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QUANTITATIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF BACITRACIN, A POLYPEPTIDE ANTIBIOTIC

KIYOSHI TSUJI, JOHN H. ROBERTSON and JOHN A. BACH

Control Analytical Research and Development, The Upjohn Company, Kalamazoo, Mich. 49001 (U.S.A.)

SUMMARY

A high-pressure liquid chromatographic (HPLC) method for the analysis of bacitracin is described. The method uses a one-meter-long stainless-steel column packed with Bondapak C_{18}/Corasil with a programmed convex gradient elution of decreasing polarity, from 5% methanol in pH 4.5 buffer to 40% methanol and 20% acetonitrile in pH 4.5 buffer, at a flow-rate of 0.90 ml/min. More than 22 components of bacitracin have been separated and analyzed in less than 40 min of chromatography. The number and proportions of the components contained in bacitracin powders varies greatly between manufacturers but remains relatively constant for each manufacturer. The antimicrobial activity of bacitracins B and C against *Micrococcus flavus*, a standard test microorganism for the quantitation of bacitracin, is 87% and 30% of bacitracin A, respectively. The relative standard deviation of the HPLC method is approximately 1%. Acute toxicity of bacitracin powder containing different proportions of the components and the effect of pharmaceutical manufacturing on sterile bacitracin are described.

INTRODUCTION

Bacitracin (Fig. 1), a polypeptide antibiotic produced by strains of *Bacillus licheniformis*, has the structure of a 7-amino acid-membered ring[1,2] and a side-chain consisting of four amino acids and a glucose moiety. Commercially produced bacitracin is a mixture of various components, many of which are identified as bacitracins A, B, C, D, E, F and G[3] with bacitracin F being the primary degradation product of bacitracin A[4]. Bacitracins A and B are the major microbiologically active components of bacitracin and their antimicrobial activities vary from microbial species to species[5]. Also, the ratio of bacitracins A and B in a sample is not always constant and may be different from that of the reference standard.

The U.S. Food and Drug Administration has adopted the microbiological cylinder cup agar diffusion assay method using *Sarcina subflava* or *Micrococcus flavus* for the analysis of bacitracin[6]. However, the microbiological assay method is not a precise method of quantitation and is influenced by various factors, such as the presence of di- or trivalent cations in the test sample or the media[7], temperature of incu-

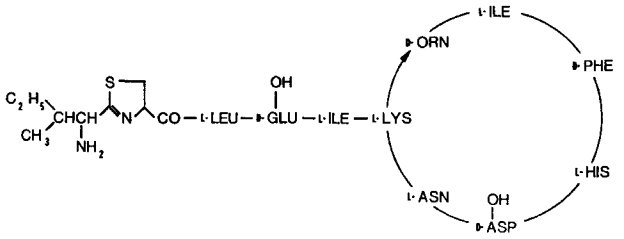

Fig. 1. Structure of bacitracin A.

bation and pH of the solution. Also, the microbiological assay method is incapable of detecting the presence of minor degradation compounds and impurities.

For the isolation and analysis of various components of bacitracin, countercurrent distribution[8,9], zone electrophoresis[10] and column chromatography using carboxymethylcellulose[11,12] or charcoal[10] have been used. However, these methods are time consuming and are not suited for a laboratory where a large number of samples have to be assayed routinely.

High-pressure liquid chromatography (HPLC) seems to be the most suitable method for the analysis of complex, high-molecular-weight compounds[13–18] such as bacitracin.

EXPERIMENTAL

Apparatus

A Laboratory Data Control (Riviera Beach, Fla., U.S.A.) modular liquid chromatograph equipped with a 254-nm UV monitor (Model 1285) was used, together with two Milton Roy (Philadelphia, Pa., U.S.A.) mini-pumps equipped with a pulse dampener (Laboratory Data Control, Model 709). One pump was a high-pressure type (max. 5000 p.s.i.) attached to the column and the other was a low-pressure type (max. 1200 p.s.i.) attached to the reference side of the flow cell.

An empty 1000 × 2.1 mm stainless-steel column (DuPont, Wilmington, Del., U.S.A.) was first rinsed with tetrahydrofuran (THF) followed by vigorous cleaning of the inside of the tubing with a cotton string pre-soaked with THF to remove loose metal particles. Chloroform was then drawn through the column and the column was dried under a stream of dry nitrogen.

A 5-μm pore size PTFE frit was fitted to the inlet end of the column and a hex-nut (DuPont, No. 820349) with stainless-steel front and back lock ferrules and cap (DuPont, No. 201724) were attached to the column. Bondapak C_{18}/Corasil (Waters Ass., Framingham, Mass., U.S.A.) was dry-packed into the open end of the column by adding a small amount of the column packing material at a time and lightly tapping the column on the floor. After the column was tightly packed, a 2-μm pore size stainless-steel frit was inserted into the outlet end of the column.

The column thus packed was attached to a septumless injector valve (Micrometrics, Norcross, Ga., U.S.A.) and to the sample side of the 254-nm UV monitor. The theoretical plates of the column thus prepared were approximately 1985 per meter for the bacitracin A peak. The efficiency of the column is comparable with that of the gas–liquid chromatographic method for the analysis of antibiotics[19–21].

Mobile phases

(A) Absolute methanol (50 ml) and water (750 ml) were poured into a 1000-ml graduated cylinder, then 200 ml of 0.1 *M*, pH 4.5 potassium phosphate buffer were added and mixed. (B) Absolute methanol (400 ml), acetonitrile (200 ml) and water (200 ml) were poured into a 1000-ml graduated cylinder and, after mixing, 200 ml of 0.1 *M*, pH 4.5 phosphate buffer were added and mixed.

Procedure

Preparation of bacitracin standard. Approximately 200 mg of the Upjohn (Kalamazoo, Mich., U.S.A.) laboratory standard bacitracin powder (55.7 units/mg, "as is") were accurately weighed and placed in a 10-ml calibrated flask.

Prior to analysis, each standard and sample was dissolved and diluted to volume with the mobile phase A.

Gradient elution. A Glenco gradient elution apparatus (Model 3130-50, Glenco Scientific, Houston, Texas, U.S.A.) was used. A 50-ml volume of each of the mobile phases A and B was placed into the two separate glass cylinders and a programmed convex mixing rate, as shown in Fig. 2, was used to chromatograph bacitracin.

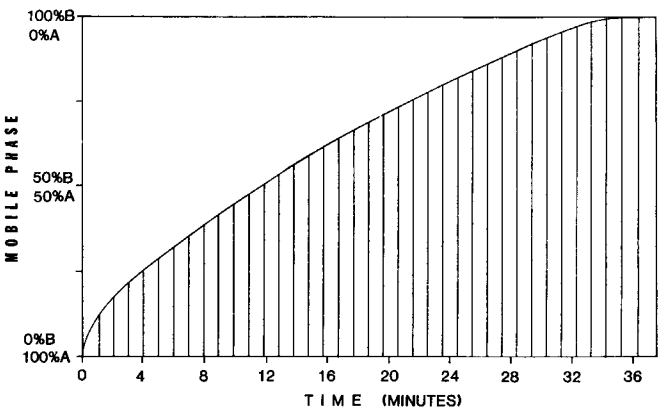

Fig. 2. Programmed convex gradient elution pattern.

In order to obtain a stable chromatographic baseline, the effluent from the gradient apparatus was split into two streams, one to the column at a flow-rate of 0.90 ml/min and the other to the reference side of the UV monitor at a flow-rate of 2.0 ml/min. The high flow-rate on the reference side was needed in order to obtain enough back-pressure to operate the mini-pump properly. A stainless-steel tube, 2.5 m long and I.D. 0.25 mm, was also attached between the low-pressure pump and the reference side of the UV monitor in order to obtain a constant back-pressure of 20 atm (300 p.s.i.).

Chromatographic conditions. The column temperature was ambient with a chart speed of 0.25 in./min. A 4.0-μl volume of the sample was injected into the column using a septumless injector with an electrometer range setting of 0.16 full-scale. The pressure of the column changed from 68 atm (1000 p.s.i.) at the beginning of chromatography to 100 atm (1500 p.s.i.) towards the end.

Column rinse. At the end of chromatography, the column was rinsed for 5 min

with mobile phase B followed by mobile phase A for a further 5 min in order to stabilize the baseline. As the actual chromatography of bacitracin took less than 40 min, samples could be assayed approximately every 50 min.

RESULTS AND DISCUSSION

Separation of bacitracin components

An HPLC chromatogram of a bacitracin bulk powder is shown in Fig. 3 (I). More than 22 components of bacitracin have been separated and analyzed in less than 40 min of chromatography. The retention times and the retention volumes of the components are shown in Table I. In contrast to the HPLC method, only 14 components were detected by the classical carboxymethylcellulose (CMC) column chromatographic procedure, as shown in Fig. 3 (II)[11], which took over 5 h to chromatograph one sample. As reported by Storm and Strominger[12], bacitracins A and B were not separated by the CMC chromatography. When the effluent from the CMC column, corresponding to the unresolved major peak, was injected into the HPLC column, it was resolved into the two separate peaks of bacitracins A and B.

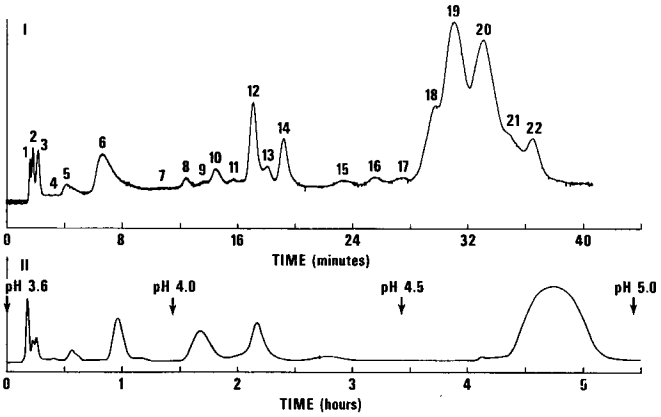

Fig. 3. Chromatogram of bacitracin. I, High-pressure liquid chromatography; II, classic carboxymethylcellulose column chromatography.

Zipax, HCP, ODS, HC-Pellosil and Vydac reversed-phase column packing materials were also examined. However, the best separation of bacitracins A and B was obtained by using the Bondapak C_{18}/Corasil packing material.

The effluents from the CMC column corresponding to each peak were collected and re-chromatographed by HPLC. The elution order of the components by the HPLC method was similar to that of the CMC method. Fig. 4 is a computer plot of the UV absorption spectra of peaks 5, 6, 19, 20 and 22 from Fig. 3 (I). A ten-fold dilution of effluent for peak 19 was made in mobile phase B for the UV absorption spectrum determination. The UV absorption spectra of peaks 1–3, 8, 10, 12 and 14 were similar to those of peaks 19 and 20 in Fig. 4. The elution profile, the UV absorption spectra and the antimicrobial activities suggest that peaks 1–3 are desamidobacitracin[3,11]; peaks 5 and 6 are bacitracins F_2 and F_1[7]; peaks 8, 10, 12 and 14 are transformation products of bacitracin (perhaps at an aspartylimidazole linkage)[11], peaks 19

TABLE I

RETENTION TIME AND RETENTION VOLUME OF BACITRACIN COMPONENTS SEPARATED BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Peak No.	*Retention time (min)*	*Retention volume (ml)*
1	1.7	1.5
2	1.8	1.6
3	2.2	2.0
4	2.7	2.4
5 (F_1)	4.3	3.8
6 (F_2)	6.6	5.9
7	10.3	9.3
8	12.5	11.3
9	13.8	12.5
10	14.6	13.1
11	15.7	14.1
12	17.1	15.4
13	18.1	16.3
14	19.1	17.2
15	23.4	21.1
16	25.6	23.0
17	27.5	24.8
18	29.8	26.9
19 (B)	31.3	28.2
20 (A)	33.2	29.9
21	35.2	31.7
22 (C)	36.7	33.1

and 20 are bacitracins B and A and peak 22 is bacitracin C. No reference to peaks 23 and 24 (not shown in Fig. 3 (I)) is available. HPLC of a relatively pure bacitracin F sample (Lot No. 585788A) obtained from Commercial Solvents (Terre Haute, Ind., U.S.A.) produced peaks with retention times identical with those of peaks 5 and 6.

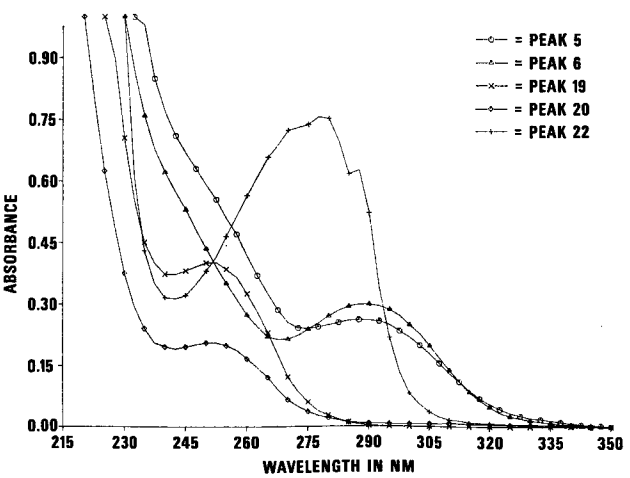

Fig. 4. UV absorption spectra of bacitracin components, peaks 5, 6, 19, 20 and 22 of HPLC (*cf.*, Fig. 3 (I)).

Fig. 5 includes chromatograms of bacitracin bulk powder purchased from three different manufacturers. Powder I, purchased from Company A, contains approximately 14% of bacitracin F and over 70% of bacitracins A and B. Powder II, purchased from Company B, consists mainly (about 90%) of bacitracins A and B. On the other hand, powder III, purchased from Company C, contains various amounts of impurities and degradation compounds with less than 60% of bacitracins A and B.

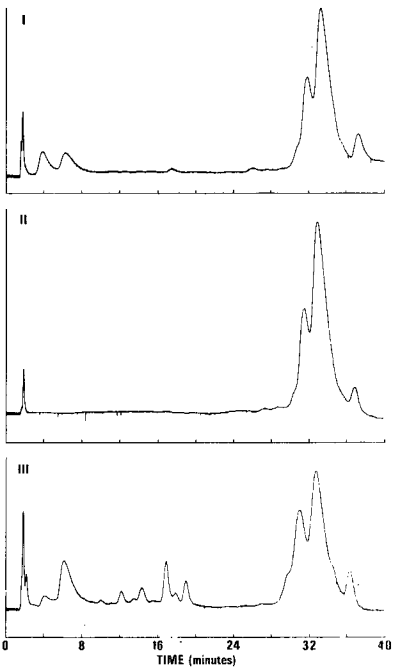

Fig. 5. Chromatograms of bacitracin bulk powders purchased from: I, Company A; II, Company B; and III, Company C.

Antimicrobial activity of bacitracin components

The bacitracin bulk powder, powder III (Fig. 5), was injected (20 units per injection) into the HPLC column and chromatographed. The column effluents corresponding to each component were collected and evaporated to dryness under a stream of dry nitrogen. Samples corresponding to bacitracins A and B were diluted 20 times and the remaining fractions with 2 ml of 1%, pH 6.0 phosphate buffer, and their antimicrobial activities were determined by the cylinder cup agar diffusion assay method using *M. flavus* as the test microorganism[6].

The results indicated that the compounds with antimicrobial activity were limited to bacitracins A, B and C and that peaks 1–18 and 21, including bacitracin F, showed no detectable activity (not more than 1% of bacitracin A activity). When the amounts of methanol and acetonitrile in mobile phase B were increased to 50% and 25%, respectively, in pH 4.5 buffer, two additional minor peaks, Nos. 23 and 24, were eluted. They comprised less than 2% of the total peak area and no antimicrobial activity was detected from these late-eluting peaks.

Samples of pure bacitracins A, B and C were obtained by HPLC using 20% methanol and 20% acetonitrile in 0.01 *M*, pH 4.5 phosphate buffer as the single mobile phase with no gradient elution technique. Although peaks 1–15 were eluted at the solvent front, this single mobile phase system gave a better separation of bacitracins A and B ($Rs = 0.9$) than by the gradient elution technique ($Rs = 0.5$). The separation factor (Rs) was calculated from the following equation[22]:

$$Rs = 2\,(t_2 - t_1)/(W_1 + W_2)$$

where

$t_2 - t_1$ = difference in retention time between bacitracins A and B;
$(W_1 + W_2)/2$ = average baseline peak width of bacitracins A and B in time unit.

The average of three consecutive days' assay indicated that the antimicrobial activities of bacitracins B and C are 87% and 30% of bacitracin A, respectively, per unit peak area. Therefore, the following equation was devised to calculate the microbiologically equivalent potency from the HPLC data:

$$\text{Bacitracin potency (unit/mg)} = \frac{R_1}{R_2} \cdot \frac{W_2}{W_1} \cdot F$$

where

R_1 = area of bacitracin A + 0.87·bacitracin B + 0.30·bacitracin C of a sample;
R_2 = area of bacitracin A + 0.87·bacitracin B + 0.30·bacitracin C of the reference standard;
W_1 = weight of a sample (mg/ml);
W_2 = weight of the reference standard (mg/ml);
F = assigned value of the bacitracin reference standard expressed in units per milligram of the reference standard.

Quantitative determination

The precision of the HPLC method for the determination of bacitracin was measured by comparing six replicate samples of the Upjohn laboratory standard material. Table II indicates that the relative standard deviation of the bacitracin

TABLE II

PRECISION OF THE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC METHOD USING THE UPJOHN LABORATORY BACITRACIN STANDARD

Sample No.	*Peak area*		*(Peak area of bacitracin A + 0.87 × bacitracin B)/wt.*
	Bacitracin A	*Bacitracin B*	
1	796.0	277.7	51.46
2	823.8	265.7	52.32
3	786.5	286.9	51.38
4	804.2	270.3	51.55
5	792.4	265.1	50.73
6	809.9	272.7	51.93
	Relative standard deviation 1.04%		

TABLE III

ANALYSIS OF CURRENT LOTS OF BACITRACIN BULK POWDER FROM VARIOUS MANUFACTURERS

— Indicates below 0.1%.

Source	*Drug content (unit/mg)*		*Percentage of components by peak area*																					
	Microbiological	*HPLC*	*1*	*2*	*3*	*4*	*5 (F_2)*	*6 (F_1)*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19 (B)*	*20 (A)*	*21*	*22 (C)*
Company A																								
1	59.2	58.5	0.3	2.8	0.3	—	6.1	8.6	—	0.1	—	—	—	0.6	—	—	0.5	0.7	—	1.4	17.9	55.6	1.4	3.8
2	57.9	52.0	1.1	3.7	—	—	6.5	7.5	—	—	—	—	—	0.4	—	—	—	0.5	—	1.7	22.7	54.2	—	1.6
Company B																								
1	61.2	59.4	0.3	1.4	—	—	—	—	—	—	—	—	—	0.9	—	—	—	0.8	0.7	2.5	21.9	67.9	—	5.9
2	55.7	55.7*	0.9	1.1	—	—	1.3	—	—	—	—	—	—	—	—	—	—	1.6	0.9	3.2	17.3	66.4	—	7.1
3	61.9	63.0	0.6	1.6	—	—	0.6	—	—	—	—	—	—	—	—	—	0.2	—	1.4	1.4	17.2	72.2	—	4.6
4	59.3	59.7	0.1	1.1	—	—	—	—	—	—	—	—	—	—	—	—	0.2	0.5	0.3	1.5	17.6	75.5	—	3.4
Company C																								
1	57.1	55.9	1.1	2.4	1.6	—	1.5	10.9	0.6	1.2	0.2	1.5	—	3.7	1.0	2.5	0.5	0.2	1.7	4.1	20.4	34.3	4.5	6.3
2	55.4	43.3	1.3	2.1	2.2	—	2.6	7.0	0.3	1.3	0.3	1.4	—	4.1	1.2	2.4	0.5	0.5	0.1	5.6	28.4	32.5	1.9	3.8
3	55.6	52.2	6.9	0.5	0.8	—	0.5	3.8	0.2	0.5	—	1.0	—	4.5	0.2	2.9	0.9	0.4	0.8	7.0	28.2	31.6	3.6	5.5
4	53.6	50.0	0.8	0.8	1.6	—	1.7	7.3	—	0.2	—	0.9	0.6	4.6	1.2	3.7	0.7	0.2	0.3	8.5	27.3	29.6	3.9	6.0
5	57.9	51.0	0.7	1.4	1.0	0.2	0.6	6.2	0.4	0.7	0.2	1.1	—	3.9	0.3	2.6	0.2	0.1	0.6	8.6	30.3	33.1	4.2	3.5

* Used as the reference standard.

determination is 1.04%. When the peak height was used for the calculation, the relative standard deviation was comparable (1.07%) with that calculated from the peak area. However, the peak area must be used to determine the percentage of each component in a bacitracin sample. Excellent precision was obtained by use of the injector valve, as there is no suitable vacant area in the chromatogram to place an internal standard, other than at the end of the chromatogram, which will significantly increase the chromatographic time. In order to obtain good precision with this assay method, it is essential to obtain a reproducible gradient elution pattern.

Eleven recent lots of bacitracin bulk powder purchased from three manufacturers were analyzed (Table III). The number of components in bacitracin varies greatly between manufacturers; however, they remain relatively consistent for each manufacturer. The calculated microbiological equivalencies of the powder purchased from Companies A and B showed no statistically significant difference ($p = 0.9$) with those of the microbiological assay method. However, the calculated values were approximately 10% lower than those of the microbiological method of powders purchased from Company C.

It may be possible that microbiologically active compound(s) have not been eluted from the HPLC column or a non-UV-absorbing compound may be contributing to antimicrobial activity. However, substantial increases in the methanol and acetonitrile concentrations in the mobile phase failed to elute any additional compound with antimicrobial activity. Also, the column effluents collected from peaks 1–18 and from peak 23 to one additional hour of chromatography showed no antimicrobial activity. These data may discount the possibility that a non-UV-absorbing compound may be contributing to the antimicrobial activity.

Increase in antimicrobial activity of bacitracin by zinc, manganese and cadmium has previously been reported[7]. In order to elucidate the possibility of cations in the powder III from Company C contributing to the 10% discrepancy in the assay results, the powders I, II and III were analyzed by X-ray fluorescence and emission spectrophotometry. The results indicated that the powders I, II, and III contained similar amounts of manganese and zinc. Therefore, the difference cannot be explained from the mineral contents in the powder.

The powders, lots 1–5, purchased approximately 4–6 months previously from Company C, were again tested by the microbiological assay method. The potencies obtained (53.0, 42.4, 48.1, 48.6 and 52.4 units/mg) are significantly lower than those of the original assay values and are comparable ($p = 0.05$) with those of the HPLC (Table III). Therefore, the 10% differences in the assay methods are probably due to the instability of these powders.

Bacitracin powders stored at room temperature for 73–85 months were then analyzed (Table IV). The results indicated that storage at room temperature caused a decrease in potency, as analyzed by the HPLC and by the microbiological methods, to about half of the original microbiological assay values. A slight decrease in the percentage of bacitracins A and B and an increase in bacitracin C and peak 21 were detected. Partial conversion of bacitracin A into bacitracin C has already been reported[8] and an increase in peak 21 with a corresponding decrease in bacitracin A can be demonstrated when bacitracin is stored in an aqueous phase near neutral pH. No apparent increase in bacitracin F was noted. The data obtained seem to indicate that both dry bacitracin powder and aqueous solutions degrade similarly; however, addi-

TABLE IV

STABILITY OF BACITRACIN BULK POWDER AGED AT ROOM TEMPERATURE

— Indicates below 0.1%.

Age (months)	*Drug content (unit/mg)*			*Percentage of components by peak area*																				
	Initial	*Aged*		*1*	*2*	*3*	*4*	*5 (F_2)*	*6 (F_1)*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19 (B)*	*20 (A)*	*21*	*22 (C)*	*23*
	Microbiological	*Microbiological*	*HPLC*																							
73	55.0	35.8	33.7	33.6	0.6	1.9	0.5	0.4	0.9	8.8	0.3	0.8	0.2	1.7	0.2	3.1	0.5	2.3	—	0.5	0.8	7.5	22.3	28.6	11.0	7.1	—
74	56.3	43.3	45.7	44.4	0.7	1.0	0.3	0.7	0.8	7.7	0.5	1.2	—	1.6	0.2	4.1	0.7	1.8	0.3	0.3	0.9	5.6	25.0	34.4	4.3	7.9	—
74	52.1	35.7	35.6	37.4	0.4	2.4	1.0	—	0.3	8.0	0.4	1.5	—	1.1	0.3	4.0	0.1	3.0	—	—	0.9	6.2	30.4	31.1	3.0	6.0	—
85	48.0	26.3	28.1	24.6	1.9	1.9	1.3	0.5	1.1	8.5	1.3	1.9	—	3.2	—	4.2	0.5	2.5	0.6	0.2	0.6	5.3	21.6	23.4	7.7	11.2	0.6

TABLE V

EFFECTS OF THE STERILE BACITRACIN USP MANUFACTURING OPERATION ON THE COMPONENTS OF BACITRACIN

— Indicates below 0.1%.

Sample	*Drug content (unit/mg)*			*Percentage of components by peak area*																					
	Microbiological		*HPLC*	*1*	*2*	*3*	*4*	*5 (F_2)*	*6 (F_1)*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19 (B)*	*20 (A)*	*21*	*22 (C)*
Bulk powder Sterile Bacitracin USP	59.2		58.5	0.3	2.8	0.3	—	6.1	8.6	—	0.1	—	—	—	0.6	—	—	0.5	0.7	—	1.4	17.9	55.6	1.4	3.8
1	59.6	56.2	58.7	0.8	2.0	0.3	—	3.5	6.6	—	0.7	—	—	—	0.8	—	—	1.0	—	—	1.3	20.9	53.7	3.5	6.1
2	59.8	56.1	57.1	1.2	2.4	0.2	—	9.4	5.8	0.7	0.4	—	—	—	0.4	—	—	0.7	0.2	—	2.0	17.6	51.6	2.7	4.6
3	60.3	55.8	57.5	0.7	2.5	—	—	6.6	5.3	—	—	—	—	—	0.7	—	—	1.0	0.6	0.4	2.5	20.1	52.1	2.2	5.4
4	59.6	56.8	58.5	1.5	2.1	0.1	—	6.6	6.3	—	—	—	—	—	1.3	—	—	0.3	0.2	—	2.3	18.6	52.3	3.5	5.0
5	60.2	56.8	58.9	0.8	2.6	0.6	—	3.6	6.6	—	1.4	—	—	—	0.4	—	—	0.4	1.1	—	2.6	20.7	53.0	3.0	2.9

tional data are needed to make definite conclusions, as data on the composition of these powders at the beginning of the storage are not available.

Effects of the manufacturing operation on the components in sterile bacitracin USP

As the manufacturing operation for the production of sterile, freeze-dried bacitracin is complex, the effects of the manufacturing operation on the composition of bacitracin were investigated. Five sterile bacitracin USP lots manufactured from one bulk lot of bacitracin powder were analyzed. It is worth noting that the common oxidative degradation product, bacitracin F, did not increase by the process. A slight (1–2%) decrease in the amount of bacitracin A with a corresponding increase in the peak 21 was observed after the manufacturing operation (Table V). Similar phenomena were observed when bacitracin was stored in an aqueous phase. However, the change was so small that neither the microbiological nor the calculated potencies were significantly changed.

Effects of impurities and degradation compounds on acute toxicity in mice

Bacitracin is nephrotoxic when administered parenterally in high doses and attempts to eliminate this toxicity by purification have not been successful[5,23]. Codington[23] noted that, as bacitracin F is antimicrobially inactive but equal in toxicity to bacitracin A, increasing the ratio of bacitracin A to bacitracin F should increase the ratio of antimicrobial activity to nephrotoxicity. Because other antimicrobially inactive components of bacitracin powders demonstrated by HPLC might also contribute to nephrotoxicity, we compared the acute toxicities of bacitracin powders containing varying amounts of inactive components.

The samples tested were powders I, II and III (Fig. 5) and a purified powder (powder IV) consisting of the material in the last, major peak of Fig. 3 (II). Powder IV was obtained by adjusting the column effluent to pH 7.0 with sodium hydroxide, extracting with *n*-butanol and evaporating to dryness at 30° under vacuum.

Acute toxicity was measured by preparing 6–8 concentrations of each powder

TABLE VI

EFFECTS OF IMPURITIES AND DEGRADATION COMPOUNDS ON ACUTE TOXICITY IN MICE

Powders I–III (*cf.*, Fig. 5).

Powder	*Experiment No.*	LD_{50} *(units/kg)*	*95% confidence interval (units/kg)*
I	1	24,200	20,900–28,100
	2	20,300	17,300–23,800
	3	32,500	28,100–37,500
II	1	19,700	17,700–21,800
	2	29,300	25,600–33,500
	3	25,100	22,100–28,500
III	1	29,000	25,400–33,200
	2	33,200	29,100–37,800
	3	35,600	31,500–40,100
IV	1	24,200	19,700–29,700

in 1% sodium chloride solution and injecting 0.5 ml into the tail vein of 8–10 20-g male strain CF-1 mice. Deaths were recorded for 14 days post-injection and the method of Spearman and Karber[24] was used to calculate the number of microbiological assay units required to kill 50% of the mice treated (LD_{50}). The LD_{50} data shown in Table VI do not show significant differences in toxicity between powders with widely different contents of inactive components. However, only large differences in toxicity could have been detected from these data because of the high variance.

Therefore, further studies are being planned to evaluate the effect of purification on minimization of nephrotoxicity.

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