DETERMINATION OF ADRIAMYCIN BY ENZYME IMMUNOASSAY*

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A solid phase immunoassay for the determination of adriamycin has been developed. Specific antibody against adriamycin was raised in rabbits after coupling adriamycin to bovine serum albumin. β-D-Galactosidase labelled adriamycin was prepared by m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as a coupling reagent. This assay system followed the double antibody solid phase method for the B/F separation of antibody-bound and free adriamycin. By this method, from 0.5 to 100 ng of adriamycin could be detected. Using this assay system, drug levels were determined in serum of mice, following administration of adriamycin in a single dose of 15 mg/kg i.p., the peak appearing at 20 min after administration. The change of serum levels was found to be biphasic, and the half-life of β-phase was about 5 h.

Keywords—adriamycin; enzyme immunoassay; double antibody solid phase method; drug monitor; β-D-galactosidase

INTRODUCTION

Adriamycin is an anthracycline antibiotic used extensively for the treatment of leukemia and various malignant tumors. At therapeutically useful levels, adriamycin can sometimes produce several side effects. The most serious of these is cardiotoxicity which can lead to congestive heart failure. In order to prevent cardiotoxicity, the most simple method is limited-dose administration, and careful use is essential in long-term administration. Thus, the rapid, and specific assay system is necessary to monitor the serum concentration after administration. Adriamycin in biological fluids and tissues has been determined by bioassay, fluorescence methods, isotope methods and high pressure liquid chromatography. Furthermore, effectiveness of immunological assay of the drug is supported by evidence from the reports concerning radioimmunoassay (RIA) and enzyme immunoassay (EIA) for adriamycin. In these assay procedure, the B/F separation was performed according to the double antibody method, in which the centrifugation step is necessary. Therefore, we attempted to perform B/F separation by use of goat anti-rabbit IgG-antibody coated polystyrene balls as immobilized antibody to the principle of the double antibody solid phase method. To conclude, we confirmed the availability of this method by the determination of adriamycin concentration in the serum of mice after administration.

MATERIALS AND METHODS

Materials—Adriamycin hydrochloride was obtained from Kyowa Hakko Co. Ltd. (Japan). β-D-Galactosidase from Escherichia coli was purchased from Boehringer Mannheim Co. (Germany). Bovine serum albumin (BSA) was from Seikagaku Kogyo Co. Ltd. (Japan), and ovalbumin and goat anti-rabbit IgG-antibody were from Miles-Yeda Ltd. (Israel). 4-Methylumbelliferyl-β-D-galactopyranoside was from Koch-Light Laboratories Ltd. (U.S.A.).

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Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) was from Pierce Chemical Co. (U.S.A.). Glutaldehyde (Glu) was from Kishida Chemical Industry Co. Ltd. (Japan). Polystyrene balls (3.2 mm in diameter) were the products of Ichiko Laboratories (Japan). \(N-(\gamma\text{-Maleimidobutyryl})\)-succinimide (GMBS) was synthesized according to the method described by Kitagawa \textit{et al.}\textsuperscript{13} Other chemicals used were of analytical grade available.

\textbf{Animals} — Male ddY mice were obtained from Shizuoka Agricultural Co. Association of Laboratory Animals (Japan). The animals were provided with a standard diet and water.

\textbf{Buffers} — 0.01 M Sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl\(_2\), 0.1% NaN\(_3\) and 0.05% ovalbumin was used for the measurements of enzyme activity (buffer A). 0.06 M Sodium phosphate buffer (pH 7.4) containing 0.01 M EDTA and 0.05% ovalbumin was used for the EIA method to dilute the antiserum against adriamycin (buffer B). 0.01 M Sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20 was used for the EIA method in order to wash the polystyrene balls in the B/F separation (buffer C).

\textbf{Antiserum against Adriamycin} — Immunon-antigens, adriamycin-BSA conjugate (ADR-BSA), were prepared with Glu,\textsuperscript{12} GMBS,\textsuperscript{13} These conjugates were purified by gel filtration. The immunization was done according to the method described by Kitagawa \textit{et al.}\textsuperscript{13}

\textbf{Preparation of Enzyme-Labelled Adriamycin} — Adriamycin-\(\beta\)-D-galactosidase conjugate (ADR-\(\beta\)-Gal) was prepared according to the method described by Kitagawa \textit{et al.}\textsuperscript{13} with MBS as a coupling reagent. The ADR-\(\beta\)-Gal was diluted with buffer A and this solution was used in the EIA system.

\textbf{Preparation of Goat Anti-rabbit IgG-antibody-coated Polystyrene Balls} — The polystyrene balls coated with goat anti-rabbit IgG-antibody for the B/F separation were prepared as follows: polystyrene balls were washed with a detergent (Scat 20X-PF) and rinsed completely with deionized water. The balls were then immersed in 0.25 M sodium phosphate buffer (pH 7.5) containing goat anti-rabbit IgG-antibody (0.1 mg/ml) at room temperature overnight. Next, the antibody solution was taken off and 0.01 M sodium phosphate buffer (pH 7.4) containing 0.85% NaCl and 0.5% ovalbumin was added. After the balls were shaken at 37°C for 2 h and the added solution was discarded. Last, the balls were washed with Buffer A several times and stored in the same buffer at 4°C.

\textbf{Standard Assay Procedure} — Enzyme immunoassay for adriamycin was performed by the double antibody solid phase method as follows: 5 \(\mu\)l of ADR-\(\beta\)-Gal, 10 \(\mu\)l of an appropriate amount of adriamycin and 5 \(\mu\)l of normal mouse serum, or 10 \(\mu\)l of deionized water and 5 \(\mu\)l of test sample, and 150 \(\mu\)l of the antiserum diluted 1 : 100000 with Buffer B were mixed and allowed to stand at room temperature overnight. Then, one of the second antibody coated polystyrene balls was added to the mixture and incubated at 37°C for 4 h with shaking. Next, the ball was washed with 1.5 ml of Buffer C twice and the ball was transferred into another test tube for the measurement of the bound enzyme activity.

\textbf{Enzyme Activity} — Three hundred \(\mu\)l of 0.1 mM 4-methylumbelliferyl-\(\beta\)-D-galactopyranoside solution was added to each tube containing above polystyrene ball. These samples were incubated at 37°C for 10 min, then 2.5 ml of 0.1 M glycine-NaOH buffer (pH 10.3) was added to terminate the enzyme reaction. Enzyme activities of these solutions were determined by measuring the 4-methylumbellifere released by a fluorescence spectrophotometer with the excitation at 360 nm and the fluorescence monitoring at 448 nm.

\textbf{Fluorescence Method of Adriamycin} — The fluorescence of adriamycin was measured after the extraction from the serum according to the procedure described by Rosso \textit{et al.}\textsuperscript{5}

\textbf{RESULTS}

\textbf{Preparation of Anti-adriamycin Antibody}

The ADR-BSA conjugates were prepared by use of Glu and GMBS as coupling reagents, respectively. Antisera collected at biweekly inter-
vals after the first immunization were investigated. The binding capacity of antiserum against the ADR-BSA coupled with Glu to the ADR-β-Gal was examined. Typical binding curves are shown in Fig. 1. The binding capacity of the antiserum to the ADR-β-Gal increased progressively by the injection of booster at biweekly intervals after the first immunization, and reached maximum at 10 weeks after the first immunization, and reached maximum at 10 weeks after the first immunization. The antisera from the ADR-BSA coupled with GMBS did not show such a high potency as the antisera prepared by the Glu method. From these results, we used anti-ADR-BSA antiserum prepared by Glu method (antiserum obtained 10 weeks after the first immunization) for the following EIA.

**Enzyme Labelling of Adriamycin**

MBS was used for the acylation of adriamycin, and MBS acylated adriamycin was coupled with β-galactosidase. Next, the conjugates was applied on a column of Sepharose 6B to remove the free adriamycin which remained. The elution profiles of total and immunoreactive activities of β-galactosidase are shown in Fig. 2. In addition, competitive reaction characteristic of enzyme labelled adriamycin was investigated by EIA using 20 ng of adriamycin. The sensitivity of EIA in the former peak fractions was superior to the latter peak fractions. Fraction numbers (16 - 20) were chosen as enzyme labelled adriamycin in the EIA system.

**Enzyme Immunoassay for Adriamycin**

Experimental conditions for the EIA were chosen after investigation of optimum concentration of antiserum against ADR-BSA coupled with Glu and of ADR-β-Gal. It was found that a 1 : 100000 dilution of antiserum obtained 10 weeks after the first immunization (Fig. 1) and 5 μl of ADR-β-Gal (120 μU) were the optimum concentrations. Optimum incubation times for the immune reaction of anti-ADR-BSA antiserum with the ADR-β-Gal, and for the reaction with second antibody-coated polystyrene ball were investigated, and it was found that overnight incubation of the antiserum with the antigen at room temperature and further 4 h incubation with a second antibody coated polystyrene ball provided as optimum assay system. However, because 5 μl of serum sample slightly affected on the standard curve (in the presence of 5 μl of serum, fluorescence intensity was decreased by about 10%), the addition system of 5 μl of normal mouse serum was developed. A working curve for adriamycin in the presence of 5 μl of normal mouse serum in the assay system is shown in Fig. 3. when 0.5 ng of adriamycin was added in the assay system, the fluorescence intensity of

![FIG. 1. Dilution Curve of Anti-adriamycin Antiserum](image)

**FIG. 1. Dilution Curve of Anti-adriamycin Antiserum**

Antisera 1, 2, 3, 4 and 5 were collected 4, 6, 8, 10 and 12 weeks after the first immunization of ADR-BSA coupled with Glu. The sera were diluted with 0.06M phosphate buffer (pH 7.4) containing 0.01% EDTA and 0.05% ovalbumin. — ○ — : normal rabbit serum diluted with the above buffer.
Enzyme Immunoassay of Adriamycin

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The final product (4-methylumbelliferone) was different from the control at the $p < 0.05$. From this result, the lower limit of detection by this assay system was 0.5 ng of adriamycin per tube, and the working range was shown to be $0.5 - 100$ ng per tube.

In this EIA, within assay variations were examined and shown in Table I. The coefficient of variation was 5.0 % ($n=15$).

Then, adriamycin was added to a series of sera to study the rate of recovery. The results are given in Table II. The average recovery was 96.9 % from sera.

The effect of anticancer drugs on the EIA system of adriamycin was also examined. The following drugs had no effect on the determination of adriamycin at concentration of 0.1 mM of mitomycin C; 5-fluouracil, cyclophosphamide, neocarzinostatin and cytarabin.

**Correlation of the Proposed Method and the Fluorometric Method**

Correlation of the EIA and the fluorometric method was investigated by the assay of adriamycin in serum ($0.35 - 18$ μg/ml, $n=12$). As shown in the result, the correlation was good.

![Fluorescence intensity vs Absorbance at 420 nm](image)

**FIG. 2. Elution Profile of the Enzyme-Antigen Conjugate from a Sepharose 6B Column**

- • • : Immunoreactive enzyme activity determined by the EIA described in "Materials and Methods" expected that $5 \mu l$ of the conjugate in the absence of adriamycin and mouse serum; - △ △ : competitive immunoreactive enzyme activity determined in the presence of $20$ ng of adriamycin by the same manner; - ○ ○ : β-D-Dalactosidase activity determined by the ONPG method described.
with the correlation coefficient of 0.990 ($n = 12$).

**Determination of Adriamycin Using the EIA Method with Adriamycin-Injected Mice**

Fifteen mg/kg of adriamycin was administered to mice by intraperitoneal injection. Blood was collected from the tail vein before administration of the drug and at certain intervals after dosing. Adriamycin content in the serum was determined directly by EIA, using 5 µl of the serum. The results are shown in Fig. 4. Adriamycin showed the maximum concentration at about 20 min after administration, followed by a slow elimination phase. Initial and subsequent half-lives of adriamycin were about 20 min and 5 h, respectively, according to the curve.

**DISCUSSION**

Adriamycin has a broad spectrum in clinical therapy against several types of cancer.$^{1,2}$ However, its use is limited due to its severe side effect, cardiotoxicity. Therefore, when it is administered, the adriamycin level in serum should be carefully monitored. RIA and EIA for adriamycin have been investigated,$^{13,14}$ but RIA has inherent drawbacks such as radiation hazards and short half-life. EIA procedure reported by Kitagawa et al.$^{13}$ was performed using the double antibody method which necessiated the centrifugation step for the B/F separation. We developed an EIA for adriamycin using the double antibody solid phase method, in which the B/F separation was more easily performed using immobilized goat anti-rabbit IgG-antibody coated on polystyrene balls. Moreover, the pro-

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**TABLE I. Reproducibility for the Determination of Adriamycin Concentration in Serum by the Proposed Method**

<table>
<thead>
<tr>
<th>Experimental number</th>
<th>Serum (ng/ml)</th>
<th>Experimental number</th>
<th>Serum (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400</td>
<td>10</td>
<td>396</td>
</tr>
<tr>
<td>2</td>
<td>380</td>
<td>11</td>
<td>400</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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<td>390</td>
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<td>404</td>
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<td>400</td>
</tr>
<tr>
<td>7</td>
<td>396</td>
<td>X</td>
<td>402</td>
</tr>
<tr>
<td>8</td>
<td>396</td>
<td>C.V. (%)</td>
<td>5.0</td>
</tr>
<tr>
<td>9</td>
<td>400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE II. Recovery of Adriamycin Added to Mouse Serum

<table>
<thead>
<tr>
<th>Number of sample</th>
<th>Adriamycin (ng/tube)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Recovered</td>
</tr>
<tr>
<td>1</td>
<td>19.0</td>
<td>18.0</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>1.12</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The proposed method can be used to analyze adriamycin concentration without prior extraction of adriamycin from sera. Under laboratory conditions, the lower limit of detection was 0.5 ng per tube. The immunoassay was used to determine the levels of adriamycin in the serum of mice after intraperitoneal injection. A rapid biphasic disappearance of adriamycin from the mouse serum was observed with a short initial half-life of the drug, followed by a relatively long period of elimination. Thus the behavior of adriamycin in serum was similar to that stated in the previous report, in which adriamycin was measured by high pressure liquid chromatography. The EIA procedure for adriamycin presented here is, reproducible, simple, safe to perform, and adaptable to analyses of large numbers of samples. This EIA will be a great asset in clinical trials of adriamycin. Since it was difficult to obtain sera from patients receiving chemotherapy for cancer, we conducted experiments on mice and confirmed the change of serum levels of adriamycin. In the near future, the EIA for adriamycin will be applied to clinical trials to monitor drug concentration in serum.

**FIG. 4. Serum Levels of Adriamycin in Mice after a Single Intraperitoneal Injection Determined by the EIA.**

*Data show mean ± S.D. of 3 determinations.*

**REFERENCES**