Enzyme Immunoassay for Pepleomycin, a New Bleomycin Analog

Kunio Fujiwara, Motomi Yasuno, and Tsunehiro Kitagawa
Faculty of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi 1-14, Nagasaki 852, Japan

ABSTRACT

An antibody directed toward pepleomycin, a new antitumor antibiotic related structurally to bleomycin, has been produced in rabbits by immunization with a pepleomycin-protein conjugate which was prepared by a novel procedure of coupling pepleomycin to mercaptosuccinylated bovine serum albumin using N-(γ-maleimidobutryloyloxy)succinimide as a coupling agent. The antiserum was monospecific to pepleomycin and showed almost no cross-reactivity with a variety of other bleomycin analogs. An enzyme immunoassay for pepleomycin has been developed utilizing this antiserum and β-galactosidase-labeled pepleomycin. The lower limit of detection by this assay, which involves a double antibody technique for the separation of antibody-bound and free antigen, was 50 pg of pepleomycin per tube. Using this assay, drug levels were easily determined in blood and urine of rabbits following administration of pepleomycin in a single dose of 1.2 mg/kg i.v. This assay is also suitable for measuring pepleomycin in the presence of other drugs since the assay is not significantly affected by any other antineoplastic agents tested. Since pepleomycin is now undergoing clinical trial, the enzyme immunoassay of the drug will be a valuable tool in clinical pharmacological studies.

INTRODUCTION

PEP is a semisynthetic second-generation bleomycin analog which differs from bleomycin in its terminal side-chain structure (Chart 1). PEP has been shown to be more potent than bleomycin in the N-methyl-N'-nitro-N-nitrosoguanidine-induced rat gastric carcinoma and AH 66 ascites hepatoma and has also been shown to be 3 times more concentrated in the stomach than was bleomycin in the distribution study (26). Bleomycin sometimes produced several side effects, the most serious of which is pulmonary fibrosis (3, 5, 7). PEP has shown lower pulmonary toxicity in experimental animals (22, 25, 26). These advantages may allow PEP to have an antitumor effect superior to that of the present bleomycin, and the drug has been on clinical trial in Japan.

Investigations concerning PEP concentration in patients' plasma and tissues may provide a quantitative method for the individualization of dosage regimens to afford maximum chemotherapeutic benefit and to avoid toxicity. A sensitive and specific nonisotopic EIA procedure would be a valuable tool in clinical pharmacological studies.

This report describes the production in rabbits of an antibody specific to PEP using a novel procedure for preparing the immunogen, the labeling of PEP with β-Gal (EC 3.2.1.23) to act as a tracer, and the use of the rabbits in an EIA capable of detecting 50 pg of PEP. Optimal assay conditions were established, and the method was used to follow levels of PEP in the serum and urine of rabbits treated with the drug.

MATERIALS AND METHODS

Materials

PEP, bleomycins A₂, A₂'-b, A₆, B₂, and clinically available bleomycin HCl were kindly supplied by Dr. W. Tanaka, Nippon Kayaku Co., Tokyo, Japan. Bleomycin A₂'-b contained copper. However, the other bleomycin analogs and PEP were copper free. S-Acetylmercaptoasuuccinic anhydride, T-β-o-galactopyranosyl(1→4)-methylcoumarin, and BSA were purchased from the Nakarai Chemical Co., Kyoto, Japan. β-Gal (Lot 127818) from Escherichia coli was obtained from the Boehringer Mannheim Co., Mannheim, Germany. MBS was synthesized by the method of Kitagawa and Aikawa (14).

Synthesis of the Cross-Linking Agent

GMBS was synthesized from N-maleolaminobutyric acid and N-hydroxysuccinimide by a condensation reaction using N,N'-dicyclohexylcarbodiimide (21). To a solution containing γ-maleoylaminobutyric acid (204 mg, 1 mmol) in 5 ml of tetrahydrofuran, N,N'-dicyclohexylcarbodiimide (227 mg, 1.1 mmol) in 5 ml of methyldrofuran, N,N'-dicyclohexylcarbodiimide (227 mg, 1.1 mmol) was added dropwise at 4° for 20 min; this was followed by overnight stirring. After the precipitate (dicyclohexylurea) was removed by filtration, solvent was evaporated off, and the residue was subjected to silica gel column chromatography using dichloromethane as an eluent solvent. Evaporation of the fraction containing the desired product left 224 mg (80%) of GMBS in crystalline form (m.p. 130-132°). The values of elemental analyses of the product were entirely in agreement with the theoretical values.

Preparation of the Immunogen

The reactions involved in the preparative process used in this study are shown in Chart 2.

Chemical Modification of BSA. BSA was chemically modified with S-acetylmercaptoasuccinic anhydride essentially by the method of Klots and Heiney (17). The resulting compound, AMS-BSA, was purified by column chromatography on Sephadex G-75 with deionized water as an eluent solvent; this was followed by lyophilization to give almost quantitative yield. The number of thiol groups introduced into BSA was estimated spectrophotometrically according to the method of Ellman (8) and was found to be 17 ± 0.3 (S.D.).

Coupling Reaction. The conjugation reaction, involving the formation of covalent bonds between amino groups of PEP and thiol groups of chemically modified BSA, is done using the heterobifunctional cross-linking agent, GMBS. Ten mg (6.2 μmol) of PEP in 1 ml of 0.1 M phosphate buffer, pH 7.0, were allowed to react with 0.52 mg (1.85 μmol) of GMBS in 0.5 ml of methyldrofuran at 25° for 140 min. The reaction mixture was concentrated to about half-volume in a vacuum, and the aqueous solution was washed twice with 3 ml of an ether.
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Chart 1. Structures of PEP and some bleomycin analogs. The terminal amine component (R) is linked to the carboxy group of bleomycinic acid by an amide bond.

PEP-LEOMYCIN-NH2 + BSA-NH2

\[ \text{GMBS} \]

\[ \text{AMS, BSA} \]

\[ \text{AMS-} \text{BSA} \]

\[ \text{GMBS-acylated PEP} \]

\[ \text{BSA-NHCOCH2CHCOOH} \]

\[ \text{GMBS-acylated PEP} \]

\[ \text{PEP-GMBS-MS-BSA} \]

Chart 2. Chemical coupling of protein to PEP using thiolated BSA and PEP conjugated with a maleimide group.

dichloromethane (2:1, by volume) to remove any remaining free GMBS. At the same time, 10 mg of AMS-BSA (approximately 0.15 µmol) were incubated in 0.12 ml of 0.1 M hydroxylamine, pH 7.2, at 25° for 30 min to remove the protecting acetyl group. The resulting product (MS-BSA) was diluted with 3 ml of 0.1 M phosphate buffer, pH 7.0; added immediately to the GMBS-acylated PEP solution; and then incubated for 30 min with stirring. The mixture was applied to a 2.8 × 47-cm column of Sephadex G-100 equilibrated with 0.1 M phosphate buffer, pH 7.0, containing 3 M urea and developed with the same buffer. Then, the purified conjugate was examined spectrophotometrically and estimated to contain about 2 molecules of PEP per BSA molecule on the basis of its UV spectrum compared to those of PEP and BSA.

The Antiserum

Three white female rabbits were given s.c. and i.m. injections of 1.5 ml of the immunoien (approximately 1.0 mg of protein) emulsified in an equal volume of complete Freund's adjuvant. Booster injections were then given 3 times at biweekly intervals, using one-half the amount of the dose of the first immunization. The animals were bled from the ear vein every 2 weeks, and the samples of serum collected were stored at -30°.

Preparation of PEP-β-Gal

PEP-β-Gal was prepared using MBS as a coupling agent according to the method reported previously (1). PEP (0.23 mg; 0.15 µmol) in 1.0 ml of 0.1 M phosphate buffer, pH 6.0, and MBS (2.2 µg; 7.0 nmol) in 30 µl of tetrahydrofuran were mixed and incubated at 25° for 30 min. Then, 78 µg of β-Gal [0.14 µmol; a molecular weight of approximately 540,000 (6)] in 1.0 ml of 0.1 M phosphate buffer, pH 6.0, were further added, and the mixture was stirred for 30 min. The reaction mixture was applied to a Sephadex G-100 column (2 × 38 cm) equilibrated with 0.02 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% BSA, and 0.1% NaN₃ (Buffer A) and eluted with the same buffer. Both enzyme and immune activities in 5 µl of each fraction (3 ml/tube) were measured by the methods described below.

Measurement of β-Gal Activity

Enzyme activity was measured by the method of Kato et al. (11). Five µl of diluted enzyme solution were incubated with 0.15 ml of 0.1 M 7-β-D-galactopyranosyloxy-4-methylcoumarin in Buffer A at 30° for 30 min. The reaction was stopped by the addition of 2.5 ml of 0.2 M glycine-NaOH buffer, pH 10.3, and the 7-hydroxy-4-methylcoumarin liberated was measured spectrophuorometrically. The amount of conjugate was expressed in units of β-Gal activity, defining 1 unit of enzyme activity as the amount that hydrolyzes 1 µmol of the substrate per min.

EIA Method

EIA of PEP was performed by the double antibody method (27) and standardized as follows. Five µl of a 10% PEP-β-Gal (186 microunits), 50 µl of PEP or sample (as appropriate), and 50 µl of a 1:5000 solution of the antiserum in Buffer B (0.06 M sodium phosphate, pH 7.4, containing 0.01 M EDTA and 0.1% BSA) or, as a background control, 50 µl of a solution of normal rabbit serum diluted in Buffer B were mixed, giving a final reaction volume of 105 µl, and the mixture was incubated at 25° for 6 hr. Then 50 µl of a 5% solution of goat antirabbit IgG and 50 µl of 0.33% solution of normal rabbit serum were added. After an additional incubation for 3 hr, the immune precipitate was washed twice by addition of 1.0 ml of Buffer A and centrifuged at 3000 x g for 20 min in a refrigerated centrifuge. The supernatant was decanted, and the enzyme activity in the immune precipitate was measured.

RESULTS

Preparation of PEP-GMBS-MS-BSA. GMBS, a heterobifunctional cross-linking agent with 2 selective reactivities, was used to conjugate PEP with MS-BSA. The maleimide group was introduced onto the amino group of PEP by reaction with the succinimidyl ester of GMBS. AMS-BSA was converted to MS-BSA by treatment with 0.1 M hydroxylamine. Subsequently, after its dilution with a phosphate buffer, the MS-BSA was coupled to GMBS-acylated PEP. The compound produced (PEP-GMBS-MS-BSA) was readily water soluble and was purified by column chromatography on Sephadex G-100. The conjugate contained 2 mol of PEP per mol of BSA.

Labeling of PEP. MBS was used for the PEP acylation reaction at a MBS:PEP molar ratio of 1:22. Then, MBS-acylated PEP was coupled immediately with β-Gal in a molar ratio of 50:1 (total MBS:β-Gal). There was no loss of enzyme activity during the coupling reaction. The amount of immunoreactive enzyme activity of the conjugate formed was 30% of the enzyme activity of the conjugate formed.
Enzyme Immunoassay of PEP

The enzyme activity of the conjugate, measured by the use of 5 μl from each fraction at 30° for 5 min; O, immunoreactive enzyme activity, determined by the EIA described in "Materials and Methods" except that 5 μl of the conjugate and a 1:1000 solution of anti-PEP serum were used in the absence of PEP; D, competitive immunoactive enzyme activity, determined in the presence of 200 pg of PEP by the same manner.

Antibody Response. Antibodies against PEP were produced in each of 3 rabbits immunized with the PEP-GMBS-MS-BSA conjugate. Samples of serum collected 2 weeks after each booster injection were tested for their ability to bind to PEP-β-Gal. The PEP-β-Gal was incubated with solutions of the antiserum (or prebled serum) for 6 hr; this was followed by further incubation for 3 hr with the second antibody (goat anti-rabbit IgG and normal rabbit serum). The uptake of PEP-β-Gal in the precipitate served as a quantitative measure of bound antibody. Typical binding curves are shown in Chart 4 for serum from bleedings of one of the 3 rabbits. If the serum dilutions required to bind 20 microunits are compared as an indicator of antibody titer, the potency increased over 20-fold from the second booster injection (1:5,000 dilution; Bleeding 3) to the final bleeding (1:100,000 dilution; Bleeding 5). There is no significant binding when comparable dilutions of prebled serum were tested.

EIA for PEP. Experimental conditions for the EIA were decided by determining an optimal concentration of anti-PEP serum and of PEP-β-Gal and an optimal incubation time for the immune reaction of anti-PEP antibody with the labeled PEP (in the presence or absence of unlabeled PEP) and with a second antibody. After several sets of assay conditions were tried, it was found that a 1:5,000 solution of the antiserum obtained 8 weeks after the first immunization (Chart 4, Bleeding 5), a 1:10 solution of PEP-β-Gal (186 microunits), a 6-hr incubation of the antiserum with the antigen, and a further 3-hr incubation with

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**Table 1**

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<th>Added (pg/tube)</th>
<th>Estimated (pg/tube)</th>
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a Assay number of experiment. b Mean ± S.D.
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a second antibody provided an effective assay system at 25°.

A standard calibration curve of PEP quantification is presented in Chart 5. The lower limit of detection by the assay was at 50 pg of PEP per tube, and the working range was shown to be from 50 to 800 pg per tube. Quality control data within assay and between assay are shown in Table 1, revealing that this newly developed EIA is a reproducible technique. The coefficients of variation within assays and between assays, at 5 different levels of PEP each, ranged from 4.4 to 21.0% with an overall mean of 10%. These data were derived from 70 assays, or 140 determinations, since each assay was done in duplicate. The effect of normal human serum on the EIA is also shown in Table 1, proving that 50 μl of serum do not affect the EIA systems.

The antibody specificity was determined by measuring the displacement of bound PEP-β-Gal by several bleomycin analogs such as bleomycins A₂, A₂′-b, A₅, and B₂ and a clinically available bleomycin mixture. As indicated in Chart 5, all of these analogs showed almost no cross-reactivity (0.0018, 0.060, 0.027, 0.0055, and less than 0.0013% of PEP at the amount of the drug for a 50% inhibition for bleomycins A₂, A₂′-b, and B₂, the clinical mixture, and A₅, respectively.

We also examined the effect of other anticancer drugs on the EIA for PEP. The following drugs had no effect on PEP-β-Gal binding at a concentration of 0.15 mM: daunomycin, Adriamycin, actinomycin D, mitomycin C, and neocarzinostatin. Experiments with a microbiological assay using Bacillus subtilis EVO were also done in order to compare the sensitivity of PEP detection with that of the EIA, and they showed that the lower limit of detection by microbiological assay was such that PEP, 1 μg/ml in a 0.08-ml volume, could be measured. This is 2000 times less sensitive than this immunoassay.

Quantification of PEP in Rabbit Serum by EIA. Four mg of PEP were administered by rapid injection into the marginal ear vein of 2 rabbits, each weighing 3.4 kg. Blood was obtained from the ear vein before administration of the drug and at intervals thereafter. PEP content in the serum was determined directly by this EIA using 0.1 to 3 μl of serum within 6 hr. Samples of serum from 6 hr after administration, which therefore contained a much lower amount of PEP were treated with trichloroacetic acid prior to performing the assay to remove rabbit γ-globulin since the second antibody system, i.e., the goat anti-rabbit γ-globulin, used in this assay, would not precipitate the labeled drug-bound rabbit antibody quantitatively if more than 10 μl of rabbit serum were present (27). To each serum sample, an equal volume of 10% trichloroacetic acid was added, and the sample was centrifuged in a refrigerated centrifuge at 2000 x g for 15 min. The supernatant was then neutralized with 0.5 n NaOH; this was followed by quantification by this immunoassay method. The results in Chart 6 show a rapid initial clearance (distribution), followed by a slower elimination phase. Estimated from these curves, PEP initial and terminal serum half-lives were 46.5 ± 1.5 and 276 ± 12 min, respectively. Rabbit urine was also collected and assayed for PEP by the EIA. The 22-urine sample of 2 animals contained an average of 20.4 ± 3.52 μg of PEP per ml, and the excretion accounted for 55 ± 9.5% of the dose administered.

DISCUSSION

A novel preparation method of an immunogen to PEP, an antitumor antibiotic, was investigated. This method is based on the reaction of a maleimide group coupled to a hapten and a sulfhydrol group introduced into a protein by thiolation (Chart 2), as originally developed by several investigators (11, 12, 18) and by us (14). A carrier protein and a cross-linking agent were decided upon from the expectation that a hapten-protein conjugation product would be water soluble and stable, so as to act as an immunogen effectively in animals.

Carrier Protein. One of the most commonly used carrier proteins, BSA, was chemically modified to introduced AMS groups mainly onto the ω-amino group of lysine. The AMS-BSA can be stored safely at 4° for more than 1 year without any loss of thiol groups and can be changed easily and quantitatively into its deacetylated derivative MS-BSA by treatment with hydroxylamine. The MS-BSA with 17 sulfhydrol groups was highly water soluble and was coupled, immediately after dilution with buffer, to GMBS-acetylated PEP.

Cross-Linking Agent. GMBS with 2 selective functional groups of maleimide and succinimidyl ester was newly synthesized on the basis of the consideration that the maleimide group linked to the aliphatic carbon atom is relatively stable with an optimal reactivity to thiol groups in a near-neutral pH (10), and it would be sufficiently satisfactory for the conjugation reaction for preparation of the immunogen.

The present method is very mild and does not result in extensive (intra- or intermolecular) self-coupling of PEP or protein as occurs when other commonly used coupling agents are used (such as glutaraldehyde, disiocyanates, bisdiazonium salts, and carbodlimides). The conjugate thus prepared, PEP-GMBS-MS-BSA, with 2 mol of PEP per mol of BSA, elicited the production of a highly specific and sensitive antibody to PEP in each of 3 rabbits immunized.

In the EIA using antibody elicited with the immunogen containing a cross-linking agent, use of another cross-linker for conjugating the hapten to the enzyme as a tracer is desirable to avoid the possibility of cross-reaction of the antibody with

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**Chart 6.** Serum PEP levels in rabbits after a single i.v. injection of the drug. Two rabbits, each weighing about 3.4 kg., were given injections of 4 mg of PEP. At 11 time intervals, blood was collected, and the PEP content of the serum was determined.

![chart image](chart.png)

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cross-linking parts in the enzyme:hapten conjugate (23). A structurally different cross-linker, MBS, was therefore used to couple PEP to β-Gal in consideration of the fact that the anti-PEP antibody binds specifically to the PEP group and not to the β-Gal or cross-linking parts. The maleimide group was introduced first onto an amino group of PEP by the reaction with MBS and then coupled directly with thiol groups of β-Gal without affecting the enzyme activity, as can be done with several other hormones and antibiotics (1, 13–16). The conjugate thus obtained was stable in Buffer A at 4 °C for more than 1 year during which no loss of the enzyme and immune activities was observed using the EIA determinations.

Using anti-PEP serum and PEP-β-Gal as the tracer, a sensitive double-antibody EIA for quantification of PEP is described in this report. By varying the concentration of these reagents and the incubation periods, an optimal assay procedure has been established. This nonisotopic EIA is sufficiently sensitive to detect 50 pg of PEP per tube and is reproducible, as is shown by an overall mean coefficient of variation of 10% for within assays and between assays at 5 different levels of PEP. This assay is quite specific to PEP and does not detect bleomycin analogs which differ from PEP only by the substitution of the amine component on bleomycinic acid (Charts 1 and 5). This presumably indicates that the physicochemical forces involved in the determination of the specificity of the antibody include both steric hindrance and charge differences imposed by the substitutions of these amine components. However, Strong et al. have recently reported data concerning the specificity of antiserum produced in animals in bleomycin radioimmunoassay, showing that the antibody produced cross-reacted significantly with a variety of bleomycin analogs (4, 24). This EIA appears to be about 30 times more sensitive than the bleomycin radioimmunoassay at the amount of the drug required to inhibit immune binding by 50% [EIA, 0.089 pmol PEP; radioimmunoassay, 3.45 pmol bleomycin sulfate (24)]. It was also found that with this EIA no cross-reactivity existed with other anticancer drugs (daunomycin, Adriamycin, mitomycin C, actinomycin D, and neocarzinostatin), indicating that the EIA for PEP is appropriate for use in multiple chemotherapy since anticancer drugs are often used with other chemotherapeutic agents.

The immunoassay was used to follow the levels of PEP in the serum collected from rabbits at different times after the drug was injected i.v. A rapid biphasic disappearance of PEP from the rabbit serum was observed with a short serum initial half-life of the drug followed by a relatively long period of elimination. The excretion of PEP equivalents in the urine (based on a total dose administered) was found in a wide range, with an average of 55 ± 9.5% in 22 hr. This excretion rate is relatively high among antibiotics, and excretion appears to serve as a major route for disappearance of the antibiotic from the host, as in the case of bleomycin (2, 9, 19).

The EIA procedure for PEP presented here is highly specific, sensitive, reproducible, simple, nonisotopically safe to perform, and adaptable to analyses of a large number of samples. This is the first report to quantify PEP by a highly sensitive immunoassay method. This EIA will be a valuable tool in clinical pharmacological studies during clinical trials of PEP.

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