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# EFFICIENT ENDOTOXIN REMOVAL WITH A NEW SANITIZABLE AFFINITY COLUMN: AFFI-PREP POLYMYXIN

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## SUMMARY

A new affinity column packing for removal of endotoxins has been prepared by coupling USP drug-quality polymyxin B to Affi-Prep, a novel synthetic macroporous polymer. Affi-Prep Polymyxin binds endotoxins from a number of different strains of gram-negative bacteria. Endotoxin binding is not significantly affected by 10 mg/ml of bovine serum albumin or human immunoglobulin G, by 1 mg/ml sodium dodecyl sulphate, or by 1 mg/ml deoxycholate. Affi-Prep Polymyxin is stable to treatment with 1.0 *M* sodium hydroxide, an important property for sanitizing the resin. The resin shows a high ligand stability, since no leakage of polymyxin B from the packing could be detected. Affi-Prep Polymyxin exhibited the highest endotoxin binding efficiency when compared with several commercial agarose affinity packings.

## INTRODUCTION

Endotoxins are the pyrogenic lipopolysaccharide (LPS) components of gram-negative bacteria. They can have potent biological effects in man and in many animal species when administered systematically. In addition, endotoxins have been shown to perturb *in vivo* and *in vitro* experiments, even at concentrations of a few ng/ml[1,2]. Thus, the elimination of endotoxins is crucial for many *in vivo* studies and in the purification of drugs intended for injection in man.

As therapeutic and pharmacologically important products are developed in the biotechnology and pharmaceutical industries, the need for efficient chromatographic columns for endotoxin removal has become apparent. Besides removing endotoxins with high efficiency and selectivity, these columns should be able to withstand the treatments necessary for adequate sanitation before use.

Although a number of methods have been used to remove endotoxins[3], the most efficient and practical method utilizes polymyxin B (PMB) affinity chromatography[4]. Polymyxin B is the generic name for a group of chemically related fatty acyl peptide antibiotics produced by *Bacillus polymyxa* and related species. These compounds are generally administered at 2.5 mg/kg/day in the treatment of certain gram-negative bacterial infections, where PMB is thought to disrupt the membrane structure by a high affinity binding to the lipid A moiety of the bacterial lipopolysaccharides[5,6].

This report describes the evaluation of a new affinity packing prepared by

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coupling USP drug-grade polymyxin B to a novel macroporous synthetic polymer. This is the first publication giving a detailed evaluation of a commercial polymyxin B affinity material in terms of specificity, binding conditions, and ligand stability.

## MATERIALS AND METHODS

### *Materials*

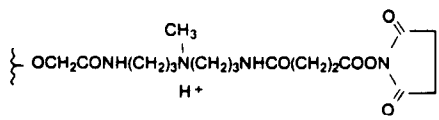
PMB sulfate from the following sources was utilized: Sigma (St. Louis, MO, U.S.A.), Calbiochem (La Jolla, CA, U.S.A.) and Pharma Tek (Huntington, NY, U.S.A.). Polymyxin E (colistin methanesulfonate), human immunoglobulin G (IgG), and bovine serum albumin (BSA) were obtained from Sigma. The following lipopolysaccharide preparations were obtained from Sigma: *Salmonella abortus equi, S. minnesota, Escherichia coli* 055:B5, *E. coli* 0127:B8, *E. coli* 0111:B4, *E. coli* 0128:B12 and *Serratia marcescens*. Deoxycholic acid (DOC) and *E. coli* 055:B5 were obtained from Calbiochem. Sodium dodecyl sulfate (SDS, electrophoresis grade) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Sterile, pyrogen-free water (PF-water) was obtained from Cadillac Medical Supplies (Richmond, CA, U.S.A.). Affi-Prep 15 was obtained from Bio-Rad Labs. All buffers used in coupling reactions, wash solutions and endotoxin binding assays were prepared with the PF-water. Phosphate-buffered saline (PBS) consisted of 10 m*M* phosphate buffer (pH 7.2), containing 140 mM sodium chloride. The binding buffer was 10 m*M* phosphate buffer (pH 6.0), containing 100 m*M* sodium chloride. The following affinity packing materials have been tested: polymyxin B-agarose from Sigma; Detoxi-Gel from Pierce (Rockford, IL, U.S.A.); Actigel-polymyxin B from Sterogene Biochemicals (San Gabriel, CA, U.S.A.); and polymyxin B-agarose from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

### *High-performance liquid chromatographic (HPLC) analysis of PMB*

PMB was analyzed on a Bio-Sil ODS 5S column (250 × 4 mm I.D.), connected to a Bio-Rad isocratic HPLC system, equipped with a 100-$\mu$l injection loop, a Bio-Rad Model 1330 pump and a Bio-Rad Model 1306 UV monitor, set at 200 nm. Elution was carried out at 1 ml/min with 100 m*M* sodium triphosphate, containing 22% acetonitrile, adjusted to pH 3.0 with orthophosphoric acid.

### *Coupling of PMB to Affi-Prep*

PMB was coupled to the N-hydroxylsuccinimide activated ester of Affi-Prep 15 which contained a spacer arm (succinylmethyliminobispropylaminocarboxymethyl group).




The activated resin was transferred to a coarse-frit sintered glass funnel and the material washed free of 2-propanol with ten bed volumes of ice cold 10 m*M* sodium acetate buffer (pH 4.5). PMB (5 mg/ml), in one bed volume of 0.1 *M* HEPES buffer (pH 8.0) was added immediately to the activated Affi-Prep. The coupling was carried

out at room temperature by allowing this PMB solution to filter through the Affi-Prep material over 10–15 min. The coupled material was washed with one bed volume of 0.1 *M* HEPES buffer (pH 8.0). The effluents from the coupling and the buffer wash were collected and saved for the HPLC analysis of PMB. The Affi-Prep Polymyxin was then washed with two bed volumes of 0.2 *M* ethanolamine (pH 8.5) followed by ten bed volumes each of 0.1 *M* glycine buffer (pH 9.0) containing 1 *M* sodium chloride and 0.1 *M* acetate buffer (pH 4.5) containing 1 *M* sodium chloride. The amount of PMB coupled to Affi-Prep was determined by the HPLC analysis of the initial and effluent PMB solutions. Under these coupling conditions the Affi-Prep Polymyxin contained *ca.* 2.5 mg PMB per ml Affi-Prep.

A control Affi-Prep, lacking PMB, was prepared as described above except PMB was omitted from the coupling reaction.

*Endotoxin binding assays*

*Batch assay.* A uniform suspension in water (30–50%, v/v) of the Affi-Prep Polymyxin resin was prepared, from which 30–100 μl of resin was pipetted into a 12 × 75 mm glass test tube, containing 1–10 μg of purified endotoxin in 1.0 ml buffer, usually binding buffer. A control tube was included which contained no resin. The tubes were capped and rotated at room temperature end-over-end for 4–24 h. To determine the amount of endotoxin not bound to the resin, serial 10-fold dilutions in PF-water were made of the supernatant solutions, which were assayed for endotoxin by the limulus ameobocyte lysate (LAL) assay. The total input of endotoxin in each experiment was determined from the total endotoxin units (EU) in the control tube. The results are expressed as the total unbound EU and sometimes as the percent endotoxin removal (bound EU × 100/total EU).

*Equilibrium binding capacity.* This assay is similar to the batch procedure described above, except that an excess of LPS was added. Approximately 0.5 ml of a 50% suspension of the Affi-Prep Polymyxin was transferred to a test tube. The resin was washed with 5 ml PF-water, and the volume of the solution was adjusted to give an approximately 25–35% suspension. The suspension was stirred and aliquots of 100 μl were added to 12 × 75 mm glass test tubes containing 0.9 ml of binding buffer and 200–800 μg of *E. coli* 055:B5 LPS (Calbiochem). The test tubes were capped, sealed and rotated end-over-end for 14–20 h at room temperature. Serial 10-fold dilutions were made to final dilutions of $10^6$–$10^7$. The amount of LPS bound to the resin was determined by analyzing the supernatant solution and control sample for endotoxin, using the LAL assay. To determine the exact quantity of Affi-Prep Polymyxin added, duplicate 200-μl aliquots of the same 25–35% slurry were added to tared test tubes. These were then dried in an oven for 1 h at 100°C and from the resin dry weight an accurate resin volume was calculated, using a swelling factor of 2.5. The endotoxin bound was calculated from the difference between the total EU added and the unbound EU in the supernatant of the Affi-Prep Polymyxin treated samples.

*Column.* Poly-Prep columns (10 × 8 mm I.D.) prepared from 0.4–0.6 ml of Affi-Prep Polymyxin were washed with 5 ml of PF-water and two 5-ml aliquots of binding buffer. To each column 2–3 ml of LPS (0.2–1 μg/ml) in binding buffer was applied. The effluent solutions were collected and analyzed for endotoxin.

*LAL assay for endotoxin*

The amount of endotoxin present in a solution was determined by a modified LAL assay and a synthetic color producing substrate. The assay kits were from Whittaker M.A. Bioproducts (Walkersville, MD, U.S.A.). The assays were performed in microtiter plates according to the instructions supplied with the kits. The LAL assay was linear between 0.1–1.0 EU/ml. 1 EU is approximately equal to 0.1 ng of *E. coli* 055:B5 LPS.

## RESULTS

*Coupling of PMB to Affi-Prep*

PMB was coupled to a new rigid macroporous polymeric resin[8] (Affi-Prep 15) which contains the same spacer arm and N-hydroxysuccinimide ester as previously reported for agarose gels (Affi-Gel® 15). Under the reaction conditions the coupling of PMB to Affi-Prep was rapid, being essentially complete within 10 min. Affi-Prep Polymyxin typically contained 2.5 mg PMB/ml Affi-Prep.

*Endotoxin binding*

The analysis of multiple samples for endotoxin binding was generally performed using the batch assay described in Materials and methods. This measures equilibrium binding using inputs of $10^3$–$10^4$ EU. This requires 10- to 100-fold dilutions of the supernatant before assaying for endotoxin by a standard LAL test. Table I shows the effect of some incubation conditions, such as buffer strength, salt concentration, and incubation time on the endotoxin binding of Affi-Prep Polymyxin. Comparable binding was observed in water, 10 and 50 m*M* phosphate buffer (pH 7.0) and 10 m*M* phosphate (pH 7.0), containing 100 m*M* sodium chloride. Only in the 250 m*M* phosphate buffer was there a decrease, the binding efficiency dropping from *ca.* 99.5 to 92%. Under most conditions the binding after 48 h was more efficient than after 4 h. As a control for the endotoxin binding experiments, Affi-Prep containing no

TABLE I

EFFECT OF INCUBATION CONDITIONS ON ENDOTOXIN REMOVAL

Affi-Prep Polymyxin (50 μl) was incubated for the indicated times in 1.0 ml of the indicated solutions in the presence of 6500 EU/ml. The supernatants were assayed for unbound endotoxin. The endotoxin removal is expressed as the total EU not bound and as % removal (values in parentheses) (bound EU × 100/total EU).

| *Binding conditions* | *Unbound (EU)* | |
|---|---|---|
| | *Incubation time* | |
| | *4 h* | *48 h* |
| Water | 4 (99.9) | 2 (99.9) |
| 10 m*M* Phosphate buffer (pH 7.0) | 10 (99.8) | 6 (99.9) |
| 10 m*M* Phosphate buffer (pH 7.0) 100 m*M* Sodium chloride | 20 (99.6) | 4 (99.9) |
| 50 m*M* Phosphate buffer (pH 7.0) | 32 (99.5) | 5 (99.9) |
| 250 m*M* Phosphate buffer (pH 7.0) | 500 (92.3) | 360 (94.4) |
| 50 m*M* Acetate buffer (pH 5.6) | 32 (99.5) | 2 (99.9) |

TABLE II

EFFECT OF PROTEINS AND DETERGENTS ON ENDOTOXIN REMOVAL

Aliquots of 70 μl of Affi-Prep Polymyxin were rotated at 20°C for 16 h in 1.0 ml of PBS-containing endotoxin and the indicated concentrations of SDS, DOC, BSA and IgG. The supernatants were assayed for endotoxin.

| *Addition* | *Concentration (mg/ml)* | *EU Removal (%)* | *Unbound EU* |
|---|---|---|---|
| None | – | 99.9 | 4.8 |
| SDS | 0.06 | 99.7 | 15 |
| SDS | 0.25 | 99.8 | 10 |
| SDS | 1.00 | 99.9 | 4.1 |
| DOC | 0.06 | 99.8 | 11 |
| DOC | 0.25 | 99.9 | 4.8 |
| DOC | 1.00 | 99.9 | 2.2 |
| | | Total added, 6000 EU | |
| None | – | 99.9 | 2.2 |
| BSA | 0.10 | 99.9 | 2.0 |
| BSA | 1.0 | 99.9 | 2.2 |
| BSA | 10.0 | 99.9 | 2.1 |
| IgG | 0.10 | 99.9 | 2.4 |
| IgG | 1.0 | 99.9 | 4.1 |
| IgG | 10.0 | 99.9 | 6.4 |
| | | Total added, 6700 EU | |

polymyxin was incubated in binding buffer for 16 h with 10000 EU. This control Affi-Prep left 5600 EU unbound while Affi-Prep Polymyxin was able to bind all but 12 EU.

The addition of up to 10 mg/ml of BSA or human IgG in the binding assay (Table II) had little or no effect on the equilibrium values of endotoxin binding of Affi-Prep Polymyxin. Table II also shows the influence of two different detergents,

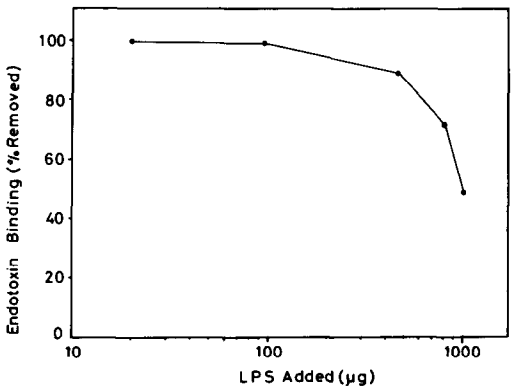



Fig. 1. Endotoxin binding *versus* LPS load. The indicated amounts of LPS (*E. coli* 055:B5) were rotated for 16 h at 20°C with 75 μl of Affi-Prep Polymyxin in 1.0 ml of binding buffer. The percentage removal was calculated from the unbound endotoxin in the supernatant and the total in the respective controls containing no resin.

SDS and DOC. At concentrations of 1 mg/ml there was no inhibition of binding, more than 99.9% of the added endotoxin being bound. However, there was a small but consistent decrease in the unbound endotoxin with dilution.

Fig. 1 shows a curve of endotoxin binding efficiency *versus* added endotoxin. Under conditions of excess endotoxin, theoretical binding capacities of more than 6 mg LPS per ml resin have been obtained in the equilibrium binding assay decribed under Materials and methods.

*Binding of different endotoxins by Affi-Prep Polymyxin and Affi-Prep PME*

In addition to PMB, a second type of polymyxin is commercially available, colistin or polymyxin E (PME). PMB and PME were coupled separately to Affi-Prep and evaluated for their ability to bind the purified endotoxins isolated from a number of different strains of gram-negative bacteria. The results, presented in Table III, show that both affinity packings were able to bind the LPS molecules from all the strains tested, PMB being superior to PME. In a separate experiment (not shown) the PMB derivative had about twice the endotoxin binding capacity of the PME packing.

*Stability*

Affi-Prep Polymyxin does not appear to lose endotoxin binding activity after short treatments (5–15 min) with 1% SDS, 1% DOC, 70% aqueous ethanol and 100% 2-propanol. Affi-Prep Polymyxin was examined for its stability toward sodium hydroxide, a standard reagent used to sanitize chromatographic columns. Table IV depicts the binding efficiency of 0.5-ml columns of Affi-Prep Polymyxin, which had been washed with two cycles of sodium hydroxide of different concentrations. No significant loss in binding was observed, even after the two washes with 1 *M* sodium hydroxide, all samples exhibiting binding efficiencies of greater than 99.9%. Time-course experiments (not shown) further indicated little or no loss in endotoxin binding even after 1 h incubation with 1 *M* sodium hydroxide.

TABLE III

BINDING OF ENDOTOXINS FROM DIFFERENT BACTERIAL STRAINS

Approximately 1 μg of the LPS specimens shown were rotated for 16h in 1.0 ml of 10 m*M* phosphate buffer (pH 7.0), containing 70 μl of AP-PMB (Affi-Prep Polymyxin B) or AP-PME (Affi-Prep Polymyxin E). The supernatants were assayed for unbound endotoxin. The endotoxin removal is expressed as the total EU not bound.

| *Bacterial strain* | *Input (EU)* | *Unbound (EU)* | |
|---|---|---|---|
| | | *AP-PMB* | *AP-PME* |
| *E. coli* 055:B5 | 3800 | 10 | 60 |
| *E. coli* 0111:B4 | 2100 | 3 | 5 |
| *E. coli* 0127:B8 | 5300 | 3 | 16 |
| *E. coli* 0128:B12 | 2800 | 9 | 20 |
| *Salmonella abortus equi* | 4000 | 5 | 35 |
| *Salmonella minnesota* | 1600 | 17 | 20 |
| *Serratia marcescens* | 3000 | 10 | 35 |

TABLE IV

EFFECT OF SODIUM HYDROXIDE ON ENDOTOXIN REMOVAL

Poly-Prep columns, containing 0.5 ml of Affi-Prep Polymyxin, were washed over a 5–10 min period with 3.0 ml of sodium hydroxide at the indicated concentrations. The columns were then washed with 4 ml of water, followed by two 4-ml portions of 10 m*M* phosphate buffer (pH 6.0), containing 100 m*M* sodium chloride. Each column was loaded with 3.0 ml of LPS in the above buffer and the eluates were analyzed for unbound endotoxin. The sodium hydroxide wash and assay were repeated on the same columns. The endotoxin removal is expressed as the total EU not bound by the columns.

| *Sodium hydroxide concentration (M)* | *Unbound (EU)* | |
|---|---|---|
| | *1st Wash* | *2nd Wash* |
| 0 | 1.1 | 0.6 |
| 0.1 | 0.8 | 1.2 |
| 0.2 | 1.3 | 1.2 |
| 0.5 | 1.3 | 1.5 |
| 1.0 | 1.8 | 1.5 |
| Total added | 13 500 EU | 11 000 EU |

*Leakage of PMB from Affi-Prep Polymyxin*

Commercial preparations of PMB have been separated by HPLC into several major components[7]. The leakage of PMB from the affinity columns was examined by HPLC. Fig. 2A shows the chromatogram of a PMB standard (1 μg/ml), which has two major peaks.

Suspensions of Affi-Prep Polymyxin were rotated overnight at room temperature with one-half volume of 0.1 *M* glycine buffer (pH 10.0). The chromatogram of the resultant supernatant shown in Fig. 2B indicates the absence of any PMB in this 0.1 *M* glycine wash. PMB which had been incubated overnight under these conditions

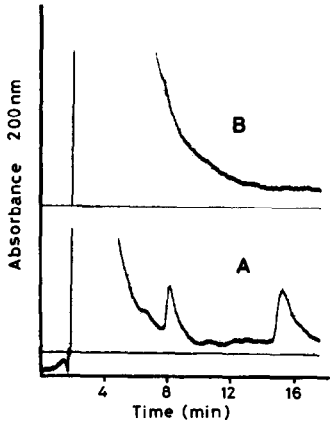



Fig. 2. HPLC analysis of the pH 10 glycine wash of Affi-Prep Polymyxin. PMB was analyzed at an absorbance scale of 0.005 on a Bio-Sil ODS 5S column, as described under Materials and methods. (A) 100 μl of PMB standard (1.0 μg/ml) in 0.1 *M* glycine buffer (pH 10.0). (B) 100 μl of a supernatant, following an overnight incubation at 20°C of Affi-Prep Polymyxin with one-half volume of 0.1 *M* glycine (pH 10.0).

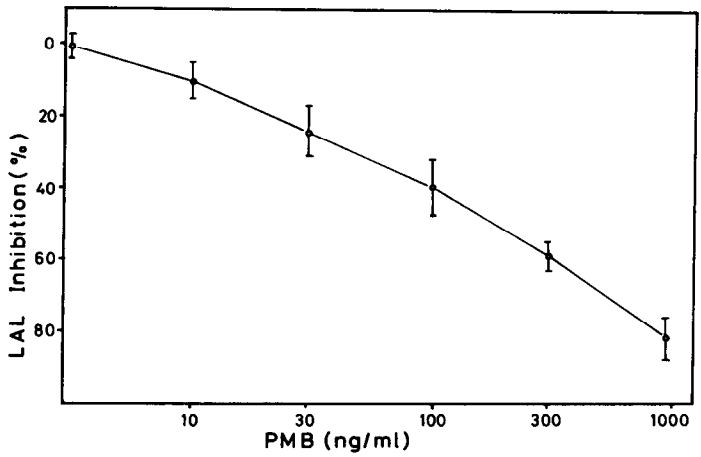



Fig. 3. Inhibition of the LAL assay by PMB. Aliquots of 100 $\mu$l of the indicated concentrations of PMB in PBS were incubated for 60 min at 20°C with 75 $\mu$l of an endotoxin standard (2 EU/ml in PF-water). Duplicate 50-$\mu$l samples were then assayed for endotoxin by the standard assay. The control (0% inhibition), containing only PBS, had an absorbance at 410 nm of 0.4. The values represent the mean of three analyses $\pm$ standard deviation.

as a control was fully recoverable by HPLC (data not shown). With a detecton limit of 0.25 $\mu$g/ml this would correspond to a leakage of $< 125$ ppm PMB from the packing. In a similar experiment, no PMB was detected in a supernatant following a 2-h incubation with 0.01 *M* sodium hydroxide at room temperature.

A more sensitive assay for PMB was developed based on an inhibition of the LAL assay for endotoxin. Fig. 3 shows a typical standard inhibition curve where increasing concentrations of PMB were incubated for 60 min with a constant amount of endotoxin (0.4 EU/ml) before the LAL assay. Half of the maximal inhibition typically occurs between 80–150 ng/ml, with a detection limit of *ca.* 20 ng/ml. Affi-Prep Polymyxin, which was endotoxin-free, was incubated for 16 h at 37°C with one volume

TABLE V

COMPARISON OF DIFFERENT AFFINITY PACKINGS

Poly-Prep columns containing *ca.* 0.45 ml of the respective packing were equilibrated in 10 m*M* phosphate buffer (pH 6.0), containing 100 m*M* sodium chloride, and then loaded with 2.0 ml of LPS solutions. The eluates were assayed for endotoxin. In the batch experiments, 30 $\mu$l of the resins were incubated overnight in 1.0 ml of the above buffer containing (A) 10 000 EU and (B) $1.2 \cdot 10^6$ EU. The supernatants were assayed for unbound endotoxin. Values in parentheses represent percentage removal.

| *Packing* | *Column* | *Unbound (EU)* | |
|---|---|---|---|
| | | *Batch A* | *Batch B* |
| Affi-Prep Polymyxin | 2 (99.9) | 5 (99.9) | $3 \cdot 10^3$ (99.8) |
| PMB-Agarose (Sigma) | 26 (99.8) | 90 (99.1) | $4.5 \cdot 10^4$ (96.3) |
| Detoxi-Gel | 34 (99.7) | 200 (98.0) | $6.5 \cdot 10^5$ (45.8) |
| Actigel-PMB | 100 (99.2) | 60 (99.4) | $6 \cdot 10^5$ (50.0) |
| PMB-Agarose (BM) | 100 (99.2) | 1400 (86.1) | $9 \cdot 10^5$ (25.0) |
| Added (EU) | 13 000 | 10 000 | $1.2 \cdot 10^6$ |

of PBS. Using the inhibition assay, no PMB was detected in the supernatant, which corresponds to a leakage of < 8 ppm of PMB or < 0.5 ppm/h.

*Comparison with agarose affinity columns*

Several commercially available agarose affinity packings, designed to remove endotoxin, have been compared with Affi-Prep Polymyxin in column and in batch assays at two different endotoxin levels (Table V). Columns (0.45 ml) of all packing materials were able to remove over 99% of the added endotoxin, Affi-Prep Polymyxin showing the most efficient binding (>99.9%). In the batch assay with low endotoxin levels, all except one material exhibited over 98% endotoxin removal. At the high endotoxin load of 1.2 million EU significant differences were noted between some of the packings probably reflecting differences in the binding capacities. Under all test conditions, Affi-Prep Polymyxin was superior to the agarose materials.

*Applications of Affi-Prep Polymyxin*

In addition to binding purified endotoxins, Affi-Prep Polymyxin can also remove "naturally" occurring endotoxins from various contaminated solutions. Passage of 20 ml of distilled water containing 15 EU/ml through an Affi-Prep Polymyxin column (10 × 8 mm I.D.) yielded a solution containing less than 0.05 EU/ml. The endotoxin level (500 000 EU/ml) of crude culture filtrates from *E. coli* cells could be reduced by >99.5% after incubation with Affi-Prep Polymyxin. HPLC cartridges containing Affi-Prep Polymyxin have also been used to reduce the endotoxin level of HPLC eluates.

Another application is the removal of endotoxin from antibody solutions. As an example, two monoclonal antibodies from ascites fluids have been purified and treated with the affinity material. An anti-human chorionic gonadotropin (HCG) was purified in a Bio-Rad HRLC 800 system by using sequentially, Affi-Prep cation-exchange (ABCM) and Affi-Prep Protein A cartridges. The other antibody against horseradish peroxidase (HRP) was purified on a conventional Affi-Prep Protein A column (1.0 ml). After elution from the Protein A columns with citrate buffer both antibodies were dialyzed against 20 m*M* phosphate buffer (pH 6.5). A 3.0-ml aliquot of the anti-HRP dialyzate, containing 2.1 mg protein/ml and 20 EU/ml was passed through a 0.5-ml column of Affi-Prep Polymyxin. This reduced the endotoxin level 98.5% to 0.3 EU/ml without affecting the protein. The same treatment of the anti-HCG dialyzate, containing 0.5 mg protein/ml and 61 EU/ml, yielded a solution with the same protein content and 6 EU/ml, representing a 90% reduction of endotoxin.

## DISCUSSION

Polymyxin B is not a pure compound, but a mixture of closely related chemical substances. Commercial preparations have been separated by HPLC into 10–13 components[7]. The polymyxin B used in the preparation of Affi-Prep Polymyxin is of USP drug-quality, and this could be important in its application for affinity chromatography in purifying products for pharmaceutical applications where leakage of PMB could cause problems. Leakage of nanogram quantities of a substance administered as a drug in milligram quantities may not be objectionable. It should be added here that Affi-Prep Polymyxin appears to have a high ligand stability.

The Affi-Prep matrix has been designed to satisfy the scale-up requirements of industrial production. It is a rigid, macroporous polymer exhibiting excellent pressure/flow characteristics as well as excellent handling properties[8]. In addition, this resin is able to stand the harsh treatments needed for sterilization. The Affi-Prep Polymyxin support is not affected functionally by treatment with 1 *M* sodium hydroxide.

The endotoxin removal by Affi-Prep Polymyxin has been examined under a variety of conditions. Probably the most efficient removal of endotoxins is achieved in the batch mode, where typically 50 $\mu$l of resin can bind more than 99.5% of 5000–10 000 EU (0.5–1.0 $\mu$g). Efficient binding was also observed in column chromatography (Table V). High salt and buffer concentration appear to inhibit the endotoxin binding somewhat (Table I). However, binding efficiency was not affected by the addition of up to 10 mg/ml of BSA or IgG (Table II).

Endotoxin binding can be carried out in the presence of up to 1 mg/ml SDS or deoxycholate. In several experiments the highest efficiency was observed at concentrations of 1 mg/ml SDS. With dilution, there was a slight but significant decrease in binding efficiency. This may be related to the critical micelle concentrations (CMC) of these detergents. Around the CMC (1–2 mg/ml), the detergents may dissociate the LPS aggregates, making the molecules more accessible to the Affi-Prep Polymyxin resin. The use of a surfactant has recently been reported to reduce contamination by protein-bound endotoxin[9].

In addition to PMB, a second type of polymyxin (PME) is commercially available. If these two polymyxins would exhibit different binding patterns on different endotoxin molecules, it might be advantageous to incorporate a mixture of polymyxin molecules in an affinity support. To test this, PMB and PME were coupled to Affi-Prep and evaluated for their ability to bind the purified endotoxins isolated from a number of different strains of gram-negative bacteria. Both materials were able to bind the molecules from all the strains tested, with Affi-Prep Polymyxin being superior in all cases to the PME support. This result confirms that PMB alone is sufficient and further demonstrates the broad binding specificity of Affi-Prep Polymyxin, suggesting its applicability under a wide variety of conditions.

An important aspect of any affinity packing is the potential leakage of the ligand from the material contaminating the product(s) of interest. We have tested for PMB leakage under several incubation conditions, using two different assays for PMB. Using an HPLC assay with a detection limit of 250 ng/ml, no PMB was found in 0.1 *M* glycine (pH 10) or in 0.01 *M* sodium hydroxide washes. This corresponds to a leakage of $<125$ ppm PMB from the resin. If these values are calculated on the basis of unit time, they would be $<7.5$ ppm/h or $<0.13$ ppm/min. It is difficult to translate these rather long, harsh treatments to more normal chromatographic conditions, such as neutral pH and shorter contact time.

A more sensitive assay for PMB was developed, based on the ability of PMB to inhibit the LAL assay for endotoxin. Using this assay, little or no PMB was detected in the supernatant of a 16-h incubation at 37°C with PBS. This would correspond to a leakage of $<20$ ng/ml or $<8$ ppm of PMB from the column material. These results indicate that under these conditions there is little PMB leakage from the packing —a high ligand stability indeed.

Affi-Prep Polymyxin was superior in terms of endotoxin removal when

compared to four commercial agarose materials designed to bind endotoxins. The basis for this better performance is not known. It is probably not due to the PMB, as the source of PMB as well as the amount coupled does not appear to be critical for the binding efficiency of the Affi-Prep Polymyxin material. It is possible that the better performance may be due to the polymer matrix or to a synergism of the Affi-Prep matrix with the PMB.

Although the Affi-Prep Polymyxin has been tested mainly on purified endotoxins, it has been used in several practical applications, such as the efficient removal of endotoxin from water and from purified monoclonal antibodies. Affi-Prep Polymyxin treatment resulted in a 98.5% removal of endotoxin from the anti-HRP and only a 90% removal from the anti-HCG. This suggests that some endotoxin may be more tightly bound to some proteins than to others. It is possible that the use of Affi-Prep Polymyxin in combination with detergents, which may dissociate endotoxin and protein, may aid in the removal of tightly bound endotoxins.

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