SEPTEMBER 1979
VOLUME 68 NUMBER 9

JOURNAL OF PHARMACEUTICAL SCIENCES

RESEARCH ARTICLES

Effect of ^{60}Co-Irradiation on Penicillin G Procaine in Veterinary Mastitis Products

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Received January 8, 1979, from *Control Analytical Research and Development, The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication March 2, 1979.

Abstract □ The effect of ^{60}Co-irradiation on penicillin G procaine in a peanut oil-based veterinary mastitis product was examined by reversed-phase high-performance liquid chromatography (HPLC). The HPLC method is capable of separating and quantifying procaine, penicillin G, and various degradation compounds. Penicillin G recovery from a placebo formulation was 100.4% with a relative standard deviation of <1%. When irradiated at 4.0 Mrads, the penicillin G in two product lots decreased slightly (~1–2%) with a corresponding increase in two compounds inherently present in bulk penicillin G powder. These compounds may be formed in abundance by treating penicillin G in an acidic solution. From the mass spectrometric analysis and the relative retention data with authentic compounds, these compounds were identified as benzylpenilloaldehyde and benzylpenaldic acid. Values obtained by the HPLC method on the product irradiated and stored at various temperatures correlated well with those of the microbiological assay. No significant decrease in the procaine was detected even after 4.0-Mrad irradiation. The HPLC method is applicable for analysis of other β-lactam antibiotics.

Keyphrases □ Penicillin G procaine—effect of cobalt irradiation, veterinary mastitis products, sterilization □ Cobalt irradiation—effect on penicillin G procaine, veterinary mastitis products, sterilization □ Sterilization—penicillin G procaine, veterinary mastitis products, cobalt irradiation

The Ministry of Agriculture, Food, and Fisheries in the United Kingdom has served notice that all new veterinary mastitis products to be marketed in the United Kingdom must be sterile. Moreover, sterility is required for existing veterinary mastitis products at their reregistration time. Since the efficacy and toxicity of ethylene oxide as a sterilization agent are being questioned by both the Food and Drug Administration (1) and the Environmental Protection Agency in the United States (2), ^{60}Co-irradiation was examined as a reliable and economical means to sterilize terminally a veterinary mastitis product. The ^{60}Co-irradiation has been recognized as the method of choice for sterilization of medical devices and has been accepted for terminal sterilization of ophthalmic ointments (3). In many countries, products irradiated at 2.5 Mrads by cobalt 60 may be released as sterile without the final sterility testing.

Although the official microbiological cylinder cup–agar diffusion assay may be used as a stability-indicating method for penicillin G (4), a more precise and accurate method is desirable for monitoring the effect of ^{60}Co-irradiation treatment. Since a reversed-phase high-performance liquid chromatographic (HPLC) assay of β-lactam antibiotics (5) was improved by use of a column packed with 5–10-μm particle size C_{18} (6) and used in this laboratory for many years, this method was selected to assess the effect of ^{60}Co-irradiation on procaine and penicillin G in a veterinary mastitis product. Similar HPLC assays for β-lactam antibiotics have appeared (7–16).

EXPERIMENTAL

Apparatus—A modular liquid chromatograph equipped with a 254-nm UV monitor[1], a high-pressure pump[2], and a 20-μl fixed-loop injector[3] were used. A low C_{18}-loaded reversed-phase column[4] with a stainless steel precolumn[5], 51 × 2.1 mm i.d., was used to separate procaine and penicillin G.

Reagents—For the mobile phase, acetonitrile–water–0.2 *M* ammonium acetate buffer (20:70:10) was used. The pH was adjusted to 6.0 with acetic acid. The 0.2 *M* ammonium acetate buffer was prepared by weighing 15.5 g of ammonium acetate into a 1-liter graduated cylinder and adding water to volume. The mobile phase was filtered through a membrane filter[5].

The mobile phase was pumped through the column at a flow rate of ~1.0 ml/min.

Reference Standard Solutions—*Penicillin G*—The USP penicillin G potassium reference standard was used "as is" at 1590 units/mg. Ap-

[1] Model 1203 UV monitor III, Laboratory Data Control, Riviera Beach, Fla.
[2] Model 6000 A chromatographic pump, Waters Associates, Milford, Mass.
[3] Model 70-10, Rheodyne, Berkeley, Calif.
[4] Partisil PXS 10/25 ODS, Whatman Inc., Clifton, N.J.
[5] Catalog No. FHLP04700 fluoropore filter, Millipore Corp., Bedford, Mass.

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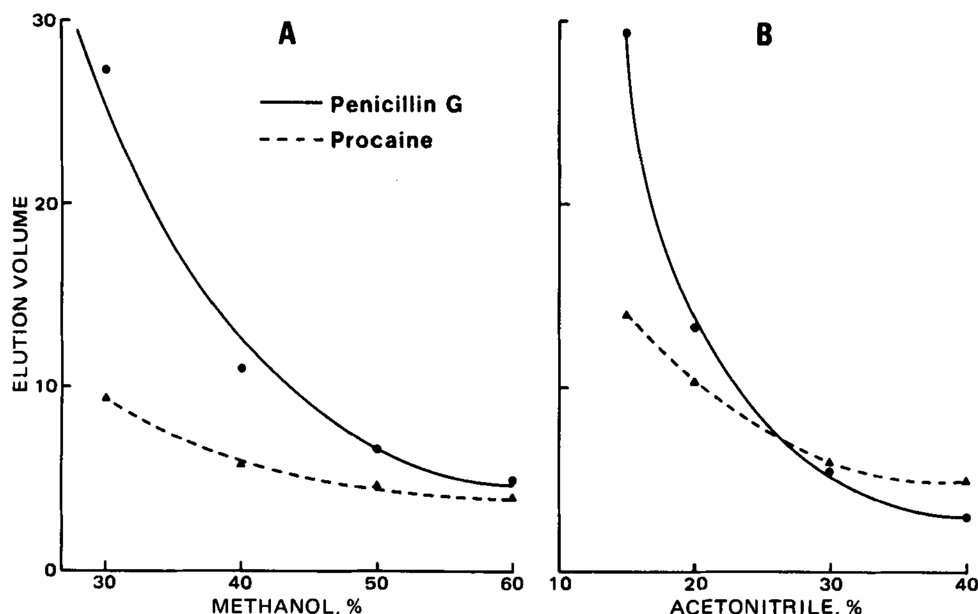


Figure 1—*(A) Effect of methanol concentration in the mobile phase on elution volumes of penicillin G and procaine using a μBondapak C_{18} column. (B) Effect of acetonitrile concentration in the mobile phase on elution volumes of penicillin G and procaine using a μBondapak C_{18} column.*

proximately 6.7 mg of the reference standard was weighed accurately[6] and placed in a 10-ml volumetric flask. The standard was dissolved and diluted to volume with the mobile phase. The reference standard solution thus prepared was transferred to a 35-ml disposable vial, and 10 ml of isooctane (2,2,4-trimethylpentane) was added. The vial was capped and shaken gently for 5 min on an automatic reciprocal shaker[7]. The top isooctane layer was aspirated off, and 5 ml of isooctane was again added. The vial was shaken again for 5 min and then centrifuged at 1000×g for 1 min. The top half of the sample was aspirated off, and the sample was sonicated just prior to chromatographic analysis.

Procaine—Approximately 4.5 mg of procaine hydrochloride USP was weighed accurately[6] and placed in a 50-ml volumetric flask. The standard was dissolved and diluted to volume with the mobile phase. The standard solution was transferred to a 125-ml separator containing 50 ml of isooctane. The flask was shaken gently for 5 min on an automatic reciprocal shaker[7].

The top isooctane layer was aspirated off, and 25 ml of isooctane was added. The flask was shaken again for 5 min. Approximately 10 ml of the lower phase was transferred into a 35-ml disposable vial and centrifuged at 1000×g for 1 min. The top half of the sample was aspirated off, and the sample was sonicated just prior to chromatographic analysis.

Sample Preparation—A plastet containing the peanut oil-based mastitis product was weighed. After the plastet was shaken vigorously, the contents were expelled into a 250-ml separator containing 100 ml of the mobile phase. The empty plastet was weighed to calculate by difference the weight of the content being assayed. Approximately 100 ml of isooctane was added, and the flask was shaken gently for 5 min on a reciprocal shaker[7].

Approximately 75% of the top layer was aspirated off, and 50 ml of isooctane was added. The flask was shaken again for 5 min. Approximately 10 ml of the lower phase was transferred into a 35-ml disposable vial and centrifuged for 1 min at 1000×g. The top half of the sample was aspirated off.

Table I—Precision of the HPLC Assay for Penicillin G in Peanut Oil-Based Mastitis Product

Net Weight of Product, g	Height of Penicillin G Peak, cm	Peak Height of Penicillin G/ Weight of Product[a]
8.16	27.0	3.31
8.21	27.0	3.29
8.19	27.2	3.32
8.25	27.6	3.35
8.36	28.2	3.37
8.33	28.0	3.36

[a] *RSD* = 0.94%.

[6] Model G electrobalance, Cahn Instrument Corp., Paramount, Calif.
[7] Eberbach, Ann Arbor, Mich.

Penicillin G—For the penicillin G assay, the sample solution was chromatographed.

Procaine—The sample solution was diluted 1:5 with the mobile phase and chromatographed.

Calculations—*Penicillin G*—The penicillin G content in a plastet was calculated from:

$$\text{penicillin G content} = \frac{P_{sm}}{W_{sm}} \times \frac{W_{std}}{P_{std}} \times \text{Sp.G.} \times 100 \times 1590 \quad \text{(Eq. 1)}$$

where P_{sm} is the peak height or peak area of the sample, W_{sm} is the weight of the sample in grams, W_{std} is the weight of the standard in milligrams per milliliter, P_{std} is the peak height or peak area of the reference standard, Sp.G. is the specific gravity of the mastitis product, and 1590 is the potency of the penicillin G USP reference standard (issue F).

Procaine—The procaine content per plastet may be calculated from:

$$\text{procaine content} = \frac{PK_{sm}}{W_{sm}} \times \frac{W_{std}}{PK_{std}} \times \frac{F_1}{F_2} \times F_3 \times F_4 \times F_5 \times \text{Sp.G.} \times P \quad \text{(Eq. 2)}$$

where PK_{sm} is the peak height or peak or peak area of the sample; W_{sm} is the weight of the sample; PK_{std} is the peak height or peak area of the reference standard; W_{std} is the weight of the standard; F_1, F_2, and F_3 are volumes of the sample solution, the reference standard solution, and the plastet, respectively; F_4 is the final volume of the sample; F_5 is the conversion factor from procaine hydrochloride to procaine base (0.866); Sp.G. is the specific gravity of the mastitis product; and P is the purity of the procaine USP reference standard.

RESULTS AND DISCUSSION

Factors Affecting Resolution of Penicillin G and Procaine Peaks—During the evaluation of several silica with bonded octadecylsilane[8] columns, the resolution of penicillin G from the procaine peak

Table II—Recovery of Penicillin G from Mastitis Product

Penicillin G Added, mg/plastet	Penicillin G Recovered, mg/plastet	Recovery, %
67.63	68.91	101.9
67.13	66.58	99.2
66.79	66.91	100.2
Average		100.4

[8] μBondapak C_{18}, Waters Associates, Milford, Mass.

Table III—HPLC Analysis of Penicillin G in Mastitis Product Irradiated with Cobalt 60

Lot	Penicillin G, units/plastet			
	0 Mrad	1.5 Mrads	2.5 Mrads	4.0 Mrads
A	112,400	109,200	107,600	108,500
	114,700	107,500	106,800	108,000
	108,400	108,800	107,500	107,900
	107,500	109,500	106,000	109,800
Average	110,800	108,800	107,000	108,600
B	117,300	115,300	115,500	113,600
	117,600	115,800	115,500	113,400
	110,500	112,300	112,400	112,400
	111,400	113,500	113,500	113,000
	114,400	114,400	112,900	112,900
	113,600	114,900	112,500	111,200
Average	114,100	114,400	113,700	112,800

was difficult to reproduce. Factors such as the acetonitrile and methanol concentrations in the mobile phase were examined to obtain the optimum resolution of the two compounds.

As shown in Fig. 1A, an increase in the methanol concentration decreased the elution volume of both the penicillin G and the procaine peaks. The effect on the penicillin G peak was more pronounced than on procaine. The peak elution order, however, remained the same.

Acetonitrile, on the other hand, drastically affected the penicillin G peak elution volume (Fig. 1B). The effect of the acetonitrile concentration on the procaine elution volume was less. In fact, the elution order of

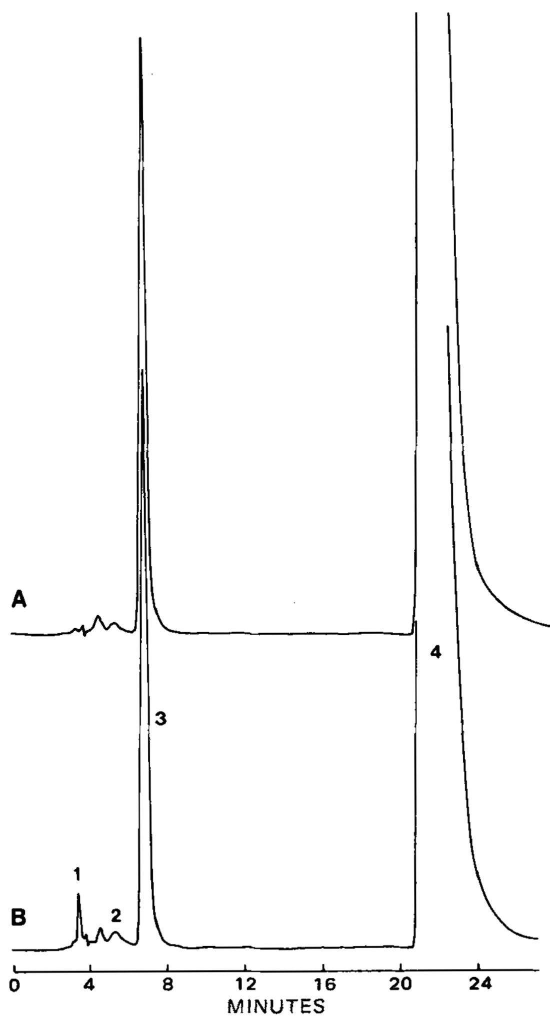


Figure 2—*HPLC analysis of penicillin G procaine irradiated at 0 Mrads (A) and 4.0 Mrads (B) in a mastitis product using a ODS-1 column. The mobile phase was acetonitrile 0.2 M ammonium acetate–water (20:10:70), pH 6.0. Key: 1, degradation compound I; 2, degradation compound II; 3, penicillin G; and 4, procaine.*

Table IV—HPLC Analysis of Procaine in Mastitis Product

Lot	Procaine Concentration, mg/plastet			
	0 Mrad	1.5 Mrads	2.5 Mrads	4.0 Mrads
A	45.0	43.1	44.3	43.5
	42.0	43.8	44.0	43.1
Average	43.5	43.5	44.2	43.3
B	43.5	43.7	43.3	43.1
	43.4	43.1	43.2	43.1
	43.8	43.7	43.5	43.0
	43.7	44.3	43.8	42.8
Average	43.6	43.7	43.5	43.0

penicillin G and procaine crossed over at the acetonitrile concentration of ~27–28%. This intersecting point varied from one column to the next. Since the majority of the penicillin G degradation compounds elute earlier than the penicillin G peak, the chromatographic conditions should be adjusted such that penicillin G elutes faster than the procaine peak.

This adjustment cannot always be reliably reproduced, however, especially between columns, and such attempts often caused the penicillin G peak to elute too close to the solvent front. Therefore, various commercially available C_{18} reversed-phase columns[9] were evaluated. In general, use of a highly C_{18} loaded column (more than 10%) resulted in near complete merger of the two peaks. Only a low C_{18} loaded column[4] (about 5%) consistently achieved a complete separation of the two peaks;

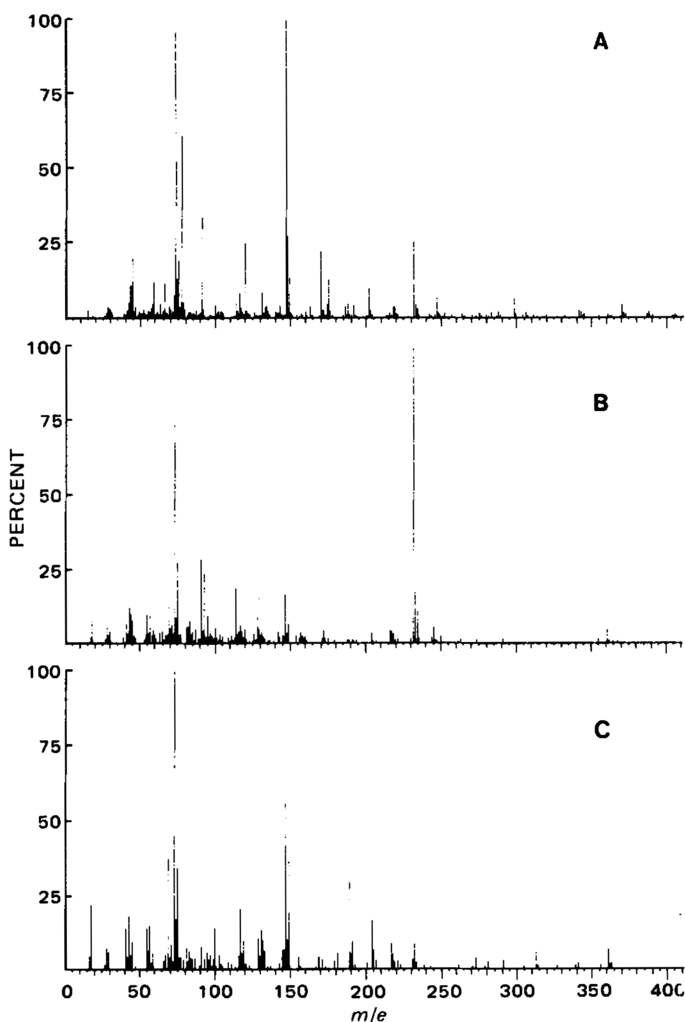


Figure 3—*Mass spectrum of silylated penicillin G and radiolytic compounds. Key: A, trimethylsilylpenicillin G; B, peak 1; and C, peak 2.*

[9] Waters C_{18}, Brown Lee RP-18, E. M. Merck RP-18, Varian C_{18}, Whatman ODS-1, and Whatman ODS-II.

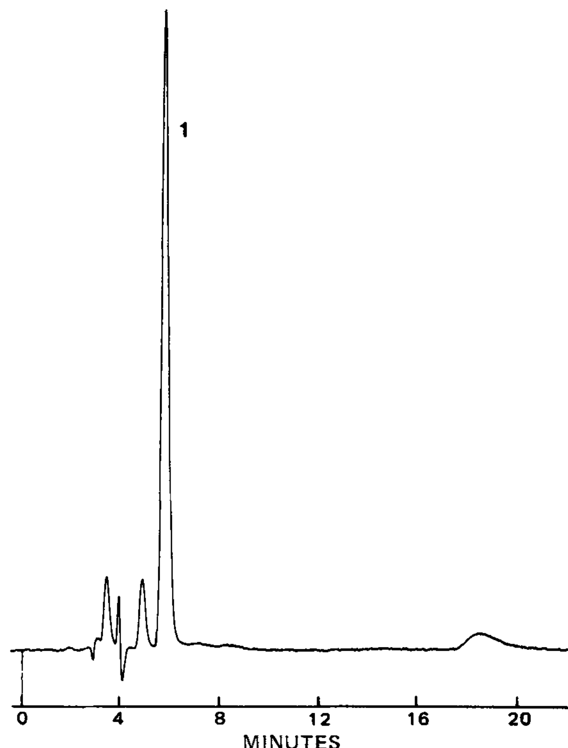


Figure 4—*HPLC analysis of ampicillin (1) in an oral formulation using a μBondapak C_{18} column. The mobile phase was acetonitrile–0.2* M *ammonium acetate buffer–water (15:10:85), pH 6.0.*

more than 12 min of elution time separated the procaine and penicillin G peaks (Fig. 2A). It may be theorized that the underivatized hydroxyl groups on the silica column support exhibit strong affinity toward the procaine molecule, resulting in longer procaine retention and the separation of the two compounds.

Precision and Recovery of Penicillin G from Peanut Oil-Based Mastitis Product—Precision of the HPLC assay was evaluated by individually weighing and extracting six replicate mastitis product samples. The relative standard deviation of the HPLC method was <1% (Table I).

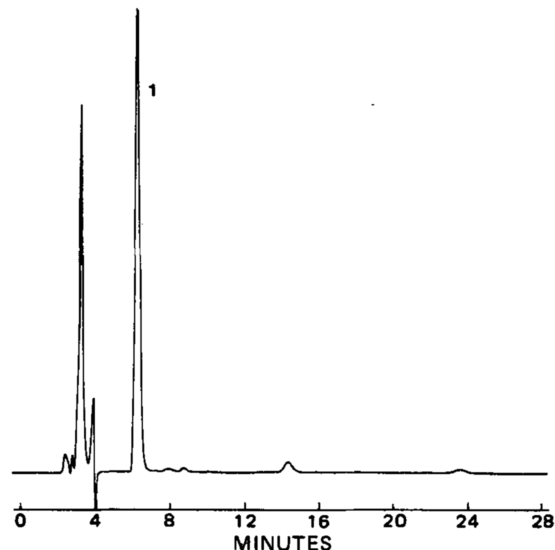


Figure 6—*HPLC analysis of penicillin V (1) in an oral formulation using a μBondapak C_{18} column. The mobile phase was acetonitrile–0.2* M *ammonium acetate–water (30:10:60), pH 6.0.*

The extraction technique efficiency for penicillin G recovery from the product was evaluated by adding three independently weighed penicillin G USP reference standard samples to the product base. The penicillin G recovery from the product base was 100.4% (Table II).

Penicillin G Stability to ^{60}Co-Irradiation—Penicillin G in a peanut oil-based veterinary mastitis product was relatively stable to ^{60}Co-irradiation. Even after 4.0 Mrads irradiation, the maximum irradiation level targeted for product sterilization, penicillin G in two product lots showed decreases in potency of only 2.0 and 1.1% (Table III).

Although several impurity peaks were present in unirradiated penicillin G procaine (Fig. 2A), only two peaks were increased by ^{60}Co-irradiation (Fig. 2B). Since these degradation compounds are inherently present in the untreated penicillin G procaine powder, these compounds are not formed uniquely by ^{60}Co-irradiation. These degradation compounds can be formed in abundance when penicillin G powder is treated in a low pH solution (pH 3.0) at 37° for 1 hr.

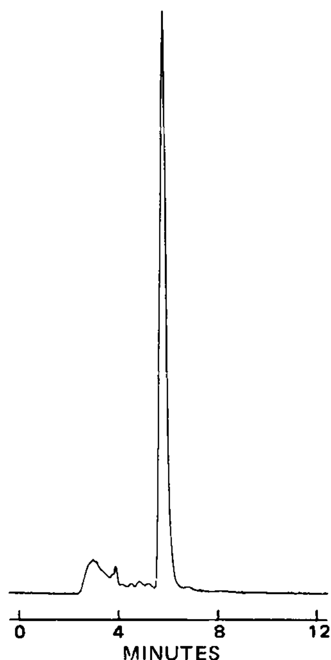


Figure 5—*HPLC analysis of penicillin O using a μBondapak C_{18} column. The mobile phase was acetonitrile–0.2* M *ammonium acetate–water (25:10:65), pH 6.0.*

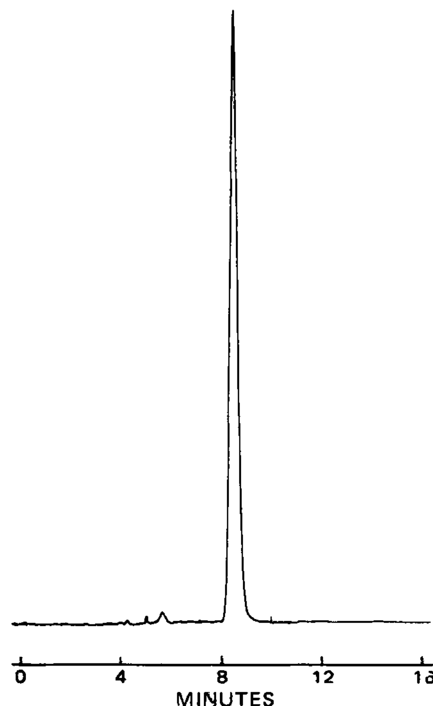


Figure 7—*HPLC chromatogram of cephalothin using a μBondapak C_{18} column. The mobile phase was acetonitrile–0.2* M *ammonium acetate–water (25:10:65), pH 6.0.*

Table V—Correlation between HPLC and Microbiological Assay Methods for Penicillin G in Mastitis Product

Lot	Irradiation, Mrads	Storage	Penicillin G, units/plastet HPLC	Microbiological
A	0	Initial	100,500	108,000
		25°, 3 months	100,100	101,000
		40°, 3 months	54,300	41,000
	2.5	Initial	103,600	107,000
		25°, 3 months	99,100	103,000
		40°, 3 months	61,500	62,000
	4.0	Initial	104,600	109,000
		25°, 3 months	100,900	101,000
		40°, 3 months	59,500	34,000
B	0	Initial	105,100	111,000
		25°, 3 months	101,300	101,000
		40°, 3 months	64,200	65,000
	2.5	Initial	105,800	108,000
		25°, 3 months	99,700	104,000
		40°, 3 months	64,800	58,000
	4.0	Initial	106,400	97,000
		25°, 3 months	101,700	102,000
		40°, 3 months	61,600	53,000

Approximately an eightfold increase in degradation compound I (peak 1) was noted, with less than a twofold increase in peak 2, by 4 Mrads irradiation. Increase in the area under peaks 1 and 2 was too great to be accounted for by the decrease in the penicillin G peak. This discrepancy may be attributed to the difference in the molar absorptivity of these compounds at 254 nm.

Degradation Compound Identification—As stated previously, the impurity compounds (peaks 1 and 2) that were increased by ^{60}Co-irradiation also may be formed in abundance by treating a penicillin G powder in a pH 3.0, 0.02 *M* phosphate buffer at 37° for 1 hr. The fact that the retention of peak 2 is greatly influenced by change in the mobile phase

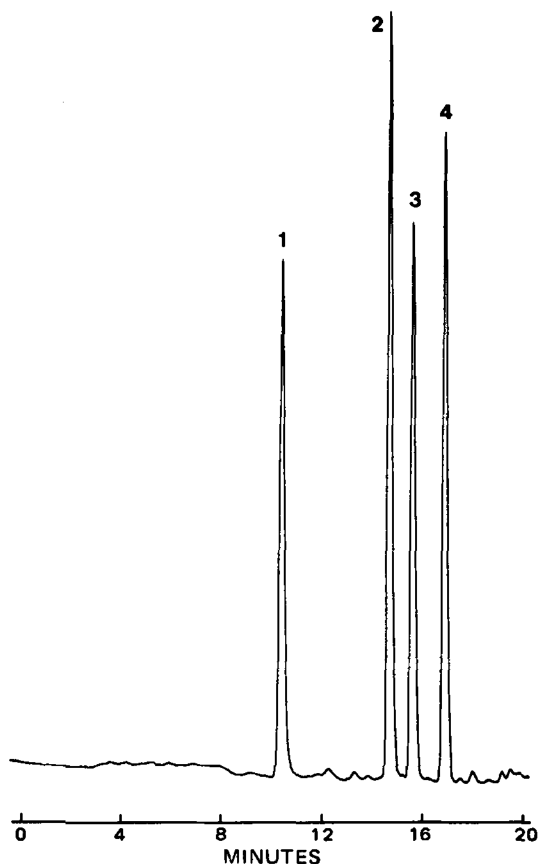


Figure 8—*HPLC gradient elution chromatogram for tentative identification of β-lactam antibiotics using a μBondapak C_{18} column. The linear gradient was from 10:10:80 (acetonitrile-0.2* M *ammonium acetate-water), pH 6.0, to 50:10:40, pH 6.0, in 15 min. Key: 1, ampicillin; 2, penicillin O; 3, penicillin G; and 4, penicillin V.*

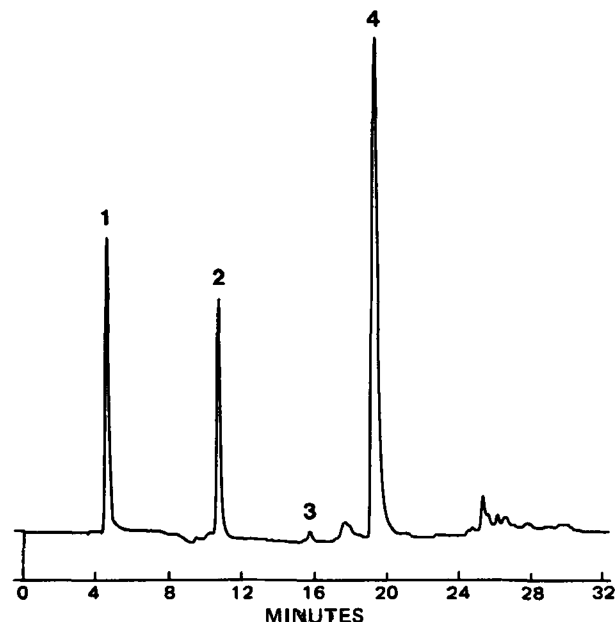


Figure 9—*HPLC gradient elution chromatogram indicating separation of an ampicillin prodrug, bacampicillin, from process intermediates. The convex gradient elution was from mobile phase A, 5:10:85 (acetonitrile-0.2* M *ammonium acetate-water), pH 4.0, to mobile phase B, 80:10:10, pH 4.0, in 30 min. Key: 1, phenylglycine; 2, ampicillin; 3, penicillin G; and 4, bacampicillin.*

pH indicates the presence of acidic radical(s) in the molecule. The retention of peak 1 was not affected by the pH change.

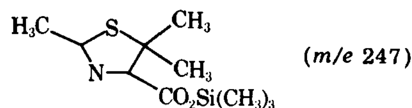
Formation of benzylpenicilloic acid, benzylpenilloic acid, and hydroxybenzylpenicillin in an aqueous penicillin G solution irradiated by cobalt 60 has been reported (17). These radiolytic compounds also may be formed by an alkaline treatment (18). However, the relative retention of these alkaline (pH 10.0) hydrolyzed compounds failed to match the two peaks of interest.

To identify the two degradation compounds, a small preparative liquid chromatograph was constructed with a 122-cm × 7-mm i.d. stainless steel column packed with C_{18}[10].

About 30 mg of penicillin G sodium USP, irradiated at 6.0 Mrads, was dissolved in 10 ml of the mobile phase and introduced onto the column *via* a 100-μl loop injector. The column effluents corresponding to the two peaks were collected in 250-ml round-bottom flasks immersed in a methanol-dry ice bath. The procedure was repeated 10 times. The effluents were freeze dried overnight.

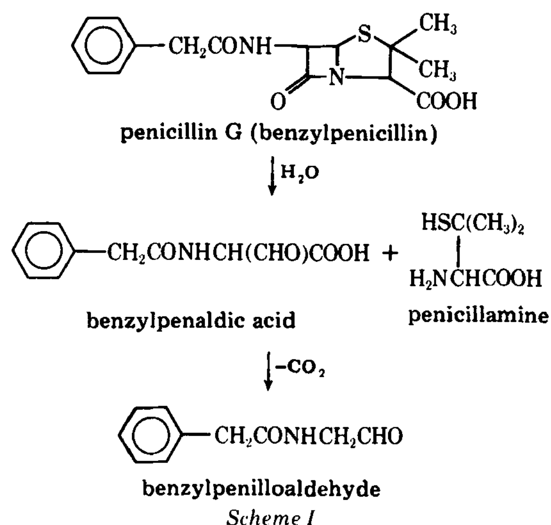
The submilligram quantities of samples thus collected were dissolved in acetonitrile, transferred into microreaction vials[11], and dried under a nitrogen stream. About 100 μl of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane[12] was added to the two dried samples and penicillin G powder and allowed to react 3 hr at room temperature. The silylated samples were placed in capillary glass tubings and dried under vacuum. The samples were analyzed by mass spectrometry using the electron-impact ionization mode.

The mass spectra of penicillin G and two degradation compounds are shown in Fig. 3. The penicillin G mass spectrum (Fig. 3A) showed that both *m/e* 478 [M^+, bis(trimethylsilyl)penicillin G] and 406 [mono(trimethylsilyl)penicillin G] were weak, indicating instability of the silylated compounds. Another weak peak was observed at *m/e* 306 [M − $C(CH_3)_2CHCO_2Si(CH_3)_3$]. Much stronger peaks were seen at *m/e* 463 (M − 15), 387 (M − $CH_3C_6H_5$), and 247. An intense peak was shown at *m/e* 232 (*m/e* 247 − CH_3).



(*m/e* 247)

[10] Bondapak C_{18} Porasil B, Waters Associates, Milford, Mass.
[11] Reacti-Vial, Pierce Chemical Co., Rockford, Ill.
[12] Regisil RC-2, Regis Chemical Co., Morton Grove, Ill.



Scheme I

The mass spectrum of the column effluent corresponding to peak 1 (Fig. 3B) is essentially identical to that of benzylpenilloaldehyde and showed the weak molecular ion at *m/e* 250 (M^+) and a strong peak at *m/e* 232 (M − 18). Weak peaks were found at *m/e* 158 ($M - CH_2C_6H_5$), 130 ($M - CH_2C_6H_5CO$), and 43 ($M - CH_2C_6H_5CONH$). A much stronger peak was observed at *m/e* 149 [$M - COSi(CH_3)_3$].

The mass spectrum of Compound II (Fig. 3C) is that of benzylpenaldic acid. The molecular ions at *m/e* 437 [tris(trimethylsilyl) derivative] and 291 [mono(trimethylsilyl) derivative] were relatively weak, indicating incomplete silylation. Much stronger peaks were shown at *m/e* 232 [$M - CH_2C_6H_5CONSi(CH_3)_3$] and 207 [M − CH(COO-trimethylsilyl)-$COSi(CH_3)_3$]. The presence of an acidic radical in this compound was indicated previously.

From the identification, it may be speculated that penicillin G may undergo a slight degradation by ^{60}Co-irradiation as shown in Scheme I. Penicillamine cannot be detected by monitoring at 254 nm.

Procaine Stability to ^{60}Co-Irradiation—The effect of ^{60}Co-irradiation on the stability of procaine is shown in Table IV. Although 0.5 and 1.5%, decreases were noted in two lots of the product irradiated at 4.0 Mrads, these decreases were not statistically significant. Careful examination of the chromatogram of procaine irradiated at 4.5 Mrads failed to detect any peak other than procaine.

Correlation between HPLC and Microbiological Methods—A correlation between the HPLC and the microbiological methods for the penicillin G assay was examined by use of two simulated product lots ^{60}Co-irradiated at 2.5 and 4.0 Mrads and stored at 25 and 40° for 3 months. The microbiological cylinder cup-agar diffusion assay used was as described in the Code of Federal Regulations (4) (Table V). A cross-classified analysis of variance was used to test for lot, radiation dosage, and time-temperature main effects and for all two-way interactions between the main effects. The analysis of variance showed that only the time-temperature effect was statistically significant ($p<0.05$) due to the instability of penicillin G at 40°.

Thus, the HPLC method correlated well with the microbiological assay and was stability indicating.

Other β-Lactam Antibiotics—With a slight modification of the mobile phase, the isocratic reversed-phase HPLC method is applicable to many β-lactam antibiotics (Figs. 4–7) with a relative standard deviation of ~1.0%.

The reversed-phase HPLC method using a gradient elution technique also is applicable for tentatively identifying β-lactam antibiotics (Fig. 8) and for assessing the stability of the prodrug, bacampicillin (Fig. 9).

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ACKNOWLEDGMENTS

The authors thank D. G. Pope for the supply of ^{60}Co-irradiated products, L. Baczynskyj for the mass spectrometric analysis, K. A. Gusciora for technical assistance, and A. R. Lewis for statistical analysis.