SOLID PHASE ENZYME IMMUNOASSAY OF TOBRAMYCIN

HIDEHIKO SUZUKI, HIROMI KATO, NAOKO NAKANO, SABURO YANO
and TSUNEHIRO KITAGAWA*

First Department of Internal Medicine, Toyama Medical and Pharmaceutical University Medical School, Sugitani, Toyama 930-01, Japan
*Department of Microbiological Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi, Nagasaki 852, Japan

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We have developed a competitive enzyme immunoassay (EIA) using co-polymer of styrene and maleate as the solid phase and glutaraldehyde for binding of the antibody to the solid phase. This assay showed a striking efficacy in its use for the assay of tobramycin (TOB). Furthermore, the pharmacokinetics of TOB were studied in six healthy male volunteers after the administration of 60- or 90-mg doses intramuscularly.

Several procedures have been developed for enzyme immunoassay (EIA) using polystyrene tubes[1], glass rods[2], or silicone pieces[3], as the solid phase. The solid phases are usually coated with antigens or antibodies by a simple adsorption[1,2]. On the other hand, glutaraldehyde has been shown to be a potent activation agent for immunoadsorbents[4,5]. In addition, KATO *et al.*[2] have reported that glass rods aminoalkylated with 3-aminopropyltriethoxysilane were strongly coupled with rabbit IgG in the presence of glutaraldehyde.

In this report we describe an EIA using cylinders of styrene and maleate co-polymer, with or without glutaraldehyde treatment, as the solid phase and its application to the assay of tobramycin (TOB).

Materials and Methods

Materials

Bovine serum albumin (BSA), Sephadex G-100, Sepharose 6B (Sigma Chemical Co., St. Louis, Mo. U.S.A.), β-D-galactosidase (Boehringer-Mannheim GmbH, Mannheim, Germany), glutaraldehyde, 4-methylumbelliferone (4-MU), 4-methyl-umbelliferyl-β-D-galactoside (Nakarai Chemical Ltd., Kyoto, Japan,), complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.), DEAE-cellulose (DE-52, Whatman Biochemicals Ltd., U.S.A.), and sorbitan mono-oleate (Tween 80) (Wako Pure Chemical Industries Ltd., Osaka, Japan) were obtained as indicated. Cylinders of styrene and maleate copolymer (8×5 mm) were kindly provided by Eiken Kizai Co., Tokyo, Japan. TOB was a gift from Shionogi Pharmaceutical Co., Ltd., Osaka, Japan.

Anti-TOB Antibodies

BSA-TOB conjugates were prepared by using *N*-(*m*-maleimide-benzoyloxy)succinimide (MBS) as a coupling reagent[6]. In brief, an 8 M urea solution of reduced BSA (0.3 μmole) was incubated with MBS-acylated TOB (50 μmole) for 2 hours at room temperature. The reaction mixtures were then chromatographed on a Sephadex G-100 column. The protein fraction eluted from the column was used for BSA-TOB conjugates. The conjugates were emulsified with complete Freund's adjuvant and injected intramuscularly several times into rabbits at monthly intervals. Anti-TOB antibodies were purified from the sera of the rabbits by ammonium sulphate precipitation, DEAE-cellulose chromatography, and lyophilization.

β-D-Galactosidase-*N*-(3-maleimido-propionyl-glycyloxy)succinimide (MPGS)-TOB Conjugates

The enzyme labelling was performed with the reagent MPGS in the place of MBS[6]. The MPGS-

acylated TOB (1 nmole) dissolved in 0.1 ml of 0.05 M phosphate buffer (pH 7.0) was incubated with β-D-galactosidase (97 pmole) for 2 hours at room temperature. The reaction mixtures were chromatographed on a Sepharose 6B column. The eluted peak fraction of the enzyme activity was used for the EIA of TOB.

Anti-TOB-coated Cylinders

The cylinders of styrene and maleate co-polymer were treated with anti-TOB antibodies in the presence of glutaraldehyde. The cylinders were immersed into 0.1 M phosphate buffer (pH 6.8) containing 0.01 mg/ml of IgG fraction of anti-TOB sera and 0.2% glutaraldehyde for 2 hours at room temperature. After treatment, the cylinders were washed twice with 0.01 M phosphate buffer (pH 7.0) supplemented with 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% NaN_3, 0.1% BSA (referred to as Buffer A), and stored in the Buffer A at 4°C until use. Anti-TOB-coated cylinders without glutaraldehyde treatment were prepared by procedures similar to those described above.

Human Volunteers

Six healthy male volunteers ranging in age from 24~44 years and in weight from 55~65 kg were studied. Their renal and liver function tests performed during this study were normal. The 60 mg or 90 mg doses of TOB dissolved in 1.5 ml of sterile distilled water were injected intramuscularly. Blood samples were drawn from an cubital vein at 0, 0.25, 0.5, 1, 1.5, 2.5, 4, 6, and 8 hours after injection.

EIA of TOB

The anti-TOB-coated cylinders were incubated with 50 μl of the β-D-galactosidase-MPGS-TOB conjugates (1: 40 diluted with Buffer A) and various amounts of TOB or serum samples in 0.5 ml final volume of 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl, 0.05% Tween 80, 0.02% NaN_3, and 1% BSA (referred to as Buffer B). After incubation for 5 hours at room temperature, each cylinder was washed twice with Buffer B and incubated with 5×10^{-5} M 4-methyl-umbelliferyl-β-D-galactoside in a final volume of 0.5 ml Buffer A at 30°C for 50 minutes. The reaction was stopped by adding 2 ml of 0.1 M glycine-NaOH buffer (pH 10.3), and the fluorescence intensity of 4-MU released was measured. The wave lengths used for excitation and emission analysis were 365 and 448 nm, respectively.

Bioassay of TOB

The concentrations of TOB were determined by the agar diffusion method with *B. subtilis* (ATCC 6633) as test organism and Difco antibiotic medium 5[7]. The holes punched into the agar (6 mm in diameter) were filled with 0.02 ml of serum or standard solution diluted in refrigerated normal pooled human serum (Consera, Nissui Seiyaku Co., Tokyo, Japan).

Results

Standard curves for the EIA of TOB are shown in Fig. 1. A strikingly good dose-response correlation was observed between 100 pg and 50 ng TOB in the glutaraldehyde-treated assays, although a small dose response was also demonstrated in the assays without glutaraldehyde treatment. However, the enzyme activity was negligible when β-D-galactosidase-MPGS-TOB conjugates were incubated with not antibody-coated cylinders in the presence or absence of glutaraldehyde (not shown).

The effect of the incubation time on the amounts of β-D-galactosidase-MPGS-TOB conjugates binding to the glutaraldehyde-treated cylinders was tested. As shown in Fig. 2, maximal binding of the enzyme activity was obtained at 5 hours and reached a plateau thereafter.

The precision of the assay was evaluated by assaying two sample sera at concentrations of 53.2 μg/liter and 3.9 μg/liter (Table 1). The standard deviations and coefficients of variation found were 7.4 μg/liter (13.9%) and 0.97 μg/liter (24.9%), respectively.

Fig. 3 shows serum concentration time curves for 60 mg TOB administered intramuscularly to human volunteers. The highest serum levels observed were 3.3 μg/ml at 60 minutes in EIA and 3.1 μg/ml at 30~60 minutes in bioassay, respectively. The half-lives in the elimination phase were 65.1 minutes

Fig. 1. Competitive EIA of TOB.

Anti-TOB-coated cylinders were incubated with 50 μl of the enzyme-labeled TOB (1 : 40 diluted with Buffer A) and various amounts of unlabeled TOB for 5 hours at room temperature. After washing, they were incubated with 5×10^{-5} M 4-methylumbelliferyl-β-D-galactoside for further 50 minutes. Intensity of fluorescence was measured as described in Materials and Methods. The assay with glutaraldehyde-treated cylinders (●), the assay with glutaraldehyde-nontreated cylinders (○). One unit of fluorescence intensity corresponds to that of 1×10^{-9} M 4-MU. Each point represents the mean and standard deviation of five assays.

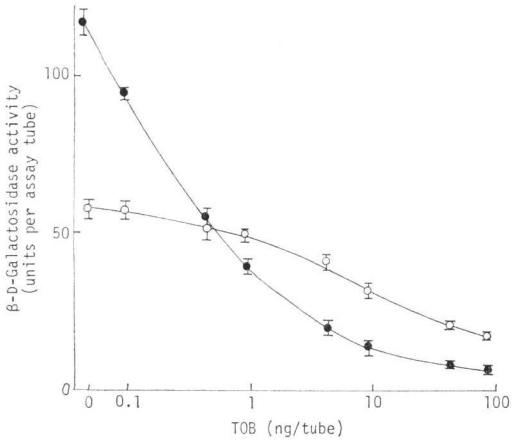


Fig. 2. Binding amounts of β-D-galactosidase-MPGS-TOB conjugates as a function of incubation time.

Anti-TOB-coated cylinders treated with glutaraldehyde were incubated with the enzyme-labeled TOB for the indicated times. At each point, the cylinders were incubated with 5×10^{-5} M 4-methylumbelliferyl-β-D-galactoside for further 50 minutes. One unit of fluorescence intensity corresponds to that of 1×10^{-9} M 4-MU.

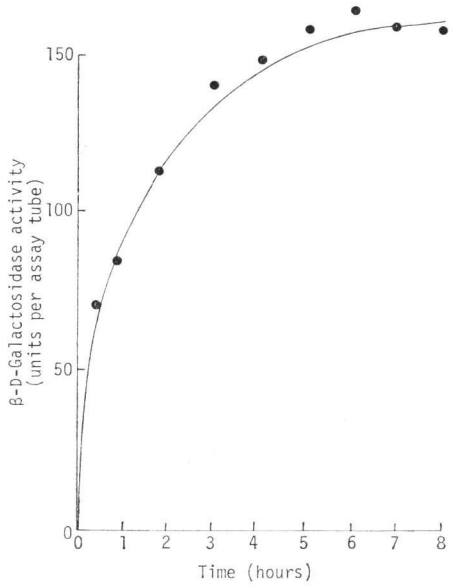
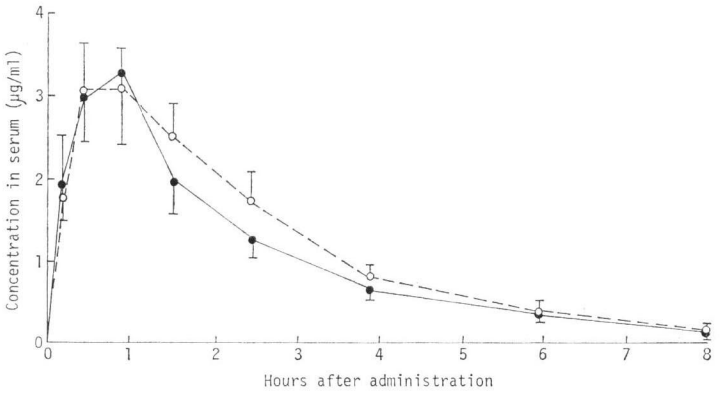


Fig. 3. Serum concentration time curves for 60 mg-TOB administered intramuscularly to healthy volunteers.

Each point represents the mean and standard deviation of serum samples from six volunteers. EIA (●), bioassay (○).



in EIA and 87.9 minutes in bioassay. In the case of 90 mg of TOB, the highest serum levels observed were 4.3 μg/ml at 30 minutes in EIA, and 4.7 μg/ml at 60 minutes in bioassay (Fig. 4). The half-lives in the elimination phase were 68.1 minutes in EIA and 89.7 minutes in bioassay.

Table 1. Precision of TOB assay.

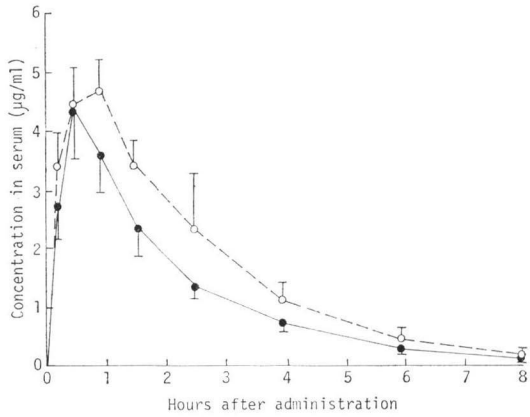
Inter-assay	n	x	SD	CV(%)
Sample 1	8	53.2 (μg/liter)	7.4	13.9
Sample 2	8	3.9 (μg/liter)	0.97	24.9

SD: standard deviation, CV: coefficient of variation.

The reliability of the present assay method was evaluated by comparison with the results obtained from bioassay with serum samples. The coefficient of correlation was r=0.92. The best line to fit the data was expressed by the equation: Bioassay=0.91×EIA+0.31, (n=96).

Fig. 4. Serum concentration time curves for 90 mg TOB administered intramuscularly to healthy volunteers.

Each point represents the mean and standard deviation of serum samples from six volunteers. EIA (●), bioassay (○).



Discussion

The assay we describe here is convenient for assaying of TOB. Firstly, centrifugation procedures are not required for separation of antibody-bound from free enzyme-TOB conjugates. Secondly, the assay can be carried out in the absence of excess second antibody and solid-phase second antibody (in the competitive EIA for antigen). In addition, the cylinders prepared in the present study are effective when stored for more than 2 months. We have also observed that non-specific binding of the enzyme-TOB conjugates was small enough to be negligible.

Glutaraldehyde has been found to be a coupling agent for proteins[8], an activator of immunoadsorbents[4,5], and an effective fixative for cellular preparations to be examined by electron microscopy[9]. KATO *et al.*[2] have reported that glutaraldehyde markedly enhanced the binding capacity of antibody-coated aminoalkylsilyl glass for antigens. Also, we have found that the cylinders lacking amino residues used here were apparently capable of binding antibodies in the presence of glutaraldehyde. Further experiments are in progress to examine the effects of glutaraldehyde on the binding of antibodies to various solid phases.

Pharmacokinetics of TOB have been usually examined by bioassay[7,10,11]. BLACK and GRIFFITH[11] have reported that peak serum concentrations of 1.14, 2.09, and 2.7 μg/ml were found within 1 hour after the intramuscular injection of 25, 50, and 75 mg doses of TOB. MEYERS *et al.*[10] also reported that an average peak serum level was 3.77 μg/ml following 100 mg intramuscular doses. In the present study, we have used the newly developed EIA to study the serum concentrations achieved after the intramuscular injection of 60 or 90 mg TOB. It was found that average peak serum levels were 3.3 μg/ml and 4.3 μg/ml, respectively. Our findings by the EIA showed that the half-lives of 60 or 90 mg TOB following intramuscular injection were 65.1 and 68.1 minutes, respectively, which is considerably shorter than previously reported[7,10]. In conclusion, this EIA offers a convenient and sensitive method for analysis of pharmacokinetics of TOB in the clinical study.

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