A SODIUM-CONTAINING POLYMYXIN DERIVED FROM POLYMYXIN-COMPLEX DURING CHROMATOGRAPHY

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(Received for publication July 11, 1984)

Commercial colistin (polymyxin E)-complex was separated into two major components (colistins A and B) on a preparative scale by HPLC (alkyl bonded silica and aqueous-organic mobile phase containing 0.2 M NaCl-HCl buffer (pH 2.0)). Desalting of the colistins A and B fractions was completed by reversed-phase adsorption and elution using methyl alcohol. In these experiments, it was inadvertently found that prolonged elution with water gave two hydrophilic peptides, which were tentatively named colistins A_H and B_H, respectively. Further elution with methyl alcohol produced two lipophilic peptides which were named colistins A_L and B_L. Colistin B_H showed higher potency than colistin A_H, A_L or B_L, and it was also effective *in vivo*. The fatty acid and amino acid composition of colistin B_H was identical with that of colistin B_L, but colistin B_H contained a relatively large amount of nonionic sodium which scarcely responded to the sodium ion-selective electrode of an ion meter; colistin B_H had a slightly lower molar extinction coefficient than the colistin B_L which contained negligible amount of nonionic sodium. Colistin A_H also contained nonionic sodium. Potassium containing colistins could also be derived from colistin-complex. These compounds could not be formed merely by adding sodium chloride or potassium chloride to colistin-complex solution. To obtain the sodium-containing compounds, contact with some hydrophobic stationary phase, such as alkyl-bonded silica or styrene-divinylbenzene copolymer, was necessary. It is postulated that one of the antibacterial mechanisms of polymyxin is associated with its complex-forming action on monovalent cations after contact with the lipid layer of the bacterial outer membrane.

Colistin, one of the polymyxin group antibiotics, consists of two major components, colistin pro-A and pro-B. Colistin pro-A is converted to colistin A and, similarly, colistin pro-B to colistin B during counter-current distribution[1]. Recent years have seen developments in the application of HPLC for analysis of polymyxin antibiotics using reversed-phase column and aqueous organic solvent with an inorganic salt[2,3,4]. We found that colistins pro-A and pro-B could also be converted to colistins A and B in this manner. During isolation of colistins A and B from colistin-complex by preparative scale HPLC, a hydrophilic peptide was found in the colistin A fraction, and a similar peptide in the colistin B fraction. Since their chromatographic behavior was different from that of colistins A and B, these peptides were suspected to be artifacts of colistins A and B. We report here the isolation, chemical analyses and antibacterial activities of these two peptides compared with those of colistins A and B.

Materials and Methods

Reagents

Colistin (19,500 U/mg) sulfate was kindly supplied by Banyu Pharmaceutical Co., Ltd. HPLC-grade methyl alcohol, acetonitrile and all other chemicals were purchased from Nakarai Chemicals, Japan. All aqueous solutions containing reagents were passed through a membrane filter (0.22 μm).

Apparatus

HPLC: a Jasco Model Twincle equipped with a Model UVIDEC 100-II variable-wavelength UV detector (Japan Spectroscopic Co., Ltd., Japan). Ion meter: Model N-8M (Horiba Ltd., Japan). Polarized zeeman atomic adsorption spectrophotometer: Model 180-60 (Hitachi Ltd., Japan). Mass spectrometer: Model JMS-DX 300 fitted with a fast atom bonbardment (FAB) gun (Jeol Ltd., Japan). Amino acid analyzer: Model KLA-3B (Hitachi Ltd., Japan). GC: Model GC-7A (Shimadzu Ltd., Japan) equipped with a FID.

Column Packing and Mobile phases

The following systems were applied: for micro analysis, Ultrasphere ODS (Altex, U.S.A.) (25 cm×4.6 mm I.D.) and MeCN - 0.05 M Na_2SO_4-H_2SO_4 buffer (pH 2.2) (1: 4) as mobile phase; for preparation, YMC-pack SM 343 (Yamamura Chemical Lab., Japan) (25 cm×2 cm I.D.) and MeOH - 0.2 M NaCl-HCl buffer (pH 2.0)(1: 1); for desalting, Amberlite XAD-2 (100~120 mesh) (Rohm and Haas, U.S.A.) (16 cm×8 mm I.D.) and methyl alcohol following distilled water as eluate; for gel permeation chromatography, TSK-gel G2000H (60 cm×7.5 mm I.D.) (Toyo Soda, Japan) and dimethylformamide; and for gas chromatography of fatty acids, capillary columns of Apiezon-L (50 m× 0.25 mm I.D.) and a carrier gas of nitrogen.

Preparation of Colistins A_H, A_L, B_H and B_L

One gram of colistin sulfate in 7.5 ml of water was added to the YMC-pack SM column (Fig. 2). The eluates of the two major peaks in the chromatogram were collected in separate tubes. Each fraction was desalted by adsorption on an Amberlite XAD-2 column, washing with water, and elution with methyl alcohol. In this procedure, prolongation of the water washing caused a new peptide (colistin A_H or B_H) to elute (Fig. 3b). Further elution with methyl alcohol produced two lipophilic peptides which were named colistins A_L and B_L. Colistins A_H, A_L, B_H and B_L were obtained as white amorphous powders by removal of the volatile eluent *in vacuo* and lyophilization. The yield was 130 mg of colistin A_H hydrochloride 90 mg of colistin A_L sulfate, 200 mg of colistin B_H hydrochloride and 100 mg of colistin B_L sulfate.

In Vitro Antibacterial Activity

The antibiotic potency determination was based on the agar-well method, modified from the official assay method for antibiotic preparation suggested by British Pharmacopoeia (1973). The minimum inhibitory concentrations of colistins A_H, A_L, B_H and B_L were determined by a two-fold serial agar dilution method recommended by the Japan Society of Chemotherapy. The medium was Müller-Hinton agar.

In Vivo Activity

The *in vivo* antibacterial activity of colistin B_H was evaluated in mice experimentally infected by *Klebsiella pneumoniae*. Mice were challenged intraperitoneally with a 316 LD_{50} dose of the pathogen in a 5% suspension of hog gastric mucin, and the treatment consisted of a single dose administered subcutaneously immediately after inoculation.

Results and Discussion

Previously, methods were developed in our laboratory for analyzing polymyxin antibiotics by HPLC. In our recent experiments, a combination of Ultrasphere ODS column and aqueous acetonitrile containing 0.05 M Na_2SO_4-H_2SO_4 buffer (pH 2.2) has given more favorable separation than a

method reported by TERABE *et al.*[2], which used Nucleosil C_{18} column and a mobile phase containing tartrate buffer (pH 3.0) with sodium 1-butanesulfonate as the paired-ion reagent for analysis of colistin-complex (Fig. 1). A large scale column (YMC-pack SM 343 (ODS)) with MeOH - 0.2 M NaCl-HCl buffer (pH 2.0) (1: 1) as the mobile phase was adopted for preparation of colistins A and B from commercial colistin, a crude polymyxin sulfate complex (Fig. 2). Desalting of the colistins A and B fractions was completed by the reversed-phase adsorption-elution method but not by gel filtration (Sephadex G-15). Fig. 3a shows the chromatogram of colistin A desalted by the usual technique. It was inadvertently found that prolonged elution with water gave a hydrophilic peptide as shown in Fig. 3b. The fatty acid and amino acid composition of this peptide was identical with that of colistin A, but this peptide contained an appreciable amount of sodium. Similarly, another hydrophilic peptide was isolated from the colistin B fraction. These hydrophilic peptides were tentatively named colistins A_H and B_H. In these experiments, the compounds obtained by elution with methyl alcohol were named colistins A_L and B_L to distinguish them from colistins A and B. It was found that colistin A which was prepared by HPLC using a mobile phase containing an inorganic salt is a mixture of colistins A_H and A_L, and also colistin B was a mixture of colistins B_H and B_L. The ratio of colistins A_H and A_L, or colistins B_H and B_L varied slightly in each experiment. These results may help to explain the lack of uniformity in the antibacterial potency of different samples of colistins A or B. During the chromatography containing chloride ion in mobile phase, both colistin A_H and B_H formed hydrochloride salt from colistin-complex sulfate, although colistins A_L and B_L remain unchanged sulfate. 2,4-Dinitrophenyl-(DNP)-derivatives of colistins A_H and A_L were

Fig. 1. Chromatogram of colistin on Ultrasphere ODS (5 μm) column (for micro analysis).
Column size: 25 cm×4.6 mm I.D. Mobile phase: MeCN - 0.05 M Na_2SO_4-H_2SO_4 buffer (pH 2.3) (1: 4). Flow-rate: 1.0 ml/minute. Temp: ambient. Detector: UV (210 nm). Sample size: 50 μg.

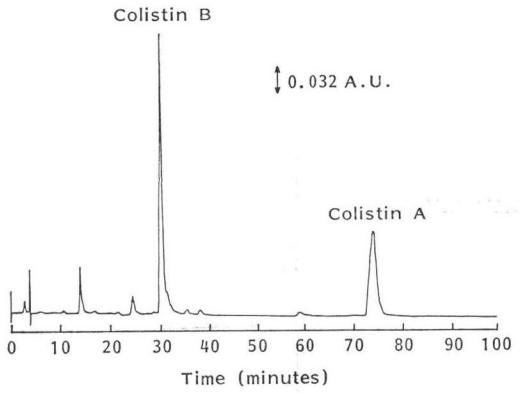


Fig. 2. Chromatogram of colistin on YMC-pack SM 343 (ODS) column (for preparation).
Column size: 25 cm×2 cm I.D. Mobile phase: MeOH - 0.2 M NaCl-HCl buffer (pH 2.0) (1: 1). Flow-rate: 8.0 ml/minute. Temp: ambient. Detector: UV (235 nm). Sample size: 1 g.

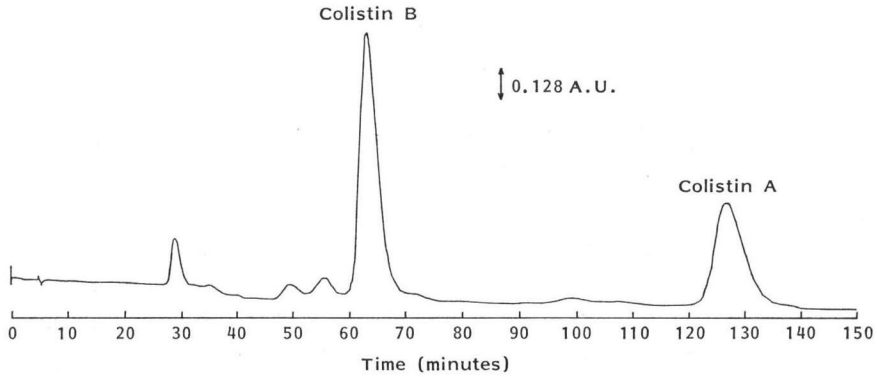
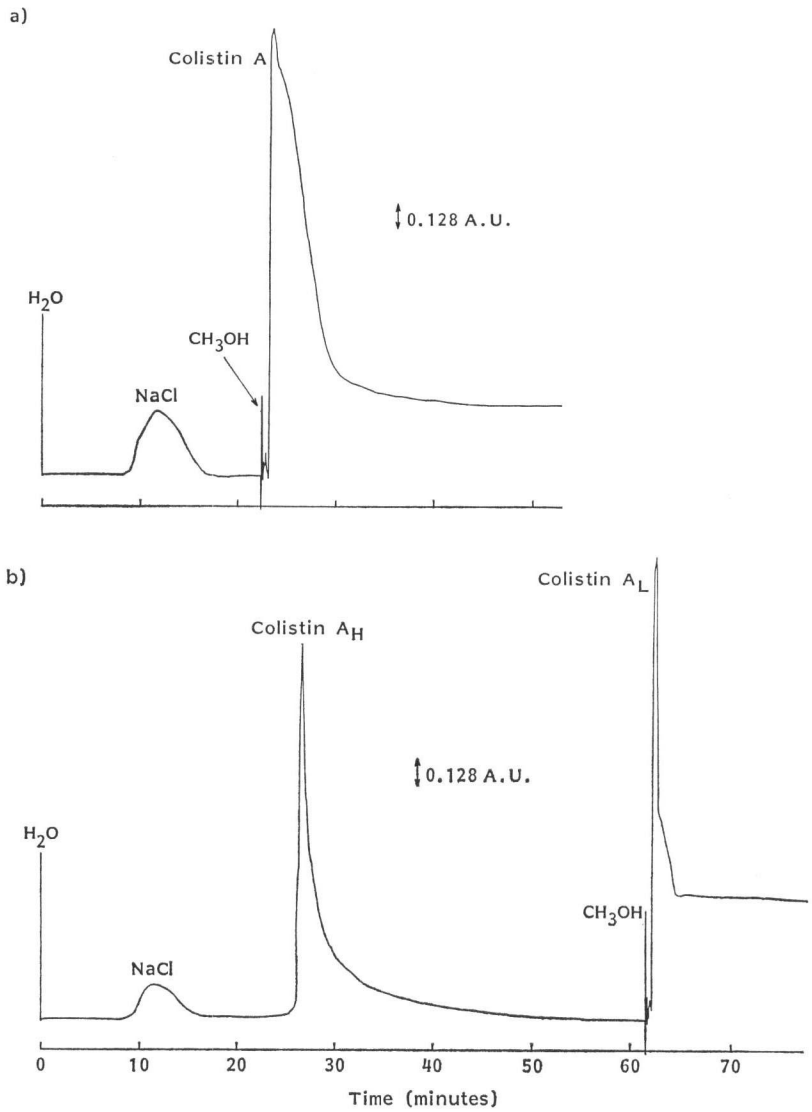


Fig. 3. Desalting of colistin A fraction by reversed-phase adsorption-elution method.
a) The usual technique, b) an improved technique.
Column: Amberlite XAD-2 16 cm×8 mm I.D. Detector: UV (210 nm).



prepared by a method previously described[4]. Their molecular weights by gel permeation chromatography using TSK-gel G2000H column were identical and smaller than that of colistin pro-A. Furthermore, colistins A_H and A_L gave the same protonated molecular ion $(M+H)^+$ at *m/z* 1,169 by mass spectrometry (FAB); similarly, colistins B_H and B_L had the same protonated molecular ion at *m/z* 1,155. However, the FAB mass spectra of colistins A_H and B_H showed a barely detectable cationated molecular ion $(M+Na)^+$. Their sodium content measured by atomic adsorption spectrophotometer was approximately 1.0 molar for colistin A_H and 0.5 molar for colistin B_H, although they both measured 0.065 molar using the sodium ion-selective electrode of an ion meter. On the other hand, the sodium content of colistin A_L was approximately 0.2 molar and that of colistin B_L was 0.1 molar,

Table 1. Sodium contents and amino acid composition of colistins A_H, A_L, B_H and B_L and their physicochemical properties.

Sample	Amino acids ratio			Fatty acid	Sodium contents (%)		$[\alpha]_D^{18}$ (c 0.4, H_2O)	UV $\lambda_{max}^{H_2O}$ nm (ε)	MS (FAB) m/z (M+H)
	A_2bu	Thr	Leu		Atomic adsorption spectrophotometer	Ion meter			
Colistin A_H	2.81	0.97	1.00	a-C_9	1.64	0.11	−59.6°	197.2 (22,800)	1,169
Colistin A_L	2.95	1.02	1.00	a-C_9	0.36	0	−52.5°	198.5 (24,500)	1,169
Colistin B_H	3.11	1.09	1.00	i-C_8	0.83	0.11	−63.3°	198.8 (23,200)	1,155
Colistin B_L	3.17	1.08	1.00	i-C_8	0.20	0	−50.4°	200.8 (26,300)	1,155

A_2bu: α,γ-Diaminobutyric acid, a-C_9: 6-methyloctanoic acid, i-C_8: 6-methylheptanoic acid.

Table 2. The antimicrobial spectrum and potencies of colistins A_H, A_L, B_H, B_L and colistin complex.

Test organisms	MIC (μg/ml)				
	Colistin A_H	Colistin A_L	Colistin B_H	Colistin B_L	Colistin complex
Staphylococcus aureus 209P	100	100	>100	>100	100
Shigella flexneri 2a	0.2	1.56	0.2	0.78	0.39
S. sonnei I	0.1	0.39	0.1	0.2	0.2
Escherichia coli NIHJ	0.78	0.78	0.2	0.39	0.39
E. coli O-111	0.78	0.78	0.2	0.78	0.39
Klebsiella pneumoniae NIHJ	0.78	0.78	0.39	0.78	0.39
Pseudomonas aeruginosa IFO 3456	0.78	0.78	0.2	0.39	0.39
P. aeruginosa P2	0.2	0.78	0.2	0.39	0.39
P. aeruginosa P3	0.2	0.78	0.1	0.39	0.39
Potency (u/mg)	23,000	14,500	24,400	16,200	19,500

Medium: Modified Müller-Hinton agar (Nissui).
Inoculum: One loopful of 10^8 cfu/ml suspension of each strain.

although neither sample responded at all to a sodium ion-selective electrode. The difference in chemical structure between colistins A and B is merely a $-CH_2-$ unit in the fatty acyl moiety. This difference could not account for the difference in sodium content between colistins A_H and B_H. Presumably, there is some other difference in chemical structure between colistins A and B other than covalent bond. These sodium-containing compounds could not be formed merely by adding sodium chloride to colistin-complex solution. To obtain the compounds, contact with some hydrophobic stationary phase, such as alkyl-bonded silica or styrene-divinylbenzene copolymer, was necessary. Probably, some lipophilic compound, such as colistin-lipid[4], is situated in a position of colistin complex is replaced by sodium ion after contact with them. Complexation of sodium ion with colistin yields a slight changes in UV spectra, decreasing of molar extinction coefficient and shift of maximum adsorption wavelength. A potassium-containing colistin could also be derived from colistin-complex. We observed that polymyxins B and M form a similar complex with sodium or potassium in the same manner. KESSLER *et al.*[5] have reported that potassium forms a weak complex with a cyclic decapeptide, cyclo (Pro-Phe-Gly-Phe-Gly)$_2$. It is postulated that one of the antibacterial mechanisms of poly-

Table 3. Protective effect of colistins B_H and B_L on *K. pneumoniae* infection in mice.

Sample	Survival rate					PD_{50} (mg/kg)
	Dose (mg/kg)					
	4	2	1	0.5	0.25	
Colistin B_H	6/6	4/6	3/6	2/6	0/6	1.0
Colistin B_L	6/6	4/6	2/6	0/6		1.4
Colistin complex	6/6	4/6	3/6	1/6	0/6	1.12

Infection: Intraperitoneally with 0.5 ml of bacterial suspension in 5% mucin (316 LD_{50}).
Treatment: Subcutaneously as a single dose immediately after inoculation.
Mice: Clea, ICR♂, 4 weeks, 23~25 g.

myxin is associated with its complex-forming action on monovalent cations after contact with the lipid layer of the bacterial outer membrane. From their studies of vertebrate peripheral nerve axons, KENDIG *et al.*[6] suggest that polymyxin forms or opens sodium and potassium-permeable channels in nerve membrane. Recently, KATSU *et al.*[7] demonstrated that the efflux of potassium ion from membrane vesicles of *Escherichia coli* was induced by polymyxin B and discussed his results in terms of deformation of the cytoplasmic membrane structure induced by polymyxin molecules.

The antibacterial spectra of colistins A_H, A_L, B_H and B_L, and colistin-complex are shown in Table 2. Generally, sodium-complex colistin, colistins A_H and B_H were more active than non-sodium colistin, colistin A_L and B_L against Gram-negative bacteria, in particular, *Pseudomonas aeruginosa* and colistin B_H showed a more prominent antibacterial spectrum and higher potency than colistin A_H. In addition, colistin B_H was effective *in vivo* as same as *in vitro* in experimental infections of mice caused by *K. pneumoniae*. A group of 6 mice was used for each dosage level administered subcutaneously with animals being observed for 4 days to determine the median protective dose (PD_{50}) and Table 3 shows the results. Thus, colistin B_H, is hydrophilic on comparing with colistin-complex and more biologically active than that. These results suggest that colistin B_H is to be expected for the more useful antibiotic against Gram-negative bacteria than colistin-complex and a complexation of cyclic peptide with metal is valuable for the improvement of biological activity.

Acknowledgments

The authors are grateful to Emeritus Professor T. SUZUKI (Kyoto University & Osaka University) for his encouragement and to Dr. T. AOYAMA (Jeol Ltd.) for the measurements of mass spectra.

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