VOL. 54 NO. 7, JULY 2001 THE JOURNAL OF ANTIBIOTICS pp. 595 — 599

Isolation and Structural Characterization of Colistin Components

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(Received for publication March 2, 2001)

Preparative-scale separation of colistin sulphate bulk sample was carried out on a preparative poly(styrene-divinylbenzene) stationary phase. Isocratic elution with acetonitrile - sodium sulphate solution (0.7% m/v; pH adjusted to 2.5 with TFA) - water (16 : 50 : 34, % v/v/v) was carried out at a flow rate of 4.0 ml min^{-1}. Six colistin components were isolated and characterized using ^{1}H and ^{13}C NMR. The molecular weights were confirmed by mass spectrometry. The structures of 2 components were determined for the first time. Polymyxin E_7 was identified as having the same composition as polymyxin E_1, except that the fatty acid moiety was 7-methyloctanoic acid. Isoleucine polymyxin E_8 was characterized as having the same composition as isoleucine polymyxin E_1 with 7-methylnonanoic acid as the fatty acid moiety.

Colistin, also known as polymyxin E, is a complex mixture of closely related polypeptides isolated from *Bacillus polymyxa* var. *colistinus*. Colistin A (polymyxin E_1) and colistin B (polymyxin E_2) are the major components and their structures were elucidated by SUZUKI *et al.* after enzymic and chemical hydrolysis[1,2]. These structures were recently confirmed using fast atom bombardment (FAB) mass spectrometry after isolation by high-speed countercurrent chromatography[3]. Colistins have a general structure (Fig. 1) composed of a cyclic hepta-peptide moiety attached to a tripeptide side chain with a fatty acyl residue on the *N*-terminus. Polymyxins of the E_1 type contain 6-methyloctanoic acid, E_2 6-methylheptanoic acid, E_3 octanoic acid and E_4 heptanoic acid. The relative composition of polymyxins E_1, E_2, E_3 and E_4, obtained from fatty acid analysis by GLC has been reported[4] and this test was included in the monograph for colistin of the British Pharmacopoeia 1980[5]. Isolation and structural characterization of E_3 and E_4 however has not been described in literature nor did any of the components we isolated correspond to E_3 or E_4.

Many attempts have been made to characterize further the commercial product, colistin sulphate. Preparative isolation on Amberlite XAD-2 with linear gradient elution resulted in the separation of colistins A, B and C. The structure of colistin C revealed the presence of one valine and one leucine instead of two leucine moieties in the ring peptide. The fatty acyl moiety of colistin C was however not elucidated[6]. Preparative isolation of colistin components was also achieved on LiChrosorb Si 100 ODS stationary phase. Norvaline polymyxin E_1 (Nva-E_1) and valine polymyxin E_2 (Val-E_2) were characterized by fatty acid and amino acid analysis, though the exact positions of the amino acid replacements were not elucidated[7].

Preparative high-speed countercurrent chromatography was used to isolate colistins A and B. Fast atom bombardment (FAB) mass spectrometry was used to confirm the nature of colistins A and B[3]. The structures, including the absolute configuration of the constituent amino acids of valine polymyxin E_1 (Val-E_1), isoleucine polymyxin E_1 (Ile-E_1), valine polymyxin E_2 (Val-E_2) and isoleucine polymyxin E_2 (Ile-E_2), were proposed based on the results of LC/MS analysis and MS/MS and amino acid analysis. The structures of these minor components were

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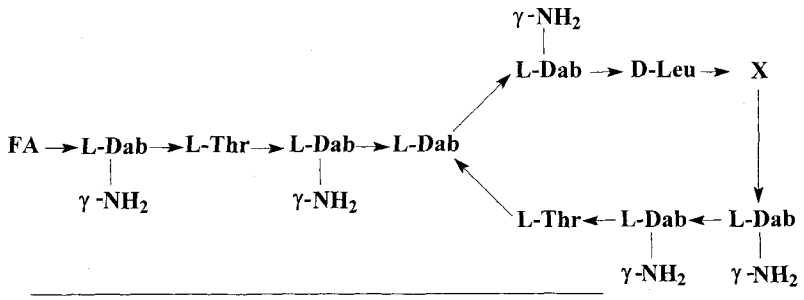
Fig. 1. Structures of already known and new colistins.



Polymyxin	X	FA
E_1*	L-leucine	6-MOA
Ile-E_1*	L-isoleucine	6-MOA
Nva-E_1	L-norvaline	6-MOA
Val-E_1	L-valine	6-MOA
E_2	L-leucine	6-MHA
Ile-E_2	L-isoleucine	6-MHA
Nva-E_2	L-norvaline	6-MHA
Val-E_2*	L-valine	6-MHA
E_3	L-leucine	OA
E_4	L-leucine	HA
E_7**	L-leucine	7-MOA
Ile-E_8**	L-isoleucine	7-MNA

* Isolated compounds
** New compounds

Dab, diaminobutyric acid; FA, fatty acid; 6-MOA, 6-methyloctanoic acid; 6-MHA, 6-methylheptanoic acid; OA, octanoic acid; HA, heptanoic acid; 7-MOA, 7-methyloctanoic acid; 7-MNA, 7-methylnonanoic acid.

the same as those of the main component colistin A or B except that one L-leucine was replaced by L-valine or L-isoleucine[8].

In this study, isolation and purification of single colistin components is described. ^{1}H and ^{13}C NMR was used to characterize isolated pure components of colistin. Molecular weights were confirmed by mass spectrometry.

Experimental

Sample, Solvents and Reagents

Acetonitrile HPLC Grade S was from Rathburn chemicals (Walkerburn, UK). Anhydrous sodium sulphate was from Merck (Darmstadt, Germany). TFA was from Acros Organics (Geel, Belgium). Phosphoric acid solution (6.8% v/v) was prepared from 85% m/m phosphoric acid (Acros Organics). Sulphuric acid (98% m/m) and ammonia solution (25% m/m) were from BDH Laboratory Supplies (Poole, England). *n*-Butanol was distilled before use. Dimethylpropane sulfonic acid, sodium salt (DSSA) was from Merck (Darmstadt, Germany). Colistin sulphate bulk powder was from Asahi Kasei Shiraoi, Hokkaido, Japan. Water was distilled twice.

Apparatus

The preparative work was carried out on a Varian model 5000 liquid chromatography system comprising a reciprocating pump, a UV detector set at 215 nm and a Varian 4270 integrator (Walnut Creek, CA, USA). A Valco injector was equipped with a 500 μl loop. The apparatus used for evaporating the fractions comprised a Rotavapor (Büchi Labortechnik, Postfach, Switzerland), a Laboport vacuum pump (KNF Neuberger, Germany) and a circulating Digital Temperature Controller (Poly Science, Illinois, USA) filled with ethylene glycol - water (1 : 1) as the cooling agent at −20°C. A preparative column 250×12.5 mm i.d., packed with PLRP-S, 8 μm, 1000 Å (Polymer Laboratories, Church Stretton, Shropshire, UK) was maintained at 60°C by means of a water bath heated by a Julabo EM thermostat (Julabo, Seelbach, Germany). A semi-preparative column, Supelcosil LC-ABZ C-18, 5 μm (Supelco, Bellefonte, PA,

USA), 250×10 mm i.d. was used for re-purification of minor components. YMC-Pack Pro C-18, 5 μm (YMC, Wilmington, NC, USA), 250×4.6 mm i.d. column was used for analytical work. For analytical work, the LC system consisted of a L-6200 intelligent pump (Merck-Hitachi, Damstadt, Germany), a Merck Hitachi model 655A-40 autosampler set to inject 100 μl, a Merck Hitachi model L-4000 variable UV detector set at 215 nm and an electronic integrator HP 3396 Series II (Hewlett-Packard, Avondale, PA). Mass spectrometry (MS) was performed with a LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). Nuclear magnetic resonance (NMR) spectra were determined using 200 MHz Varian Gemini apparatus (Palo Alto, USA).

Results and Discussion

Preparative Separation of Colistin

A preparative LC system using large bore PLRP-S stationary phase, developed for the separation of polymyxin B components was adapted for the isolation of colistin components with adjustment in the concentration of the organic modifier in the mobile phase. The mobile phase comprised acetonitrile - sodium sulphate solution (0.7% m/v; pH adjusted to 2.5 with TFA) - water (16 : 50 : 34%, v/v/v) at a flow rate of 4.0 ml min^{-1}. Detection was by UV at 215 nm. Colistin sulphate was dissolved in water to give a concentration of 60 mg ml^{-1}. This solution (500 μl) was injected repeatedly for separation on preparative PLRP-S 1000 Å, 8 μm, 250×12.5 mm i.d. stationary phase. The eluates from each peak were collected and combined. After evaporation to dryness, the isolated fractions were desalted by transformation of the sulphates in aqueous solutions into the poorly soluble bases using ammonium hydroxide. The salt-free base was transformed back to colistin sulphate in solution by neutralization using sulphuric acid and then evaporated to dryness at 40°C, under reduced pressure.

Purified components of colistins A and B were obtained by re-chromatography under the same conditions as the first preparative chromatography. The minor colistin components, which were still very impure after the first chromatography, were purified by re-chromatography on semi-preparative Supelcosil LC-ABZ C-18, 5 μm, 250×10 mm i.d. column maintained at 30°C. The mobile phase comprised acetonitrile - sodium sulphate solution (0.7% m/v; pH adjusted to 2.5 with TFA) - water (20 : 50 : 30, % v/v/v) at a flow rate of 4.0 ml min^{-1}. This base deactivated (BDS) stationary phase was found to be more selective than the polymer stationary phase. The amount of sample that could be loaded onto the BDS stationary phase was, however less (5~7 mg injected mass) than the amount loaded on the PLRP-S stationary phase. The eluent collected therefore contained less colistin, which could not precipitate by transformation of the sulphates in aqueous solutions into poorly soluble bases using ammonium hydroxide. The purified fractions were therefore desalted by *n*-butanol extraction at pH 10.0, adjusted with ammonium hydroxide. The eluents and the *n*-butanol were cooled during the extraction procedures, in order to limit degradation of colistin under the basic conditions. After evaporation of the *n*-butanol, the salt-free base was transformed back to colistin sulphate in solution by neutralization using sulphuric acid and then evaporated to dryness at 40°C, under reduced pressure.

Colistin was fractionated into 10 fractions, yielding only 6 compounds in sufficient amounts after re-purification. From a total of 10 g colistin sulphate injected mass, the yield for E_1 and E_2 was 3 g each, 0.2 g Ile-E_1, 50 mg Val-E_2, 20 mg of an unknown compound later identified as E_7 and 100 mg of an unknown compound later identified as Ile-E_8. A previously developed analytical LC method for separation of colistin components on YMC-Pack Pro C-18 stationary phase[9] was used to examine the purity of isolated colistin components. The mobile phase comprised acetonitrile - sodium sulphate (0.7% m/v) - phosphoric acid (6.8% v/v dilution of phosphoric acid 85% m/m) - water (21.5 : 50 : 5 : 23.5, % v/v/v/v) at a flow rate of 1.0 ml min^{-1}. Solutions (0.5 mg ml^{-1}) of the purified compounds were injected (100 μl) for chromatography. The purity calculated by normalization was 95% for E_1, 95% for E_2, 92% for Ile-E_1, 89% for Val-E_2, 87% for E_7 and 90% for Ile-E_8. Fig. 2 shows the analytical profile of a commercial colistin sample. The peaks with areas below 0.5% after normalization are not numbered on the chromatogram.

Structure Determination

Identification of the different colistin components was achieved by NMR spectroscopy. ^1H and ^{13}C NMR spectra were determined in D_2O with DSSA as external standard. Methyl, methylene and methine carbons were differentiated by ^{13}C NMR using the full DEPT (distortionless enhancement through polarization transfer) technique, as implemented in the Varian VNMR Software version 5.3b.

Data from the methyl region in the ^1H NMR spectra of polymyxin E components (0~2 ppm) revealed the presence of two leucine residues in the ring peptide instead of one leucine and one phenylalanine residue in polymyxin B. The structures of the isolated compounds were further derived

Table 1. Diagnostically important ^{13}C NMR chemical shifts assignments for colistin components.

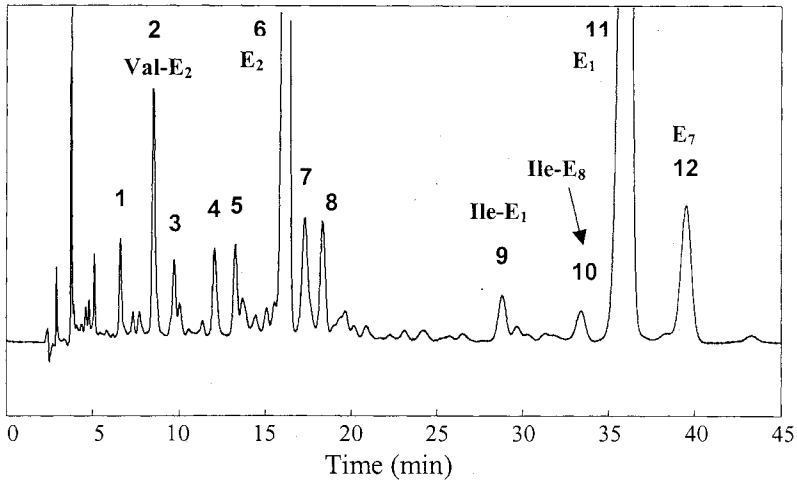
Carbon	E_1	Ile-E_1	E_2	Val-E_2	E_7	Ile-E_8
FA moiety						
CH	36.3 (6)	36.3 (6)	29.9 (6)	29.8 (6)	29.8 (7)	36.3 (7)
CH_2	38.1 (5)	38.0 (5)	40.6 (5)	40.5 (5)	40.8 (6)	38.2 (6)
	31.3 (2)	31.3 (2)	31.3 (2)	31.3 (2)	31.2 (2)	31.2 (2)
	31.6 (7)	31.6 (7)	28.8 (4)	28.7 (4)	28.5 (5)	31.2 (4)
	28.5 (4)	28.5(4)	28.3 (3)	28.2 (3)	28.9 (4)	31.7(8)
	28.3 (3)	28.3 (3)			28.0 (3)	28.6 (5)
						28.3 (3)
CH_3	21.3 (6')	21.2 (6')	24.7 (6')	24.6 (6')	24.6 (7')	21.4 (7')
	13.4 (8)	13.4 (8)	24.7 (7)	24.6 (7)	24.6 (8)	13.4 (9)
L-Leu moiety						
CH	56.1 (α)		56.2 (α)		56.0 (α)	
	27.1 (γ)		27.1 (γ)		27.0 (γ)	
CH_2	41.6		41.6		41.6	
CH_3	25.2		25.3		25.2	
	22.7		22.7		22.6	
L-Ile moiety						
CH		60.7 (α)				60.7 (α)
		38.2 (β)				38.1 (β)
CH_2		26.9				26.9
CH_3		18.1				18.1
		13.1				13.5
L-Val moiety						
CH				61.4 (α)		
				31.8 (β)		
CH_3				21.5		
				19.5		

The numbers in parentheses represent the position of the carbons on the fatty acid (FA) moiety, always counted from the carbonyl group attached to the amino acid.
6' and 7' denotes the carbon atoms of the methyl group at position 6 or 7 on the fatty acid moiety.
The α, β, γ denote the position on the amino acids, the α carbon is linked to the carbonyl group forming the chain.

from ^{13}C NMR spectroscopy. The assignments of the ^{13}C NMR resonances, which allowed structural identification of colistin components, are summarized in Table 1. The assignments for the different amino acid residues was greatly facilitated by use of the full DEPT technique, which divides the peaks into sub-spectra enabling clear interpretation. Only the signals of the amino acid and fatty acid carbons, which are different from those of the main colistin component, are shown in Table 1. The isoleucine moiety of the compound isolated from peak 9 (Fig. 2) was characterized from the downfield shifted α CH carbon at 60.7 ppm and the upfield shifted CH_2 carbon at 26.9 ppm. The other signals were similar to those of E_1 and therefore the structure of isoleucine polymyxin E_1 (Ile-E_1) was confirmed as was previously proposed[8]. For the compound isolated from peak 10 (Fig. 2), in addition to the presence of the isoleucine moiety, a 10-carbon fatty acid branched at position 7 is present. The name isoleucine polymyxin E_8 (Ile-E_8) was proposed. Another new polymyxin E component was characterized from the ^{13}C NMR data of the compound isolated from peak 12 (Fig. 2). This component has the same amino acid composition as E_1 except that its 9-carbon fatty acid is branched at position 7. The name polymyxin E_7 was proposed. Polymyxins E_1 and E_2 were also isolated and their structures confirmed by ^1H and ^{13}C NMR analysis.

A summary of the structures of the known (E_1, Ile-E_1, Nva-E_1, Val-E_1, E_2, Ile-E_2, Nva-E_2, Val-E_2, E_3 and E_4) and new (E_7 and Ile-E_8) colistin components is presented in Figure 1. The molecular masses of the isolated components were confirmed by mass spectrometric analysis (E_1, *m/z* 1169.8; E_2, *m/z* 1155.6; Ile-E_1, 1169.7; Val-E_2, 1141.7; E_7, 1169.7). The mass of Ile-E_8 could not be measured because Ile-E_8 was clogging the capillary of the LCQ.

Fig. 2. Chromatogram of 50 μg colistin sulphate commercial sample.



Conditions: YMC-Pack Pro C-18, 5 μm, 250×4.6 mm i.d. maintained at 30°C. Mobile phase of acetonitrile-sodium sulphate (0.7% m/v)-phosphoric acid (6.8% v/v dilution of 85% m/m phosphoric acid)-water (21.5 : 50 : 5 : 23.5, % v/v/v/v) at a flow rate of 1.0 ml min^{-1}. Detection was by UV at 215 nm. **2**, valine polymyxin E_2; **6**, polymyxin E_2 (colistin B); **7**, valine polymyxin E_1; **8**, norvaline polymyxin E_1; **9**, isoleucine polymyxin E_1; **10**, isoleucine polymyxin E_8; **11**, polymyxin E_1 (colistin A); **12**, polymyxin E_7; other peaks are of unknown identity.

Conclusion

Preparative-scale separation of colistin was successfully achieved on preparative PLRP-S and BDS reversed-phases with isocratic elution. The chromatographic purity was determined by analytical LC. Structural characterization of the purified single components was carried out using ^1H and ^{13}C NMR. The molecular weights were confirmed by mass spectrometry. Two major components, colistin A (polymyxin E_1) and colistin B (polymyxin E_2), and two minor components (Val-E_2 and Ile-E_1) were confirmed to have previously proposed structures. Two other minor components (E_7 and Ile-E_8) were isolated and characterized for the first time.

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