Comparison of High-Performance Liquid Chromatography and Radioimmunoassay in the Determination of Content Uniformity of Digoxin Tablets

MARTIN W. BEASLEY, PAUL SKIERKOWSKI *, ROBERT W. CLEARY ˣ, ALAN B. JONES, and ARTHUR H. KIBBE

Received February 1, 1982, from the *Department of Pharmaceutics, School of Pharmacy, The University of Mississippi, University, MS 38677.* Accepted for publication May 14, 1982. *Present address: Department of Radiation Safety, University of Oklahoma, Norman, OK 73019.

Abstract □ Digoxin 0.25-mg tablets were dissolved and assayed by the standard high-performance liquid chromatography (HPLC) method specified in USP XX and by a radioimmunoassay (RIA) method modified for the assay of tablet solutions. For the RIA method, the filtrate was diluted to a theoretical concentration of 5 ng/ml. Aliquots of this dilution were then assayed for digoxin content using a commercial digoxin ^{125}I RIA kit. Results from both methods were extrapolated to total tablet content and compared with the labeled amount for 20 individual tablets. All tablet assay results were within the USP standards for content uniformity of individual tablets. The individual tablet deviations from labeled amount by the RIA method were smaller when compared with the USP XX-specified HPLC method. Comparison of individual tablet assays show the RIA method to be both as precise and as accurate as the USP XX-specified HPLC method.

Keyphrases □ Digoxin—determination of content uniformity, comparison of high-performance liquid chromatography and radioimmunoassay, tablets □ Radioimmunoassay—comparison with high-performance liquid chromatography in determination of content uniformity of digoxin tablets □ High-performance liquid chromatography—comparison with radioimmunoassay in determination of content uniformity of digoxin tablets

Digoxin is the most predominant digitalis cardiac glycoside prescribed for clinical use. It is a potent drug used to increase the efficiency of the circulation in the treatment of congestive heart failure and to delay the ventricular rate in the treatment of atrial fibrillation and flutter (1).

BACKGROUND

The United States Pharmacopeia (USP) established the content uniformity test for tablets of certain potent drugs in 1965 with USP XVII (2). Due to the small dose (*i.e.*, 0.25 mg) utilized to elicit pharmacological response, digoxin must be assayed on a tablet-to-tablet basis. The USP XX potency definition for digoxin tablets states that the tablets must contain not <90% or not >105% of the labeled amount (3). A representative sample of 30 tablets was selected, and 10 of these tablets were assayed individually according to the drug monograph. The limits of the test state that not more than one tablet can be outside 85–115% of the average of the potency definition, and none of the tablets can be outside 75–125% of the average of the potency definition. The remaining 20 tablets must be assayed if any tablet exceeds the limits as stated. Requirements for content uniformity are met if these 20 tablets fall within the 85–115% average of the potency definition limit (4).

The digoxin content in single tablets has been determined by various methods, including colorimetric, fluorometric, polarographic, and GLC procedures (5–8). Although these methods were adequate in sensitivity, they were relatively cumbersome—either requiring formation of derivatives or complicated extractions or were limited to quantitation of millimolar concentrations. High-performance liquid chromatography (HPLC), a versatile separation technique, is the official assay method for the determination of digoxin tablet content uniformity in USP XX (3).

A number of radioimmunoassay (RIA) procedures for the measurement of biological and drug molecules in body fluids are available. Sensitive assay systems are capable of measuring antigen in the range of femtomole concentrations (9). RIA has been employed in bioavailability studies of digoxin (10–12), and is a highly sensitive method that is able to detect digoxin in human serum at the ng/ml level. The USP does not recognize it as an official test for tablet content uniformity at the present time. Since digoxin can be measured at such dilute levels in blood, it may be possible to utilize this method to determine drug levels in a dilute solution prepared from a single tablet.

EXPERIMENTAL

Materials—Digoxin 0.25-mg tablets[1] and digoxin reference standard powder[2] were used. Acetonitrile[3] was HPLC grade, and the dilute alcohol was USP grade. A [^{125}I]digoxin kit[4] was used in the RIA of the tablets.

Apparatus—A HPLC[5] equipped with a reverse-phase column[6] was connected to a variable-wavelength detector[7] and an automatic integrator[8]. The detector was operated at 218 nm and was attenuated at 0.04 AUFS for digoxin. A single-channel analyzer and amplifier[9] were connected to a counter timer[10] and a NaI(Tl) well crystal[11] for the RIA.

A 250 × 4.6-mm i.d. column, packed with 10-μm hydrocarbon (C18) bonded to silica gel[12], was used at ambient temperature with a mobile phase flow rate of 1.17 ml/min.

The mobile phase consisted of a mixture of acetonitrile–distilled water in a 1:2 ratio. The mixture was allowed to equilibrate 30 min before transfer to the solvent reservoir.

External standard—The digoxin reference standard power was dried for 1 hr at 105° and stored under vacuum in a glass desiccator before preparation. A standard solution (39.8 μg/ml) was prepared in dilute alcohol USP and stored at ambient temperature.

Digoxin 0.25-mg Tablet Assay Solutions—Samples of 10 tablets from two lots of digoxin 0.25-mg tablets were selected at random. Each individual tablet was placed in the center of a single sheet of weighing paper. The paper was tightly folded lengthwise around the tablet to form a cylinder and a 1-cm fold was made at each end of the paper. The tablet was crushed lightly, but completely, into powder with a glass pestle. One end of the paper was opened and inserted well into a clean, dry 10-ml volumetric flask to allow the powdered tablet to slide from the paper to the bottom of the flask. The other end of the paper was opened for the addition of dilute alcohol USP. Six milliliters of dilute alcohol was added through the paper cylinder, washing the residual tablet into the flask. The flask was covered and mixed for 15 sec by a mechanical shaker[13] and then sonicated[14] for 30 min. After sonication, the flask was removed from the sonicator and allowed to cool for 15 min. The solution was brought to volume with dilute alcohol USP and mixed for 15 sec on the mechanical shaker. Immediately after mixing, the entire solution was filtered through a medium-porosity sintered glass funnel. The first five drops of filtrate were discarded, and the remaining filtrate was collected in a clean, dry 10-ml volumetric flask and covered tightly with laboratory film. Based on the labeled quantity of digoxin in the tablets (0.25 mg), theoretical concentrations of the tablet test solution were in the range of 25 μg/ml.

[1] Lanoxin, 0.25-mg tablets, lots 9L2297 and 0J2236; Burroughs Wellcome Co., Research Triangle Park, N.C.
[2] Digoxin Reference Standard, lot 48C 0239; Sigma Chemical Co., St. Louis, Mo.
[3] Burdick & Jackson Laboratories, Inc., Muskegon, Mich.
[4] RIANEN, New England Nuclear Corp., Billerica, Mass.
[5] Model 4200, Varian, Varian Aerograph, Walnut Creek, Calif.
[6] HIBAR-II, MCB Manufacturing Chemists, Inc., Cincinnati, Ohio.
[7] Model 970, Tracor, Tracor Instruments, Austin, Tex.
[8] Model 3380A, Hewlett-Packard, Avondale, Pa.
[9] Model TL 200, Tennelec, Oak Ridge, Tenn.
[10] Model TL 400, Tennelec, Oak Ridge, Tenn.
[11] Model 51SP51, Quartz and Silice, Scintibloc, Paris, France.
[12] Lichrosorb RP-18, MCB Manufacturing Chemists, Cincinnati, Ohio.
[13] Mini-Shaker, Model 58, Fisher Scientific, Pittsburgh, Pa.
[14] Sonicor, Model SC-101th, Sonicor Instrument Corp., Copiague, N.Y.

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Table I—HPLC Assay Results of Digoxin 0.25-mg Tablets

Sample	AUC	μg of Digoxin, Calculated	Average Recovery, %	Sample	AUC	μg of Digoxin, Calculated	Average Recovery, %
Digoxin, RS	2.56 2.50 (Avg. 2.53)	—	—	Digoxin, RS	3.43 3.59 (Avg. 3.51)	—	—
A1	1.53	241		B1	1.91	217	
	1.65	260	100.4		2.16	245	92.4
A2	1.50	236		B2	2.01	228	
	1.47	231	93.6		2.17	246	94.8
A3	1.50	236		B3	2.36	268	
	1.53	241	95.6		2.35	267	107.0
A4	1.50	236		B4	2.18	247	
	1.56	246	96.4		2.11	239	97.2
A5	1.29	203		B5	2.02	229	
	1.50	236	88.0		2.15	244	94.6
A6	1.43	225		B6	2.09	237	
	1.43	225	90.0		2.07	235	94.4
A7	1.56	246		B7	2.07	235	
	1.45	228	94.8		2.04	231	93.2
A8	1.48	233		B8	2.09	237	
	1.48	233	93.2		1.88	213	90.0
A9	1.61	253		B9	1.99	226	
	1.53	241	98.8		1.94	220	89.2
A10	2.02	229		B10	2.20	250	
	2.02	229	91.6		2.18	247	99.4

The solutions of the 20 individual tablets were designated A1–A10 and B1–B10.

Linearity Test of External Standard—A stock solution (100 μg/ml) was prepared with the digoxin reference standard powder in dilute alcohol USP. Dilutions of 10, 20, 30, 40, 50, 60, 70, and 80 μg/ml were prepared from the stock solution with dilute alcohol USP. Duplicate aliquots of 35-μl injections of the stock solution and each dilution were chromatographed. The average retention time was 5.52 min. Linearity ($r = 0.99708$) was observed.

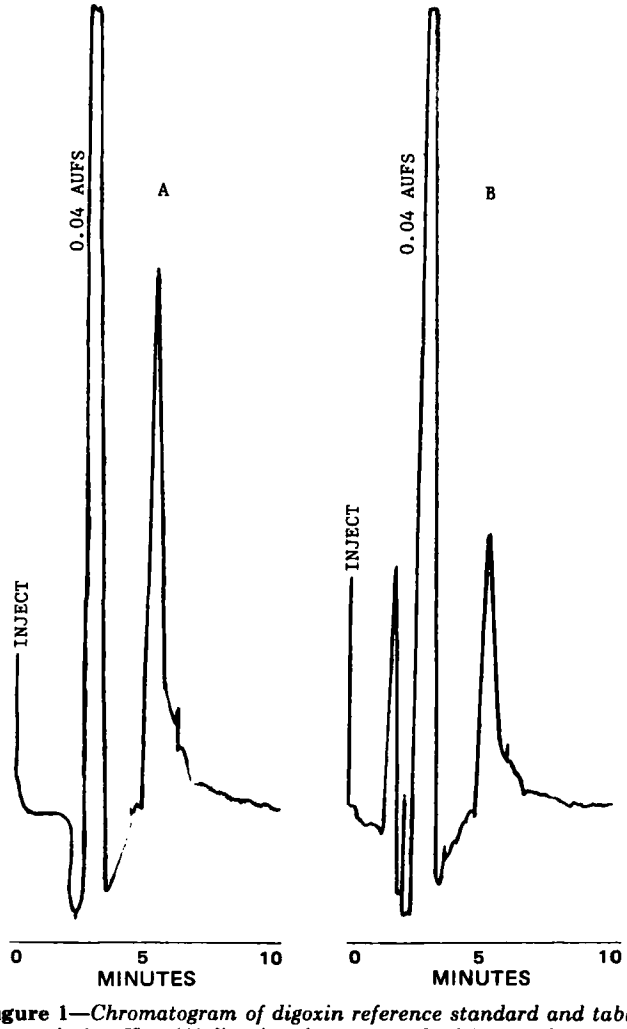

Figure 1—*Chromatogram of digoxin reference standard and tablet assay solution. Key: (A) digoxin reference standard (external standard, 39.8 μg/ml); (B) digoxin tablet assay solution (theoretical concentration of 25 μg/ml).*

HPLC Assay of Digoxin Tablet Solutions—Duplicate 35-μl aliquots of each digoxin tablet solution were chromatographed. Prior to injection of the digoxin tablet assay solutions, between the different sample lots, and after the chromatographs of all the digoxin tablet assay solutions, duplicate 35-μl aliquots of each digoxin reference standard solution (external standard, 39.8 μg/ml) were chromatographed.

Quantitation—The areas under the curve (AUC) generated by duplicate assays of each tablet solution were averaged and compared with the average of the initial duplicate digoxin reference standard solution injections by the formula specified in the USP (3): μg of digoxin = $10C(H_u/H_s)$, where 10 is the dilution volume of each tablet assay solution, C is the concentration of digoxin reference standard in μg/ml, H_u is the average AUC of the tablet assay solution, and H_s is the average AUC of the digoxin reference standard solution.

Preparation of RIA Kit—The [^{125}I]digoxin kit was prepared at room temperature as required by the manufacturer's instructions. The range of the standards for the kit was 0–8.0 ng/ml.

Dilution of Digoxin Reference Standard Solution and Digoxin 0.25-mg Tablet Assay Solutions—An exact volume (1.4 ml) of the digoxin reference standard solution (39.8 μg/ml) was pipeted into a clean, dry 100-ml volumetric flask with a 2-ml glass pipet (0.2-ml graduations). The solution was brought to volume with dilute alcohol USP and allowed to equilibrate overnight. A 1-ml glass pipet was used to transfer 1.0 ml of the equilibrated solution into another clean, dry 100-ml volumetric flask. The solution was brought to volume with dilute alcohol USP to a final concentration of 5.57 ng/ml.

Two milliliters of each tablet solution was pipeted into separate clean, dry 100-ml volumetric flasks with 2-ml glass pipets. The solutions were then brought to volume with dilute alcohol USP and allowed to equilibrate overnight. A 1-ml glass pipet was used to transfer 1.0 ml of each equilibrated solution into another 100-ml volumetric flask. Each solution was brought to volume with dilute alcohol USP. Based on the labeled

Table II—RIA Results of Digoxin 0.25-mg Tablets

Sample	Average Net cpm	μg of Digoxin in Tablet	Sample	Average Net cpm	μg of Digoxin in Tablet
A1	2305	251	B1	2370	247
A2	2323	250	B2	2216	256
A3	2237	255	B3	2383	246
A4	2325	250	B4	2245	254
A5	2266	253	B5	2229	255
A6	2353	248	B6	2312	250
A7	2220	256	B7	2154	260
A8	2137	261	B8	2547	237
A9	2318	250	B9	2240	255
A10	2284	252	B10	2356	248

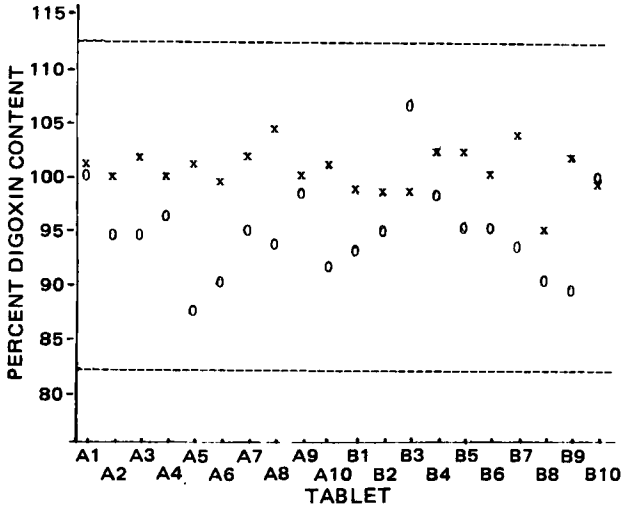

Figure 2—*Comparison of content uniformity test results of single digoxin tablets by HPLC and RIA. Key: (O) percent digoxin recovery by HPLC assay method; (X) percent digoxin recovery by RIA method; (---) limits of digoxin content uniformity.*

quantity of digoxin in the tablets (0.25 mg), theoretical concentrations of the tablet test solutions were in the range of 5 ng/ml.

Gamma Counter Calibration—A simulated iodine-125 source (iodine-129, 0.108 μCi) was utilized to calibrate the single-channel gamma analyzer. The sample[2]–background ratio for the energy peak in the differential mode was utilized to determine the optimum window setting, baseline, and high-voltage potential.

RIA of the Digoxin 0.25-mg Tablet Assay Solutions—All solutions assayed were done in duplicate. The assay consisted of a series of known digoxin standards from the kit (16 tubes), digoxin tablet solution (5 ng/ml) samples (40 tubes), and digoxin reference standard solution (5.57 ng/ml) samples (two tubes). Samples were pipeted into polystyrene 12 × 75-mm tubes[15] with an adjustable microliter pipet[16]. Known digoxin standards consisted of the following duplicate tubes: blank solution (0.02 *M* phosphate buffer, pH 7.4, with 0.5% bovine serum albumin); 0, 0.5, 1.0, 2.0, 4.0, and 8.0 ng/ml standards of digoxin in human serum; and control serum (human serum containing a digoxin concentration of 3 ± 0.3 ng/ml). The duplicate blank solution tubes contained 200 μl of 0.02 *M* phosphate buffer in place of antiserum and 50 μl of 0-ng/ml standard. Each of the remaining digoxin standard duplicate tubes consisted of 50 μl of the respective tablet solution and 200 μl of antiserum (digoxin rabbit serum albumin). The duplicate control serum tubes contained 50 μl of control serum and 200 μl of antiserum. The duplicate digoxin tablet solution samples consisted of 50 μl of the respective tablet solution and 200 μl of antiserum. The duplicate digoxin reference standard solution tubes consisted of 50 μl of digoxin reference standard solution (5.57 ng/ml) and 200 μl of antiserum. After adding 200 μl of radioactive tracer (histamine–digoxin conjugate labeled with iodine-125) to all tubes, the solutions were mixed and incubated at room temperature for 30 min. Following incubation, 1000 μl of concentrated charcoal suspension was added to each tube. The tubes were mixed and incubated again for 10 min. The unbound antigen was adsorbed on the charcoal and separated from the bound antigen by centrifugation[17] at 1200×*g* for 10 min. The supernatant solutions containing the antigen–antibody complexes from each tube were decanted into clean, dry polystyrene tubes, and the radioactivity was determined in the gamma counter.

Quantitation—The counts were averaged for each set of duplicate samples. Average net counts were calculated for all standards and samples by subtracting from each the average blank counts. The average net counts for each standard and sample were expressed as a percentage of the average net counts for the 0-ng/ml standard (normalized percent bound or percent of B/B_0) (13):

$$\text{percent of } B/B_0 = \frac{\text{Average net counts of standard or sample}}{\text{Average net counts of zero standard}}$$

Determinations of digoxin in the control and tablet solutions were made by preparing an exponential least-squares plot of percent of B/B_0 for each standard against the corresponding log concentration of digoxin in ng/ml and calculating the concentration of digoxin in ng/ml from the formula for the line of best fit. Samples were calculated as ng/ml since identical volumes were used for all standards and samples.

[15] Disposable polystyrene culture tubes, Curtin Matheson Scientific, Inc., Houston, Tex.
[16] Pipetman, Model P200D, Rainin Instrument Co., Inc., Woburn, Mass.
[17] Model K size 2, International Equipment Co., Boston, Mass.

Table III—Comparison of Digoxin Tablet Content Assayed by HPLC and RIA

Sample	High-Performance Liquid Chromatography: μg Digoxin in Tablet	HPLC: Recovery, %	Radioimmunoassay: μg of Digoxin in Tablet	RIA: Recovery, %
A1	251	100.4	251	100.4
A2	234	93.6	250	100.0
A3	239	95.6	255	102.0
A4	241	96.4	250	100.0
A5	220	88.0	253	101.2
A6	225	90.0	248	99.2
A7	237	94.8	256	102.4
A8	233	93.2	261	104.4
A9	247	98.8	250	100.0
A10	229	91.6	252	100.8
	avg. 235.6 ± 9.5	avg. 94.2 ± 3.8	avg. 252.6 ± 3.8	avg. 101.0 ± 1.5
B1	231	92.4	247	98.8
B2	237	94.8	256	102.4
B3	268	107.0	246	98.4
B4	243	97.2	254	101.6
B5	237	94.8	255	102.0
B6	236	94.4	250	100.0
B7	233	93.2	260	104.0
B8	225	90.0	237	94.8
B9	223	89.2	255	102.0
B10	249	99.4	248	99.2
	avg. 238.2 ± 13.0	avg. 95.3 ± 5.2	avg. 250.8 ± 6.6	avg. 100.3 ± 2.6

RESULTS AND DISCUSSION

A typical chromatogram of the digoxin reference standard solution and a tablet assay solution is shown in Fig. 1. The area under the curve unexpectedly increased by ~40% on sample A10. Two aliquots of digoxin reference standard solution (39.8 μg/ml) were injected and the AUC had increased almost twofold. The AUC from the nearly twofold increase of digoxin reference standard solution was utilized to calculate the amount of digoxin in tablet assay solutions A10–B10.

There was a considerable degree of tailing from each peak on all reference standard and tablet assay solutions. Amounts of digoxin calculated by the USP formula appeared to be 10–15% lower than expected due to the tailing. Adjustment of the slope sensitivity setting in the integrator did not provide any remedy. The decision was made to calculate the AUC manually by the triangle formula $A = H(W_{1/2})$. The width at mid-height is used instead of the width at baseline to reduce errors. A baseline was drawn, mid-height was determined, and height and width at mid-height were measured in centimeters (14). The assay results of the digoxin tablet solutions are shown in Table I.

The results of the RIA of the digoxin tablet solutions are shown in Table II. A comparison of each individual tablet from the two lots by the two assay methods is shown in Table III. The assay results by both methods are compared with the limits of the USP content uniformity test for digoxin tablets in Fig. 2. Statistical analysis by the paired *t* test is shown in Table IV.

The results in Tables III and IV show that the assay amounts for both methods for each digoxin tablet solution differed by significant amounts at the 95% confidence level. Assay results by HPLC were slightly higher for tablet solutions B3 and B10. RIA results were equal or higher for the remaining tablet solutions. The HPLC assay results for digoxin content ranged from 220 μg (88.0% recovery) to 268 μg (107.2% recovery). Assay results from digoxin content by RIA ranged from 237 μg (94.8% recovery) to 261 μg (104.4% recovery). Assay results of lot A indicated an average digoxin content recovery of 94.2% by HPLC as compared with an average digoxin content recovery of 101.0% by RIA. Results from lot B indicated an average digoxin content recovery of 95.3% by HPLC and an average digoxin content recovery of 100.3% by RIA. The average digoxin content recovery by RIA was 6.8 and 5.0% higher for both lots of digoxin tablets than the average digoxin content recovery by HPLC.

The RIA results for the digoxin tablet solutions were expected to be close to the results provided by the HPLC assay, because it was assumed

Table IV—Statistical Analysis of Digoxin 0.25-mg Tablet Content by HPLC and RIA with Paired t Test at 95% Confidence Level

Lot[a]	Method	Average Digoxin Content, μg	p
A	HPLC	235.6 ± 9.5	<0.05
A	RIA	252.6 ± 3.8	
B	HPLC	238.2 ± 13.0	<0.05
B	RIA	250.8 ± 6.6	

[a] $n = 10$.

that the digoxin would inhibit the radiolabeled antigen from binding with the antiserum in the same manner as if the digoxin was present in human serum. Since the drug was dissolved in USP dilute alcohol, there were no other steroid molecules present in the solutions which might cross-react with the antiserum. The filtering step prior to the assay procedure eliminates most of the excipient ingredients in the tablet dosage form.

As mentioned previously, the content uniformity test for tablets in USP XX required that each tablet must contain not <85% or not >115% of the average of the limits specified in the drug monograph. Digoxin tablets must contain <90% or not >105% of the label claim. Thus, a conforming tablet must fall within 82.9 and 112.1% of the average of the digoxin monograph limits[18].

All digoxin tablets assayed by both HPLC and RIA met the requirements of the content uniformity test.

This study showed that RIA is an accurate alternative to HPLC for content uniformity of digoxin tablets. The data showed the range of digoxin content determined by RIA was narrower than the range of digoxin content determined by HPLC. The significant difference at the 95% confidence level with the paired t test between the assay results of both methods showed that the RIA method appeared to be more precise and closer to the labeled amount than HPLC in the determination of digoxin content in the two lots of digoxin tablets.

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In-Beam Electron Ionization Mass Spectra of Penicillins

MAMORU OHASHI, ROBERT P. BARRON ˣ, and WALTER R. BENSON

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Abstract □ The characteristics of in-beam electron ionization mass spectra of 6-aminopenicillanic acid and several penicillins, which yield no detectable molecular ion peaks using a conventional direct-insertion probe, have been established. The spectra of all compounds studied, with the exception of amoxicillin, exhibited molecular ion or (M+1) peaks with spectral features similar to the reported methyl ester or amide derivatives of the compounds. The fragmentation of penicillin G on electron impact under in-beam conditions can be described on the basis of data from mass analyzed ion kinetic energy spectrometry. A desorption technique utilizing polyethylene glycol 4000 was used as a means of obtaining satisfactory spectra of ampicillin and amoxicillin.

Keyphrases □ Penicillin—in-beam ionization mass spectra β-lactam antibiotics, amoxicillin, ampicillin □ Electron ionization mass spectra, in-beam—penicillin, β-lactam antibiotics, amoxicillin, ampicillin □ Amoxicillin—in-beam electron ionization mass spectra of penicillins, β-lactam antibiotics □ Ampicillin—in-beam electron ionization mass spectra of penicillins, β-lactam antibiotics

Because of their low vapor pressure and thermal instability, penicillins, a class of β-lactam antibiotics, have generally required chemical pretreatment with formation of their esters or amides prior to mass spectrometric investigations (1, 2). Recently, isobutane and ammonia chemical ionization (CI) mass spectrometric data were published on the free acids of penicillins G and V (3), and the ammonia CI mass spectrum of the potassium salt of penicillin G was reported (4). Pyrolysis mass spectrometry was investigated (5) as a means of characterizing the compounds. The use of in-beam or extended-probe techniques to study apparently nonvolatile and thermally unstable compounds is now commonplace and well documented in the literature (6–14). Using this technique, ammonia-positive and methane-negative ion desorption CI of penicillins was reported (15); however, this report did not include any electron ionization (EI) data. Other researchers[1] have developed a technique using a mixture of the respective potassium salt and ammonium chloride to obtain the EI mass spectra of several penicillins. Accordingly, results obtained in this laboratory are presented on in-beam EI mass spectra of several penicillins, as their free acids, and their fragmentation processes based on mass analyzed ion kinetic energy spectrometric studies (16).

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