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## Note

# Analytical and preparative high-performance liquid chromatography of the papain-cleaved derivative of polymyxin B

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Polymyxins are cationic amphipathic decapeptides produced by *Bacillus polymyxa* and related species. They consist of a cyclic heptapeptide moiety, a straight tripeptide side-chain and a fatty acid attached to the N-terminus of the side-chain. Polymyxins are very effective bactericidal antibiotics against most Gram-negative bacteria.

Chihara and co-workers[1–3] showed that it is possible to remove the fatty acid part and the N-terminal amino acid from the polymyxin molecule using certain enzymes such as ficin and papain to yield a polymyxin-derived nonapeptide. This polymyxin nonapeptide lacked any bactericidal activity. It was then found by Vaara and Vaara[4–6] and confirmed by others[7–10] that polymyxin nonapeptides still have one important property left, *viz.*, they drastically damage the Gram-negative bacterial cell wall and thus increase its permeability to several antibiotics. After these findings, polymyxin nonapeptides were subjected to intensive research.

Even though during the enzymatic treatment of polymyxin and the subsequent purification scheme most of the polymyxin is cleaved and the residual polymyxin removed, it is important to control the polymyxin contamination in the final nonapeptide preparation. This is especially important in those many assays where even trace amounts of highly active polymyxin could be expected to interfere. In this paper we show that reversed-phase high-performance liquid chromatography (HPLC) is a useful method for determining such contamination levels and for purifying polymyxin B nonapeptide free from polymyxin B without affecting its biological activity.

## EXPERIMENTAL

### *Reagents and materials*

Papain-cleaved derivative of polymyxin B (polymyxin B nonapeptide, PMBN) was prepared essentially as described by Viljanen and Vaara[11] and was a kind gift from Farmos Group (Turku, Finland) (lot 8.3.84). Polymyxin B sulphate (P-1004, lot 72F-0251) was obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade water and acetonitrile were supplied by Rathburn (Walkerburn, U.K.). Trifluoroacetic acid (purum) was purchased from Fluka (Buchs, Switzerland).

The assay medium for the determination of antibacterial activity was 0.01 *M*

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sodium phosphate buffer (pH 7.2) with 0.15 *M* sodium chloride and 300 μg/ml of trypticase soy broth solid (Difco Labs., Detroit, MI, U.S.A.). Rifampicin (Sigma) stock solution (1 mg/ml) was prepared in 10% methanol as described earlier[11]; all the subsequent dilutions were made in the assay medium.

*Analytical HPLC*

The chromatographic system consisted of a Varian (Palo Alto, CA, U.S.A.) 5000 liquid chromatograph, a Rheodyne (Cotati, CA, U.S.A.) loop injector (loop size, 10 μl), a Vydac 218 TP $C_{18}$ column (25 cm × 4.6 mm I.D., particle size 5 μm, pore size 300 Å) (Separations Group, Hesperia, CA, U.S.A.), a Varian UV-100 variable-wavelength detector and a Servogor recorder. The chromatographic solvents were 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.075% (v/v) trifluoroacetic acid in acetonitrile (solvent B). The solvent gradient was a linear gradient from 10 to 80% B in 20 min. The flow-rate was 1.2 ml/min. The effluent absorbance was monitored at 215 nm.

*Preparative HPLC*

The chromatographic system, column, gradient and flow-rate were as above. After determining the void volume between the detector and the tip of the detector outlet line (four eluent drops), 300 μg of PMBN in 10 μl of water were injected, and the eluting major peak was collected manually. Such peaks from a total of seven HPLC runs were pooled. The pooled peak fractions were concentrated in an evaporator (Speed-Vac; Savant Instruments, Hicksville, NY, U.S.A.) and lyophilized (Freezemobile II lyophilizer; Virtis, Gardiner, NY, U.S.A.). The lyophilized material was dissolved in 1% acetic acid to yield a PMBN concentration of 10 mg/ml and the solution was neutralized (pH 7) with sodium hydroxide and analysed by HPLC.

*Determination of antibacterial activity*

L-broth[12] grown cells of *Escherichia coli* IH3080 (018:KI, see ref. 5) in their early exponential growth phase (Klett 40, Klett–Summerson colorimeter, red filter) were used to inoculate (with $5 \cdot 10^4$ cells per ml) the assay medium (see Reagents and materials) containing 0, 0.1, 0.3, 1, 3 and 10 μg/ml of rifampicin. Aliquots (100 μl) of this inoculated medium were pipetted into wells of a microtiter plate (Nuclon Delta, Catalogue no. 167008; Nunc, Roskilde, Denmark). Each well already contained increasing amounts of PMBN (in 10 μl of 0.01% acetic acid). The plates were incubated at 37°C for 2 h and the viable counts determined by plating onto L-agar[12].

## RESULTS

The chromatographic system we chose employs a Vydac trimethylsilane-end-capped large-pore $C_{18}$ column, gradient elution with acetonitrile in water and trifluoroacetic acid as a volatile ion-pairing agent. This system allowed us to run a sample of 100 μg of polymyxin B nonapeptide (PMBN) in an analytical column (25 cm × 4.6 mm I.D.) with high efficiency, as shown in Fig. 1A and by the calculated number of theoretical plates (*N*, see ref. 13) on the order of 18 000. In addition to the major peak, the chromatogram revealed several minor impurities.

Fig. 1B shows the chromatogram of a polymyxin B sample (100 μg). Its reten-

tion time was significantly longer than that of PMBN, obviously owing to the amphipathicity (fatty acid part) of the molecule. Also this sample showed heterogeneity. Commercial polymyxin B preparations are unpurified mixtures of the natural polymyxins $B_1$, $B_2$ and, to a lesser extent, $B_3$, each of which have a characteristic fatty acid part[14–17]. In addition, these preparations usually also contain small amounts of other polymyxins (polymyxin Ile-$B_1$, polymyxin $E_1$, polymyxin $E_2$)[18,19]. Our HPLC system was suitable for the chromatography of this polymyxin B mixture (number of theoretical plates *ca.* 26 000 for the major peak, polymyxin $B_1$) but was not optimized for maximal separation of individual polymyxins from each other. Such systems have been published earlier[14,16,17,19].

Fig. 1C represents a sample consisting of 50 μg of PMBN and 50 μg of poly-

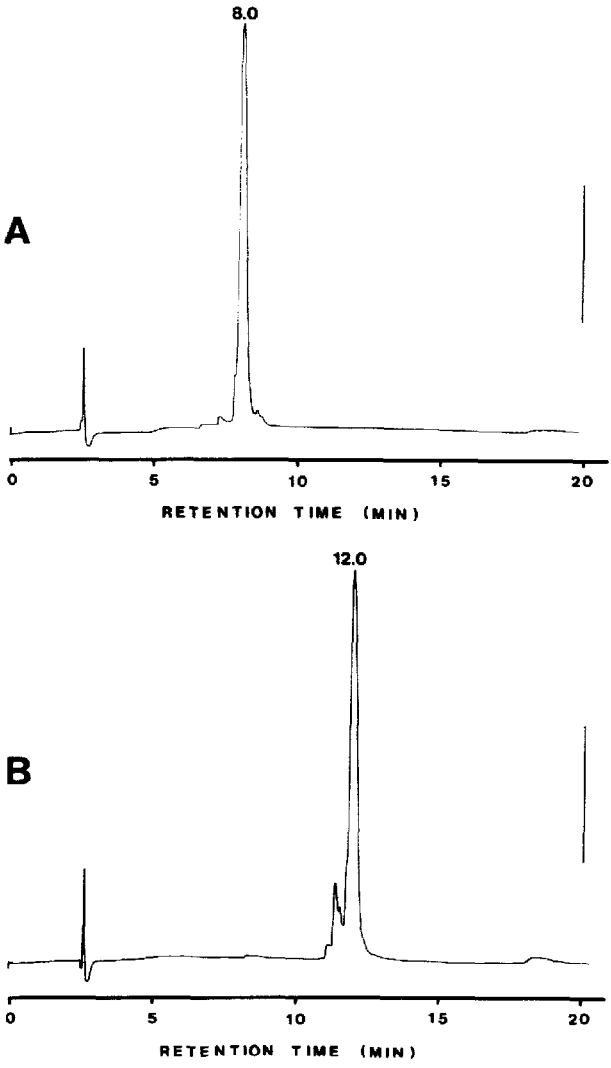


Fig. 1.

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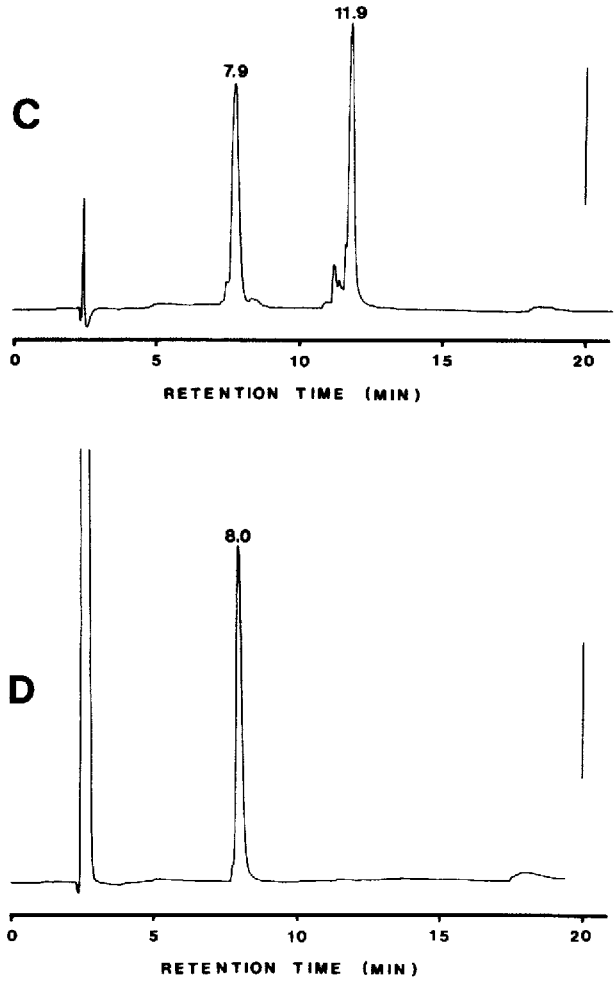


Fig. 1. Separation of polymyxin B and its papain-cleaved product polymyxin B nonapeptide (PMBN) by reversed-phase HPLC. Column, Vydac 218 TP $C_{18}$ (300 Å), 25 cm × 4.6 mm I.D. Eluents: A = 0.1% (v/v) trifluoroacetic acid in water; B = 0.075% (v/v) trifluoroacetic acid in acetonitrile. Eluent gradient, from 10 to 80% B in 20 min; flow-rate, 1.2 ml/min. Detector setting at 215 nm, 0.5 a.u./mV. The bars represent the height of 0.25 absorbance unit. (A) Crude PMBN, 100 μg; (B) polymyxin B, 100 μg; (C) crude PMBN, 50 μg and polymyxin B, 50 μg; (D) HPLC-purified PMBN, 100 μg. The peak eluting at 2.6 min in D is sodium acetate (see Preparative HPLC).

myxin B. As is evident from the chromatogram, the separation was complete. The separation factor [$\alpha = (V_2 - V_0)/(V_1 - V_0)$][13] between PMBN (retention time, 7.9 min) and the closest polymyxin-related peak (retention time, 11.3 min) was 1.66, indicating an excellent separation.

These findings prompted us to purify the major peak in the PMBN preparation further by preparative HPLC (see Experimental). As shown in Fig. 1D, this procedure removed the obvious impurities.

In the experiment reported in Fig. 2, the crude PMBN material, HPLC-purified PMBN and crude PMBN artificially contaminated with polymyxin B (degree of contamination, 2%, w/w) were run under HPLC conditions identical with those for Fig. 1, except that the sensitivity of detection was increased by a factor of 10. Now, in addition to the minor impurities already evident in Fig. 1A, a small peak having the retention time of polymyxin B was seen in the crude PMBN preparation (Fig. 2A). When this peak was compared with that produced by a 2% artificial contamination with polymyxin (Fig. 2B), it can be calculated that the polymyxin contamination in

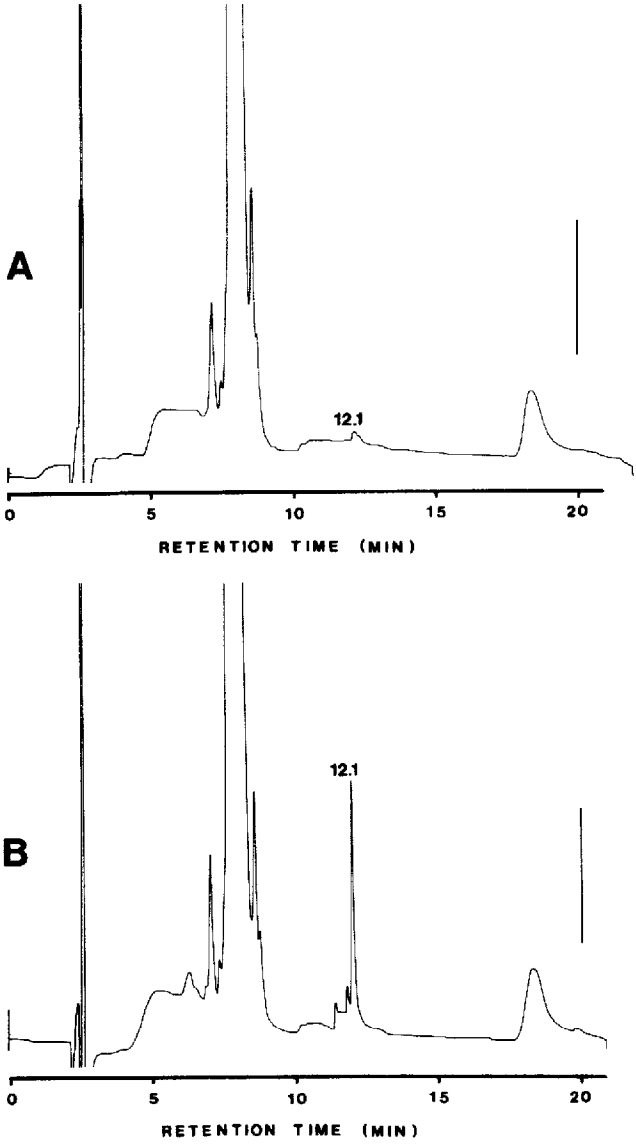


Fig. 2.

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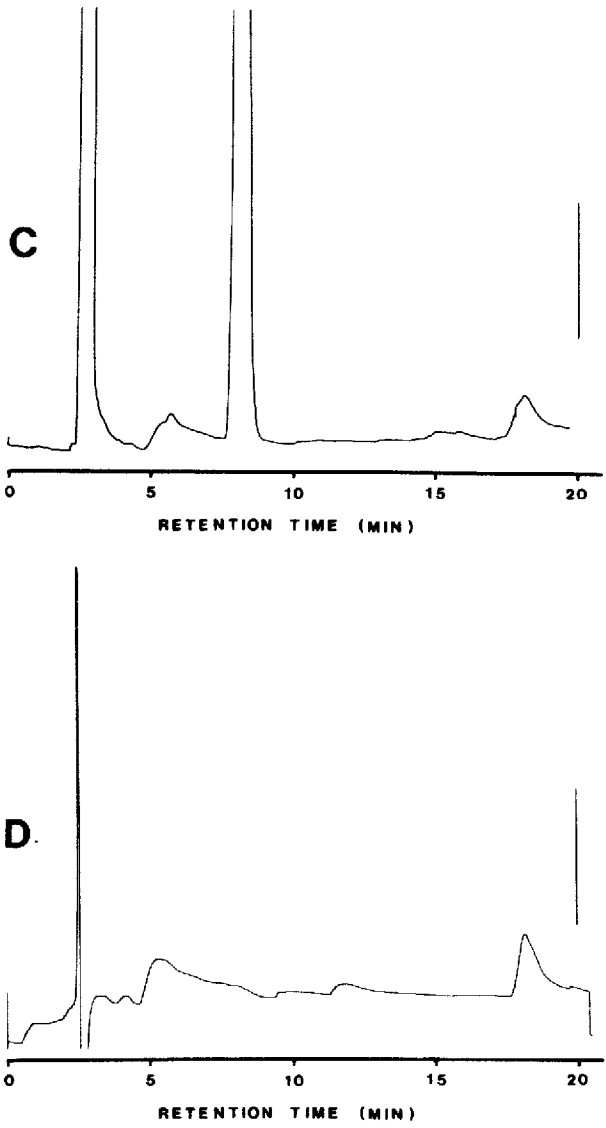


Fig. 2. Impurity analysis of the PMBN preparations. HPLC conditions as in Fig. 1 except that the detector setting was 0.05 a.u./mV. The bars represent the height of 0.025 absorbance unit. (A) crude PMBN, 100 $\mu$g; (B) crude PMBN, 100 $\mu$g and polymyxin B, 2 $\mu$g; (C) HPLC-purified PMBN, 100 $\mu$g; (D) blank run (to show the baseline at this sensitivity setting).

the crude PMBN was of the order of 0.1%. As shown in Fig. 2C, the HPLC-purified PMBN was free from contaminating polymyxin B and also the several other impurities found in the crude PMBN.

Fig. 3 shows that the crude PMBN and the HPLC-purified PMBN are equally

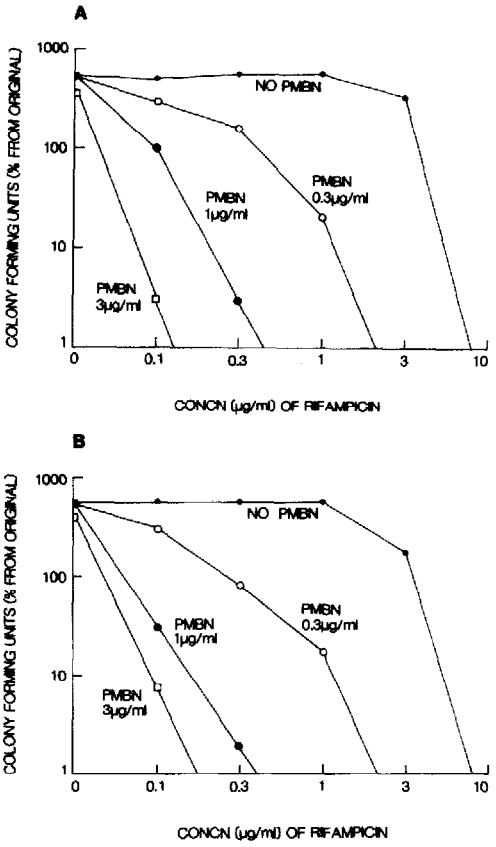


Fig. 3. The capacity of crude PMBN (A) and HPLC-purified PMBN (B) to sensitize *Escherichia coli* to the bactericidal action of rifampicin in the bacterial growth medium (incubation at 37°C for 2 h).

active in their antibacterial action. Both sensitize the target bacteria identically to the antibiotic rifampicin, thus lowering its minimum inhibitory concentration (MIC) by a factor of *ca.* 100.

## DISCUSSION

We have shown that reversed-phase HPLC is a useful method in the purity analysis of the papain-cleaved derivative of polymyxin B (polymyxin B nonapeptide, PMBN), and especially in determining how heavily it is contaminated by residual polymyxin B. Even though the PMBN preparation we studied contained only *ca.* 0.1% of polymyxin B (Fig. 2A), we still feel that much higher contamination levels are possible in scaled-up preparation processes. Thus, each lot should be analysed separately by HPLC and the contamination levels should be indicated. Because polymyxin B is a very potent bactericidal agent even at low concentrations, PMBN preparations such as those stated to contain less than 2% of polymyxin (see. *e.g.*, ref.

20) or where no purity data has been given[10] might be unsuitable for bacteriological studies, depending on the real contamination level.

In addition to polymyxin B, the HPLC of the crude PMBN revealed several other impurities. They might represent polymyxin B cleavage products other than PMBN. In the light of the results of Chihara *et al.* (Table VII in ref. 2), it is possible that up to 10% of the papain-cleaved product has a N-terminal diaminobutyric acid, thus being cleaved to an octapeptide.

When the crude PMBN was purified by HPLC to yield a single peak (Fig. 2C), it completely retained its antibacterial activity (the ability to sensitize the target bacteria to rifampicin, Fig. 3). This indicates that neither the residual polymyxin (0.1%) nor the impurities eluting distinct from the main peak were responsible for this antibacterial activity of the crude preparation. It also indicates that PMBN tolerates the conditions encountered during the preparative HPLC without any loss of biological activity. Thus, HPLC offers a convenient method for purifying PMBN for biological studies.

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