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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR POLYPEPTIDE ANTIBIOTICS AND ITS APPLICATION TO STUDY THE EFFECTS OF TREATMENTS TO REDUCE MICROBIAL LEVELS IN BACITRACIN POWDER

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SUMMARY

Improvements were made in the high-performance liquid chromatographic (HPLC) method to obtain baseline separation of chromatographic peaks of structurally similar polypeptide components in bacitracin. The improved method uses a 30-cm-long stainless-steel column packed with μBondapak C_{18}. The theoretical plates of the column are approximately 140,000 per meter for the bacitracin A peak.

The resolution function between bacitracins B_1 and B_2 and that between bacitracins A and B_2 have been improved 418 and 225%, respectively. The components of bacitracin, bacitracins A, B, C, D, E, F, and G, were fractionated by the countercurrent distribution technique. These components, together with Compound X, a compound separated on a carboxymethylcellulose column, and bacitracin F, obtained by degrading bacitracin A sample at neutral pH, were used to identify peaks in the HPLC chromatogram.

Effects of processing methods used to reduce microbial contamination levels in bacitracin powders were evaluated. Heat treatment caused a significant loss of antimicrobial activity (35% reduction), bacitracins A, B_1, and B_2 were reduced by 37, 22, and 21%, respectively. A significant increase (2.8 times) of bacitracin F, an oxidative degradation compound, was shown. Irradiation by ^{60}Co at 1.8 Mrad caused no loss of potency nor change in any of the bacitracin components. Ethylene oxide treatment, on the other hand, caused considerable (46%) reduction of potency. Substantial reduction of areas under the peaks of bacitracins A, B_1, and B_2 (50, 24 and 37%, respectively) were noted. The chromatograms showed numerous unresolved peaks around bacitracins A, B_1, and B_2; however, no significant increase in the bacitracin F peak, nor appearance of non-UV absorbing peaks were observed.

Peptide antibiotics of the polymyxin group, circulin, colistin, and polymyxin, were also analyzed using the μBondapak C_{18} column with a linear-gradient elution. A UV monitor was used for polymyxin. A moving-wire flame ionization detector was used to monitor circulin and colistin. A sample of polymyxin, circulin, and colistin may be analyzed in less than 20 min of chromatographic time.

INTRODUCTION

Commercially available polypeptide antibiotic preparations, bacitracin, circulin, colistin, and polymyxin, are mixtures of various components and degradation products.

According to Newton and Abraham[1], bacitracin preparations often contain bacitracin A, A_1, B, C, D, E, F_1, F_2, F_3, and G. The structure of bacitracin B differs from A (Fig. 1) by the replacement of isoleucin with valine. However, the exact position of the replacement is not absolutely clear. Bacitracin F, a nephrotoxic agent, is an oxidative deaminated compound having a keto-thiazole instead of an amino-thiazoline moiety[2,3]. Newton and Abraham[1] reported that acid-hydrolyzed fractions of bacitracins B, D, and E contained a strong valine spot and those of bacitracins C and G, a strong glycine spot. However, structures of bacitracins C, D, E, and G have not been elucidated. Bacitracins A and B are the major components with antimicrobial activity.

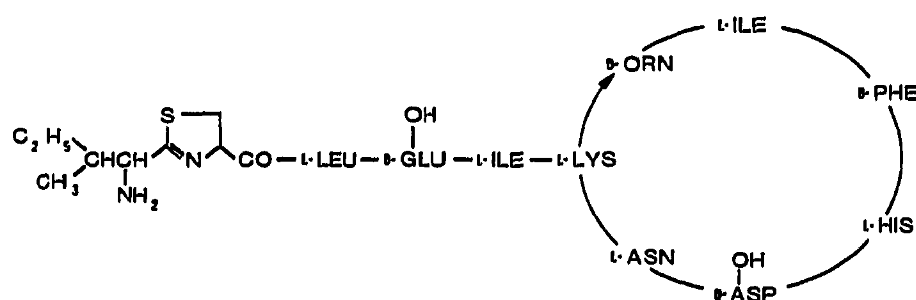

Fig. 1. Structure of bacitracin A.

For isolation and analysis of bacitracin, countercurrent distribution[4,5] and column chromatography[6,7] have often been used. However, Craig *et al.*[4] reported that a 1,000-tube countercurrent distribution failed to completely resolve the components and chromatography on a carboxymethylcellulose column may not separate bacitracins A and B. Doulakas[8] proposed a colorimetric assay method using α-amino-carboxylic acid with sodium hypobromide for determination of bacitracin in ophthalmic ointments. The method, however, is relatively non-specific and is incapable of differentiating various entities of bacitracin. For quantitative determination of bacitracin, a microbiological assay method using *Sarcina subflava* or *Micrococcus flavus*, has been recognized as the official analytical method[9]. The method, however, is not capable of quantitating a degradation compound, especially bacitracin F, a nephrotoxic agent. A high-performance liquid chromatographic (HPLC) method for qualitative and quantitative determination of bacitracin reported by Tsuji *et al.*[10] represented a significant breakthrough in the analysis of bacitracin; however, the resolution of the chromatographic peaks of bacitracins A, B, and C was not optimal.

This paper describes improvements made to the HPLC method for bacitracin.

The HPLC method was then used to evaluate the effects of processing methods, dry heat, ethylene oxide treatments, and ^{60}Co irradiation on the composition of bacitracin.

The HPLC method was further expanded to analyze polypeptide antibiotics of the polymyxin group.

EXPERIMENTAL

Apparatus

A Laboratory Data Control (Riviera Beach, Fla., U.S.A.) modular liquid chromatograph equipped with a high-pressure Milton Roy Minipump (max. 5,000 p.s.i.) with a pulse dampener (Model 709) was used. For the determination of bacitracin and polymyxin, a 254-nm UV monitor (Model 1285) was used and for circulin and colistin determination, a moving-wire flame ionization detector (Pye LCM2, Pye Unicam, distributed by Phillips Electronic Instruments, Mount Vernon, N.Y., U.S.A.) was used.

A stainless-steel column (30 cm × 4.6 mm I.D.) pre-packed with μBondapak C_{18} (Waters Ass., Milford, Mass., U.S.A.) was used. The column was attached to a septumless injector valve (Micromeritics, Norcross, Ga., U.S.A.) and to the UV monitor or to the flame ionization detector. The theoretical plates of the column were approximately 140,000 per meter for the bacitracin A peak. This column efficiency is approximately 70 times more than that of the Bondapak C_{18}/Corasil column used previously[10]. An Ultragrad gradient mixer (Model 11,300, LKB, Stockholm, Sweden) with a 0.25-ml mixing chamber (Model 11,361-1) was attached to the minipump to form the gradient elution pattern.

For bacitracin determination, the programmed convex gradient used followed the exponential equation $y = [191/(1 + e^{0.438-0.67x})] - 78.5$ from 1.5 min to 1 h, where x equals time in minutes and y equals the per cent of the mobile phase B with respect to A.

A linear gradient elution, from mobile phase A to B in 30 min, was used for circulin, colistin, and polymyxin determination. The gradient was stopped at 18 min of the elution time, thus the maximum ratio of mobile phase B attained was 60% of mobile phase A.

For circulin and colistin determination, a moving-wire flame ionization detector was used. The temperatures of the cleaner, oxidizer, evaporator, reactor, and flame ionization detector were 900, 690, 380, 490, and 175°, respectively. Flowmeter settings for hydrogen, nitrogen, and air flow-rate were 4.7, 2.2, and 1.1, respectively.

Mobile phases for bacitracin

Mobile phase A. 50 ml of absolute methanol and 850 ml of water were added into a 1,000-ml graduated cylinder. Then 100 ml of a 100 m*M*, pH 4.5 phosphate buffer were added and mixed.

Mobile phase B. 500 ml absolute methanol, 200 ml of acetonitrile, and 200 ml of water were added into a 1,000-ml graduated cylinder. After mixing, 100 ml of a 100 m*M*, pH 4.5 phosphate buffer were added and mixed.

Mobile phases for circulin, colistin, and polymyxin

Mobile phase A. 200 ml of acetonitrile and 700 ml of water were added into a

1,000-ml graduated cylinder. Then 100 ml of a 200 m*M*, pH 2.0 phosphate solution were added and mixed.

Mobile phase B. 500 ml acetonitrile, 200 ml absolute methanol, and 290 ml water were added into a 1,000-ml graduated cylinder. After mixing, 10 ml of a 200 m*M*, pH 2.0 phosphate solution were added and mixed.

The 200 m*M*, pH 2.0 phosphate solution was prepared as follows: A solution containing a suitable quantity of dibasic sodium phosphate was prepared and the pH of the solution was adjusted to pH 2.0 with phosphoric acid. Water was then added to the volume for a phosphate concentration of 200 m*M*.

Procedure

Chromatographic conditions. The column temperature was ambient with a chart speed of 6.4 mm/min. The column pressure was approximately 7.0 atm (1,000 p.s.i.) with a flow-rate of 1.0 ml/min.

For bacitracin, a 2.0-μl volume of sample was injected into the column using a septumless injector with an electrometer range setting of 0.08 absorbance units full scale.

For circulin, colistin, and polymyxin, a 8.0-μl volume of sample was injected into the column using a septumless injector. The UV electrometer range setting for polymyxin was 0.04 absorbance units full scale. The moving-wire flame ionization detector range setting for circulin and colistin was 32 $\times$ 1.0 full scale. Wire speed setting was 2$\times$.

Preparation of bacitracin reference standard. Approximately 5 mg of the Zn·bacitracin USP Reference Standard, Issue J, was accurately weighed and dissolved in 1 ml of acidified methanol (20 m*M* HCl in 80% methanol).

Preparation of bacitracin sample. Approximately 5 mg of bacitracin powder were accurately weighed. Just prior to analysis, sample was dissolved in 1 ml of 80% methanol.

Preparation of circulin, colistin, and polymyxin. Approximately 16 mg of the standard and samples were accurately weighed and dissolved in 1 ml of water.

RESULTS AND DISCUSSION

The major modification made to the original HPLC analysis method for bacitracin[10] was the use of a column packed with octadecylsilane bonded on 10-μm silica gel (μBondapak C_{18}), which necessitated the use of a higher concentration of acetonitrile in mobile phase B.

As may be seen in Fig. 2, the peaks of bacitracins A, B_1, and B_2 separated well. The theoretical plates obtained by the μBondapak C_{18} column for bacitracin A are 140,000 per meter and are approximately 70 times more than those obtained by the Bondapak C_{18}/Corasil column (Waters) used previously. The resolution function (R_s) between bacitracins B_1 and B_2 was improved from 0.33 to 1.37 and the R_s between bacitracins A and B_2 was improved from 0.58 to 1.31.

Identification of peaks

In order to help identify the peaks obtained in HPLC chromatograms of bacitracin, the components in bacitracin powder were fractionated using the counter-

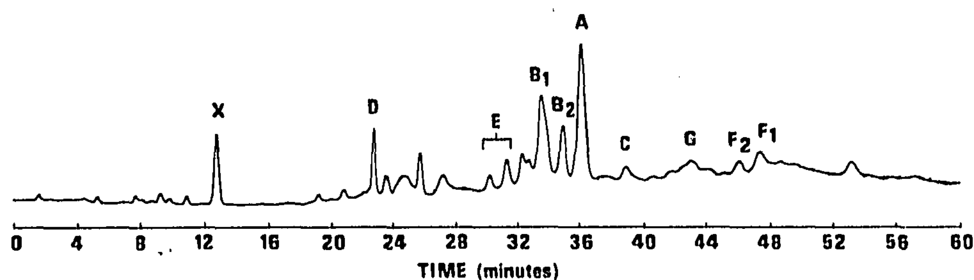

Fig. 2. An HPLC chromatogram of a commercial preparation of bacitracin powder indicating separation of bacitracin components A, B_1, B_2, C, D, E, F_1, F_2, G and X. A μBondapak C_{18} column, 300 × 4 mm I.D., was used with a convex gradient from 5% methanol to 50% methanol and 20% acetonitrile, both with a 10 m*M* phosphate buffer, pH 4.5, in 1 h. Flow-rate, 1.0 ml/min.

current distribution technique[1] with a straight 200-tube transfer method. Crude preparations of bacitracins A, B_1, B_2, C, D, E, F, and G were thus obtained. The UV absorption spectra of these preparations were similar to those reported by Newton and Abraham[1]. Since the bacitracin powder used to prepare various fractions of bacitracin contained only a small amount of bacitracin F, a bacitracin F sample was prepared from a crude bacitracin A preparation by degrading in a neutral pH solution. Compound X was obtained from N. Kantor of FDA (Washington, D.C., U.S.A.). Compound X elutes several hours after the unresolved bacitracins A and B peak from a carboxymethylcellulose column[6]. The UV absorption spectrum of Compound X was identical to that of peak 6, mislabeled as peak 22 in the original publication[10]. Compound X was not separated by the countercurrent distribution technique and was present in a fraction containing bacitracin B_1.

These bacitracin preparations were chromatographed individually and in combination with a commercially available bacitracin powder to identify peaks in HPLC chromatograms. The UV absorption spectra of crude preparations containing various components were also compared with those of chromatographic peaks of bacitracin powder. The results of the identification are shown in Fig. 2.

Antimicrobial activity of bacitracin components

Although relative antimicrobial activities of bacitracins A, B and C were reported[10], these response factors may not be reliable due mostly to inadequate separation of these compounds by the original HPLC method. Therefore, the antimicrobial activities of bacitracin components were again examined by collecting column effluent corresponding to well separated peaks from the improved HPLC chromatograms.

Approximately 10 mg of the Zn·bacitracin USP Reference Standard were accurately weighed and dissolved in 1.0 ml of acidified methanol. Eight microlitres of the solution were injected into the HPLC column. Column effluents corresponding to bacitracins A, B_1 and B_2 were collected and evaporated to dryness under a stream of dry nitrogen. The dried residue was dissolved in 1 ml of 1%, pH 6.0 phosphate buffer for each of bacitracins B_1 and B_2 fractions and 3 ml for bacitracin A fraction. The experiment was repeated three times, each using an independently weighed USP Reference Standard. The re-dissolved fractions corresponding to each compound were pooled and their antimicrobial activity was determined by the cylinder cup, agar diffusion assay method using *M. flavus* as the test microorganism[9].

TABLE I

ANTIMICROBIAL ACTIVITY OF BACITRACINS A, B_1, AND B_2 FRACTIONS IN USP Zn·BACITRACIN REFERENCE STANDARD

Weight (mg)	*Amount injected (μl)*	*Peak area*		
		A	B_1	B_2
10.040	8	192.1	56.5	36.3
10.052	8	215.7	66.3	41.2
10.180	8	218.2	65.0	46.0
Antimicrobial activity		1.18 U/3 ml	1.06 U/ml	0.82 U/ml
Corrected activity (unit/area/wt.)		0.171	0.171	0.200
Relative response factor		1.00	0.999	1.16

The results, shown in Table I, indicate that the relative antimicrobial responses of bacitracins A, B_1 and B_2, corrected by the peak area to weight ratio, were equal; the variation is well within that of the microbiological assay method. Also, all of the antimicrobial activity of the reference standard can be accounted for by these three compounds. Therefore, the peak areas under bacitracin A, B_1, and B_2 were calculated and their relative antimicrobial response factors were used to determine the microbiologically equivalent potency of bacitracin preparations from the HPLC data. As may be seen in Table II, the HPLC calculated antimicrobial potencies agreed well with those obtained by the microbiological assay method.

TABLE II

EFFECTS OF DRY HEAT, ETHYLENE OXIDE, AND ^{60}Co IRRADIATION TREATMENTS ON COMPONENTS IN BACITRACIN POWDER

Treatment	*Antimicrobial potency (unit/mg)*		*Composition (%)*					
	Microbiological	*HPLC*	*A*	B_1	B_2	*C*	F_1	F_2
USP reference standard	60*	60*	58.7	19.4	11.2	2.3	4.5	4.0
Dry heat treatment								
Control, Lot 1	48.1	52.6	49.4	17.4	11.9	0.3	7.2	1.2
Lot 2	53.4	51.9	51.4	15.4	10.9	11.2	7.1	5.1
Lot 3	54.1	54.7	54.8	16.1	11.0	1.7	3.8	5.0
Dry heat, Lot 1	30.5	32.6	27.3	12.1	9.3	4.2	10.8	2.6
Lot 2	33.4	34.5	32.7	11.3	7.6	4.8	10.6	5.2
Lot 3	38.1	42.2	38.6	14.7	9.9	3.4	10.5	4.2
Ethylene oxide treatment								
Control	52.9	52.0	53.6	11.4	12.3	3.2	4.0	2.7
Treated	28.8	28.3	27.1	8.7	7.7	4.4	5.6	6.5
^{60}Co irradiation								
Control	52.9	52.0	53.6	16.4	11.5	5.8	6.5	2.7
Treated	50.4	57.3	55.2	17.5	11.4	4.0	6.2	3.3

* Used as the reference standard.

Effects of dry heat, ethylene oxide, and ⁶⁰Co irradiation treatments

The effects of various treatments commonly used to reduce microbial contamination levels in powders are shown in Table II. The dry heat treatment at 55° for 20 days significantly (34.5%) reduced the antimicrobial potency of bacitracin powder. The treatment caused 36.9, 21.9 and 20.8% reduction of bacitracins A, B_1, and B_2, respectively. A 2.8 times increase of an oxidative deaminated compound, bacitracin F_1, was observed.

The ^{60}Co irradiation at 1.8-Mrad dose level, on the other hand, caused no loss of potency nor change in any of the components (Fig. 3).

The ethylene oxide treatment, 6.5 h under 6.5 p.s.i. ethylene oxide gas (12:88 mixture) pressure, caused significant loss (45.6%) of antimicrobial potency. Bacitracins

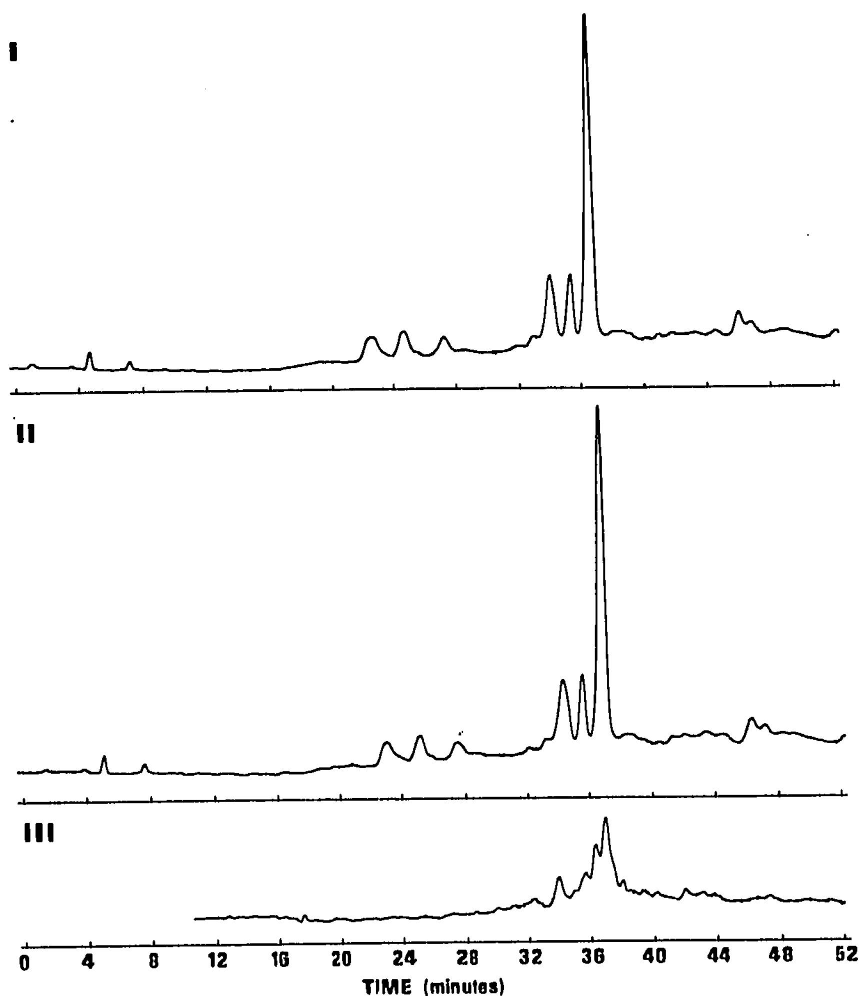

Fig. 3. Effects of ^{60}Co irradiation and ethylene oxide sterilization treatments on the composition of bacitracin powder. I, Control; II, ^{60}Co irradiation at 1.8 Mrad; III, ethylene oxide treatment.

A, B_1, and B_2 were reduced by 49.5, 23.7, and 37.4%, respectively. The chromatogram (Fig. 3) showed appearance of numerous peaks near bacitracins A, B_1, and B_2, causing loss of resolution of these peaks. No significant increase in bacitracin F nor appearance of non-UV absorbing compound, as monitored by the moving-wire flame ionization detector, was seen.

Therefore, ^{60}Co irradiation or aseptic crystallization is the treatment of choice for reducing contaminating microbial levels in bacitracin powder.

Application of HPLC to polypeptide antibiotics of the polymyxin group

With a slight modification of the HPLC method for bacitracin, polypeptide antibiotics of the polymyxin group, circulin, colistin, and polymyxin, may be analyzed by the HPLC technique. The HPLC method for polymyxins calls for the use of a linear gradient elution, from 20% acetonitrile to 50% acetonitrile and 20% methanol in 30 min. Only 16 min of the gradient was used, thus the maximum ratio of the upper mobile phase attained was approximately 60% to the lower mobile phase. This gradient elution pattern was shown to give the maximum resolution between polymyxins B_1 and B_2 (Fig. 4).

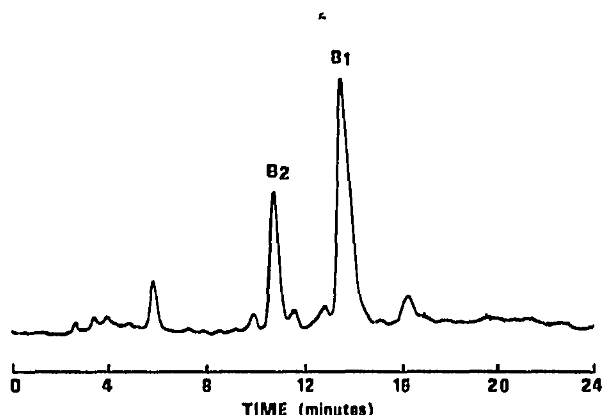

Fig. 4. Separation of polymyxin B_1 from B_2 on a μBondapak C_{18} column, 300 × 4 mm I.D., as monitored by a 254-nm UV detector. A linear gradient was used from 20% acetonitrile to 50% acetonitrile and 20% methanol, both in a pH 2.0 phosphate buffer, in 16 min. Flow-rate, 1.0 ml/min.

Although polymyxin may be monitored by a UV detector, circulin and colistin have to be monitored by a detector other than UV, since these antibiotics do not absorb UV. Therefore, a moving-wire flame ionization detector was used. The sensitivity of this detector was similar to that of the UV detector for polymyxin. HPLC chromatograms of circulin and colistin are shown in Figs. 5 and 6.

Thus, HPLC was shown to be a valuable tool in the study of high-molecular-weight polypeptide antibiotics.

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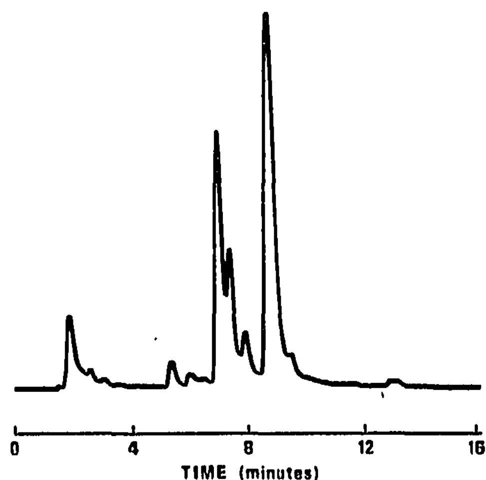

Fig. 5. An HPLC chromatogram of circulin as monitored by a moving-wire flame ionization detector. A μBondapak C_{18} column, 300 × 4 mm I.D., was used with a linear gradient from 20% acetonitrile to 50% acetonitrile and 20% methanol, both in pH 2.0 phosphate buffer, in 16 min. Flow-rate, 1.0 ml/min. Peaks eluting at 7.4 and 9.0 min are assumed to be circulins B and A.

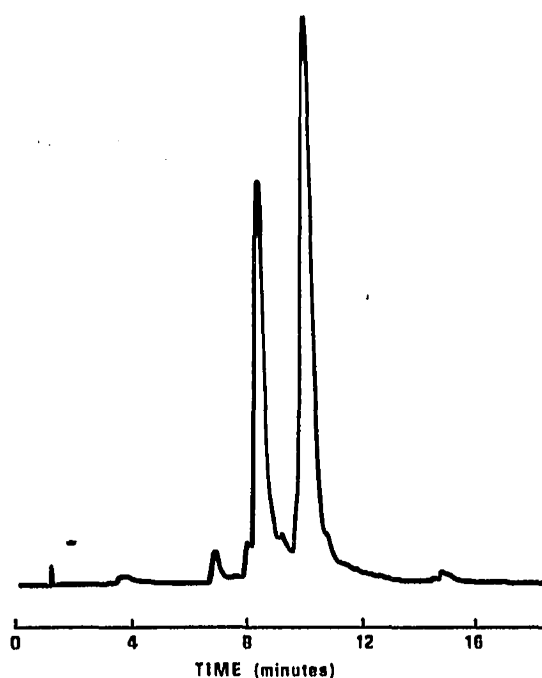

Fig. 6. An HPLC chromatogram of colistin (polymyxin E) as monitored by a moving-wire flame ionization detector. A μBondapak C_{18} column, 300 × 4 mm I.D., was used with a linear gradient from 20% acetonitrile to 50% acetonitrile and 20% methanol, both in pH 2.0 phosphate buffer, in 16 min. Flow-rate, 1.0 ml/min. Peaks eluting at 8.5 and 10.3 min are assumed to be colistin B (polymyxin E_2) and colistin A (polymyxin E_1).

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