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ANALYTICAL AND PREPARATIVE METHODS FOR POLYMYXIN ANTIBIOTICS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A POROUS STYRENE–DIVINYLBENZENE COPOLYMER PACKING

YUKIO KIMURA*, HISAMI KITAMURA, TOYOSHIGE ARAKI, KUMIKO NOGUCHI and MIYAKO BABA

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16 Edagawa-cho, Nishinomiya 663 (Japan)

and

MASATAKE HORI

Chemical Research Laboratories, Central Research Division, Takeda Chemical Industries Ltd., Yodogawa-ku, Osaka 532 (Japan)

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SUMMARY

Polymyxin antibiotics, such as polymyxin A, B, D, E, K, M and P, circulin and colistin, are a complex of two or three components. These components have been successfully separated on a porous styrene–divinylbenzene copolymer column with methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1) as mobile phase. Hitachi gel 3011 (particle size 10 μm) gave a good analytical separation of polymyxins using the isocratic mobile phase. In the preparation, all of the components of colistin and polymyxin B are separated by use of Hitachi gel 3010 (particle size 25 μm) or Amberlite XAD-2(100–200 mesh) packings. A new component of polymyxin B, named polymyxin B_0, was deduced from the results of the amino acid and fatty acid analyses.

INTRODUCTION

Since 1947, many polymyxin antibiotics have been isolated from a culture broth of *Bacillus polymyxa* and related species. Some of them have been evaluated as clinically useful antibiotics against gram-negative bacteria. Their chemical structure consists of a cyclic heptapeptide moiety and a side-chain consisting of a tripeptide with a fatty acyl residue on the N-terminus[1]. As they have four or five unmasked A_2bu (2,4-diaminobutyric acid) residues, all polymyxins are strongly basic. Each polymyxin reported to date is a complex of two or three components and can be separated into single components by counter-current distribution[2], paper partition chromatography[3] and thin-layer chromatography[4]. In our studies on screening and identifying a new polymyxin antibiotic, a more efficient method for separation into single components was required. Ten years ago, we developed an excellent liquid

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chromatographic method using an Amberlite XAD-2 column[5] and successful results for the separation of E_1 and E_2 from polymyxin E and B_1 and B_2 from polymyxin B were obtained[6]. The separation mechanism seems to be reversed-phase adsorption between the styrene–divinylbenzene copolymer and the hydrous mobile phase.

On the other hand, it is well known that porous styrene–divinylbenzene copolymer and alkyl-bonded silica, such as LiChrosorb RP-18, μBondapak C_{18} and Nucleosil C_{18}, behave in a similar manner in reversed-phase liquid chromatography. Because of this similarity, it was thought that the alkyl-bonded silica might be applied to the separation of polymyxins. Since Tsuji and co-workers' work[7,8], there have been several studies on the separation of polymyxins by high-performance liquid chromatography(HPLC) using alkyl-bonded silica packings[9,10]. The paucity of reports on the study of polymyxins by HPLC using porous styrene–divinylbenzene copolymer, such as Hitachi gel 3011 and Jasco HP-01, prompted us to investigate this method.

EXPERIMENTAL

Reagents

Several polymyxins were kindly donated, as follows: polymyxin A hydrochloride and polymyxin E sulphate by Dr. S. Wilkinson (Wellcome Research Laboratories, Beckenham, Great Britain), polymyxin D and circulin hydrochlorides by Emeritus Professor T. Suzuki and Dr. K. Hayashi (Kyoto University, Kyoto, Japan), polymyxin M hydrochloride by Professor A. B. Silaev (Moscow State University, Moscow, U.S.S.R.) and colistin sulphate by Banyu Pharmaceutical (Tokyo, Japan). Polymyxin B sulphate was purchased from Sigma (St. Louis, MO, U.S.A.). Polymyxin K and P hydrochlorides were prepared as reported by Kimura and co-workers[11,12]. Sulphates of antibiotics could be convested into the hydrochlorides by treatment with barium chloride, if necessary. All of the polymyxins designated by the same capital letter but with different arabic numeral subscript were used as mixtures.

HPLC-grade methanol and reagent-grade potassium chloride were obtained from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of analytical-reagent grade. Water was de-ionized and distilled. All aqueous solutions containing reagents were passed through a Millipore CS filter, 0.22 μm (Millipore, Bedford, MA, U.S.A.), and degassed prior to use.

Apparatus

The following types of liquid chromatographs were used: a Shimadzu Model LC-3A equipped with a Model CRB-1B chemical reaction chamber and a Model FLD-1 fluorescence detector (Shimadzu, Kyoto, Japan); a Jasco Model Familic 100-N for micro-HPLC (Japan Spectroscopic, Tokyo, Japan); Model KHD-26 reciprocating pump (Kyowa Seimitsu, Tokyo, Japan); a bellows-type damper (Umetani Seiki, Osaka, Japan); a Model KHP-UI-130 syringe-loading sample injection valve (Kyowa Seimitsu, Tokyo, Japan); and a Model UVIDEC 100 variable-wavelength UV detector (Japan Spectroscopic). In some instances a Model R-403 refractive index detector (Waters Assoc., Milford, MA, U.S.A.) was used for small preparative separations. All separations were carried out at room temperature.

Column packings and columns

The column packings used for the preparative method were Amberlite XAD-2

100–200 mesh) (Rohm and Hass, Philadelphia, PA ,U.S.A.) and Hitachi gel 3010 (25 μm) (Hitachi, Tokyo, Japan), and for the analytical method Hitachi gel 3011 (10 μm), Jasco HP-01 (10 μm) (Japan Spectroscopic) and Diaion CHP-3C (10 μm) (Mitsubishi Chemicals, Tokyo, Japan). All of these packings are porous styrene–divinylbenzene copolymers.

The column size for preparations was 55 cm × 16 mm I.D. or 50 cm × 8 mm I.D., and for analyses 20 cm × 4 mm I.D. or 20 cm × 0.5 mm I.D. (micro-HPLC). After equilibrating the gel with the mobile phase with a sonic oscilator, the columns were packed by the slurry techniques at a higher flow-rate than would be used in the chromatographic separation. An alkyl-bonded silica, Nucleosil $5C_{18}$ (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.) was packed into a PTFE micro-column (20 cm × 0.5 mm I.D.) by means of slurry technique and used for comparative experiments.

Procedures

Preparation of colistin A, B and C from colistin. After equilibrating the Amberlite XAD-2 column with mobile phase[methanol–0.2 *M* sodium chloride solution (1:1)], 500 mg of colistin hydrochloride in 5 ml of water were added to the column. The elution was performed by linear gradient elution (0.5 ml/min) with the mixing chamber containing 500 ml of methanol–0.2 *M* sodium chloride solution (1:1) and the reservoir containing 500 ml of methanol–0.01 *N* hydrochloric acid (1:1). A 5-ml volume of eluate was collected in each tube and 30 μl of each fraction were used to detect colistin by the ninhydrin colour reaction (Fig. 1). As an alternative method, Hitachi gel 3010 instead of Amberlite XAD-2 with isocratic elution of methanol–0.2 *M* sodium chloride solution (1:1) was used. The chromatogram obtained with refractive index detector (Fig. 2) is similar to Fig. 1.

After desalting of fractions by Sephadex G-15 gel filtration, each component was analysed as follows. The sample was hydrolysed with constant-boiling hydrochloric acid at 110°C for 1 h and the hydrolysate was extracted with three 1-ml portions of diethyl ether. The ethereal extract was dried over anhydrous sodium sulphate and evaporated to about 0.2 ml under a slow stream of nitrogen at 25°C. The fatty acids in diethyl ether were analysed with a Shimadzu Model GC-5A gas chromatograph equipped with a hydrogen flame-ionization detector and a glass column (2 m × 3 mm I.D.) packed with FON (10%) on Celite 545 (Wako Chemicals, Osaka, Japan) at 180°C. The flow-rate of the carrier gas (nitrogen) was 60 ml/min. The aqueous layer was evaporated to dryness and the residue hydrolysed with constant-boiling hydrochloric acid, then the hydrolysate was analysed with a Hitachi KLA-3B automatic amino acid analyser.

The antibiotic potency of the samples was based on the official assay method for antibiotic preparations issued by the Ministry of Public Welfare, Japan[13].

Preparation of polymyxin B_1, B_2 and B_0 from polymyxin B. Polymyxin B hydrochloride was separated into three components by the Hitachi gel 3010 column. The chromatogram is shown in Fig. 2(B). Each component was analysed by the same procedure as for colistin.

Analysis of polymyxin group antibiotics. One milligram of peptide antibiotics was dissolved in 1 ml of water and 25 μl of the solution were injected into a column. In micro-HPLC ,0.5 μl of a 10 mg/ml solution was injected. The flow-rates were 0.5 ml/min for the 4 mm I.D. column and 10 μl/min for micro-HPLC. Isocratic

elution was carried with methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1). The elution curves were monitored with a UV detector (210 nm) and a fluorescence detector by the post-label *o*-phthalaldehyde method (excitation, 360 nm; emission, 450-nm cut-off filter).

RESULTS AND DISCUSSION

In the course of our search for new polymyxin antibiotics, it was found that these peptides were adsorbed by Amberlite XAD-2 resin in water containing salts such as sodium chloride or sodium sulphate and eluted with a mixture of methanol and water containing a small amount of acid. This property of the resin was applied to the packing material of the chromatographic system and gave very good separations on the preparative scale, as shown in Fig. 1. Peaks 1, 2 and 3 in Fig. 1 were identified as colistin C, B and A, respectively, from results of the amino acids and fatty acids analyses and antibiotic potencies (Table I). Further, these components showed the same retention times as colistin C, B and A, which were prepared by the counter-current distribution method, but the antibiotic potencies were different from those in a previous report[1]. In contrast to the order of the antibiotic potencies of colistin A and B, the potency of colistin B obtained by this method was higher than that of colistin A. Also, the potency of colistin C was three times higher than that of colistin C. This indicates that the present method is superior to the counter-current distribution method because of the purity of the sample and the shorter time required.

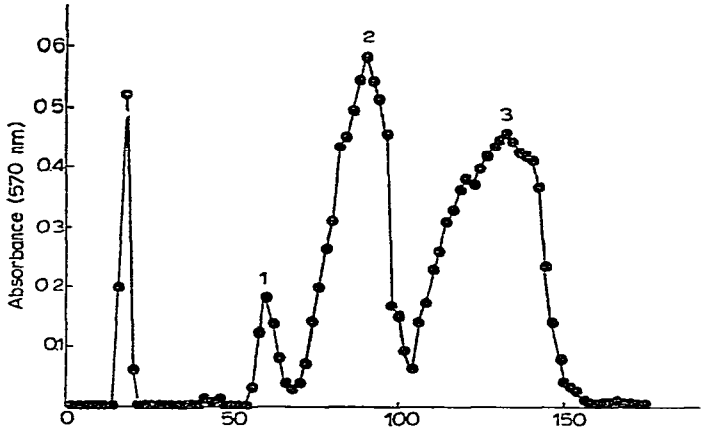

Fig. 1. Preparative separation of colistin on an Amberlite XAD-2 (100–200 mesh) column. Column size: 55 cm × 16 mm I.D. Mobile phase: linear gradient elution from methanol–0.2 *M* sodium chloride solution (1:1), with methanol–0.01 *N* hydrochloric acid (1:1) as the gradient former. Flow-rate: 0.5 ml/min. Temperature: ambient. Sample size: 500 mg of colistin hydrochloride. Peaks: 1 = colistin C; 2 = colistin B; 3 = colistin A.

Another type of styrene–divinylbenzene copolymer, Hitachi gel 3010, showed a higher affinity against polymyxins than Amberlite XAD-2. This resin gave good resolution of colistin A, B and C on the small preparative scale, even with isocratic

TABLE I

AMINO ACID AND FATTY ACID ANALYSES ON SEPARATED COMPONENTS OF COLISTIN AND POLYMYXIN B

Antibiotic	*Peak**	*Amino acid found (ratio)*					*Fatty acid***	*Potency (Unit)*	*Identification*
		A_2bu	*Thr*	*Leu*	*Val*	*Phe*			
Colistin	1	5.76	2.00	1.31	0.44	—	Unknown	15,000	Colistin C
	2	6.04	2.06	2.00	—	—	IOA	22,000	Colistin B
	3	6.02	1.98	2.00	—	—	6-MOA	19,000	Colistin A
Polymyxin B	1	5.84	2.05	0.81	0.26	1.00	Unknown	4800	Polymyxin B_0
	2	6.15	1.85	1.00	—	1.10	IOA	6400	Polymyxin B_2
	3	5.71	2.00	0.89	—	1.06	6-MOA	5600	Polymyxin B_1

* Numbered in order of elution.

** 6-MOA = 6-methyloctanoic acid; IOA = isooctanoic acid.

elution. The isocratic elution method is preferred to gradient elution because the refractive index detector can easily be used. Typical chromatograms of colistin and polymyxin B obtained under these conditions are shown in Fig. 2, where the peaks 1, 2 and 3 correspond to colistin C, B and A and polymyxin B_0, B_2 and B_1, respectively. A new component of polymyxin B, named polymyxin B_0, was determined from the results of the amino acid and fatty acid analyses and antibiotic potency. Initially,

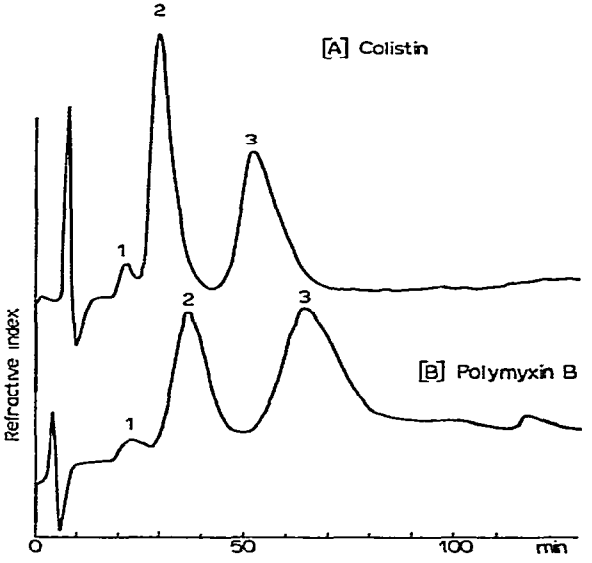

Fig. 2. Small-scale preparative separation of colistin and polymyxin B on Hitachi gel 3010 (25 μm) column. Column size: 50 cm × 8 mm I.D. Mobile phase: methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1). Flow-rate: 1.0 ml/min. Temperature: ambient. Detector: RI, ×128. Sample size: (A) 20 mg of colistin sulphate; (B) 20 mg of polymyxin B sulphate. Peaks: (A), 1 = colistin C; 2 = colistin B; 3 = colistin A; (B), 1 = polymyxin B_0; 2 = polymyxin B_2; 3 = polymyxin B_1.

peak 1 in Fig. 2(B) was identified as polymyxin B_3[14]; however, the name polymyxin B_3 was used later by Withander and Heding[15] for a component containing *n*-capric acid as a constituent fatty acid. To prevent misunderstanding, we shall change the name of peak 1 to polymyxin B_0 instead of polymyxin B_3. Analytical data and antibiotic potencies of each peak were summarized in Table I.

Several commercial micro-particulate packings made of styrene–divinylbenzene copolymer were compared with respect to their affinities for colistins, and the order was found to be Diaion CHP-3C < Amberlite XAD-2 < Hitachi gel 3011 < Jasco HP-01. An increase in the content of organic solvent and a decrease in the ionic strength and/or pH of the mobile phase also shortened the retention times of the polymyxins.

All of the attempts to use micro-HPLC method for the analysis of the polymyxins with these micro-particulate packings with different mobile phases containing methanol and aqueous buffers showed that the addition of more than 0.2 *M* of chloride or sulphate had a considerable effect on the separation of colistin A, B and C. The use of Hitachi gel 3011 column with a methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1) gave the best separation of colistin, as shown in Fig. 3(A). The Nucleosil C_{18} column also gave similar results, as shown in Fig. 3(B), but the durability of the alkyl-bonded silica with this mobile phase was poor. On the basis of these results, the analytical method adopted for polymyxin antibiotics was as described under *Procedures*. Fig. 4 shows typical chromatograms for polymyxin A, B, D, E, K, M and P, circulin and colistin obtained with isocratic elution. In this experiment, monitoring with a fluorescence detector combined with the *o*-phthalaldehyde

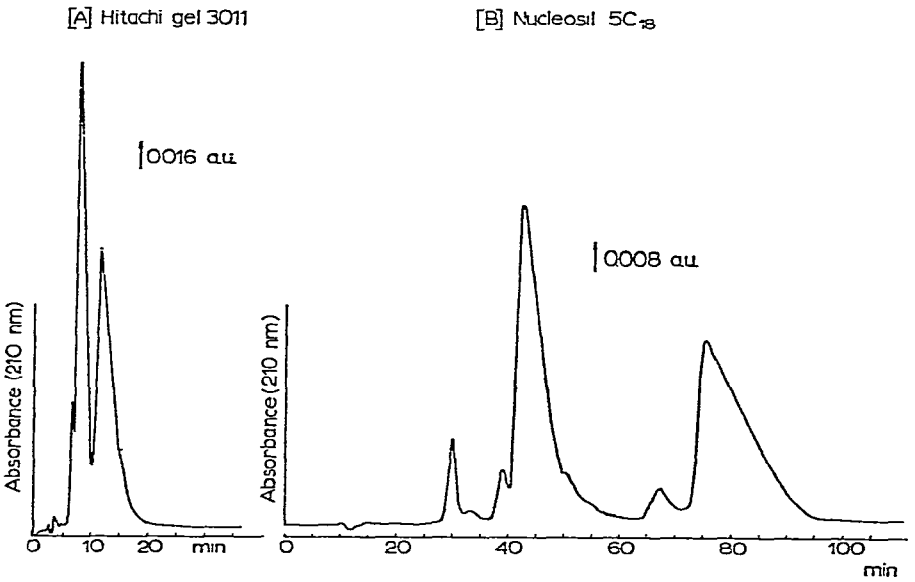

Fig. 3. Micro-column chromatogram of colistin on Hitachi gel 3011 (10 μm) and Nucleosil 5 C_{18} (5 μm). Column size: 20 cm × 0.5 mm I.D. (A) Hitachi gel 3011; (B) Nucleosil 5 C_{18}. Mobile phase: methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1). Flow-rate: 10 μl/min. Temperature: ambient. Detector: UV (210 nm). Sample size: 0.5 μl.

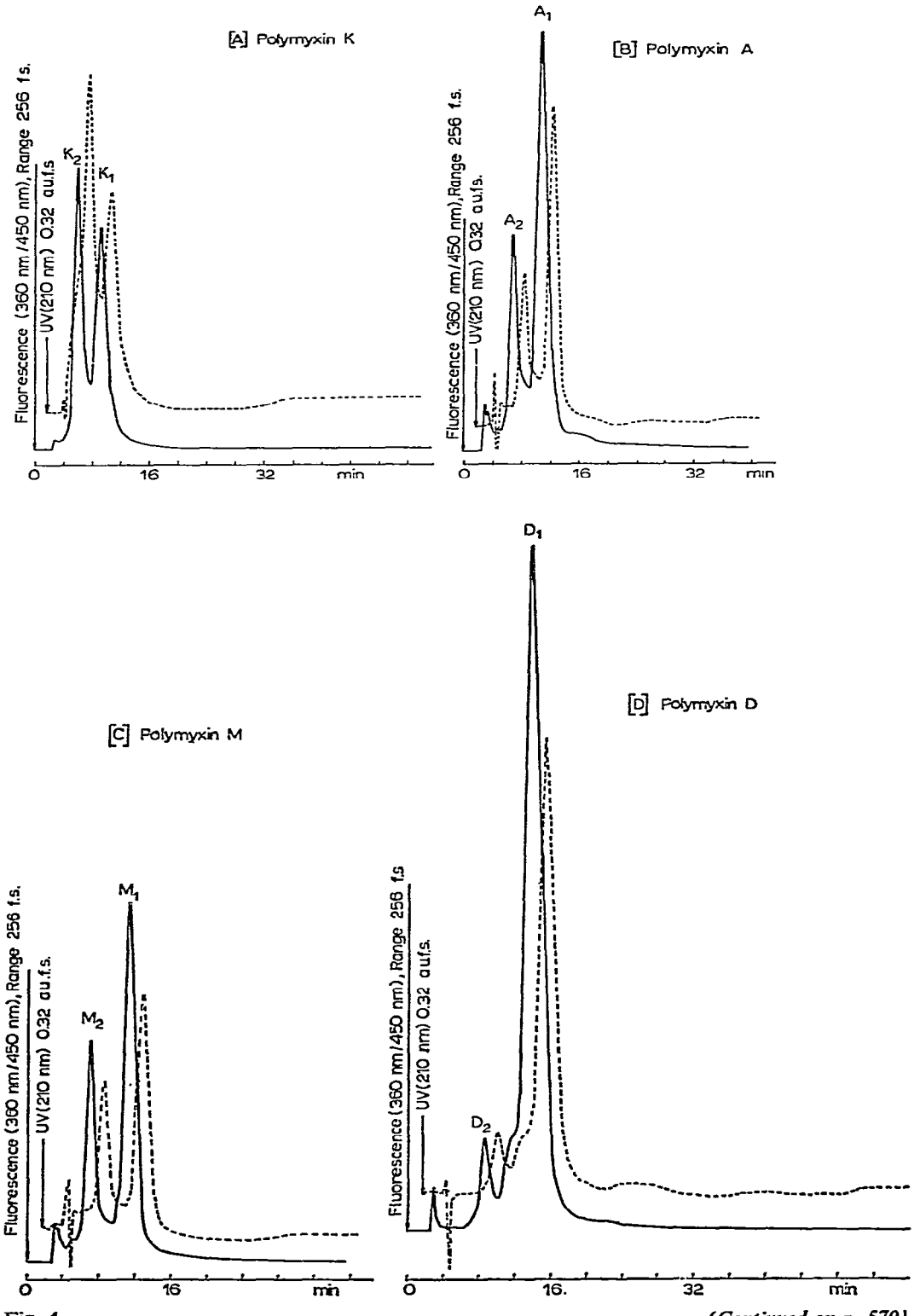

Fig. 4.

(Continued on p. 570)

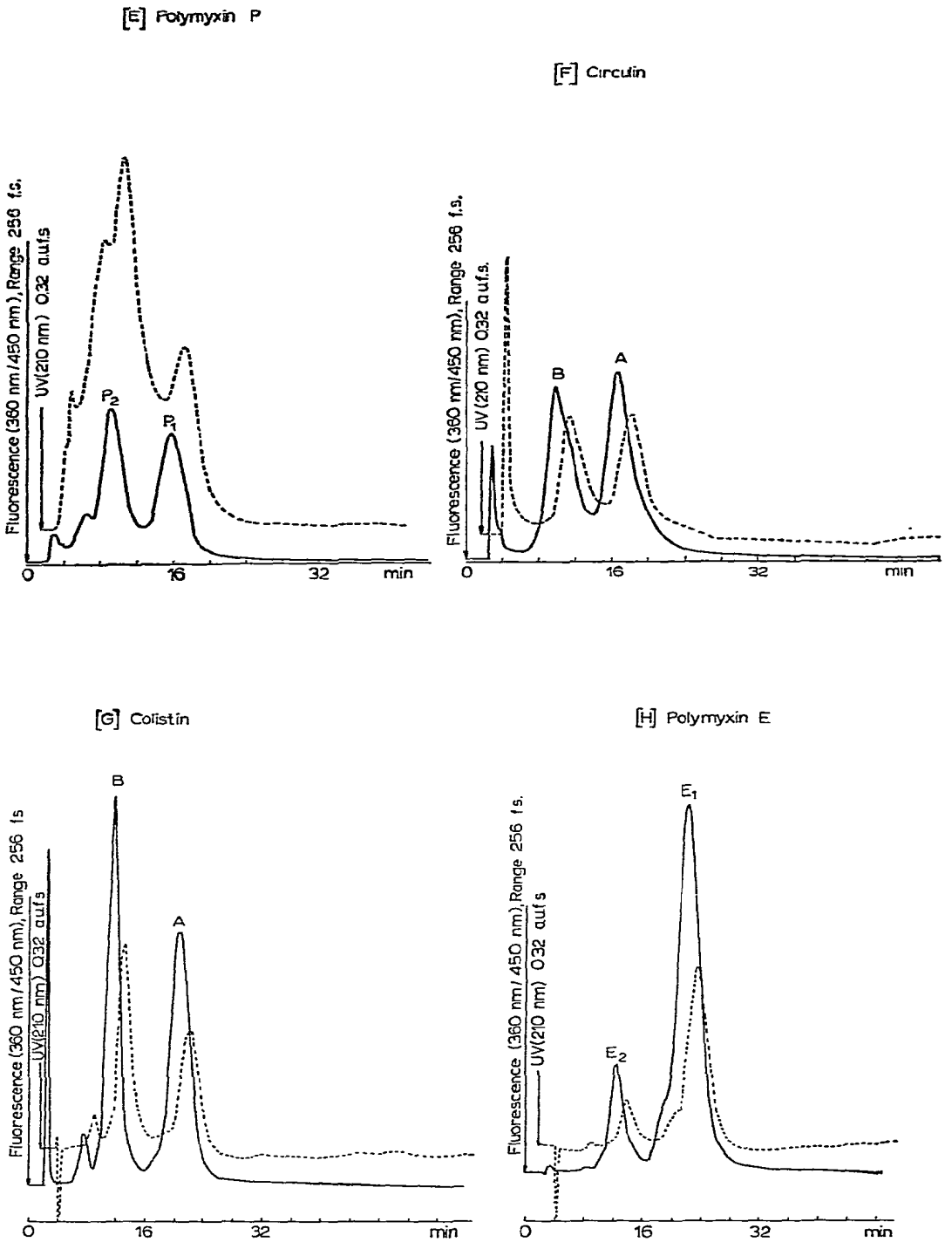
[E] Polymyxin P
[F] Circulin
[G] Colistin
[H] Polymyxin E
Fluorescence (360 nm/450 nm), Range 256 f.s.
UV(210 nm) 0.32 a.u.f.s
P2
P1
B
A
E1
E2
0
16
32
min

Fig. 4.

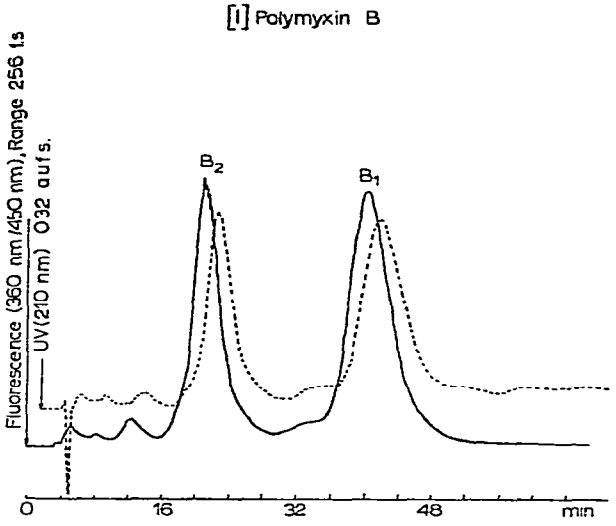

Fig. 4. Chromatogram of polymyxins on Hitachi gel 3011 (10 μm) column. Column size: 20 cm × 4 mm I.D. Mobile phase: methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1). Flow-rate: 0.5 ml/min. Temperature: ambient. Detector: fluorescence (360 nm excitation, 450 nm emission, cut-off filter) and UV (210 nm). Sample size: 25 μl. Sample: (A) polymyxin K; (B) polymyxin A; (C) polymyxin M; (D) polymyxin D; (E) polymyxin P; (F) circulin; (G) colistin; (H) polymyxin E; (I) polymyxin B.

TABLE II

AMINO ACID AND FATTY ACID COMPOSITION OF POLYMYXINS AND RETENTION TIMES ON A HITACHI GEL 3011 COLUMN

Antibiotic	*Amino acid*						*Fatty acid**		*Retention time (min)*	*Capacity factor*
	A_2bu	*Thr*	*Ser*	*Leu*	*Ile*	*Phe*	*6-MOA*	*IOA*		
Polymyxin K_1	6	3	—	1	—	—	Unknown		9.2	3.07
Polymyxin A_1	6	3	—	1	—	—	1	—	10.8	3.60
Polymyxin M_1	6	3	—	1	—	—	1	—	11.4	3.80
Polymyxin D_1	5	3	1	1	—	—	1	—	13.8	4.60
Polymyxin P_1	6	3	—	—	—	1	1	—	15.8	5.27
Circulin A	6	2	—	1	1	—	1	—	16.6	5.54
Colistin A	6	2	—	2	—	—	1	—	20.8	6.93
Polymyxin E_1	6	2	—	2	—	—	1	—	21.8	7.27
Polymyxin B_1	6	2	—	1	—	1	1	—	40.4	13.47
Polymyxin K_2	6	3	—	1	—	—	Unknown		6.0	2.00
Polymyxin A_2	6	3	—	1	—	—	—	1	6.8	2.27
Polymyxin M_2	6	3	—	1	—	—	—	1	7.0	2.34
Polymyxin D_2	5	3	1	1	—	—	—	1	8.5	2.82
Polymyxin P_2	6	3	—	—	—	1	—	1	9.2	3.07
Circulin B	6	2	—	1	1	—	—	1	9.8	3.27
Colistin B	6	2	—	2	—	—	—	1	11.8	3.94
Polymyxin E_2	6	2	—	2	—	—	—	1	12.2	4.07
Polymyxin B_2	6	2	—	1	—	1	—	1	21.0	7.00

* 6-MOA = 6-methyloctanoic acid; IOA = isooctanoic acid.

method gave better results than the UV detector at 210 nm with respect to baseline stability and sensitivity. According to the literature[16,17], colistin has been identified as polymyxin E and polymyxin A as polymyxin M; however, even though the retention times were approximately the same, some differences were noted in the amounts of the components present.

The retention times and the amino acid and fatty acid compositions of each polymyxin are given in Table II. There are two groups, polymyxin A_1, B_1, D_1, E_1, etc., containing 6-methyloctanoic acid, and polymyxin A_2, B_2, D_2, E_2, etc., containing isooctanoic acid. In each group, the retention times of the polymyxins increased in the order of integrated hydrophobicity of the amino acid residues, *e.g.*, polymyxin K_1 < polymyxin A_1 < polymyxin M_1 < polymyxin D_1 < polymyxin P_1 < circulin A < colistin A < polymyxin E_1 < polymyxin B_1.

CONCLUSION

It has been shown that all of the components of polymyxins (A, B, D, E, K, M and P), circulin and colistin can be separated successfully by the use of a styrene–divinylbenzene copolymer stationary phase and a mobile phase consisting of methanol–0.2 *M* potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1). It was found that the affinities of this resin for polymyxins increased in order Diaion CHP-3C < Amberlite XAD-2 < Hitachi gel 3011 < Jasco HP-01, and the Hitachi gel 3011 column gave the best separation. It should be emphasized that these packings have greater chemical and physical durability than alkyl-bonded silica and thus permit prolonged operation in separations with acidic and ionic mobile phases.

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