ENZYME IMMUNOAASSAY OF NEOCARZINOSTATIN USING β-GALACTOSIDASE AS LABEL*

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(Received May 18, 1981)

A novel convenient procedure was introduced for enzyme labelling of neocarzinostatin (NCS) with β-D-galactosidase using a new hetero-bifunctional reagent N-(γ-maleimidobutyryloxy)succinimide (GMBS). With the enzyme labelled NCS and a rabbit anti-NCS serum which was elicited in a rabbit immunized with NCS in complete Freund's adjuvant, a highly sensitive enzyme immunoassay which can quantify a femto mol order NCS was developed. Accuracy and precision of the assay as well as a comparison with the immunodiffusion method were studied with satisfactory results. This enzyme immunoassay of NCS was applicable to monitoring serum NCS levels of patients in clinical treatment of the antitumor agent.

Keywords — enzyme immunoassay; immunoassay; β-galactosidase; enzyme labelling; neocarzinostatin; antitumor agent; hetero-bifunctional reagent; N-(γ-maleimidobutyryloxy)succinimide; serum drug level; immunodiffusion

INTRODUCTION

An important contribution of radioimmunoassays to the biological sciences and to clinical medicine has been the development of sensitive, specific, precise, rapid and convenient assay methods for the quantitative determination of physiologically important substances in body fluids of human and experimental animals. Recent studies of enzyme labelling instead of radioisotopes for immunoassay have been extending a new field of enzyme immunoassay which may overcome serious drawbacks inherent in the radiolabelling.1-4) We have introduced a new two-step process for enzyme labelling of antigens using a hetero-bifunctional reagent.5-9) The method consists of acylation on amino groups of an antigen or antibody to introduce a maleimide group by active ester of the cross-linker, in the first step. A successive coupling of the maleimide group introduced onto the antigen, with thiol groups of enzyme β-D-galactosidase in a neutral aqueous condition is the second step. Since the maleimide group is not stable in aqueous solution,10) separation methods of the maleimide carrying antigen or antibody from the excess cross-linker, in the first step, had to be performed as quickly,5,6) or moderately7-9) as possible, to avoid decomposition of the unstable maleimide group.

Taking the results of our recent study10) on the properties of hetero-bifunctional cross-linkers into account, a modified quick enzyme labelling procedure which does not require a separation process of the excess reagent and diminishes decomposition of the unstable maleimide group was developed for enzyme immu-

* This work was presented at the 100th Annual Meeting of Japanese pharmaceutical Society held at Tokyo, April 5th, 1980.
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Enzyme Immunoassay of Neocarzinostatin

Enzyme immunoassay of neocarzinostatin (NCS), an antitumor antibiotic isolated from the culture filtrate of *Streptomyces carzinostaticus*. NCS is highly cytotoxic and in mammalian cells it inhibits DNA synthesis. Pharmacokinetic studies of NCS showed that NCS was extremely rapidly excreted into urine and rapidly inactivated by proteolysis in serum. This limits the way of treating NCS in clinical use. A clinical adult dose (1.3 mg/d) of NCS is very small and it has been given to a patient via an intravenous drip infusion method in a couple of hours in combination with other drugs. Consequently, highly sensitive and specific assay is required to measure the serum concentration of NCS in clinical use. Recently, three sensitive immunological assays of NCS, radioimmunoassay, fluorescent polarization assay and enzyme immunoassay, which uses peroxidase as the labelling enzyme, have been reported. All of them were used to determine serum NCS levels of experimental animals that were injected by one shot with a 77-to 385-times weight/kg dose of NCS used for an adult patient. The present study was undertaken to develop a highly sensitive and precise enzyme immunoassay of NCS which uses β-D-galactosidase as the labelling enzyme and can monitor the serum NCS level of clinical patients as an extension of a series of sensitive enzyme immunoassay of hormones and drugs.

MATERIALS AND METHODS

**Chemicals and Drug** — β-D-Galactosidase (Gal) from *Escherichia coli* was bought from

![FIG. 1. Elution Profiles of β-Gal-NCS Conjugate from a Sepharose CL-6B Column](image)

- Open circle: enzyme activity of the enzyme conjugate (5 μl) prepared from β-Gal and GMBSacylated NCS. Solid circle: immune reactivity of the enzyme conjugate. The reactivity was measured by enzyme immunoassay described in "MATERIALS AND METHODS" in which 5 μl of the conjugate and a 10³-fold diluted solution of anti-NCS serum was used in the absence of NCS. Solid square: competitive immune reactivity of the conjugate. The reactivity was determined in the presence of 100 ng NCS by the same manner.

![FIG. 2. Typical Standard Curve for Enzyme Immunoassay of NCS (Open Circle), together with Cross Reactivities of Various Antigens in the Assay](image)

- Enzyme activities of the bound conjugates (B/B₀) in the assay were expressed for daunomycin (■), bleomycin (△), ampicillin (□), cephalaxin (●), myoglobin (▲), cytochrome C (○), and pepsin (◇).
- B₀: enzyme activity of the bound conjugate without competition of an antigen. B: enzyme activity of the bound conjugate with competition of an antigen.
Boehringer Mannheim Corp. Mannheim, Germany. NCS was supplied by Yamanouchi Pharmaceutical Ind. Ltd. Tokyo. 7-β-D-Galactopyranosyloxy-4-methylcoumarin, mercaptotoethanol, γ-aminobutyric acid, maleic anhydride and EDTA were purchased from Nakarai Chemicals, Kyoto. Complete Freund’s adjuvant and agarose were obtained from Difco, Detroit, Michigan, U.S.A. Sepharose CL-6B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) was obtained from Sigma Chemicals, St. Louis, Mo., U.S.A. Dicyclohexylcarbodiimide and N-hydroxysuccinimide were bought from Protein Research Foundation, Minoh, Osaka. All other chemicals used were of reagent grade.

Preparation of N-(γ-Maleimidobutyryloxy)-succinimide (GMBS): γ-Aminobutyric acid was converted to γ-maleimidobutyric acid according to the method of Rich et al. 21) and then esterified with N-hydroxysuccinimide using dicyclohexylcarbodiimide applying the similar method of Kitagawa and Aikawa 5) to give GMBS as white needles, mp 130–131°C. NMR; δ ppm in CDCl₃: 6.91 (2H, s, CH =CH), 3.80 (2H, t, J =6Hz, N-CH₂⁻), 3.07 (4H, s, COCH₂-CH₂-CO), 2.81 (2H, t, J =6Hz, CH₂-COO⁻).

Buffers: Buffer A: 0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% BSA and 0.1% NaN₃. Buffer B: The phosphate-EDTA buffer, pH 7.4 (2.12 g Na₂HPO₄·2H₂O, 745 mg EDTA·2Na·2H₂O, and 200 mg of BSA were dissolved in distilled water with a final volume of 200 ml). PBS: 0.02 M sodium phosphate buffer, pH 7.0, containing

| Sample and interference                      | Added (pg/tube) | Estimated (pg/tube) | Recovery (%) | CV | n
<table>
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<tr>
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<tbody>
<tr>
<td>Variation within-run, NCS alone</td>
<td>1000/3</td>
<td>31.6 ± 4.72</td>
<td>95.0</td>
<td>14.9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>100.4 ± 10.1</td>
<td>100.4</td>
<td>10.1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1000/3</td>
<td>350.0 ± 15.2</td>
<td>99.1</td>
<td>4.6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1040.0 ± 50.0</td>
<td>104.0</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10000/3</td>
<td>3390.0 ± 300.0</td>
<td>101.3</td>
<td>8.9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>10800.0 ± 1180.0</td>
<td>108.0</td>
<td>10.9</td>
<td>10</td>
</tr>
<tr>
<td>Variation between-run, NCS alone</td>
<td>1000/3</td>
<td>32.5 ± 6.40</td>
<td>97.6</td>
<td>19.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>103.0 ± 12.7</td>
<td>103.0</td>
<td>12.3</td>
<td>3</td>
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<tr>
<td></td>
<td>1000/3</td>
<td>332.9 ± 28.4</td>
<td>100.3</td>
<td>8.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1030.0 ± 50.0</td>
<td>103.0</td>
<td>4.9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10000/3</td>
<td>3340.0 ± 380.0</td>
<td>100.2</td>
<td>11.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>10100.0 ± 1480.0</td>
<td>101.0</td>
<td>14.7</td>
<td>3</td>
</tr>
<tr>
<td>Effect of normal human plasma 10 μl</td>
<td>1000/3</td>
<td>31.4 ± 5.34</td>
<td>94.3</td>
<td>17.1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>102.7 ± 14.4</td>
<td>102.7</td>
<td>13.8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1000/3</td>
<td>318.4 ± 18.4</td>
<td>95.5</td>
<td>5.8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1005.0 ± 67.0</td>
<td>100.5</td>
<td>6.7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10000/3</td>
<td>3440.0 ± 260.0</td>
<td>103.3</td>
<td>8.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>10800.0 ± 1760.0</td>
<td>108.0</td>
<td>15.9</td>
<td>6</td>
</tr>
</tbody>
</table>

a) Values are the mean ± S.D.

b) Number of assay times.
Enzyme Immunoassay of Neocarzinostatin

0.1 M NaCl.

Preparation of Antisera — Antiserum specific to NCS (anti-NCS) was elicited in a Japanese albino female rabbit immunized with an emulsion prepared from a saline solution of NCS (1.3 mg/2 ml) and the equal volume of complete Freund’s adjuvant by multiple subcutaneous and intramuscular injections. Two booster injections were performed at a biweekly intervals with a half and one-fourth doses of that of the first for second and third injections, respectively. Ten days after the last injection, the rabbit was bled from an ear vein and the serum obtained was heated at 50°C for 30 min and then kept at −30°C until used.

Antiserum to rabbit IgG was elicited in a goat immunized with rabbit IgG emulsified in complete Freund’s adjuvant.

Preparation of Enzyme Labelled NCS — To a vigorously stirring solution of NCS (1.07 mg, 10⁻⁷ mol) dissolved in 2 ml of 0.02M sodium phosphate buffer, pH 7.0, was added 10 μl of tetrahydrofuran solution of GMBS (2.8 μg, 10⁻⁸ mol). The mixture was incubated at 30°C for 40 min. One ml of the reaction mixture was then added dropwise to a stirring solution of β-D-galactosidase (50 μg, 9.3 × 10⁻¹¹ mol) in 0.95 ml of 0.02M sodium phosphate buffer, pH7.3, and the solution was incubated at room temperature for 2 h. The reaction was terminated by an addition of 10⁻⁴M mercaptoethanol solution (50 μl) in 0.02M sodium phosphate buffer pH 7.3. Then, an equal volume of buffer A was added. The enzyme labelled NCS was chromatographed on a Sepharose CL-6B column (1.5 × 43 cm) with buffer A as the eluent. Both enzyme and immune activities in 5 μl of each fraction (3 ml/tube) were measured by the methods described below.

Measurement of Enzyme Activity — The β-D-galactosidase activity was determined as reported by Kitagawa et al. 7) The amount of enzyme labelled NCS was expressed as units of β-D-galactosidase activity, defining 1 U enzyme activity as the amount that hydrolyzes 1 μmol 7-β-D-galactopyranosyloxy-4-methylcoumarin

![Graph 3: Correlation of Values for NCS Concentration obtained by Enzyme Immunoassay and a Single Radial Immunodiffusion Method](image)

**FIG. 3.** Correlation of Values for NCS Concentration obtained by Enzyme Immunoassay and a Single Radial Immunodiffusion Method

![Graph 4: Time-course of NCS Concentrations in the Serum Samples of Three Patients measured by Enzyme Immunoassay](image)

**FIG. 4.** Time-course of NCS Concentrations in the Serum Samples of Three Patients measured by Enzyme Immunoassay

The concentrations are given logarithmic amounts (μg/ml) against time after the start of i.v. injection of the drug via drip infusion method.
per min.

**Enzyme Immunoassay** — Buffer B was used as a dilution and incubation media for enzyme immunoassay unless otherwise stated. The enzyme conjugate (13 μU) and graded amounts of NCS or samples were incubated with 100 μl of a 5 x 10^4-fold diluted solution of anti-NCS in a final assay volume of 250 μl at 25°C for 8 h. Then, 50 μl of normal rabbit serum (dilution, 1:500) and 50 μl of a 20-fold diluted anti-rabbit IgG were added. After further incubation at 25°C for 16 h, 1 ml of buffer A was added to the mixture and then, the bound enzyme conjugate was precipitated by centrifugation. The supernatant was removed and the enzyme activity of the precipitate was measured using 7β-D-glactopyranosylxy-4-methyl-coumarin as substrate. Each assay was run at least in triplicate.

**Single-radial Immunodiffusion Assay** — Single-radial immunodiffusion studies were performed according to the method of Nancini et al. with a little modification. A melted 7.5 ml of 3% agarose solution in PBS was mixed with 7.5 ml of a 15-fold diluted solution of anti-NCS in PBS and the mixture was poured onto a glass plate to give a 1 mm thick layer. Holes 2 mm in diameter were punched in the gel and 2 μl of PBS solution of NCS (1000, 800, 500, 400, 250, 200, or 125 ng) was placed in each hole. After incubation at 25°C for 20 h, the area of the resulting halo was determined.

**RESULTS**

Acylation of NCS with a hetero-bifunctional reagent GMBS was performed under a limited molar ratio 10:1 at 30°C for 40 min. GMBS-acylated NCS formed in the reaction mixture was used directly for enzyme labelling of NCS without a process to separate the unreacted cross-linker. The maleimide function of GMBS-acylated NCS was conjugated with free thiol groups of β-D-galactosidase under a mild condition. The reaction was terminated by an addition of mercaptoethanol equimolar to the cross-linker used. No reduction in the enzyme activity was observed during the enzyme labelling reaction. The labelled NCS was separated from the reaction mixture by a chromatography on a Sepharose CL-6B column with buffer A as an eluent. The elution profiles of the chromatogram assayed by both enzyme and immune reactivities are shown in Fig. 1.

The immune reactivity of enzyme conjugate in

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**TABLE II. Serum NCS Levels of Patients determined by Enzyme Immunoassay at the Graded Times after i.v. Injection of NCS (1.3 mg per 250 ml Saline) by a Drip Infusion Method was started**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Woman</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>0.026 ± 0.006</td>
<td>0.039 ± 0.005</td>
</tr>
<tr>
<td>10</td>
<td>0.067 ± 0.014</td>
<td>0.071 ± 0.008</td>
</tr>
<tr>
<td>30</td>
<td>0.061 ± 0.013</td>
<td>0.094 ± 0.001</td>
</tr>
<tr>
<td>Time</td>
<td></td>
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<tr>
<td>60</td>
<td>0.101 ± 0.021</td>
<td>0.112 ± 0.023</td>
</tr>
<tr>
<td>(min)</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.056 ± 0.011</td>
<td>0.035 ± 0.006</td>
</tr>
<tr>
<td>180</td>
<td>0.037 ± 0.007</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>Half life (min)</td>
<td>44.8</td>
<td>59</td>
</tr>
<tr>
<td>N b)</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

a) Values are the mean ± S.D.

b) Number of assay times.
the fractions estimated by enzyme immunoassay was almost parallel with its enzyme activity. The immune specificity of the enzyme conjugate to anti-NCS serum was measured also by an enzyme immunoassay procedure in which a competition with 100 ng of NCS was involved. The conjugate in the later peak fractions was found to be more specific to anti-NCS than that in the former peak fractions. Fractions 15 - 17 of the main peak were thus chosen as the labelled antigen for enzyme immunoassay of NCS.

Enzyme Immunoassay

Enzyme immunoassay of NCS which can detect 10 pg to 10 ng of the antigen was performed by a competitive binding procedure with a double antibody method. A typical calibration curve of enzyme immunoassay of NCS is shown in Fig. 2.

Experimental results for accuracy and precision of enzyme immunoassay of NCS are summarized in Table I. Recovery of their assay values was arranged over 95 to 108% for both within-run and between-run experiments. Precision of the assay was fairly good. CV values obtained under the within-run experiments, were less than 15% for NCS samples of 33 pg to 10 ng/tube. While, those values obtained under the between-run experiments were a little larger than those of the corresponding within-run experiments. The specificity of the present assay is proved by cross-reactivity experiments with the results summarized also in Fig. 2. Antitumor antibiotics, daunomycin and bleomycin, and ampicillin as a representative of penicillin and cephalaxin as that of cephalosporin together with three proteins that possess similar molecular weights with NCS were chosen as the competitors. All of them showed no appreciable cross reactivity in this assay.

Comparison of Assay Methods

A comparison of two different methods, enzyme immunoassay and single-radial immunodiffusion, for the quantitation is shown in Fig. 3. There was a good correlation between the results by the two methods (r > 0.99).

Quantitation of NCS in Human Serum by Enzyme Immunoassay

Effect of an addition of 10 μl normal human serum to this enzyme immunoassay was examined. The B/Bo values measured were a little higher for samples with the serum than without. Consequently, a standard curve of NCS determined at the presence of 10 μl normal human serum in each tube was used for quantitation of sera NCS levels of patients. Accuracy and precision of enzyme immunoassay of NCS under this condition are presented also in Table I. The results were quite satisfactory.

Three patients were injected with NCS (1.3 mg/person) dissolved in 250 ml saline via i.v. by a drip infusion method for 1 h. The blood was withdrawn from their arm veins at graded time intervals, and NCS content in 10 μl of each serum was determined. Time-courses of NCS levels of these patients after the administration of NCS was started, are shown in Fig. 4, and their exact data with standard deviations are summarized in Table II.

NCS levels in the sera samples raised rapidly to ca. 0.1 μg/ml within 10 min and the level continued until the administration of NCS was over. Then, NCS levels decreased almost linearly. The average time required for 50% reduction of the sera level from their own maximum concentration can be obtained from Fig. 4 to be 55 ±10.2 min.

DISCUSSION

A novel convenient enzyme labelling procedure was introduced for highly sensitive enzyme immunoassay of NCS. Acylation of NCS with a new cross-linker GMBS was run under the condition in which GMBS was expected to react exclusively. For this, a molar ratio of GMBS to NCS was limited to one-tenth. Since NCS is a single chain protein and it possesses two amino groups at the N-terminal alanine and lysine (position at 20) residues,20 the exact molar ratio between the acylating agent and the acylated free amino groups in NCS was 1:20. By this way, a procedure which separates the excess cross-linker from GMBS-acylated NCS after the acyla-
tion was omitted. Addition of a thiol group (or groups) of enzyme to the maleide group of GMBS-acylated NCS was performed in a neutral aqueous solution. Enzyme activity examined before and after the conjugation steps did not show any difference, suggesting full retention of the enzyme functional group. The enzyme conjugate isolated by a chromatographic procedure was stable in buffer A and its full immune and enzyme activities were retained after a storage at 4°C for more than one year as judged by enzyme immunoassay. A highly specific anti-NCS serum was elicited in a rabbit by the immunization procedure described. Using the enzyme conjugate and a 50000-fold diluted solution of anti-NCS serum, enzyme immunoassay of NCS was able to quantify 10 pg (1 fmol) to 10 ng (1 pmol). From the results of accuracy and precision studies, 35 pg per tube was found to be the minimum amount that can assay with good accuracy and precision. Results by the present method were comparable in quantitation to those for antigen determination by single radial immunoassay, though the former method used 250-fold diluted solutions of the corresponding NCS samples applied for the latter method. Previous pharmacokinetic studies of NCS in experimental animals showed that NCS was rapidly cleared from serum with a half-life of NCS level in rabbits of 7.019 and 8.020 min, while that in human serum is measured to be 55 min as described above. The main reason for the discrepancy of the half-life times of serum NCS levels between rabbit and human could be attributed to the difference in NCS administrations that gave different disappearance phases of the NCS levels. Rabbits were injected by a single shot with 10 mg19 or 2 mg20 per kg weight dose of NCS while 0.026 mg/kg was dosed for humans via i.v. drip infusion method in 1 h. Samy and Raso18 observed that the disappearance of serum level of NCS in rats dosed by a single i.v. injection had two phases; the half-life of initial phase was very short and most of the drug was removed in the first 10 min, and second mode of removal was characterized by a longer half-life. We determined the half-life corresponding to the second phase while they determined that of the first phase, since both of them could calculate the apparent distribution volume of NCS.19,20

A convenient enzyme labelling method introduced into the present study is applicable for several antigens using MBS5,7 as well as GMBS as a cross-linker (unpublished data), and enzyme immunoassay of NCS using β-D-galactosidase labelled NCS can quantify NCS with high sensitivity and precision. After this study was complete, Napier et al.24 as well as Ishida and his co-laborators25–29 reported very recently that NCS contained a nonprotein chromoprobe (NPC) which is responsible for the pharmacological activity of NCS, though NCS has been reported as a single polypeptide antibiotic.11–20,23 However, the complete chemical structure of NPC and the binding site of NPC on the NCS are not clear. The present enzyme immunoassay will be useful for investigating biological activities of NCS especially for the role of its protein component, since the protein component of NCS has been shown to enhance the antimicrobial activity of NPC26 but the relationship between the protein component and NPC for the biological activity of NCS are not elucidated.

Acknowledgement This work was supported in part by Cancer Research Grants from Ministries of Education, and Health and Welfare of Japan.

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