamine, 4.5 min; benzylpenilloic acid, 7.0 and 8.0 min; benzylpenamaldic acid, 13.0 min; benzylpenicilloic acid, 19.5 min; and benzylpenillic acid, 22.0 min. In addition, the system permits quantification using linear calibration curves.

Keyphrases □ Penicillin G potassium and degradation products—high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography—analysis, penicillin G potassium and degradation products

It has been suggested that penicillin degradation products may have a role in eliciting penicillin allergy (1). Since most analytical methods are directed at identifying and quantifying intact penicillin, a method of detection, separation, and quantification is needed to determine the presence of specific degradation products. Such a method would also be useful in pharmacokinetic studies.

Penicilloic acids have been separated from their parent penicillins using low-voltage electrophoresis (2), TLC (3), and solvent extraction (4). TLC has also been used to separate penicillin G potassium and four model decomposition products (5).

Recent reviews (6, 7) indicated that high-pressure liquid chromatography (HPLC) could be useful for the separation and quantification of compounds of pharmaceutical interest. Thus far, limited use has been made of HPLC in analyzing penicillins. The separation of the methylbenzyl ester of penicillin G from its impurities was reported (8), where a stationary polyamide phase was used with a hexane–ethanol mobile phase. An anion-exchange column with a pH 9.1 disodium borate buffer as the mobile phase was used to separate penicillin from additives in a penicillin dosage form[1]. The anion-exchange mode also was used to determine the amount of ampicillin contamination of nitrofurantoin preparations (9). The system separated ampicillin from the nitrofurantoin and other unidentified impurities.

An anion-exchange HPLC method for the separation and quantification of penicillin G potassium and five of its decomposition products was developed and is the subject of this report.

EXPERIMENTAL

Materials—All chemicals used were USP or reagent grade.

Penicillin G potassium[2], 1585 units/mg, and DL-penicillamine[3] were obtained commercially.

Benzylpenillic acid was synthesized following a procedure reported by Cook (10). Penicillin G potassium (0.05 *M*) was allowed to stand in a 0.03 *M* citric acid–0.0067 *M* disodium phosphate buffer, pH 2.70, at 25° for 24 hr. The white crystalline precipitate was collected and dried in a stream of air. Its identity was confirmed by potentiometric titration [pKa 3.25 and 7.7; literature (10) pKa 3.2–3.4 and 7.7], elemental analysis[4], IR spectroscopy, and mass spectrometry.

Benzylpenilloic acid was synthesized by a standard method (11, 12). Two grams of penicillin G potassium was placed in a round-bottom flask with 568 ml of water and 56.2 ml of 0.1 *N* hydrochloric acid (molar equivalent) and refluxed for 3 hr. The product was cooled, frozen, lyophilized[5], and recrystallized from methanol. An NMR spectrum of the product in deuterated chloroform gave signals that agree with the structure for benzylpenilloic acid.

Benzylpenamaldic acid was obtained using HPLC. Penicillin G potassium (0.05 *M*) was dissolved in the pH 2.7 citric acid–disodium phosphate buffer and aged at 37° for 2 hr. Ten-microliter samples were withdrawn and injected onto an anion-exchange[6] column in a high-performance liquid chromatograph[7]. The compounds were eluted with a mobile phase of 15.4 ml of 0.1 *M* citric acid and 7.0 ml of 0.2 *M* disodium phosphate diluted to 650 ml with double-distilled water. The fraction of eluent containing the compound with a retention time of 13 min was collected immediately and then lyophilized. The compound was identified as benzylpenamaldic acid based upon its UV spectrum, which possesses a unique ϵ_{max} in methanol at 282.5 nm, its instability to acid, and its partitioning properties (13).

Benzylpenicilloic acid was prepared using a standard synthesis (11). Exactly 70.6 ml of 0.05 *N* sodium hydroxide was added dropwise over 1 hr to a solution of 1.16 g of penicillin G potassium in 10 ml of double-distilled water. The mixture was allowed to stand at room temperature for 1.5 hr and then was brought to pH 5.6 with the addition of 1.25 ml of 0.1 *N* hydrochloric acid. The resulting solution was lyophilized and the product was shown to be benzylpenicilloic acid by a positive reaction to the arsenomolybdic acid–mercuric chloride test (4).

Instrumentation—The liquid chromatograph[7] was equipped with a UV detector operating at 254 nm and a double-beam refractive index detector. The UV detector was used for the quantitative aspects of all compounds. The double-beam refractive index detector, referenced against a cell filled with mobile phase, was used in the preparation of a calibration curve of benzylpenamaldic acid. The recorder was operated at a chart speed of 2.54 cm/5 min.

The stationary phase was an anion-exchange resin prepacked into a 0.61-m (2-ft) stainless steel column, 2.3 mm i.d.[6]. The mobile phase was a citric acid–disodium phosphate buffer prepared from stock solutions of 0.1 *M* citric acid and 0.2 *M* disodium phosphate. All injections were made on stream with a 25-μl high-pressure syringe[8].

The instrument was operated at ambient temperature at a flow

[1] Waters Associates, Inc., Framingham, Mass., personal communication.

[2] Chas. Pfizer and Co., New York, N.Y.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Midwest Microlab, Ltd., Indianapolis, Ind.
[5] Model USM-15, Virtis Co., Gardiner, N.Y.
[6] Bondapak AX/Corasil, Waters Associates, Inc., Framingham, Mass.
[7] Model ALC 202, Waters Associates, Inc., Framingham, Mass.
[8] Series B-110 Pressure-LOK liquid syringe, Precision Sampling Corp., Baton Rouge, La.

rate of 0.7 ml/min. With a single 0.61-m (2-ft) column, this flow rate was achieved with a pressure of 600–700 psi. When a pair of 0.61-m (2-ft) columns was coupled together, a pressure of 1000–1200 psi was required.

Methods—UV scans were run using a double-beam spectrophotometer[9]. IR[10] spectral samples were prepared as KBr pellets. Mass spectra[11] were obtained at 75 ev. NMR spectra[12] were determined using tetramethylsilane as the internal reference.

The least-squares regression line and standard error of estimate, $S_{y \cdot x}$, calculated using a standard program[13], were used to construct each calibration curve. The standard error of estimate has properties analogous to those of the standard deviation. For example, if lines parallel to the regression line of y on x at respective vertical distances $S_{y \cdot x}$, $2S_{y \cdot x}$, and $3S_{y \cdot x}$ were constructed, there would be included between these lines about 68, 95, and 99.7% of the sample points, respectively.

RESULTS AND DISCUSSION

Development of HPLC Separation System—An assay developed for the separation of penicillin from additives in a penicillin dosage form used an anion-exchange column, with 0.01 *M* disodium borate buffer (pH 9.1) as the mobile phase[1]. The system was the first one investigated for the separation of penicillin degradation products. Intact penicillin G potassium was separated from penicillin degradation products; however, the degradation products were not resolved.

Majors (14) suggested that the pH of the mobile phase for the separation of weakly basic or acidic solutes should be approximately 1.5 pH units above the pKa of the solute. The pKa of penicillin G is 2.76 (12), indicating that a mobile phase having a pH of approximately 4.25 would best achieve separation if the pKa's of the decomposition products were close to that of penicillin G. A 0.135 *M* citric acid–disodium phosphate buffer, which could be adjusted over a pH range of 2.2–7.8 (15), was varied from pH 3.45 to 4.60. Benzylpenicilloic acid, which had been stored at room temperature for 1 year, was used to test the efficiency of the mobile phase. The number of peaks obtained when this sample dissolved in water was injected into the column varied from four to six. The optimum separation was observed when the pH of the mobile phase was 3.58–3.80.

Once the optimum pH for the mobile phase had been established, the length of the column was increased by coupling a pair of 0.61-m (2-ft) columns. The longer column increased the number of peaks obtained from an aged benzylpenilloic acid sample from two to three.

Decreasing the buffer concentration of the mobile phase led to improved separation of the sample peaks. Optimum separation was achieved at pH 3.8 with a 0.0045 *M* mobile phase of a final composition of 15.4 ml of 0.1 *M* citric acid and 7 ml of 0.2 *M* disodium phosphate diluted to 650 ml with double-distilled water. This buffer system was used as the mobile phase with a 1.22-m (4-ft) anion-exchange column for the separations reported. The buffer salts, possibly by displacement of anionic species at the time of sample injection, produced a single peak with a retention time of 4.0 min.

Penicillin G Potassium—Accurately weighed samples of about 2 mg/ml of penicillin G potassium were dissolved in water and injected into the HPLC system. A single peak with a retention time of 17.5 min was eluted. A calibration curve was prepared each day by injecting freshly prepared samples of 1–11 μl and calculating the peak areas by multiplying peak height by the width at half-height. A least-squares linear regression program was used to construct a calibration curve. A typical calibration curve had a slope of 24.6 $mm^2/\mu g$ and an intercept of -2.5 mm^2. The correlation coefficient for the linear region from 2 to 22 μg was 0.999, and the standard error of estimate was 20.9 mm^2.

Benzylpenillic Acid—A similar technique was followed with benzylpenillic acid, except that it was dissolved in 0.05 *M* sodium hydroxide in concentrations ranging from 1 to 2 mg/ml to prepare samples for injection. It was eluted from the column as a single peak with a retention time of 22 min. Linear calibration curves over a range of 1–20 μg were obtained. Benzylpenillic acid is relatively stable in dilute alkali (16), and no chromatographic changes were observed in the freshly prepared samples used to prepare the calibration curves. Values such as 185.2 $mm^2/\mu g$ for the slope and -18.6 mm^2 for the intercept were obtained. The lowest correlation coefficient was 0.982 with a standard error of estimate of 114.4 mm^2.

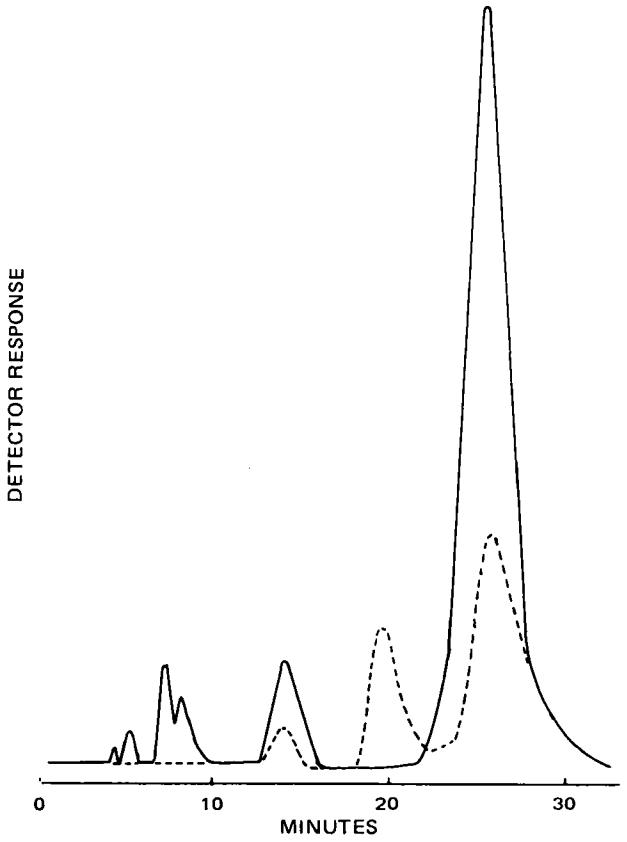

Figure 1—*Liquid chromatogram of a 0.05* M *penicillin G potassium solution aged in a 0.03* M *citric acid–0.0067* M *disodium phosphate buffer, pH 2.7, 37°; UV detector sensitivity, 254 nm; 0.04 auf except 0.08 auf for 31.5-hr benzylpenillic acid peak. Reported retention times are for single-component injections. Key: buffer salts, 4.0 min; penicillamine, 4.5 min; benzylpenilloic acid, 7.0, 8.0 min; benzylpenamaldic acid, 13.0 min; penicillin G potassium, 17.5 min; benzylpenillic acid, 22.0 min; - - - -, aged 7 min at 37°; and —, aged 31.5 hr at 37°.*

Penicillamine—Solutions of DL-penicillamine were prepared in concentrations of 0.49 and 0.90 mg/ml in 0.025 *M* NaOH, and 1–16 μl was injected. A single peak with a retention time of 4.5 min was observed. A linear calibration curve with a slope of 15.8 $mm^2/\mu g$ and an intercept of -12.49 mm^2 was typically obtained. The linear region between 0.9 and 7.8 μg had a correlation coefficient of 0.982. The standard error of estimate was 7.0 mm^2.

Benzylpenamaldic Acid—Benzylpenamaldic acid had a retention time of 13 min. It could not be isolated from the buffer component of the eluent by extraction into acetone, chloroform, octanol, or other organic solvents. Acidification of the eluent caused degradation of benzylpenamaldic acid, as evidenced by the appearance of new peaks when reinjected into the chromatograph.

To construct a calibration curve, the response of the refractive index detector to a blank solution of column eluent, which had been lyophilized and then reconstituted with water, was monitored. The buffer salts produced a single sharp peak 4 min after injection whose height was linear over a range of 50–365 μg of citric acid–disodium phosphate buffer salts. A known weight of the ly-

[9] Cary model 17 spectrophotometer, Cary Instruments, Monrovia, Calif.
[10] *Model IR-33* spectrophotometer, Beckman Instruments Inc., Fullerton, Calif.
[11] Model RMU-6A spectrometer, Hitachi Ltd., Tokyo, Japan. The mass spectra were interpreted with the help of Dr. R. G. Cooks, Director of the Mass Spectrometry Center, Purdue University.
[12] Model T-60 spectrometer, Varian Associates, Palo Alto, Calif.
[13] CAL 360-STAT-4, Wang Laboratories, Inc., Tewksbury, Mass.

ophilized benzylpenamaldic acid–buffer mixture was also redissolved in double-distilled water, and 3–20 μl was injected.

Since the refractive index response for benzylpenamaldic acid came at a retention time of 13 min, the difference between expected and actual responses for the buffer salts at retention time of 4 min was used to calculate the weight of benzylpenamaldic acid that had been injected to produce the corresponding peak from the UV detector. This information was used to construct a calibration curve of UV spectral peak areas *versus* weight of benzylpenamaldic acid. The detector response was linear over a range of 0.45–5.4 μg of benzylpenamaldic acid. A least-squares regression analysis typically gave a line of slope of 94.5 $mm^2/\mu g$, an intercept of 22 mm^2, a correlation coefficient of 0.9976, and a standard error of estimate of 27.4 mm^2.

Benzylpenilloic Acid—Aqueous solutions of benzylpenilloic acid (0.75–2.1 mg/ml) were injected in volumes ranging from 1 to 23 μl and consistently produced a pair of overlapping peaks of approximately equal size at retention times of 7 and 8 min. Florey *et al.* (12) reported that two isomers of benzylpenilloic acid are obtained from the decarboxylation of benzylpenicilloic acid, possibly accounting for the pair of peaks on the chromatograph. Because of the overlapping peaks, the total peak area of the double peak was determined using a planimeter. Linear calibration curves in the 5–27-μg range typically had a slope of 0.0021 planimeter unit/μg, an intercept of −0.0024 planimeter unit, and a correlation coefficient of 0.9950. The standard error of estimate was 0.0021 planimeter unit.

Benzylpenicilloic Acid—Benzylpenicilloic acid was eluted from the column with a retention time of 19.5 min.

CONCLUSIONS

The HPLC system is expected to have great utility in kinetic studies to monitor the occurrence of specific penicillin degradation products. For example, Fig. 1 is a chromatogram of a 0.05 *M* penicillin G potassium solution aged for 7 min and 31.5 hr at 37° in a 0.03 *M* citric acid–0.0067 *M* disodium phosphate buffer, pH 2.70. Peaks suitable for quantification are seen having retention times corresponding to penicillin G potassium, benzylpenillic acid, benzylpenamaldic acid, benzylpenilloic acid, and penicillamine. No other analytical method is available that allows the direct detection and quantification of penicillin degradation products forming during aging.

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Differentiating Nonaqueous Titration of Aspirin, Acetaminophen, and Salicylamide Mixtures

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Abstract □ Mixtures containing aspirin, acetaminophen, and salicylamide were assayed potentiometrically by nonaqueous titration. The difference in pKa values for these weak acids was sufficient to permit successful differentiation. The titrant was tetrabutylammonium hydroxide, and the titration solvent was dimethylformamide. The procedure was applied to commercial dosage forms.

Keyphrases □ Aspirin mixtures with salicylamide or salicylamide/acetaminophen—differentiating nonaqueous titration □ Salicylamide mixtures with aspirin or aspirin/acetaminophen—differentiating nonaqueous titration □ Acetaminophen, aspirin, and salicylamide mixtures—differentiating nonaqueous titration □ Titrimetry, nonaqueous, differentiating—mixtures of aspirin, salicylamide, and acetaminophen

Aspirin, acetaminophen, and salicylamide are frequently used analgesic–antipyretics in tablet dosage forms, individually, in a variety of combinations, and often with other drugs. Suitable analytical procedures are available for estimating the individual components, but generally methods are not available for