A Highly Sensitive ELISA for the Quantification of Polymyxin B Sulfate in Human Serum

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Received May 19, 1999; accepted August 26, 1999

A highly sensitive ELISA for the determination of polymyxin B sulfate (PMB) was developed which is capable of measuring as low as 32 pg/ml. Anti-PMB antibody was obtained by immunizing rabbits with PMB conjugated with mercaptosuccinyl bovine serum albumin (MS. BSA) using N-(γ -maleimidobutyryloxy) succinimide (GMBS) as a heterobifunctional coupling agent. An enzyme marker was similarly prepared by coupling PMB with horseradish peroxidase (HRP) employing GMBS. This ELISA showed very low reactivity with the PMB analogue, polymyxin E (0.05%). The values for PMB concentration detected by this assay were comparable with those detected by the bioassay. Moreover, the ELISA was about 10000 times more sensitive in detecting PMB at lower concentrations. Serum PMB concentration after the oral administration of a PMB tablet to human subjects was determined by the ELISA. PMB was rapidly absorbed from the gastrointestinal tract after the administration, then slowly decreased. These results indicate that the ELISA may be a valuable tool for studies of the pharmacokinetics and pharmacodynamics of the anti-endotoxin drug, PMB.

Key words polymyxin B; ELISA; anti-endotoxin drug

Polymyxin B Sulfate (PMB), a basic polypeptide antibiotic produced by *Bacillus polymya*, shows strong bactericidal actions against various gram-negative bacteria.¹⁾ PMB is not usually systemically administered because of its strong toxicity to the kidney,²⁾ and since it is not absorbed from the gastrointestinal tract, it is used for oral administration and local spraying.

PMB has been known to show anti-endotoxin activities, and it is reported to bind to endotoxin *in vitro* at a 1 : 1 ratio to suppress many of its activities.³⁾ An *in vivo* study showed that intramuscular injection of a small amount of PMB (12500 U $\times$ 4/d) and its oral administration (3000000 U/d) rapidly normalized the blood endotoxin concentration.^{4,5)} It has been suggested that orally administered PMB is not transported to blood, but it seems likely that a small amount of PMB does enter the blood. PMB can be measured by bioassay or HPLC,⁶⁾ but a very small amount understood in the blood cannot be detected by these methods because of their low sensitivities.

The blood endotoxin concentration is believed to be 1 ng/ml at the highest level in patients with sepsis,^{7,8)} for which the blood PMB concentration required should be very small. Despite antimicrobial activities of PMB, it is not used for systemic administration such as intravenous injection because of side effects in the kidney.²⁾ However, the amount of PMB required for anti-endotoxin is much smaller than that used as an antibiotic, so that there may be no problems of side effects. Endo *et al.*^{4,5)} reported that intramuscular administration of a small amount of PMB showed no clinical side effects. If the kinetics of PMB in human are determined, its smallest dose having a clinical effect with minimal side effects will be established. If it can be shown that a small amount of PMB administered not as an antibacterial drug but as an anti-endotoxin drug has no side effects, and if its effectiveness including the dose-effect relationship can be clinically demonstrated, PMB administration will become a major treatment method for sepsis.

This paper describes the production of a PMB-specific antibody, using N-(γ -maleimidobutyryloxy) succinimide (GMBS) as a cross-linker for preparation of the immunogen and PMB labeled with horseradish peroxidase (HRP) as a tracer, and the development of an ELISA capable of detecting as low as 32 pg PMB/ml. Using this assay, we are the first to identify the PMB kinetics in human blood after its oral administration.

MATERIALS AND METHODS

Reagents PMB tablet (Polymyxin B Sulfate Tablets[®], 1000000 U/tablet) and PMB powder were supplied by Pfizer Pharmaceuticals Inc. Polymyxin E was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HRP (for enzyme immunoassay) and 3,3',5,5'-tetramethyl-benzidine (TMB) were obtained from Boehringer Mannheim (Mannheim, Germany). GMBS was purchased from Dojin Chemical Co. (Kumamoto, Japan).

Subjects The volunteer subjects participating in the study were 2 healthy males and 1 female from all of whom informed consent was obtained. The subjects ranged in age from 24 to 34 years (an average of 28 years) and weighed 45 to 65 kg (an average of 56 kg).

Preparation of the Immunogen for PMB PMB was conjugated to bovine serum albumin (BSA), essentially by the same principle as used for the previous preparation of bleomycin immunogen (Fig. 1).⁹⁾ PMB (14 mg; approximately 10 mmol) in 1.5 ml of 0.1 M phosphate buffer (pH 7.0), was mixed with GMBS (2.8 mg; 10 μ mol) in 0.5 ml of tetrahydrofuran and incubated for 30 min at 30 °C with stirring. After removing tetrahydrofuran by passing nitrogen through the reacted mixture, the aqueous solution was washed three times with 3 ml of ether-dichloromethane mixture (2 : 1, v/v) to remove any remaining free GMBS. Acetylmercaptosuccinyl BSA (AMS. BSA; 10 mg), containing 17 acetylmercaptosuccinyl groups per BSA molecule, in 0.2 ml of 0.1 M phosphate

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Fig. 1. Scheme for Preparation of the Immunogen and Enzyme Conjugate in ELISA for PMB

FA, 6-methyloctanoyl or 6-methylheptanoyl; Dab, 2,4-diaminobutyric acid.

buffer (pH 7.0) was incubated in 50 μ l of freshly prepared 0.5 M hydroxylamine (pH 7.0) at 25 $^{\circ}$ C for 10 min to remove the protecting acetyl group. The resulting mercaptosuccinyl (MS) BSA (MS-BSA), was diluted with 3 ml of 0.1 M phosphate buffer (pH 7.0) containing 3 M urea, added immediately to the GMBS-acylated PMB solution, and then incubated at 25 $^{\circ}$ C for 30 min with vigorous stirring. The reaction mixture was dialyzed successively for 48 h against 50 and 1 mM phosphate buffer (pH 7.0) and H_2O . The purified conjugate was lyophilized and used as an immunogen for the ELISA. Using the 4,4-dithiodipyridine method to determine the thiol group,¹⁰ the conjugate was estimated to contain about 9.8 molecules of PMB per BSA molecule.

Preparation of PMB Antibody One milliliter of a saline of 1 mg of PMB-BSA conjugate was emulsified with an equal volume of complete Freund's adjuvant. White female rabbits were given multiple s.c. injections at sites along both sides of their backs. Booster injections were then given 3 times at biweekly intervals, using one-half the amount of dosage of the first immunization. The rabbits were bled from an ear vein 10 weeks after immunization began. Fractions of IgG were extracted from the sera with 50% saturated ammonium sulfate and chromatographed on a column of DEAE-Sephacel (2.1 $\times$ 23 cm) using 17.5 mM phosphate buffer (pH 6.8) as an eluent.¹¹ The fraction passed through the column was lyophilized and used as anti-PMB IgG for ELISA.

Preparation of the PMB-HRP Conjugate PMB-HRP was prepared by essentially the same 2-step procedure as the immunogen for PMB, using a cross-linker GMBS (Fig. 1). PMB (1.75 mg; approximately 1.25 μ mol) in 250 μ l of 0.1 M phosphate buffer (pH 7.0), was mixed with GMBS (0.35 mg; 1.75 μ mol) in 50 μ l of tetrahydrofuran and incubated for

30 min at 30 $^{\circ}$ C with stirring. After removing tetrahydrofuran by passing nitrogen through the reaction mixture, the aqueous solution was washed 3 times with 0.5 ml of ether-dichloromethane mixture (2:1, v/v) to remove any remaining free GMBS.

HRP (2 mg, 50 nmol) in 0.25 ml of 0.1 M phosphate buffer (pH 7.5), was mixed with *S*-acetylmercaptosuccinic anhydride (0.9 mg, approximately 5 μ mol) in 10 μ l of *N,N*-dimethylformamide (DMF) and incubated for 30 min at 30 $^{\circ}$ C with stirring to introduce thiol groups. Then, 50 μ l of 0.1 M Tris-HCl buffer (pH 7.0), 5 μ l of 0.1 M EDTA (pH 7.0) and 100 μ l of 1 M hydroxylamine (pH 7.0), were added to remove acetyl groups, and the mixture was incubated for 5 min at 30 $^{\circ}$ C. The reaction mixture was chromatographed on a column of Sephadex G-25 (2.0 $\times$ 45 cm) using 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA. Using the 4,4-dithiodipyridine method to determine the thiol group,¹⁰ the average number of thiol groups introduced per HRP molecule was 2.2.

The resulting mercaptosuccinyl HRP (approximately 0.5 mg, 12.5 nmol) in 2 ml of 0.1 M phosphate buffer (pH 6.0) was mixed immediately with the GMBS-acylated PMB solution, and then incubated for 30 min at 30 $^{\circ}$ C with stirring. The mixture was chromatographed on a column of Sephadex G-100 (2 $\times$ 40 cm) using 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1% BSA (buffer A) to remove any small molecular compounds remaining. Four-milliliter fractions were collected, and fractions 10 to 13, representing the main peak of pure enzyme activity, were chosen as a label in the ELISA. Using the 4,4-dithiodipyridine method to determine the thiol group,¹⁰ the conjugate was estimated to contain about 2.0 molecules of PMB per HRP molecule.

ELISA for Determination of PMB ELISA is based on the principle of competition between enzyme-labeled and unlabeled PMB for an immobilized antibody, followed by measurement of the marker enzyme activity of the immunocomplex bound to the solid phase. Briefly, the wells in microtiter plates (Nunc F Immunoplates I; Nunc, Reskilde, Denmark) were coated by loading 150 μ l anti-PMB IgG (0.25 μ g/ml) in 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM NaN_3 and allowing this to stand overnight at 4 °C. After the plates had been washed twice with 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% (v/v) Tween (PBS-T), they were incubated with 200 μ l of 2% BSA-PBS for 20 min at 37 °C to prevent nonspecific adsorption. The anti-PMB IgG-coated wells were then filled with 50 μ l of either PMB drug-treated samples, or PBS as a control, followed immediately by 50 μ l of the pooled PMB-HRP conjugate that had been diluted 1:500 in PBS-T. The wells were then incubated overnight at 4 °C and once again washed briskly with PBS-T.

The amount of enzyme conjugate bound to each well was then measured by the addition of 150 μ l of 0.42 mM TMB in 0.05 M acetate-citric acid buffer (pH 5.5) containing 3% dimethyl sulfoxide, 0.01% hydrogen peroxide, followed by incubation of the wells at 37 °C for a suitable period. Enzymatic activity was stopped by the addition of 50 μ l of 2.0 M H_2SO_4 to each well, and the resulting color intensity was measured spectrophotometrically at 450 nm using an ELISA analyzer (Molecular Devices; California, U.S.A.).

Bioassay Method The bioassay method for PMB was performed according to the method of United States Pharmacopeia (USP).

Pharmacokinetic Evaluation PMB tablets (1000000 U/tablet) were orally administered to the human subjects, together with 200 ml of water. Blood samples were collected at 10, 20, 30, 40, and 50 min, 1, 2, 3, 4 and 5 h post-administration, and the serum was stored at -20 °C until assayed for PMB concentration. The serum was diluted with buffer A to obtain PMB concentration appropriate for a measurement by ELISA.

RESULTS

ELISA for PMB The optimal quantities and optimal incubation time for each reaction were established. A standard dose-response curve obtained using the buffer system is shown in Fig. 2. The limits of drug detection by ELISA were between 10 pg and 10 ng/ml of PMB. For practical purposes, the working range was arbitrarily set between 32 pg and 4 ng/ml based on the precision data for the ELISA (Table 1), which reveals this developed ELISA to be a reproducible technique. The coefficients of variation for intra- and interassays between PMB concentrations of 32 pg to 4 ng/ml at four different levels each were 5.0 to 15.0% and 5.9 to 14.8%, respectively.

Detection of the drug in serum samples (50 μ l) was also carried out by adding known concentrations. The standard curve yielded was essentially the same as that in the buffer system.

Specificity of the Antibody The antibody specificity was determined by the displacement of bound PMB-HRP by other similar compounds. Values of the cross-reactivity were

Fig. 2. Standard Curve for PMB with Anti-PMB Antibody

The curve shows the amount (%) of bound enzyme activity for various doses of PMB (B) as a ratio of that bound using PMB-HRP alone (B_0). Each point represents the mean $\pm$ S.D. of 3 replicates.

Table 1. Precision of ELISA for PMB

	Added (pg/ml)	Estimated (pg/ml)	C.V. (%)
Intraassay	32.0	30.0 $\pm$ 4.5	15.0
	160.0	169.0 $\pm$ 8.5	5.0
	800.0	784.0 $\pm$ 77.0	9.8
	4000.0	4100.0 $\pm$ 500.0	12.2
Interassay	32.0	31.0 $\pm$ 4.6	14.8
	160.0	162.9 $\pm$ 10.9	6.7
	800.0	793.2 $\pm$ 46.6	5.9
	4000.0	3900.0 $\pm$ 500.0	12.8

Values represent the mean $\pm$ S.D. of a total of 5 experiments. C.V.; coefficient of variation.

defined as the ratio of each compound to PMB in the concentrations required for 50% inhibition of PMB-HRP binding to the antibody. The anti-PMB antibody showed 0.05% cross-reaction with polymyxin E. No detectable cross-reaction, however, was found in D-phenylalanine or L-2,4-diaminobutyric acid (L-Dab) (Table 2).

Comparison of ELISA and Bioassay The ELISA method was compared with a bioassay method using specific quantities of PMB in human serum. The bioassay technique analyzed 11 samples of various concentrations of PMB ranging from 0.5 to 50 mg/ml, and showed a linear relationship between the diameter of the inhibition circle and the standard PMB concentration.

ELISA determination was made using these PMB samples, and properly diluted to the drug-concentration range detectable by ELISA. Figure 3 shows that there was good correlation between the values determined by the 2 methods, and the plot was linear as predicted by the equation $y = 0.81x - 0.54$, where y is the concentration value determined by bioassay analysis and x is that determined by ELISA; the correlation coefficient was 0.996 ($n = 11$). The bioassay, however, was less accurate when PMB was present in serum, owing to the effect of serum proteins. The ELISA sensitivity of 10.0 pg/ml as described above is about 10000 times greater than a bioassay.

Measurement of PMB in Human Serum by ELISA after Oral Administration of PMB Figure 4 shows the

Table 2. Specificity of Anti-PMB IgG

Compound structure and name		% Cross-reaction (50%)
$\text{FA-L-Dab-L-Thr-L-Dab-L-Dab}$ $\begin{cases} \text{L-Dab-D-Phe-L-Leu} \\ \text{L-Thr-L-Dab-L-Dab} \end{cases}$	PMB	100
$\text{FA-L-Dab-L-Thr-L-Dab-L-Dab}$ $\begin{cases} \text{L-Dab-D-Leu-L-Leu} \\ \text{L-Thr-L-Dab-L-Dab} \end{cases}$	Polymyxin E	0.05
$\text{C}_6\text{H}_5\text{-CH}_2\text{-CH(NH}_2\text{)-COOH}$ D-Phenylalanine	D-Phenylalanine	<0.004
$\text{H}_2\text{NCH}_2\text{CH}_2\text{CH(NH}_2\text{)-COOH}$ L-2,4-Diaminobutyric acid	L-2,4-Diaminobutyric acid	<0.004

Fig. 3. Correlation of PMB Quantification by the Present ELISA and Bioassay Methods

time course of serum concentrations of PMB following its oral administration at a dose of 1000000 U/tablet to human subjects. PMB was rapidly absorbed, reaching mean peak concentration in serum of 47.3 ± 16.3 ng/ml at 30 min after dosing, then slowly decreased.

DISCUSSION

From evaluation in past studies,^{4,5)} administration of a small amount of PMB as an anti-endotoxin drug is believed to be a promising treatment for endotoxemia. For this method to be established, it must be quantitatively demonstrated that the administration of a small amount of PMB has a clinical effect without side effects; this requires micromasurement of the blood PMB concentration. However, since the lower limit of detection of PMB by the methods currently available is in the order of $\mu\text{g/ml}$, only a small amount of PMB in the blood cannot be measured. We therefore developed a highly sensitive ELISA for PMB to study its pharmacokinetics.

Fig. 4. Serum PMB Levels in Human Subjects ($n=3$) after Oral Administration of PMB (1000000 U/ Tablet)

At each interval, blood was collected and the serum PMB was measured by ELISA. Each point represents the mean $\pm$ S.D. of 3 subjects.

PMB immunogen and PMB-HRP conjugate (as a tracer) were prepared using GMBS with 2 selective, functional succinimide and maleimide groups as a cross-linking agent (Fig. 1). For the linear conjugation of PMB with MS.BSA, a molar ratio of PMB to GMBS of 1 : 1 was chosen for the first step in order to introduce the maleimide group of GMBS into one of the amino groups of PMB. PMB contains five free γ -amino groups from the L-Dab units. Srinivasa and Ramachandran¹²⁾ reported that there are differences in the susceptibility of the 5 amino groups of PMB to formylation, and the amino groups of Dab₈ and Dab₉ of PMB get formylated more readily than the other amino groups. Judging from this finding, the site of acylation of PMB by GMBS was predicted possibly to be at the Dab₈ or Dab₉ position of the cyclic heptapeptide moiety of the PMB molecule. In the second step, the maleimide group of GMBS carrying PMB was directly coupled to the thiol group of MS. BSA by thiolation under aqueous neutral conditions. Thereafter the PMB-BSA conjugate was easily separated from unreacted small compounds by dialysis. The conjugate thus synthesized elicited the production of anti-PMB antibody in both rabbits immunized, the highest titer being obtained 2 weeks after the third

booster injection.

PMB-HRP conjugate was also prepared by essentially the same 2-step procedure with GMBS, to eliminate production of antibody binding to the cross-linkage region of HRP. The conjugate thus obtained was stable in buffer A for more than 12 months during which no loss of enzyme or immuno activity was seen.

Using both anti-PMB IgG and PMB-HRP conjugate, an ELISA was developed capable of measuring as low as 32 pg/ml PMB (Fig. 2, Table 1). This sensitivity appears to be 10000 times more sensitive than a bioassay.

The specificity of this ELISA is shown in Table 2. The antibody specificity was directed mainly toward PMB, although there is a negligible cross-reactivity (0.05%) with polymyxin E which is structurally different only in the D-phenylalanine of cyclic heptapeptide moiety, and no cross-reactivity with D-phenylalanine or L-Dab. These results indicate that the antibody-recognition sites are both a moiety which is adjacent to D-phenylalanine and the D-phenylalanine in cyclic heptapeptide moiety of the PMB molecule.

PMB is usually used for intestinal sterilization by oral administration of 3000000 U/d PMB to adult patients with leukemia. It was reported that the blood endotoxin concentration is rapidly normalized by this dose of PMB.^{4,5} It is generally thought that orally administered PMB is not transported to the blood, but it is likely that a small amount of PMB may enter the blood even when it is orally administered. Therefore, we measured the serum concentration of PMB after its oral administration to human subjects using this ELISA (Fig. 4). After oral administration, PMB was rapidly absorbed from the gastrointestinal tract, reaching a mean peak concentration in serum of about 50 ng/ml at 30 min after dosing. Using this ELISA, we first determined the PMB kinetics

in human blood after oral administration of the drug. This ELISA may be sensitive enough to quantify PMB for pharmacokinetics study in human.

To the best of our knowledge, the metabolism of PMB has not been identified. Therefore, it is unclear whether the PMB detected in the blood was PMB itself or one of its metabolites. Further detailed work is necessary to understand the metabolism of PMB and cross-reactivity of the anti-PMB antibody to the PMB metabolites. However, this ELISA may be a valuable tool for studies of the pharmacokinetics and the pharmacodynamics of the anti-endotoxin drug, PMB.

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