A METHOD FOR SEPARATING COMMERCIAL COLISTIN COMPLEX INTO NEW COMPONENTS: COLISTINS PRO-A, PRO-B AND PRO-C

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Commercial colistin was separated into three major components as well as lipid components by reversed phase adsorption chromatography on Diaion HP-20 AG, a macroreticular styrene-divinylbenzene copolymer, without any inorganic salts or detergents, in aqueous-organic solvent as mobile phase. These expected components were colistins A, B and C; there were, however, appreciable differences between these components and colistins A, B and C, isolated by countercurrent distribution. The newly isolated components showed slightly higher potency than colistins A, B and C; their molecular weights, as determined by gel permeation chromatography of 2,4-dinitrophenyl derivatives on TSK-GEL G2000H (mobile phase: dimethylformamide), were also slightly higher. Accordingly, they were tentatively named colistins pro-A, pro-B and pro-C. During purification by countercurrent distribution (solvent system: *sec*-BuOH - *n*-BuOH - 0.1 N HCl, 30: 6: 40), colistin pro-A was converted to colistin A. Similarly, colistin pro-B was converted to colistin B, and colistin pro-C to colistin C. Therefore, we concluded that colistins A, B and C are artifacts.

Colistin-complex isolated from the culture broth of *Bacillus polymyxa* var. *colistinus*, has been thought to be a mixture of three major components, colistins A, B and C, which could be separated into single components by paper chromatography[1], countercurrent distribution (CCD)[2], thin-layer chromatography (TLC)[3] and recently by high-performance liquid chromatography (HPLC) using reversed-phase mode[4~10]. Chemically, colistins contain a cyclic heptapeptide moiety and a side chain consisting of a tripeptide with an acyl residue[11], and have been identified as variants of polymyxin E, one of the polymyxin antibiotics.

Various methods for preparing a single entity from the complex have been reported: The CCD method was used at first; the application of large-bore columns packed with a silica-bonded hydrocarbonaceous stationary phase[5,9] or with a macroreticular styrene-divinylbenzene copolymer[10] was also reported. These techniques used mobile phases which contained inorganic salts, detergents or strong mineral acids. It seemed to be possible that the CCD method might have caused some structural change in colistin during several days in a mixture of *sec*-BuOH - *n*-BuOH - 0.1 N HCl (30: 6: 40). Liquid chromatography with salts or detergents in the mobile phase, is complicated by the necessity for additional desalting. Accordingly, it was thought to be profitable to attempt improvements in the chromatographic condition by which colistin-complex is separated into its components by omitting salts and mineral acid from the mobile phase.

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Using only an aqueous-organic solution and reversed-phase adsorption packing, we have developed a new method which can separate colistin into three active components as well as lipid components. We expected that these components ought to be colistins A, B and C, respectively. Nevertheless, these newly isolated components showed slightly higher potency than colistins A, B and C, and their molecular weights, based on gel permeation chromatography of 2,4-dinitrophenyl derivatives on TSK-GEL G2000-H (mobile phase: dimethylformamide), were higher than those of colistins A, B and C. These three major components were named tentatively colistins pro-A, pro-B and pro-C to distinguish them from colistins A, B and C. The new method of isolation and some properties of colistins pro-A, pro-B and pro-C are reported in this paper.

Materials and Methods

Reagent

The samples of colistin sulfate were kindly supplied by Banyu Pharmaceutical, Kayaku Antibiotics Research and Meiji Seika Kaisha, Tokyo, Japan. Polymyxin E sulfate was the generous gift of Dr. S. WILKINSON (Wellcome Research Laboratories, Beckenham, Great Britain). HPLC-grade methanol, acetonitrile and dimethylformamide were obtained from Wako Chemicals, Osaka, Japan, and all other chemicals were reagent grade, purchased from Nakarai Chemicals, Kyoto, Japan. All aqueous solutions containing reagents were passed through a membrane filter (0.22 μm) and degassed prior to use.

Apparatus

The following types of liquid chromatograph were used: a Shimadzu Model LC-3A equipped with chromatopak C-R1A and a Model UVIDEC 100-II variable-wavelength UV detector (Japan Spectroscopic, Tokyo, Japan); a Jasco Model Familic 100-N for micro-HPLC (Japan Spectroscopic).

Column Packings and Mobile Phases

The following systems were applied: HPLC of reversed phase mode with alkyl-bonded silica used Unisil C_{18} (5 μm), 25 cm×4 mm I. D. for analysis or 30 cm×8 mm I.D. for preparation (Gaskuro Kogyo, Tokyo, Japan), and methanol - 0.2 M potassium chloride-hydrochloric acid buffer (pH 2.0) (1 : 1) as mobile phase. HPLC of reversed-phase adsorption mode used macroreticular styrene-divinylbenzene copolymer, Jasco HP-01 15 cm×0.5 mm I.D. for micro analysis (Japan Spectroscopic) or Diaion HP-20 AG for preparation (Mitsubishi kasei, Tokyo, Japan) 70 cm×8 mm I.D., and a mixture of methanol - acetonitrile - water (1 : 1 : 8).

The gel permeation chromatography (GPC) was performed with TSK-GEL G2000H, 60 cm×7.5 mm I. D. (Toyo Soda) and dimethylformamide as mobile phase.

Preparation of Colistins pro-A, pro-B and pro-C

One hundred mg of colistin sulfate in 1 ml of water was added to the Diaion HP-20 AG column equilibrated with the mobile phase described above, prior to use. The isocratic elution was performed with a flow rate of 2 ml/minute. The eluate was monitored with a UV detector (230 nm) and each peak was collected in tube. The contents of the three major peaks were named colistins pro-C, pro-B and pro-A according to the order of elution. They were obtained as white powders by removal of the volatile eluent *in vacuo* and lyophilization. 26.8 mg of colistin pro-A (mp 226°C (dec.), $[\alpha]_D^{20}$ −60° (*c* 1.0, H_2O)), 59.1 mg of colistin pro-B (mp 220°C (dec.), $[\alpha]_D^{20}$ −65° (*c* 1.0, H_2O)) and 7.2 mg of colistin pro-C (mp 229°C (dec.), $[\alpha]_D^{20}$ −35° (*c* 1.0, H_2O)) were obtained.

After replacing the mobile phase by methanol, monitoring by UV (210 nm) detected some eluates, which were colistin-lipids. They were obtained as light brown oils with a pungent smell after removal of the methanol. Yield; 2 mg, UV_{max}^{MeOH} 263 nm. IR($CHCl_3$) 2950, 1740, 1700, 1600 and 1460 cm^{-1}.

Preparation of Colistins A, B and C

Colistins A, B and C were obtained by CCD (solvent system: *sec*-BuOH - *n*-BuOH - 0.1 N HCl (30 : 6 : 40)) from a commercial sample of colistin complex as previously reported by SUZUKI *et al.*[2]

2,4-Dinitrophenylation of Colistins pro-A, pro-B, A and B

To a solution of the sample (2.2 mg) in a few drops of water was added 0.05 ml of 5% 1-fluoro-2,4-dinitrobenzene solution in ethanol. To this was added 0.5 ml of 0.1% aqueous triethylamine and the mixture was shaken for 20 minutes at pH 6.0 to 7.0 in a dark place. The reaction mixture was diluted with water and extracted three times with ether. The aqueous layer was then evaporated to dryness *in vacuo*. Dinitrophenol, a by-product, was removed by vaccum sublimation (10^{-1} mmHg) for 10 minutes at 60°C. The residue was homogeneous as judged by TLC (Silica-gel 60 F_{254}, solvent system, chloroform - methanol (6: 1)), and was used as the sample of gel permeation chromatographic analysis.

Antibacterial Activity

The antibiotic potency of the sample was based on the official assay method for antibiotic preparation issued by the Ministry of Health and Welfare, Japan. The minimum inhibitory concentration (MIC) was determined by the agar dilution method as recommended by the Japan Society of Chemotherapy. The media were Müeller-Hinton broth and Müeller-Hinton agar. Bacterial cultures containing approximately 10^8 viable cells/ml were prepared from overnight cultures. One loopful of the culture was inoculated on the agar plates containing antibiotics. The agar plates were incubated at 37°C for 18 to 24 hours and the MIC value was determined.

Amino Acid and Fatty Acid Analyses

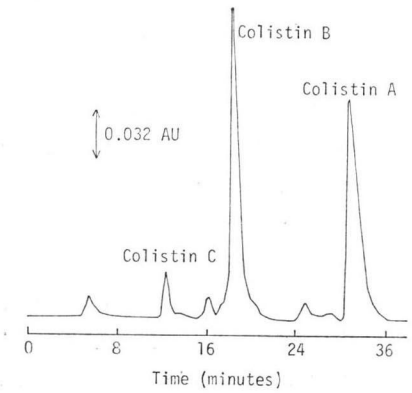
The sample was hydrolyzed with constant-boiling hydrochloric acid at 110°C for 1 hour and the hydrolysate was extracted with three 1-ml portions of diethyl ether. The ether extract was dried over anhydrous sodium sulfate and evaporated to about 0.2 ml under a slow stream of nitrogen at 25°C. The fatty acids in diethyl ether were analyzed 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/minute. The aqueous layer was evaporated to dryness and the residue hydrolyzed with constant-boiling hydrochloric acid; the hydrolysate was then analyzed with a Hitachi KLA-3B automatic amino acid analyzer.

Results and Discussion

Previously, methods were developed in our laboratory[10] for analyzing and preparing polymyxin antibiotics by reversed-phase adsorption chromatography using macroreticular styrene-divinylbenzene copolymer, Amberlite XAD-2 or Hitachi gel #3011, and an aqueous-organic mobile phase containing 0.2 M KCl-HCl buffer (pH 2.0). In contrast to this, various methods have been reported for analysis and preparation of colistin components using silica-bonded hydrocarbonaceous stationary phase and acidic aqueous-organic mobile phase containing a high concentration of salt: TSUJI *et al.*[4] used μBondapak C_{18} and linear gradient in phosphate buffer (pH 2.0); TERABE[5] *et al.*, Nucleosil C_{18} and tartrate buffer (pH 3.0) with sodium 1-butanesulfonate and sodium sulfate; WHALL[6], Ultrasphere I.P. and phosphate buffer (pH 3.0); FONG *et al.*[7], Hypersil-ODS and tetra-methylammonium chloride (pH 1.6);

Fig. 1. Chromatogram of colistin on Unisil C_{18} (5 μm) column.

Column size: 25 cm×4 mm I. D. Mobile phase: methanol - 0.2 M potassium chloride - hydrochloric acid buffer (pH 2.0) (6: 4). Flow-rate: 0.4 ml/minute. Temperature: ambient. Detector: UV (210 nm). Sample size: 20 μg.



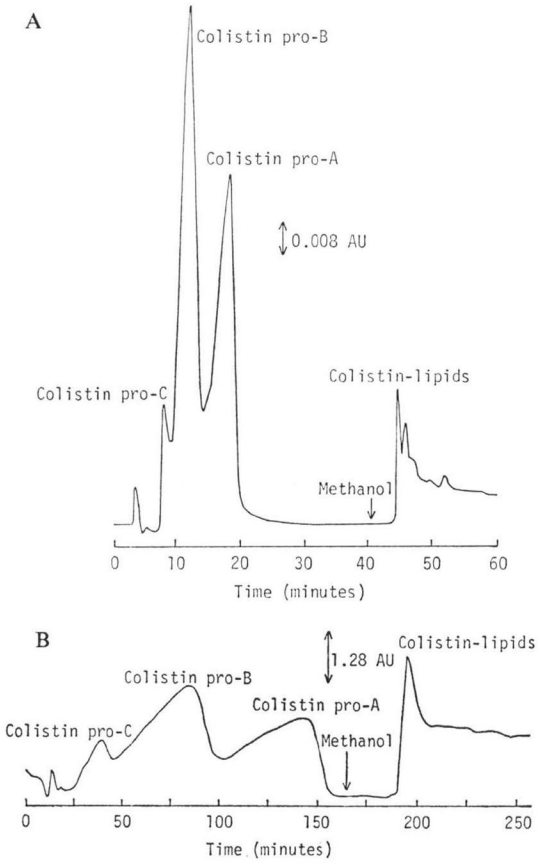
THOMAS *et al.*[8], Spherisorb ODS and tartrate buffer (pH 3.0) with detergent and salt; ELVERDAM *et al.*[9], Nucleosil C_{18} and phosphate-acetate buffer (pH 2.5) with sodium sulfate and triethylamine. Recently, KALASZ and HORVATH[12] were successful in preparative-scale separation of polymyxins using displacement chromatography; they used a LiChrosorb RP-8 column, a carrier of 10% acetonitrile in water, and a displacer of 0.05 M dodecyloctyldimethylammonium chloride in 10% acetonitrile in water.

Accordingly, we decided to test the usefulness of a mobile phase system of aqueous-organic solution containing 0.2 M KCl-HCl buffer (pH 2.0) on silica-bonded hydrocarbonaceous stationary phases. Among several silica-bonded packings tested, Unisil C_{18} gave a good resolution for colistin components, as shown in Fig. 1. The preparative-scale column of Unisil C_{18} was also useful for separating colistin into three components, without extensively impairing the selectivity. After neutralization with potassium hydroxide and removal of potassium chloride by gel filtration (Sephadex G-25), three biologically active components were obtained.

Fig. 2. Chromatogram of colistin; for analysis (A), for preparation (B).

(A) Column: Jasco HP-01, 15 cm×0.5 mm I.D. Mobile phase: acetonitrile - methanol - water (1: 1: 8). Flow-rate: 10 μl/minute. Temperature: ambient. Detector: UV (210 nm). Sample size: 20 μg.

(B) Column: Diaion HP-20 AG, 70 cm×8 mm I. D. Mobile phase: acetonitrile - methanol - water (1: 1: 8). Flow-rate: 2 ml/minute. Temperature: ambient. Detector: UV (230 nm). Sample size: 100 mg.



Since it is simpler to use an aqueous-organic mobile phase not containing KCl-HCl buffer for preparing these components, suitable packing was selected. As a result, we used a Jasco HP-01 column for analysis, a Diaion HP-20 AG column for preparation (which are both macroreticular styrene-divinylbenzene copolymer packings), and a mixture of methanol - acetonitrile - water (1: 1: 8) as the mobile phase. This gave a separation of colistin into three active components, although the resolution was poorer and the peaks were broader compared to the chromatogram of the salt-containing mobile phase, as shown in Figs. 2(A) and 2(B).

While studying polymyxin acylase, which had been thought to hydrolyze only the *N*-terminal fatty acyl bond of polymyxins, we found that no lipid could be detected in the ether extract from the colistin solution, yet an appreciable amount of lipid, other than fatty acids, was present in the ether extract from the enzyme hydrolysate. Our findings suggested the presence of lipids in the structure of polymyxins and/or lipids in noncovalent bond with polymyxins. Therefore, an attempt was made to elute the lipids which may have been adsorbed on the column by replacing the mobile phase with methanol. As depicted in Figs. 2(A) and 2(B), lipid components eluted

according to our expectation. These lipid components were contained not only in more than ten samples of colistin (including polymyxin E) supplied by four manufacturers, but also in the samples of polymyxin B and polymyxin M. To confirm that these lipids were not from the antifoaming agent, we carried out fermentation studies of colistin and related antibiotics without antifoaming agent. Analysis of the result indicates these antibiotics contained an appreciable amount of lipids. We assume that these lipids may affect the formation of the polymyxin complex. The lipids which we called colistin-lipids were unstable and the re-chromatography by reversed phase HPLC (Unisil C_{18}, and methanol - water (95: 5)) then gave several new peaks which may have been caused by polymerization. Colistin-lipids were soluble in methanol, ethanol, chloroform and acetone, but insoluble in water. The thin-layer chromatographic mobilities under a variety of solvent system were listed in Table 1. They did not have antibacterial activity in the range of 100 μg/ml on minimum inhibitory concentration, however, they showed a weak antifungal activity against *Trichophyton mentagrophytes*.

Table 1. Thin-layer chromatography of lipids.

Solvent system*	Rf
1. Hexane - ether - acetic acid (80: 30: 1)	0.41 (0.27, 0.19)
2. Chloroform - methanol - water (65: 20: 0.5)	0.93 (0.34)
3. Chloroform - methanol - aqueous ammonia (130: 70: 10)	0.96
4. Propanol - water (14: 6)	0.85

* Pre-coated silica gel F-254 plates (Merck) were used. Brackets show minor components.

The three major components were expected to be colistins A, B and C; however, there are appreciable differences between our newly isolated components and colistins A, B and C isolated by the CCD method. Table 2 compares the antimicrobial spectrum and the potencies of these newly isolated components, colistins pro-A, pro-B and pro-C and colistins A, B, C and the complex. Colistins pro-A, pro-B and pro-C showed slightly higher potencies than colistins A, B and C, respectively. We believe that colistin A which contains the longer 6-methyloctanoic acid is more active than colistin B which contains 6-methylheptanoic acid. This claim was confirmed by using formerly isolated colistin. Nevertheless, colistins pro-A and A were less active than colistin pro-B and B. In spite of the difference of potencies, the results of amino acid analysis, Rf values of TLC, Rm values of paper electrophoresis for all these samples were similar, as shown in Tables 3 and 4.

Table 2. The antimicrobial spectrum and potencies of colistins pro-A, pro-B, pro-C, A, B, C and colistin-complex.

Test organisms	MIC (μg/ml)						
	Colistin pro-A	Colistin A	Colistin pro-B	Colistin B	Colistin pro-C	Colistin C	Colistin complex
Staphylococcus aureus	25	25	12.5	25	100	100	25
E. coli NIHJ	0.39	0.78	0.39	0.78	0.39	1.56	0.78
Klebsiella pneumoniae	0.39	0.78	0.39	0.39	0.39	0.78	0.78
Pseudomonas aeruginosa (T)	1.56	1.56	0.78	1.56	0.78	3.12	1.56
Pseudomonas aeruginosa (P_2)	1.56	1.56	1.56	1.56	0.39	3.12	1.56
Pseudomonas aeruginosa (P_3)	1.56	1.56	1.56	0.78	1.56	3.12	1.56
Potency (U/mg)	22,000	19,100 (20,500)	26,400	22,400 (18,100)	27,500	16,800 (5,000)	19,500

Brackets show potencies of formerly isolated colistins A, B and C.

On the other hand, chromatograms of colistin on TSK-GEL G3000SW, which is a microparticulate silica gel chemically bonded with hydrophilic compounds, suggest that there are appreciable differences of molecular weights between colistin pro-A and colistin A, as well as between colistin pro-B and colistin B. Accordingly, many kinds of gel filtration techniques, using Sephadex G-25 and G-50, Toyopearl HW40, Bio-gel P2, P6 and P10, Shodex OH paks B-804 and Q-802, were tested to reveal this difference of molecular weight. These results were equivocal because polymyxins showed some affinity for these packings, but the use of

Table 3. Amino acid and fatty acid analyses on separated components of colistin.

	Amino acids ratio			Fatty acid
	A_2bu	Thr	Leu	
Colistin pro-A	3.01	0.99	1.00	C_9
Colistin pro-B	3.07	1.08	1.00	C_8
Colistin pro-C	2.92	1.04	1.00 (+Val)	C_8

A_2bu; α-γ-Diaminobutyric acid.
C_8; 6-Methylheptanoic acid.
C_9; 6-Methyloctanoic acid.

Table 4. Approximate Rf values and Rm values of colistins pro-A, pro-B, pro-C, A, B, C and colistin-complex in thin-layer chromatography and paper electrophoresis.

	Rf value[1)]	Rm value[2)]
Colistin pro-A	0.24	1.00
Colistin pro-B	0.23	1.02
Colistin pro-C	0.20	1.04
Colistin A	0.26	0.99
Colistin B	0.23	1.00
Colistin C	0.22	1.01
Colistin-complex	0.23	1.00

1) Silica-gel 60 F_{254}(Merck), solvent system; upper layer of *n*-BuOH - acetic acid - water (4: 1: 3), containing 1/20 volume-pyridine.
2) Paper strip of CAMAG electrophoresis (No. 2043A), 20 cm × 40 cm, buffer system; pyridine - acetic acid - acetone - water (2: 4: 15: 79), 3000 volt, 4 mA/cm, 20 minutes, colistin-complex as standard.

Fig. 3. Chromatogram of DNP-colistins pro-A and A on TSK-GEL G2000H column.
Column size: 60 cm × 7.5 mm I. D. Mobile phase: dimethylformamide. Flow-rate: 1.0 ml/minute. Temperature: ambient. Detector: UV (340 nm).

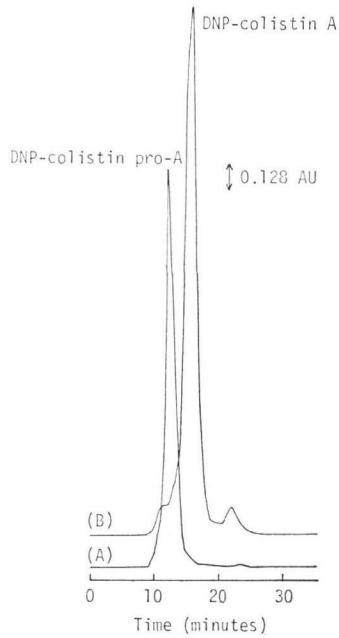
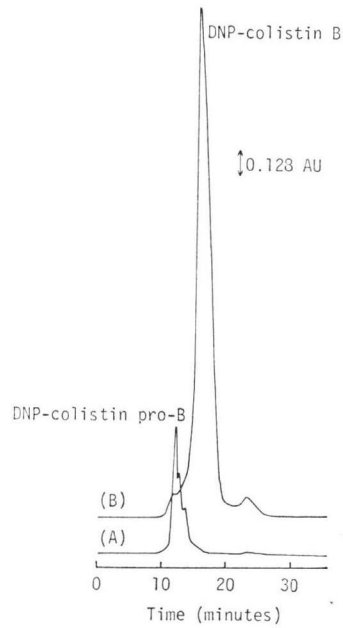


Fig. 4. Chromatogram of DNP-colistins pro-B and B on TSK-GEL G2000H column.
Column size: 60 cm × 7.5 mm I. D. Mobile phase: dimethylformamide. Flow-rate: 1.0 ml/minute. Temperature: ambient. Detector: UV (340 nm).



TSK-GEL G2000H for 2,4-dinitrophenyl derivatives of colistins gave good separation as shown in Figs. 3 and 4. Comparison of Fig. 3(A) and 3(B) shows that the molecular weight of colistin A is lower than of colistin pro-A, and/or that colistin A has more affinity for the packing than does colistin pro-A. Whichever is true, it is safe to say that colistin A differs markedly from colistin pro-A.

The normal retention time of DNP-colistin pro-A is 12 minutes. However, after several hours at pH 9~10, followed by neutralization, the solution showed a retention time of 15.5 minutes when treated with 1-fluoro-2,4-dinitrobenzene. This corresponds exactly with the retention time of DNP-colistin A.

Similarly, comparison of Fig. 4(A) and 4(B) shows the difference between colistins pro-B and B. After prolonged exposure to alkaline conditions, colistin pro-B showed a retention time identical with that of DNP-colistin B.

On the other hand, during purification by CCD (solvent system: *sec*-BuOH - *n*-BuOH - 0.1 N HCl, 30: 6: 40), colistin pro-A was converted to colistin A; in fact, the colistin pro-A prepared by our new method gave a single component in CCD. After isolation and treatment with 1-fluoro-2,4-dinitrobenzene, the resulting DNP-peptide showed a 3.5-minute longer retention time on TSK-GEL G2000H; this is identical to the retention time of colistin A. In addition, after similar treatment, colistin pro-B was converted to colistin B, and colistin pro-C, to colistin C. Thus, we have confirmed that a GPC column, made of cross-linked styrene-divinylbenzene polymer, with dimethyl formamide as the mobile phase was just as satisfactory as reversed phase chromatography for analysis and preparation of peptides.

The other polymyxin antibiotics, such as polymyxin B and polymyxin M, showed the same behavior as colistin, and their profiles on the GPC column were very similar. The details will be reported elsewhere.

Conclusion

Each polymyxin antibiotic reported to date, such as colistin or polymyxin B, has been thought to be a mixture of two or three major components which could be separated by the CCD method. However, there are appreciable difference between the colistin components separated by the CCD method and those separated by our newly-developed technique using reversed phase adsorption liquid chromatography. The newly isolated components, colistins pro-A, pro-B and pro-C, showed slightly higher potencies and higher molecular weights on the GPC column than did colistins A, B and C. They were converted to colistins A, B and C, respectively. As a result, we concluded that colistins A, B and C are artifacts from colistins pro-A, pro-B and pro-C.

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