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Studies on Viomycin. XV. Comparative Study on the Specificities of Two Anti-viomycin Antisera by Enzyme Immunoassay[1)]

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A new viomycin-bovine serum albumin conjugate was characterized, and 19 viomycin molecules were found to be coupled with one molecule of the carrier protein. Enzyme labelling of viomycin with β-D-galactosidase was performed by a continuous two-step process using a maleimide succinimidyl ester type cross-linker. The optical conditions for enzyme immunoassay of viomycin using the new anti-viomycin antiserum and the enzyme labelled viomycin (double antibody method) were studied and a procedure with satisfactory accuracy and precision was developed. The specificities of two anti-viomycin antisera were compared by the enzyme immunoassay technique. Evidence that the enzyme immunoassay procedure is preferable to radioimmunoassay for a comparative study is also presented.

Keywords——enzyme immunoassay; bovine serum albumin; viomycin; hapten-protein conjugate; β-D-galactosidase; bifunctional reagent; hapten enzyme labelling; cross-reaction; anti-viomycin antiserum

In the past decade, drug-specific antibodies have been used extensively in the development of a variety of immunological assay procedures for measurement of the concentrations of drugs in the body fluids of man and of experimental animals.[2)] The development of immunoassays for proteins and for large peptide hormones is facilitated by the fact that most such substances are capable of eliciting specific antibody production in experimental animals. Small molecules are not usually antigenic by themselves, but can be made antigenic by covalent conjugation with a high molecular weight carrier molecule such as a protein.[2d,3)]

In the previous paper on the enzyme immunoassay of viomycin (VM)[4)] we reported a method for the selective conjugation of VM to bovine serum albumin (BSA) by a five-step process, essentially by a modification of the method of Erlanger *et al.*[3,5)] Although the VM-BSA conjugate obtained was fairly homogeneous, the coupling reactions were not easy. This experience let us study a new method for easy preparation of homogeneous hapten-protein conjugates to obtain specific antisera to haptens, and a part of the work has been presented.[6)] Further studies were performed to characterize the new VM immunogen and the new anti-VM antiserum and also on a convenient enzyme labelling of VM. In the present paper, we report these studies together with a comparative study by the enzyme immunoassay procedure on the specificities of the two anti-VM antisera prepared by the new and previous methods.

Materials and Methods

Materials——β-D-Galactosidase (Gal) from *Escherichia coli* was purchased from Boehringer Mannheim Co., Mannheim, Germany. Antibiotics, capreomycin and cephalexin (Shionogi Pharmaceutical Ind., Osaka), ampicillin (ABPC) and dihydrostreptomycin (Takeda Chemical Ind., Osaka) and tuberactinomycin (Toyojozo Co., Shizuoka), used were commercial products, VM,[7)] acetylviomycin,[7)] 1-monoacetylviomycin,[8)] succinylviomycin,[4)] *N*-(*m*-maleimidobenzoyloxy)succinimide (MBS),[4,9)] and *N*-(γ-maleimidobutyryloxy)succinimide (GMBS)[10)] were prepared according to the methods cited. Other reagents used in this work were of reagent grade.

Preparation of New VM-Immunogen (VM-MBS-BSA Conjugate)——VM-MBS-BSA conjugate was prepared by a three-step process using a hetero bifunctional reagent, MBS, as a cross linker and BSA as a carrier

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protein according to the reported method.[6a)]

Assay of the Number of Viomycin Molecules introduced per Molecule of BSA——Two methods were employed for the assay: Method A: ultraviolet (UV) spectroscopic method. The absorbance of a 6 M urea solution of VM (1 mg/ml) was 28.8 at 268 nm, while that of a 6 M urea solution of BSA (1 mg/ml) was 0.45. Thus, the amount of MBS-acylated VM or BSA was calculated from the absorbance (X=6.23) at 268 nm of the conjugate dissolved in 6 M urea solution (1 mg/ml), and the number of VM molecules introduced (Y) per molecule of BSA was calculated from the following equation, which assumes the molecular weight of BSA to be 67000 and that of VM derivative coupled to BSA to be 885:

$$Y = \frac{(X-0.45)/28.8}{885} \div \frac{1-(X-0.45)/28.8}{67000} = 19.0$$

Method B: Calculation based on the molecular weight determined by SDS polyacrylamide gel electrophoresis. VM-MBS-BSA conjugate gave almost a single band on electrophoresis and its molecular weight was estimated according to the method of Dunker and Rueckert[11)] by comparison of its migration distance with those of reference proteins to be 83000 ± 3000. The number of coupled VM molecules (Y) was then calculated from the following equation:

$$Y = (\text{molecular weight of the conjugate} - 67000)/885 = 18.1 \pm 3.5$$

Immunization——Two albino female rabbits were injected subcutaneously and intramuscularly with 1.0 ml of the conjugate (approximately 1.0 mg protein) emulsified in an equal volume of complete Freund's adjuvant. Four booster injections were given at biweekly intervals, each at a dose equal to half that of the first injection. The rabbits were bled from an ear vein every ten days and the sera were stored at −30°C.

Preparation of Viomycin-β-D-Galactosidase (VM-Gal) Conjugate——VM-Gal conjugate was prepared with GMBS as the coupling reagent by a convenient enzyme labelling method.[10)] A solution of VM (0.5 mg) in 0.02 M sodium phosphate buffer (pH 7.0, 1 ml) was incubated with 30 μl of tetrahydrofuran solution of GMBS (50 μg) at 25°C for 30 min and then the mixture was dropped into a solution of Gal (50 μg) in 0.1 M phosphate buffer (pH 7.0, 1 ml) with vortex mixing. After further incubation at 30°C for 2 h, the reaction mixture was directly chromatographed on a Sepharose 6B column (1.8×30 cm) with 0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 N NaCl, 1 mM $MgCl_2$, 0.1% BSA and 0.1% NaN_3 (buffer A) as an eluent. The enzyme activity of a 5 μl aliquot of each VM-Gal conjugate fraction (4.7 ml/tube) was measured by the method described below.

Enzyme Immunoassay (EIA) of VM——The assay of VM was performed by the double antibody method[12)] and standardized as follows: 50 μl of VM-Gal (50 μunits) and 50 μl of an appropriate amount of VM or unknown sample and 100 μl of antiserum diluted 1: 10000 with buffer B (0.05 M sodium phosphate, pH 7.4, containing 0.01 M ethylenediaminetetraacetic acid (EDTA) and 0.1% BSA) were mixed and incubated at room temperature for 3 h. Then 50 μl of 20-fold diluted goat anti-rabbit IgG and 50 μl of 300-fold diluted normal rabbit serum were added. After further incubation for 12 h, the immune precipitate was washed twice by addition of 1 ml of buffer A and centrifugation at 3000×g for 20 min in a refrigerated centrifuge. The supernatant was decanted and the enzyme activity in the immune precipitate was measured.

Measurement of Enzyme Activity——Enzyme activity was measured spectrofluorometrically with 7-β-D-galactopyranosyloxy-4-methylcoumarin as a substrate.[4)] The amount of VM-Gal was expressed in units of Gal activity, and 1 unit of enzyme activity was defined as the amount that hydrolyzes 1 μmol of the substrate per min.

Cross-Reactivity——The relative amount of VM analog required to reduce the initial binding of VM-Gal with both anti-VM antisera (both dilution, 1: 10000) by half was calculated from standard curves determined by the EIA procedure, in which the mass of VM was arbitrarily taken as 100%.

Results

Physico-Chemical Properties of VM-Immunogen

VM–MBS–BSA conjugate prepared according to the reported method showed on SDS polyacrylamide gel electrophoresis a homogeneous single band which was clearly distinguished from that of BSA. The number of VM molecules coupled per BSA was assumed to be about 19 by the UV absorption method and 18±3.5 from the molecular weight of the conjugate as described in "Materials and Methods."

Enzyme Labelling of VM

Acylation of VM with a heterobifunctional reagent, GMBS, was performed at a molar ratio of 10: 1 at 30°C for 30 min. GMBS-acylated VM formed in the reaction mixture was *used directly* for enzyme labelling of VM without separation of the unreacted cross-linker. The maleimide function of GMBS-acylated VM was conjugated with free thiol groups of

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Table I. Precision and Accuracy of Enzyme Immunoassay of Viomycin

	Added (ng/tube)	Estimated[a] (ng/tube)	Recovery (%)	CV (%)	n[b]
Variation, within-run	0.1	0.0951±0.0685	95.1	72.0	10
	0.3	0.329±0.108	109.7	32.8	10
	1.0	0.997±0.128	99.7	12.8	10
	3.0	2.97±0.19	99.0	6.4	10
	10	9.83±0.43	98.3	4.4	10
	30	29.4±1.8	98.0	6.1	10
Variation, between-run	0.1	0.1037±0.0421	103.7	40.5	7
	0.3	0.32±0.122	106.7	38.1	7
	1.0	0.989±0.378	98.9	38.2	7
	3.0	3.02±0.22	100.7	7.3	7
	10	9.94±0.78	99.4	7.8	7
	30	30.1±1.7	103.3	5.6	7

a) Values are means±S.D.
b) Number of assays.

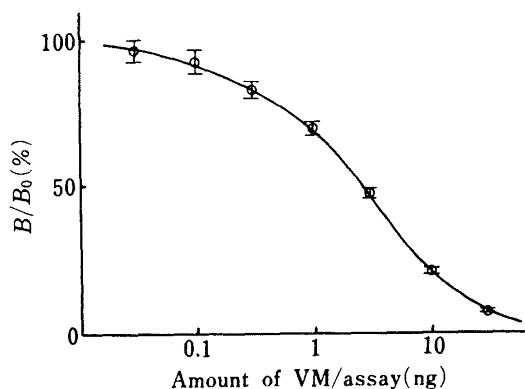
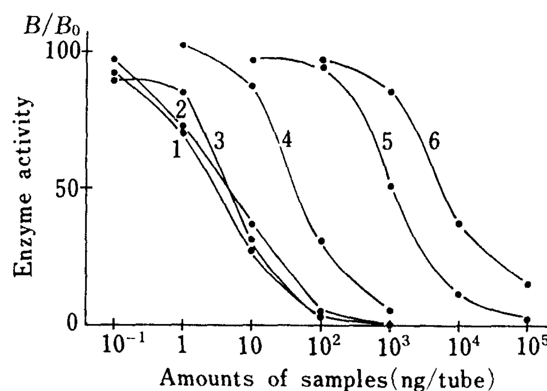


Fig. 1. Standard Curve for Enzyme Immunoassay of Viomycin

Enzyme activity of the bound conjugate is expressed as B/Bo %, where Bo is without and B is with viomycin competition.



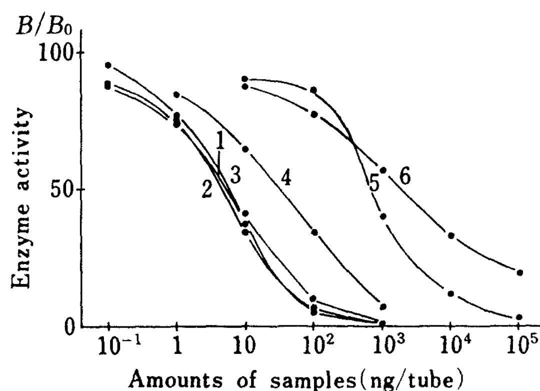


Fig. 2. Standard Curves for Enzyme Immunoassay of Viomycin Analogs

Upper and lower figures show the standard curves against anti-VM-MBS-BSA and anti-VM-suc-BSA antisera, respectively. Gurves in the figures are as follows; 1, viomycin; 2, 1-monoacetylviomycin; 3, succinylviomycin; 4, acetylviomycin; 5, tuberactinomycin; 6, capreomycin.

β-D-galactosidase under mild conditions. No reduction in the enzyme activity was observed during the enzyme labelling process. The reaction mixture was chromatographed on a Sepharose 6B column with buffer A as an eluent and the peak fraction of enezyme activity was used for enzyme immunoassay of VM.

Enzyme Immunoassay

Inhibition of the binding of VM–Gal conjugate to antisera by VM is shown in Fig. 1. Addition of increasing quantities of the drug resulted in a progressive decrease in the amount of the conjugate precipitated.

Precision and accuracy data for EIA of VM are summarized in Table I. Recoveries of various doses of VM ranging from 0.1 to 30 ng were satisfactory (95 to 110%). The coefficients of variation of within-run or between-run experiments were less than 7.8% for doses of 3.0 to 30 ng and higher than 10% for samples ranging from 0.1 to 1 ng.

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Antibody Specificity

The specificities of the present and the previous[4] anti-VM antibodies were compared by cross-reaction studies applying EIA of VM. Since the tuberculostatic antibiotic VM has been used clinically in combination with other antibiotics, interference with the EIA by the antibiotics dihydrostreptomycin, ampicillin and cephalexin was first examined; all of them showed negligible interference (data not shown). Cross-reactivities of five VM analogs against the present and previous anti-VM antisera were studied by the EIA procedure. Standard curves of VM analogs were almost parallel to that of VM as shown in Fig. 2. Fifty percent cross-reaction values for VM analogs are listed in Table II.

Table II. Specificities of the Two Anti-viomycin Sera

Sample	Per cent cross-reaction (CR 50%)	
	Anti-VM–MBS–BSA	Anti-VM-suc-BSA
Viomycin	100	100
1-Monoacetylviomycin	54.5	119
Acetylviomycin	29.0	89.3
Succinylviomycin	36.7	82.0
Tuberactinomycin	1.69	4.79
Capreomycin	0.013	0.256

The cross-reaction values were determined as described in "Materials and Methods." Anti-VM-MBS-BSA and anti-VM-suc-BSA antisera were elicited in rabbits immunized with VM-MBS-BSA[6a] and VM-suc-BSA[4] conjugates, respectively.

Every VM analog cross-reaction value with the present antiserum is smaller than that with the previous one. Consequently, the present antibody is concluded to be more specific for VM than the previous one.

Discussion

Enzyme labelling of VM molecules was performed by the improved enzyme labelling method[10] in a continuous two-step process using another cross-linker, GMBS. This differs from MBS by the substitution of *N*-butyric acid for benzoic acid, which avoids a cross-reaction of VM antibody with MBS, since MBS has been used as the cross-linker for the preparation of VM-immunogen.[6a] Enzyme immunoassay of VM and its analogs was performed according to the procedure for EIA of VM.[4] VM–GMBS–Gal (50 μunits) as the tracer and 1: 10000 dilution of newly prepared anti-VM antiserum were used instead of the corresponding reagents in the standard procedure. In the previous papers for EIA of VM,[4,6a] the methods for the assay have been reported, but not the precise conditions for accurate assay of VM. Thus, the optimal conditions for EIA of VM as well as its precision and accuracy were examined and the results are reported here; they are satisfactory. The specificities of the present and previous anti-VM antisera against VM were compared by means of the immunoassay using structurally related analogs of VM as common inhibitors in cross-reactions. All standard curves measured for the VM analogs with both of the anti-VM antisera were almost parallel to the corresponding standard curves of VM. Similar tendencies have been observed for cross-reactions of angiotensin I[13] or penicillin[6b] analogs measured by EIAs. This feature of the EIA method is advantageous for estimation of the specificity of an antiserum as compared with the radioimmunoassay method. Thus, the cross reaction values, which depend largely on the standard and on competitive analog concentrations in radioimmunoassay,[14] are less dependent in EIA. The reason for this could be that separation of free antigen from the labelled antigen is easy and complete with the enzyme label because of the large difference in molecular weights, while this is not the case with the isotope label. The contaminating antigen molecules in the isotope label, even though the amount may be small, must affect the immunological competi-

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Table III. Cross Reaction of Capreomycin at Different B/Bo Values

B/Bo (%)	Per cent cross-reaction Amount of contaminating VM in the label 0	50 pg	100 pg
80	0.017	0.0088	0.0065
70	0.014	0.0081	0.0058
50	0.013	0.0069	0.0029
30	0.015	0.0042	0.0009
10	0.015	0.0021	Not detected

The values were determined using anti-VM-MBS-BSA and the enzyme-labelled viomycin with (50 and 100 pg) and without viomycin contamination.

tion between the labelled antigen and the tested analog against the antiserum. The standard curves of the analogs, especially those showing very low cross reactivities, would not parallel that of the standard sample in radioimmunoassay.

To confirm this, the effect of VM contamination (50 and 100 pg) in the enzyme-labelled VM on the cross-reactivity of capreomycin was studied, since it showed the lowest cross-reaction value (Table II), and the results are summarized in Table III. The values at B/Bo % from 80 to 10 are gradually diminished by contamination, and the reduction is greater with 100 pg than with 50 pg, while the original corresponding values are almost constant. Judging from this, we concluded that contamination of the cold molecules in the label disturbs the measurement of the true cross-reactivity of an analog against the standard in the immunoassays. Although the specificity of the anti-VM-MBS-BSA and that of the anti-VM-suc-BSA were quite similar, a comparison of their immune specificities by the present EIA method showed that the present anti-VM antiserum is more specific to VM than the previous one, in terms of the crossreaction values of the VM analogs.

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