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ISOLATION AND CHARACTERIZATION OF THREE NEW POLYMYXINS IN POLYMYXINS B AND E BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two polymyxin antibiotics, polymyxins B and E (colistin), have been separated analytically into ten to thirteen components on a commercial reversed-phase material by isocratic elution with a mixture of acetonitrile, phosphate/formate and acetate buffer containing sodium sulphate and triethylamine. The analytical system was transferred to a preparative system, using a C_{18}-bonded stationary phase, without extensive impairing of the selectivity. The major components of each product were isolated and characterized by high-performance liquid chromatography, amino acid analysis and identification of the fatty acid. Three components were isolated and characterized for the first time. The fatty acid was also identified in some of the minor components.

INTRODUCTION

Polymyxin is the generic name of a group of chemically related fatty acyl decapeptide antibiotics, produced by *Bacillus polymyxa* and related species[1]. The general structure (Table I) comprises a cyclic heptapeptide moiety with a straight tripeptide side chain. The N-terminal amino-group in the side chain is acylated.

Polymyxin B was discovered in 1948 and colistin in 1950. Following the elucidation of the corresponding structures in the mid-sixties[1], and the establishment of the identity of polymyxin E and colistin in 1965[5], many attempts have been made to characterize further the commercial products polymyxin B sulphate and colistin sulphate. The complex composition of these products has been demonstrated by thin-layer chromatography (TLC), on silanized silica gel by Thomas and Holloway[6] and on cellulose by Junge[7]. Junge separated polymyxin B sulphate into ten fractions, five of which were microbiologically active. High-performance liquid chromatography (HPLC) has also been used for this purpose[8–11]. Thus, Tsuji and Robertson[8] separated polymyxins B_1 and B_2 and E_1 and E_2 by reversed-phase chromatography with

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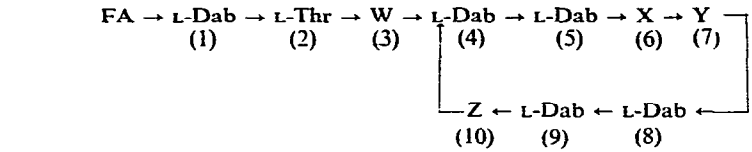
TABLE I

STRUCTURES OF CYCLIC DECAPEPTIDES OF THE POLYMYXIN GROUP OF ANTIBIOTICS[2-4]



Numerical suffix division of the polymyxins by fatty acyl radical (FA): 1, (+)-6-methyloctanoyl (MOA); 2, 6-methylheptanoyl (isooctanoyl, IOA); 3, octanoyl (OA); 4, heptanoyl (HA). Dab = α,γ-Diaminobutyric acid.

Compound	*W*	*X*	*Y*	*Z*
Circulin	L-Dab	D-Leu	L-Ile	L-Thr
Polymyxin A (M)	L-Dab	D-Leu	L-Thr	L-Thr
Polymyxin B	L-Dab	D-Phe	L-Leu	L-Thr
Polymyxin C (or P)	Dab	Phe	Thr	Thr
Polymyxin D	D-Ser	D-Leu	L-Thr	L-Thr
Polymyxin E (colistin)	L-Dab	D-Leu	L-Leu	L-Thr
Polymyxin S	D-Ser	D-Phe	L-Thr	L-Thr
Polymyxin T	L-Dab	D-Phe	L-Leu	L-Leu
Polymyxin F: Dab (5), Thr (1), Ser (1), Ile (1), Leu(2)				

a linear gradient elution. Terabe *et al.*[9] developed an isocratic ion-pair reversed-phase chromatography method which was applied by Thomas *et al.*[4] to separate polymyxin B sulphate into eleven components and colistin into thirteen components. Recently, Kimura *et al.*[11] separated the polymyxins into two or three components, both analytically and preparatively, using a porous styrene–divinylbenzene copolymer packing.

This paper describes an analytical (A-HPLC) and preparative (P-HPLC) isocratic reversed-phase chromatography method, by which polymyxin E sulphate can be separated into ten or eleven components and polymyxin B sulphate into twelve or thirteen components. The capacity ratios of the major components are shown to be dependent on the concentration of acetonitrile in the mobile phase. The relationships between the order of elution and the amino acid and fatty acid compositions are discussed. Differences in the compositions of commercial products of polymyxin B sulphate and colistin sulphate are reported.

EXPERIMENTAL

Reagents

Acetonitrile (HPLC S grade) was obtained from Rathburn (Walkerburn, Great Britain) and triethylamine (zur Synthese) from Merck-Schuchardt (Darmstadt, G.F.R.). All other solvents, except water, and reagents were obtained in analytical grade from E. Merck (Darmstadt, G.F.R.). Water was purified by using an ion-exchange column and by redistillation. The polymyxins investigated were obtained as follows: A, colistin sulphate, Lot No. 162107 (Dumex, Copenhagen, Denmark); B, colistin sulphate, Japanese source; C, polymyxin B sulphate, Lot No. 136307 (Dumex); D, polymyxin B sulphate, American source.

Apparatus

For the analytical work a DuPont Model 830 liquid chromatograph equipped with a Model 837 spectrophotometer was used. The extinction was recorded by means of a Hewlett-Packard Model HP 3380 A integrator. The column (15 cm × 4.6 mm I.D.) was packed with 5-μm Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) by a slurry technique.

The preparative work was carried out on a Jobin-Yvon column (25 × 4 cm I.D.), with a modified column-head, fitted with a six-port injection valve, Whitey SS-43Y6FS2, and a PTFE loop of 25 ml. The solvent was delivered by a Lewa FD pump, connected to a pressure transducer for pressure recording. A polarimeter, Perkin-Elmer 141, fitted with a recorder outlet, was used as detector. Recordings were made on a WW-recorder Series 316 (W & W Electronics, Basle, Switzerland). The packing material consisted of 125 g LiChrosorb Si 100 (10 μm), substituted with octadecyldimethylchlorosilane, carbon content 16.62%, corresponding to a coverage of 3.16 μmol/m^2.

Procedure

The polymyxins were dissolved in distilled water at a concentration of 0.4–5 mg/ml for analytical work and 100 mg/ml for preparative work. The amounts of sample injected were 20 μl (8–100 μg) and 5–10 ml (500–1000 mg) respectively.

For the analytical column the flow-rate was 0.9 ml/min and the inlet pressure was about 83 bar. UV detection at 220 nm was used. For the preparative column the flow-rate was about 20 ml/min and the inlet pressure 4–5 bar. Optical rotation detection at 365 nm was employed.

The mobile phase was prepared by mixing acetonitrile with a buffer: 0.023 *M* in phosphoric acid, 0.01 *M* in acetic acid, 0.05 *M* in sodium sulphate and triethylamine, pH 2.5. In the preparative rechromatographing of the components the phosphoric acid was replaced by 0.07 *M* formic acid. Acetonitrile and buffer were filtered through a membrane filter (0.45 μm) before mixing. The acetonitrile content in the mobile phase is indicated in the legends to the figures.

After the first preparative elution, the acetonitrile in the collected fractions was removed by evaporation, and the purity was checked by A-HPLC. In order to concentrate the fractions, the aqueous buffer solutions of the components were pumped on to the preparative column and then rechromatographed. The resulting fractions were concentrated by evaporation, freeze-dried and desalted by methanol extraction and the components were analysed as follows.

The samples were hydrolyzed in 6 *N* hydrochloric acid in evacuated, sealed vials for 24 h at 110°C. The acid was then removed under reduced pressure. The hydrolysates were redissolved in 0.1 *N* hydrochloric acid. Part of the hydrolysates was extracted with dichloromethane, and the fatty acid content in the organic phase was analyzed with a Pye 104 gas chromatograph equipped with a flame-ionization detector and a Hewlett-Packard Model 3380 A integrator. The column (1.8 m × 4 mm I.D., glass) was packed with OV-351 (10%) on Chromosorb W AW DMCS, 80–100 mesh (Ohio Valley Specialty Chemical Inc., OH, U.S.A.), and nitrogen was used as carrier gas. The remainder of the hydrolysates was used for amino acid analysis, carried out on a Kontron Liquimat III, using a standard procedure for protein hydrolysates. Finally, the chromatographic purity was checked by A-HPLC.

RESULTS AND DISCUSSION

Polymyxin B sulphate and polymyxin E sulphate are mixtures of several fatty acyl decapeptides, and the composition of the commercial products differs from one producer to another, as seen in Figs. 1–4. This may be the reason for the well known problems with the microbiological assay of potency. In order further to elucidate these problems, we isolated as many of the major components of each polymyxin as possible. For the subsequent tests of the biological characteristics of these components, it was necessary to use a mobile phase containing anions which do not interfere with these tests. Our method uses only hydrophilic anions, and provides a resolution at least as good as that Terabe *et al.*[9] obtained using tartrate buffer and sodium 1-butanesulphonate. The separation was strongly dependent on the concentration of acetonitrile in the mobile phase, as seen in Table II.

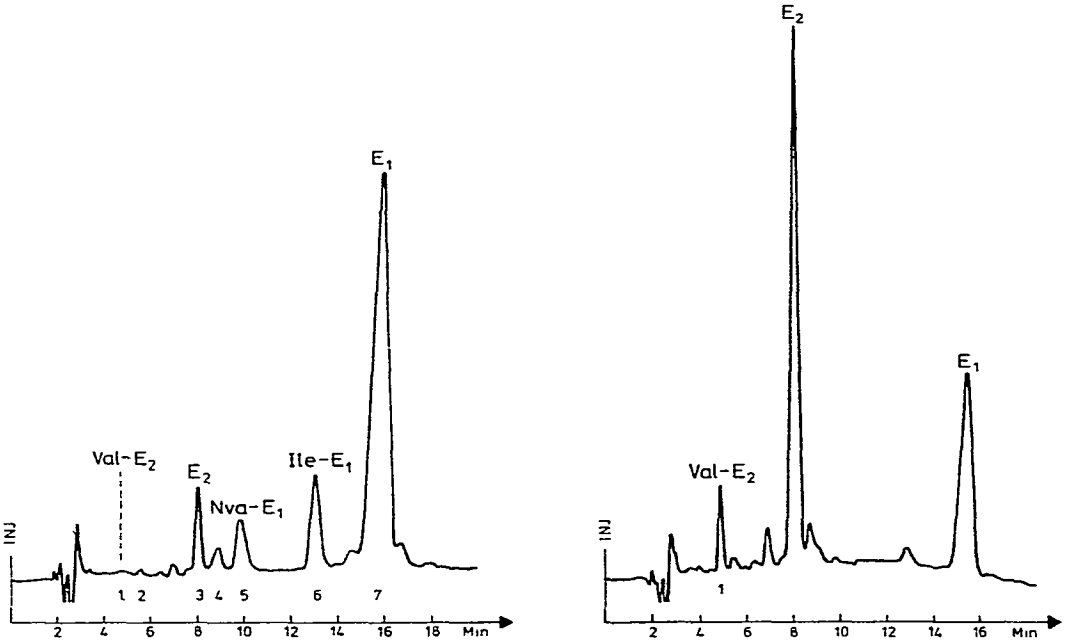


Fig. 1. Chromatogram from an analytical run of 100 μg colistin sulphate (product A) on a 150 × 4.6 mm column containing Nucleosil 5 C_{18}. Mobile phase: 22% acetonitrile in 0.023 *M* phosphoric acid, 0.01 *M* acetic acid, 0.05 *M* sodium sulphate buffer adjusted to pH 2.5 by means of triethylamine; flow-rate 0.9 ml/min. Detection: UV, 220 nm.

Fig. 2. Chromatogram from an analytical run of 100 μg colistin sulphate (product B). Conditions as in Fig. 1.

Transfer of the analytical system to a 25 × 4 cm I.D. column, chromatographing 1 g of the products, was accomplished without any problems, and no change of the mobile phase was necessary. Figs. 5 and 6 show chromatograms of 1.12 g colistin sulphate and 0.56 g of polymyxin B sulphate, respectively. The selectivity of the prepared C_{18} phase is illustrated in Fig. 7, which shows the first part of a preparative chromatogram of 1 g of polymyxin B sulphate.

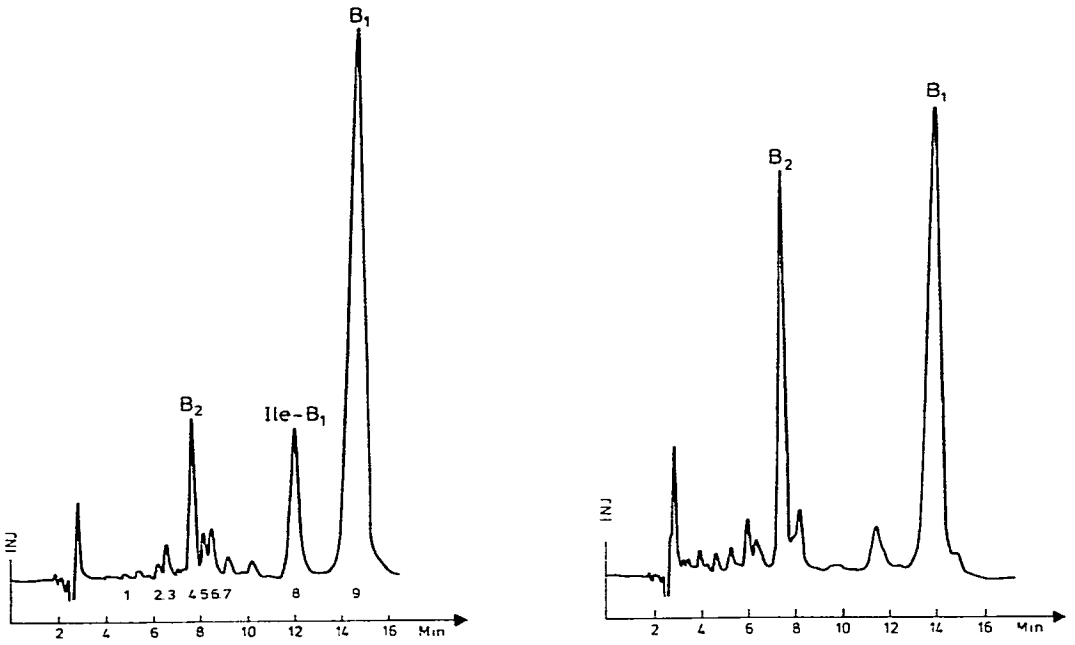


Fig. 3. Chromatogram of an analytical run of 100 μg polymyxin B sulphate (product C) on a 150 × 4.6 mm column containing Nucleosil 5 C_{18}. Mobile phase: 23% acetonitrile in 0.023 *M* phosphoric acid, 0.01 *M* acetic acid, 0.05 *M* sodium sulphate buffer adjusted to pH 2.5 by means of triethylamine; flow-rate 0.9 ml/min. Detection: UV, 220 nm.

Fig. 4. Chromatogram from an analytical run of 100 μg polymyxin B sulphate (product D). Conditions as in Fig. 3.

The effect of overloading on the capacity ratios is shown in Table II. This effect is relatively small at low capacity ratios, because the components have been separated in a non-overloaded state, whereas the effect on the major components is very large.

The amino acid and fatty acid compositions are listed in Tables III and IV, and it is seen that all the isolated components are fatty acyl decapeptides. The numbering corresponds to that in Figs. 1–3.

In the case of α,γ-diaminobutyric acid (Dab) the error in the estimation is relatively large since the peak of Dab coincides with a buffer change in the chromatogram.

TABLE II

CAPACITY RATIOS OF POLYMYXIN E COMPONENTS AS A FUNCTION OF THE ACETONITRILE CONTENT IN THE MOBILE PHASE

% Acetonitrile	*Pol. E_2*	*Ile-pol. E_1*	*Pol. E_1*
22 (A-HPLC)	1.92	3.84	4.94
23 (A-HPLC)	1.37	2.78	3.48
23 (P-HPLC, 0.56 g)	1.70	3.50	5.86
23 (P-HPLC, 1.12 g)	1.78	3.68	7.18

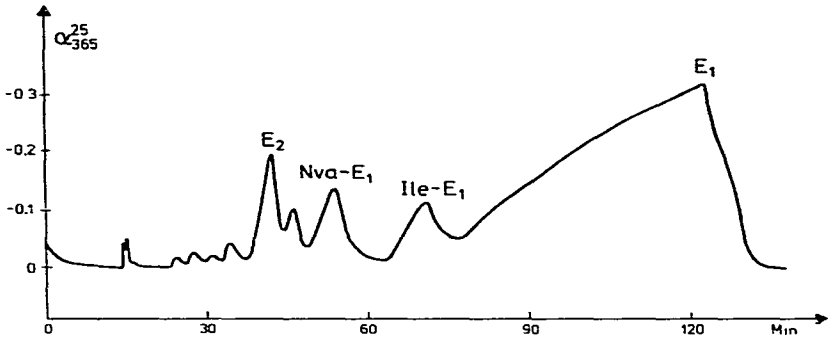


Fig. 5. Chromatogram from a preparative run of 1.12 g colistin sulphate (product A) on a 25 × 4 cm column containing octadecyldimethylsilyl substituted LiChrosorb Si 100, 10 μm. Mobile phase: 23% acetonitrile in 0.023 *M* phosphoric acid, 0.01 *M* acetic acid, 0.05 *M* sodium sulphate buffer adjusted to pH 2.5 by means of triethylamine; flow-rate 20 ml/min. Detection: optical rotation measured in a 10-cm cell at 365 nm.

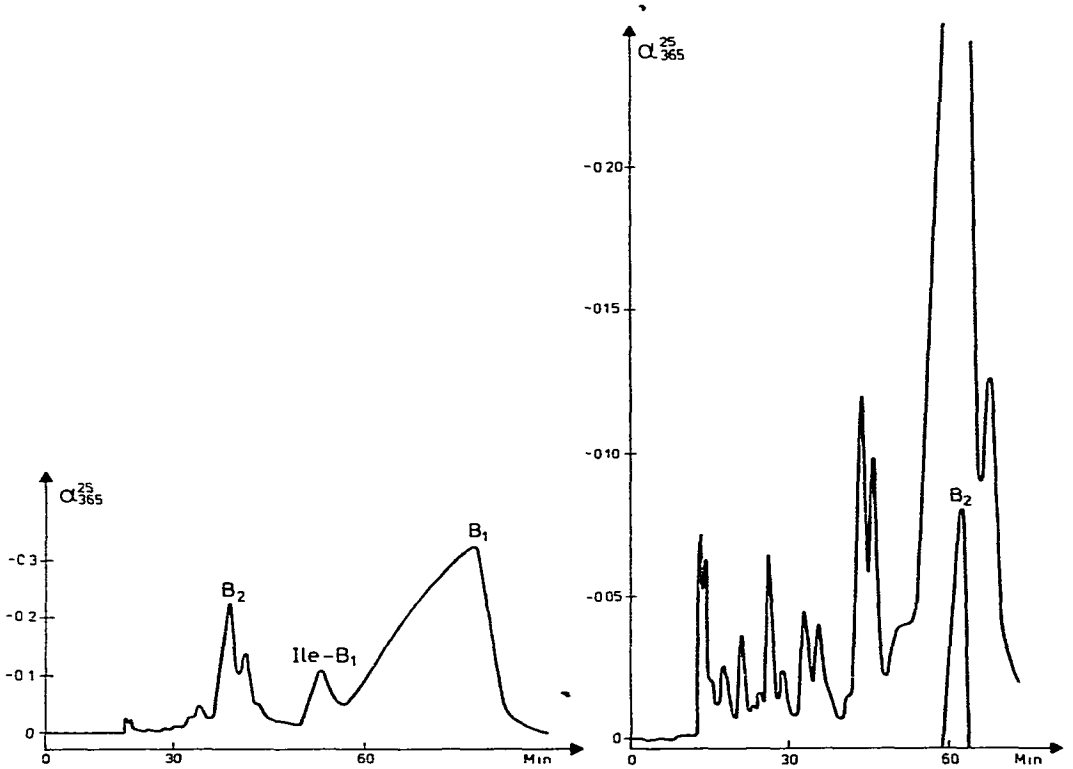


Fig. 6. Chromatogram from a preparative run of 0.56 g polymyxin B sulphate (product C) on a 25 × 4 cm column containing octadecyldimethylsilyl substituted LiChrosorb Si 100, 10 μm. Mobile phase: 25% acetonitrile in 0.023 *M* phosphoric acid, 0.01 *M* acetic acid, 0.05 *M* sodium sulphate buffer adjusted to pH 2.5 by means of triethylamine; flow-rate 20 ml/min. Detection: optical rotation measured in a 10-cm cell at 365 nm.

Fig 7. Chromatogram from a preparative run of 1.0 g polymyxin B sulphate (product D). Conditions as in Fig. 5. The chromatography was interrupted after the component B_2 was obtained.

TABLE III

AMINO ACID AND FATTY ACID ANALYSES ON SEPARATED POLYMYXIN B COMPONENTS

The peaks are numbered in elution order, compare Fig. 3. ID = Identified without quantitation.

Peak	*Amino acid found (ratio)*					*Fatty acid*	*Identification*
	Dab	*Thr*	*Phe*	*Leu*	*Ile*		
1.						IOA	
2.						IOA	
3.						IOA	
4.	5.92 (6)	1.89 (2)	1.01 (1)	1		IOA	Pol. B_2
5.	ID	ID	ID	ID		OA	Pol. B_3
6.						MOA	
7.						MOA	
8.	5.77 (6)	1.98 (2)	1		0.88 (1)	MOA	Ile-Pol. B_1*
9.	5.54 (6)	1.73 (2)	0.98 (1)	1		MOA	Pol. B_1

* New polymyxin.

Initially norvaline was identified as methionine due to the closeness of the peaks of these amino acids. However, a control analysis was carried out after performic acid oxidation prior to hydrolysis. This showed only trace amounts of methionine sulphone in the sample, and left the "methionine peak" unchanged in the chromatogram. The retention time of a norvaline standard was identical with that of the unknown amino acid appearing in the sample. We ascribe the "methionine peak" to norvaline, on account of these results and the fact that differences in amino acid composition are often found among the non-polar amino acids.

The composition of Ile-pol. E_1 is the same as for circulin A, but the identity of these two compounds has to be demonstrated by sequence analysis. Three of the components, Ile-pol. B_1, Nva-pol. E_1 and Val-pol. E_2, have not been isolated before. The chromatographic purity of the components is shown in Figs. 8 and 9.

TABLE IV

AMINO ACID AND FATTY ACID ANALYSES ON SEPARATED POLYMYXIN E COMPONENTS

Peaks are numbered in elution order, compare Figs. 1 and 2. Nva = Norvaline.

Peak	*Amino acid found (ratio)*						*Fatty acid*	*Identification*
	Dab	*Thr*	*Leu*	*Ile*	*Val*	*Nva*		
1.	5.78 (6)	1.81 (2)	1		0.84 (1)		IOA	Val-pol. E_2*
2.							IOA	
3.	5.96 (6)	2.00 (2)	2				IOA	Pol. E_2
4.	ID	ID	ID				OA	Pol. E_3
5.	6.01 (6)	1.83 (2)	1			0.86 (1)	MOA	Nva-pol. E_1*
6.	5.48 (6)	1.64 (2)	1	0.85 (1)			MOA	Ile-pol. E_1
7.	5.85 (6)	1.60 (2)	2				MOA	Pol. E_1^4

* New polymyxin.

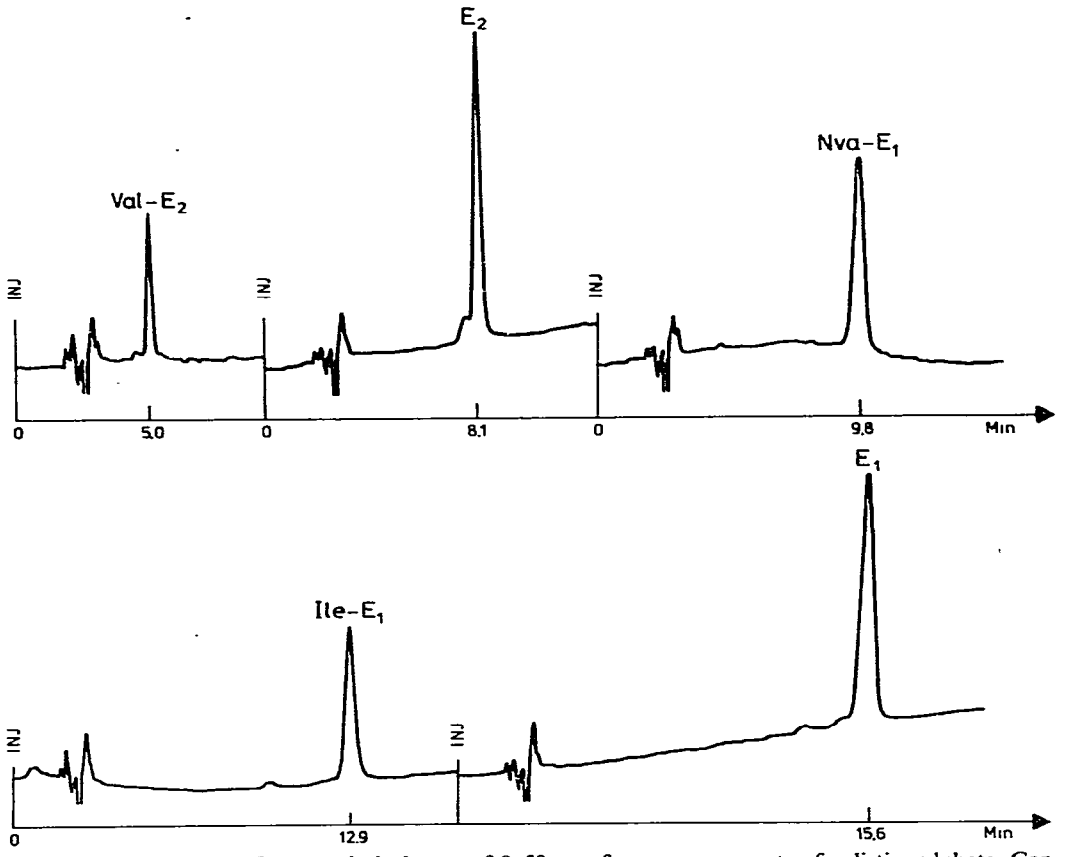


Fig. 8. Chromatograms from analytical runs of 8–50 μg of pure components of colistin sulphate. Conditions as in Figs. 1 and 2.

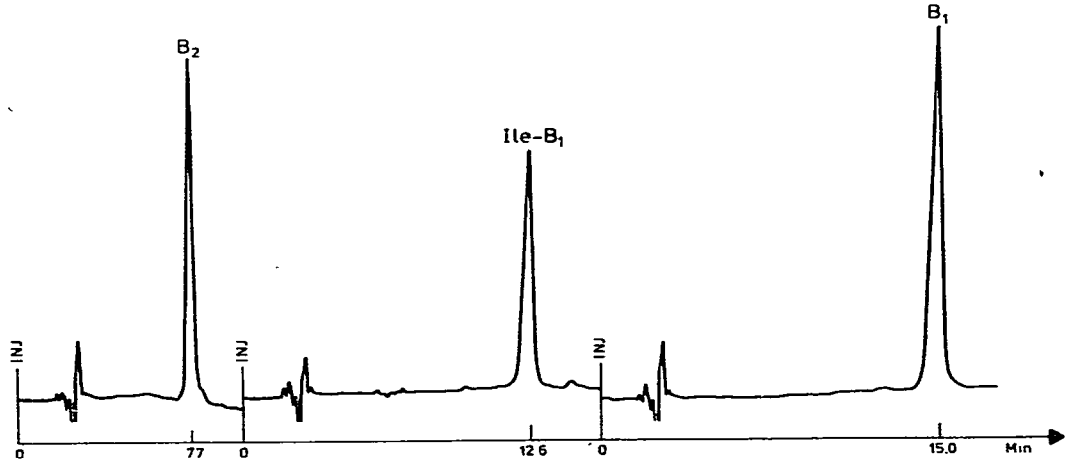


Fig. 9. Chromatograms from analytical runs of 8–50 μg of pure components of polymyxin B sulphate. Conditions as in Figs. 3 and 4.

The fatty acid analyses (Tables III and IV) revealed that several components have the same fatty acid residue. Consequently, the test for colistin sulphate composition given in ref. 12 is a test for the relative distribution of the fatty acid residues in the compound rather than for the fatty acyl decapeptide composition.

By comparing Figs. 1–3 with Tables III and IV, it is seen that the elution order of the components is primarily dependent on the fatty acid residue, less obviously so on the amino acid composition. The retention time increases in the order isooctanoyl (IOA) < octanoyl (OA) < (+)-6-methyloctanoyl (MOA). Regarding the amino acid composition, the retention time increases in the order Nva < Ile < Leu in fatty acyl decapeptides with the fatty acid residue MOA, and Val < Leu in those with the fatty acid residue IOA.

The structures of the polymyxins are shown in Table I. Four variable positions in the molecules have been found, but at the present time it is not known how the amino acid position affects the elution order.

CONCLUSIONS

It has been shown that polymyxins B and E can each be successfully separated, both analytically and preparatively, into ten to thirteen components using a chemically bonded C_{18} stationary phase and a mobile phase consisting of a mixture of acetonitrile and phosphate/formate and acetate buffer, containing sodium sulphate and triethylamine. The major components were isolated and identified by HPLC and analyses of the amino acids and fatty acids. None of them appeared to be degradation products. Three of the components represent new polymyxins, which have not been isolated before. We suggest the names polymyxin I_1 (= Ile-pol. B_1), O_1 (= Nva-pol. E_1) and L_2 (= Val-pol. E_2). The fatty acid analysis revealed that several components have the same fatty acid residue.

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