A New Technique to Analyze for Polymyxin B and its Nonapeptide Derivative

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Polymyxin B and polymyxin B nonapeptide were characterized by a new technique in which samples were acid-hydrolyzed and residues quantitated by an amino acid analyzer. Relative concentrations of the primary amino acids in polymyxin B (2,4-diaminobutyrate, threonine, phenylalanine, leucine) were; 5.94, 1.33, 0.63, and 0.58 respectively. Molar ratios among these amino acids were stoichiometric between 10 and 1200 μg of polymyxin B and between 30 to 600 μg of nonapeptide. Analyses by high-performance liquid chromatography gave similar results. A standard curve for 2,4-diaminobutyrate (amino acid analyzer data) was used to identify and quantitate polymyxin B complexed with rough and deep rough form lipopolysaccharides. The molar ratio of polymyxin B bound to lipopolysaccharides remained constant in reaction mixtures ranging from 2 to 200 ml. This procedure is appropriate for determining either free polymyxins or complexes of polymyxins/lipopolysaccharides aggregates formed with either rough or deep rough form lipopolysaccharides.

INTRODUCTION

The polymyxins are a closely related group of cyclopeptides which may be distinguished from each other by differences in amino acid and fatty acid composition (*1*). This heterogeneity has been clearly demonstrated in the two polymyxins used therapeutically, polymyxin B (PMB) and polymyxin E (colistin), which have been separated by high-performance liquid chromatography (HPLC) into ten and thirteen components respectively (*2*). Accordingly, antibacterial activity must be individually standardized for each lot of commercially prepared polymyxins.

Enzymatic removal of the PMB acyl side chain and adjacent 2,4-diaminobutyrate (DAB) residue by ficin results in a relatively nontoxic nonapeptide derivative (PMBN), which, unfortunately, has little or no bactericidal activity (*3*). PMBN is, however, a highly efficient permeabilizer which sensitizes normally resistant bacteria to hydrophobic antimicrobials (*4*) and has been used to study permeability fluctuations associated with asymmetric bilayers in the outer membrane (*5*). PMB, colistin, and their nonapeptide derivatives are capable of binding to lipopolysaccharides (LPS) and neutralizing the endotoxic shock associated with gram negative septicemia (*6*). This suggests that these versatile antimicrobials may have yet another significant clinical application.

In addition to these antimicrobial effects, PMB exerts profound effects on eukaryotic metabolism. For example PMB inhibits selected LPS-related immune functions to include: binding of monoclonal antibodies to LPS, mitogenicity and activation of B-cells, activation of serum complement, and the Shwartzman reaction (*7*). PMB influences other mammalian physiological processes such as bronchial provocation by mast cell degranulation (*8*) and enhancement of lipoprotein catabolism (*9*).

A number of analytical procedures have been used to characterize polymyxins. These methods include microbiological assays (*10*), dansyl-polymyxin conjugates (*11*), and high- performance liquid chromatography (HPLC) (*2*). The microbiological assays are influenced by multiple variables and results are difficult to reproduce. The dansyl-conjugate technique is a sensitive and accurate method but utilizes derivatives whose biological effects may differ from those exerted by the parent compounds. The HPLC procedure is accurate and has been successfully used to identify free polymyxins. However, the utility of this procedure to analyze polymyxins complexed with other substances is unknown.

This study's purpose was to develop an accurate and sensitive procedure to identify

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and quantitate PMB and PMBN with ultimate application as a research/clinical tool to determine either free or complexed polymyxins. The development of alternative analytical techniques for polymyxins is especially apropos since none of the currently available procedures are appropriate for all of these possible applications.

MATERIALS AND METHODS

Cultural conditions and LPS extraction. *Escherichia coli* EH100 (rough form of LPS) and *Salmonella minnesota* Re595 (deep rough form of LPS) are from the collection of the Max-Planck-Institut für Immunbiologie (Freiburg, Germany). LPS were extracted from aerobically grown cells by the phenol-chloroform-petroleum ether procedure described by Galanos et al. (*12*).

Analytical methods. PMB (sulfate form) and DAB were obtained from Sigma (St. Louis, MO). PMBN was purchased from Boehringer (Mannheim, Germany). All other chemicals were of the highest quality commercially available. Aqueous solutions of PMB (20 mg/ml), PMBN (15 mg/ml), and LPS (4 mg/ml) were stored at minus 20 °C until use. All samples were flushed with nitrogen and sealed in glass tubes before acid digestion (4 M HCl, 100 °C 18 h, 1 ml). Amino acids and amino sugars were determined with a Kontron Chromacon 500 amino acid analyzer (Eching, Germany) equipped with an Anacomp 220 computer. Free amino groups were treated with ninhydrin and the resulting products were quantitated by visible light absorbance at 590/440 nm. The stainless steel column (4 mm i.d.×200 mm) contained CK 10F (Mitsubishi) stationary-phase cation exchanger. The loading buffer contained (per liter H_2O) 58.82 g of sodium citrate and 0.50 g of phenol (pH 2.20). In the standard program, amino compounds were eluted by successive applications of buffers A, B, C, alkali solution, and buffer A. Buffer compositions (per liter H_2O) were as follows: Buffer A contained NaOH (5.50 g), citric acid (17.40 g), methanol (80 ml), and phenol (0.50 g, pH 3.20). Buffer B contained sodium citrate (12 g) and phenol (0.50 g, pH 4.75). Buffer C contained sodium citrate (15 g), sodium chloride (45 g), methanol (100 ml), and phenol (0.50 g, pH 5.00). The alkali solution contained 16 g of NaOH per liter. The regular elution program was as follows: buffer A (0-25 min), buffer B (25-50 min), buffer C (50-87 min), alkali solution (87-97 min), and buffer A (97-107 min). The initial column temperature of 49 °C was maintained for 31 min and then raised as rapidly as possible to 65 °C. After 55 min the temperature was raised further as rapidly as possible to 75 °C and then maintained at that temperature for the remainder of the program.

In the modified elution program, buffer C was adjusted to pH 4.70. The elution protocol was: buffer A (0-25 min), buffer B (25-40 min), buffer C (40-87 min), alkali solution (87-97 min), and buffer A (97-107 min). The column starting temperature of 47 °C was raised as rapidly as possible to 60 °C after 31 min. After 55 min the temperature was then raised as rapidly as possible to 65 °C and maintained at that temperature for the remainder of the run. Amino compounds were identified by comparison with authentic standards from commercial sources and by cochromatography of samples and standards. Statistical analyses were performed by standard procedures using Microsoft Excel version 4.0.

Analyses by reverse-phase HPLC used an 860 computer system in conjunction with two Waters 510 pumps, a Wisp 712 automatic sample processor, a Pico-Tag reverse-phase C18 column, a Column Heater/Temperature Control Module (38 °C and a Model 440 Absorbance Detector set at 254 nm (Waters Associates, Milford, MA). Hydrolyzed samples were taken to dryness in N_2 and reacted with a molar excess of phenyl isothiocyanate reagent to form the phenylthiocarbamyl derivatives. Sample diluent contained (per liter of water) 710 mg of Na_2HPO_4 titrated to pH 7.40 with 10% phosphoric acid. Fifty ml of acetonitrile were added to 950 ml of this solution for a final concentration of 5% by volume. Eluent A contained 19 g of sodium acetate trihydrate and 0.5 ml of triethylamine per liter of water. The pH was adjusted to 6.40 with glacial acetic acid. Sixty ml of acetonitrile were added to 940 ml of filtered solution and the mixture used without further treatment. Eluent B contained 600 ml of acetonitrile and 400 ml of water which were mixed and degassed under vacuum prior to use.

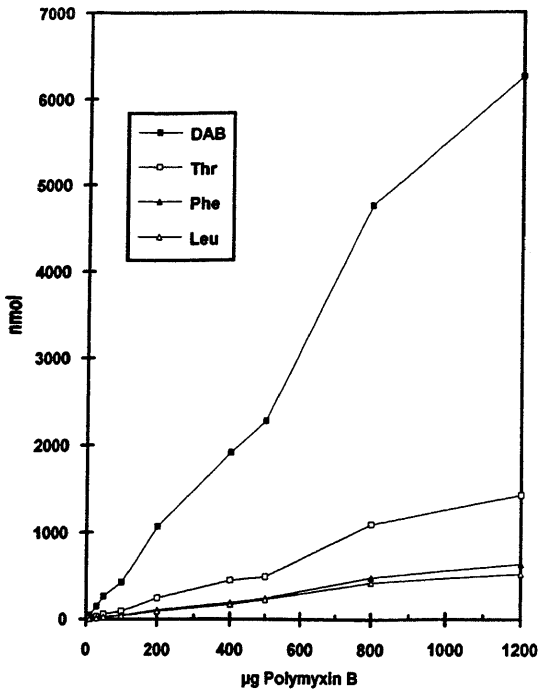

Figure 1. Standard curves of amino acids liberated from polymyxin B by acid hydrolysis. Digestion and analyses are described in Methods.

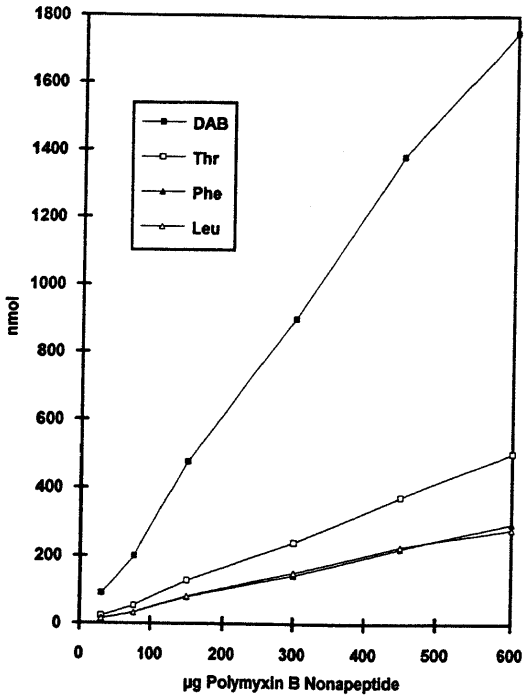

Figure 2. Standard curves of amino acids liberated from polymyxin B nonapeptide by acid hydrolysis. Digestion and analyses are described in Methods.

Analyses of PMB/LPS aggregates. Stock solutions of *S. minnesota* Re595 LPS (deep rough form) and *E. coli* EH100 LPS (rough form) were thawed, briefly sonicated, and diluted with the appropriate amount of water prior to the addition of PMB. Each reaction mixture contained 4 mg of LPS and 20 mg of PMB in final volumes of 2, 10, 25, 50, 100, and 200 ml. Controls were 4 mg of either LPS or PMB in 50 ml of water. Precipitation proceeded for two h at ambient temperature with occasional stirring. Precipitates were collected by preparative centrifugation (10^4×g, 10 min, 4 °C), washed three times with 10 ml of water, and left overnight in one ml of water at 4 °C. The sediments were centrifuged, resuspended in one ml of water, freeze-dried, and acid-digested as previously described.

RESULTS AND DISCUSSION

Analyses of PMB and PMBN with Amino Acid Analyzer. The molar ratio of amino acids in acid-digested PMB (Figure1) were: DAB (5.94), threonine (1.33), phenylalanine (0.63), and leucine (0.62). The expected ratios based on previous structural studies would be 6:2:1:1, respectively (*1*). The molar ratio (Figure 2) of these same amino acids in PMBN were; DAB (3.10), threonine (0.73), phenylalanine (0.45): and leucine (0.45) whereas the expected ratios would be 5:2:1:1, respectively. Minor amounts of isoleucine and serine were detected in PMB and PMBN (data not shown). Summations of the primary amino acids found accounted for 78.29±5.88% of PMB and 57.29±3.81% of PMBN. Molar ratios among the different amino acids showed similar stoichiometry between 10 to 1200 μg of PMB and between 30 to 600 μg of PMBN. Correlation coefficients were greater than 0.992 in all analyses.

Role of DAB in Polymyxin Analyses. Standard curves of DAB were used to identify and quantitate PMB and PMBN. The criteria for selecting DAB included acid stability and infrequent occurrence in nature (*13*). The acid stability of DAB was demonstrated by increasing the digestion time of PMB from 18 to 24 h. The molar ratio of DAB was only slightly reduced (5.94 to 5.69) whereas the ratios of the other amino acids decreased from 12 to 27% (data not shown). DAB liberated from PMB and PMBN by acid hydrolysis eluted coincidentally with the authentic

DAB standard in both the standard and modified elution programs. Comparative retention times (min) of representive basic amino acids as determined by the modified elution method were: lysine (43.78±0.37), DAB (45.13±0.90), ammonia (47.67±0.29), histidine (50.18±0.26) and arginine (61.34±0.34). The relative rareness of DAB in nature makes it an unlikely contaminant in most potential physiological/clinical samples. By contrast analyses based on either threonine, phenylalanine, or leucine could be compromised by even minor protein contamination since they are common components of most proteins.

Analyses of PMB/LPS Aggregates. One of the primary objectives of this study was to develop a procedure applicable to the analysis for complexed polymyxins. The potential of the amino acid analyzer technique to quantitate polymyxins in biological complexes was established by the following series of experiments. Suspensions of rough form *E. coli* EH100 and deep rough form *S. minnesota* Re595 LPS (4 mg each) were precipitated by an abundance of PMB (20 mg) in reaction mixtures ranging from 2 to 200 ml (Table 1). Precipitates were digested as described. A standard curve of DAB (5.94 residues/PMB) was used to quantitate PMB. Molar concentrations of LPS were calculated by correlating glucosamine content (amino sugars analyzed concomitantly) with previously elucidated LPS structures of *S. minnesota* Re595 (*14*) and *E. coli* EH100 (*15*) shown to have two and three glucosamine residues, respectively. The average PMB/LPS molar ratio was 1.68±0.03 when PMB was complexed with the Re595 deep rough LPS and 3.87±0.43 in complexes formed with EH100 rough form LPS. The relative ratios of PMB and glucosamine were in general agreement regardless of dilution factor. However, fluctuations in the absolute concentrations, indicate that careful standardization is essential for any analysis. The differences in PMB binding are consistent with structural differences between these rough and deep rough LPS forms. The truncated deep rough *S. minnesota* Re595 LPS lacks the phosphate groups (potential binding sites) found on the core of *E. coli* EH100 LPS (*14,15*). In addition Re595 LPS has a 4-aminoarabinose substituent on one of its lipid A phosphate groups, something not found in *E. coli* LPS.

TABLE 1. Analyses of PMB/LPS aggregates precipitated in different concentrations of reactants.

LPS source (LPS form)	V_r[a]/ml	Aggregate composition PMB[b]	GlcN[c]	Molar ratio[d] PMB/LPS
S. minnesota Re595 (deep rough)	2	322	386	1.67
	10	300	356	1.69
	25	344	418	1.65
	100	379	459	1.65
	200	261	303	1.72
	Ctrl[e]	--	538	--
E. coli EH100 (rough)	2	366	314	3.52
	10	226	182	3.70
	25	304	240	3.80
	50	367	306	3.60
	100	386	300	3.86
	200	372	237	4.71
	Ctrl[e]	--	354	--

a Final volume of reaction mixtures which contained 4 mg of LPS and 20 mg of PMB. Precipitates were washed, digested and analyzed as described in Methods.
b nmol of polymyxin B per mg of sample. Determined from standard curve of DAB in which 5.94 residues of DAB represent one molecule of PMB.
c nmol of glucosamine per mg of sample. Includes glucosamine and glucosamine phosphate.
d Molar concentration of LPS calculated on basis of one deep rough molecule containing two glucosamines, mass ~2500 Da, and one rough molecule containing three glucosamines, mass ~4000 Da.
e Four mg of LPS in 50 ml of water without PMB.

TABLE 2. Comparative analyses for amino acids liberated from PMB and PMBN as determined by amino acid analyzer and HPLC procedures.

Antibiotic Anyl proc[a]	Amino acid residues (concentrations relative to leucine) Leu[b]	Phe[b]	Thr[b]	DAB[b]
Polymyxin B				
AA analyzer	1.00	1.12	2.42	10.95
HPLC	1.00	1.16	1.87	13.50
Nonapeptide				
AA analyzer	1.00	0.96	1.60	5.90
HPLC	1.00	1.06	1.70	10.61

a Analytical procedure
b Leu = leucine; Phe = phenylalanine; Thr = threonine; DAB = diaminobutyrate

Comparison of Amino Acid Analyzer and HPLC Procedures. The amino acid profiles of acid-digested PMB and PMBN were compared by the amino acid analyzer and HPLC procedures (Table 2). The relative concentrations of the primary amino acids were equated by setting the concentration of leucine to 1.00 in each of the respective hydrolysates. By this criteria, the concentrations of leucine and

phenylalanine in PMB and PMBN were similar in either procedure. Likewise, the levels of threonine were similar in all samples except for PMB; assayed by the amino acid analyzer technique. The relative ratios of DAB in PMB and PMBN appeared somewhat higher in samples analyzed by the HPLC procedure. These apparent differences may be attributed to the chemical procedure used to prepare free amino acids for HPLC analysis. DAB has two primary amino groups which react with the phenyl isothiocyanate reagent (Waters Associates Technical Manual), resulting in a disubstituted chromophore with a correspondingly higher response than monosubstituted chromophores. It is likely that polymyxin determinations by the HPLC procedure based on DAB content would need additional standardization measures not required in the amino acid analyzer technique. Otherwise, both techniques have similar levels of sensitivity. Although we did not determine the utlility of the HPLC method in analyzing complexed polymyxins, these preliminary results suggest that this procedure could be useful for measuring free polymyxins.

Our results indicate that the amino acid analyzer technique is an accurate and appropriate method for analyzing polymyxins which are either free or complexed with physiological/clinical specimens. The procedure could most likely be extended to include analyses of other DAB-containing substances. The development of this method was facilitated by the presence of an uncommon acid-stable component (DAB) in the parent compound (polymyxin). This basic concept is possibly applicable to the analyses of other drugs which may contain unique and acid-stable compounds.

In summary we have developed an accurate and sensitive method to identify and quantitate PMB and PMBN. Analysis of polymyxins by the amino acid analyzer procedure is based on a standard curve of DAB. This procedure may be used to determine either free polymyxins or complexes of PMB/LPS aggregates formed with either rough or deep rough LPS.

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