Radioimmunoassays for Adriamycin and Daunomycin

Helen Van Vunakis, John J. Langone, Louis J. Riceberg, and Lawrence Levine

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

SUMMARY

Antibodies directed toward adriamycin have been produced in rabbits, monkeys, and goats by immunization with adriamycin:protein conjugates. The antisera react equally well with adriamycin and daunomycin but differ in their reaction with adriamycin aglycone and other anthracycline derivatives. Radioimmunoassays, which utilize immune precipitation or nitrocellulose membrane filtration for separation of antibody-bound and free antigen, were developed for adriamycin and daunomycin. The lower limit of detection is 2 pmoles of drug equivalents per ml of unextracted plasma or urine. The sensitivity of the assay is such that serological activity could be detected in human plasma several days after adriamycin was administered. The distribution of adriamycin administered to rabbits and a monkey was determined. In the monkey, adriamycin was found in the formed elements of blood at equal or greater concentrations than in the plasma. Some adriamycin remained in the blood cells even after they were washed four times with buffer. The distribution of adriamycin in the tissues of a rabbit sacrificed 24 hr after administration of the drug was also determined.

INTRODUCTION

Adriamycin and daunomycin, the anthracycline antibiotics isolated from Streptomyces peucetius are effective in treating several different types of neoplastic diseases (4, 9, 10). At therapeutically useful levels, these drugs can sometimes produce side effects, the most serious of which is cardiotoxicity which can lead to congestive heart failure.

The pharmacokinetics of these antibiotics in physiological fluids and their distribution in tissue have been studied by the use of isotopic and spectrofluorometric techniques (5, 6, 10, 15, 21). With labeled drugs, the distribution of radioactivity can be readily determined but characterization of the radioactive components requires that they be isolated and identified by other procedures. The fluorescent techniques require extraction of the physiological fluid or tissue to separate the drug and its metabolites from components that could interfere with the assay. The extract then is chromatographed to separate the fluorescent components prior to determining their concentration by fluorescence. There are inherent difficulties in the use of total fluorescence to measure drug equivalents in extracted samples that have not been fractionated by chromatography. In samples obtained soon after the drug is administered, the contribution to total extractable fluorescence by endogenous components may be relatively small. However, at later times when drug levels are low, the amount of fluorescence due to endogenous compounds may account for a large fraction of the extractable fluorescence. In addition, the contribution from endogenous components may fluctuate during the time course of the experiments, but there is no direct way to estimate and correct for such changes in samples that have not been fractionated.

RIA procedures that have been developed for the estimation of drugs and other biologically important molecules (7, 20) are specific and sensitive. They have proven especially useful when the levels in biological fluids are low and when other techniques require large quantities of samples that must be processed extensively prior to analysis. In this study, we report the production of antibodies to adriamycin in experimental animals. These antisera have been used to develop sensitive RIA's capable of detecting as little as 2 pmoles of adriamycin and daunomycin per ml of serum or plasma. Two RIA's are described. In one assay, antibody-bound and free antigen are separated by immune precipitation with a 2nd antibody (17, 19). In the other more rapid method, they are separated by filtration through nitrocellulose filters (11, 19). The distribution of adriamycin administered to rabbits and monkeys has been determined by RIA.

MATERIALS AND METHODS

Adriamycin-HCl was given to us by Dr. T. Borsos of the National Cancer Institute. Daunomycin-HCl, N-acetyl-daunomycin, and daunomycin benzoylhydrazone were generous gifts from Dr. N. R. Bachur of the Baltimore Cancer Research Center of the National Cancer Institute. Adriamycinone, m.p. 216—218° (literature, 223—224°), was prepared according to the procedure used to obtain the aglycone of daunomycin (1). The red solid was homogeneous by thin-layer chromatography (Rf 0.90) using CHCl3:methanol:acetic acid:H2O, 100:50:14:6, as solvent. On the same plate, adriamycin had an Rf of 0.45. The UV spectrum (H2O) was identical to that of the procedure used to obtain the aglycone of daunomycin (1). The red solid was homogeneous by thin-layer chromatography (Rf 0.90) using CHCl3:methanol:acetic acid:H2O, 100:50:14:6, as solvent. On the same plate, adriamycin had an Rf of 0.45. The UV spectrum (H2O) was identical to that of

---

1 Publication 960 from the Graduate Department of Biochemistry, Brandeis University. Supported in part by Research Contract NCI E-72-3243 from the National Cancer Institute and by Research Grant IC-10M from the American Cancer Society, Inc.

2 Research Career Awardee (Award K06-AI02372 from the National Institute of Allergy and Infectious Diseases).

3 American Cancer Society Professor of Biochemistry (Award PR7-21).

Received March 19, 1974; accepted June 14, 1974.

---

The abbreviations used are: RIA, radioimmunoassay; HSA, human serum albumin; CDI, 1-ethyl-3-(dimethylaminopropyl)carbodiimide.
adiamycin at pH values of 5.0 and 12 (2). The giant keyhole limpet hemocyanin was purchased from Schwarz/Mann, Orangeburg, N.Y. The sources of other reagents are the same as those mentioned for preparation of similar conjugates (11, 18).

Conjugates for Immunization. Adriamycin was conjugated through its amino group to HSA or to hemocyanin by the use of CDI to form an amide bond (7, 12) or by the use of glutaraldehyde to cross-link the hapten to the amino groups of the carrier (7, 14, 18).

For preparation of the conjugates with CDI, 20 mg of CDI were added to a solution containing 10 mg of adriamycin-HCl and 10 to 20 mg of the macromolecule in 1.0 ml H2O, pH 7.0. After the solution was stirred at room temperature for 18 hr, the hapten conjugate was separated from unreacted adriamycin and CDI by molecular sieve chromatography on a column of Sephadex G-50M (35 x 1.5 cm) by elution with buffer (0.15 M NaCl:0.005 M phosphate, pH 7.0). The conjugates were characterized on the basis of their UV and visible spectra compared to that of adriamycin (2). The incorporation of adriamycin ranged between 10 and 15 moles/mole of HSA and 8 to 10 moles/100,000 g hemocyanin.

The glutaraldehyde conjugates were synthesized as follows. To a solution of 20 mg of HSA and 6 mg of adriamycin-HCl in 2 ml of 0.1 M phosphate buffer, pH 7.0, was added dropwise 1.0 ml of 0.25% aqueous glutaraldehyde. After 2 hr, 1.0 ml of 1 M neutralized lysine was added to consume unreacted glutaraldehyde; then the reaction mixture was chromatographed as above to separate the adriamycin:HSA conjugate from low-molecular-weight species. From spectral measurements, it was found that approximately 10 moles of adriamycin were incorporated per mole of HSA.

Antiserum. Six rabbits, 2 monkeys, and 1 goat were immunized with adriamycin conjugates. Each rabbit received 1 injection of 1 mg of conjugate emulsified in complete Freund’s adjuvant in the toepads and i.m. After 2 weeks, the animals were again given injections and they were bled 1 and 2 weeks later. They were given an identical booster dose the following week and were bled again after 1 and 2 weeks. This booster injection was repeated in 2 months. The 2 rabbit antisera used in this study were obtained at the end of this immunization schedule. The monkeys received similar primary and 2 booster injections of HSA (glutaraldehyde) adriamycin i.m. The goat received 2 i.m. injections containing 2 mg of hemocyanin (CDI) adriamycin emulsified in complete Freund’s adjuvant and were bled 3, 4, and 5 weeks later. Two months later an identical booster shot was given and the goat was bled after 7, 9, 11, and 14 days. The bleedings after the booster injection were used in this study.

Radioisotopic Hapten. N-(p-Hydroxyphenylacetyl)adriamycin was prepared by coupling adriamycin (5.0 mg; 8.6 μmoles) and p-hydroxyphenylacetic acid (5.0 mg; 30 μmoles) with CDI (5.0 mg; 26 μmoles) in 30% dimethylformamide:water. After 2 hr at room temperature, the product (Rf 0.42 to 0.62) was separated from unreacted adriamycin (Rf 0.00) and p-hydroxyphenylacetic acid (Rf 0.72 to 0.78) by preparative thin-layer chromatography (silica gel) using ethyl acetate:methanol:acetic acid, 85:10:5, as solvent. The adriamycin derivative was labeled enzymatically with 125I by the use of lactoperoxidase (13, 16). The reaction was carried out at room temperature. To 50 μl of water were added 25 μg N-(p-hydroxyphenylacetyl)adriamycin as 25 μl of a 1.0-mg/ml solution in ethanol; 25 μl 0.5 m sodium phosphate buffer, pH 7.5; 3.35 mCi Na125I as 10 μl of a 335 mCi/ml solution in 0.1 N NaOH; 10 μl of a 1:3000 dilution of 30% hydrogen peroxide; and 10 μl of a 1.0-mg/ml aqueous solution of lactoperoxidase. After 30 min, 0.5 ml of aqueous KI (5 mg/ml) was added and the reaction mixture was chromatographed on a column of Sephadex G-10 (10 x 1.5 cm). After free 125I plus added cold iodide (detected by the formation of yellow silver iodide upon addition of ethanolic silver nitrate) had been eluted with buffer (1.5 M NaCl:0.05 M sodium phosphate, pH 7.5:0.2% gelatin), 125I-labeled N-(p-hydroxyphenylacetyl)adriamycin was eluted with 50% dimethylformamide:0.3 M NaCl. The specific activity of the product was approximately 10 Ci/mmoles.

RIA Procedures. For assay with the rabbit antisera by the double antibody technique (19), 0.1 ml of the adriamycin-125I derivative (approximately 20,000 cpm), 0.1 ml of EDTA gel:Tris buffer (0.01 M EDTA:0.14 M sodium chloride:0.01 M Tris:0.1% gelatin, pH 7.5) or 0.1 ml inhibitor serially diluted 2-fold or 0.1 ml of the biological fluid suitably diluted, and 0.1 ml of antiserum at the appropriate dilution were mixed and incubated at 37° for 1 hr. Goat anti-rabbit γ-globulin (0.1 ml known to be in region of antibody excess) was added, the solution was mixed and incubated at 2–4° overnight. After centrifugation at 1,000 X g for 30 min at 4°, the supernatant fluid was decanted and the walls of the tubes were wiped. The precipitates were counted in a Packard Auto-Gamma spectrometer. The same procedure was used with the monkey antiserum, except that rabbit anti-monkey γ-globulin was used as the 2nd antibody to precipitate the monkey γ-globulin. With the goat antiserum, rabbit anti-goat γ-globulin was used for precipitation. The volume was increased from 0.3 to 1.3 ml for the 1st reaction to permit analysis of adriamycin in 0.1 ml undiluted monkey, rabbit, or human serum. The rabbit anti-goat γ-globulin was added as before and the mixture was incubated overnight in the cold (19).

The RIA procedure for adriamycin by nitrocellulose membrane filtration will be described in detail under “Results.”

RESULTS

Specificity of Antisera. Antibodies that bind the adriamycin-125I derivative were produced in 2 rabbits immunized with HSA:adriamycin conjugate and in 1 rabbit and 1 goat immunized with hemocyanin:adriamycin conjugate synthesized with carbodiimide. Three rabbits and 2 monkeys immunized with HSA:adriamycin conjugate (prepared with glutaraldehyde) also produced antibodies. Before immunization, adriamycin-125I derivative was not bound by the sera from any of these animals. The antibody response in all 9 animals increased with time of immunization. The small number of animals and lack of variation of the dose of each immunogen precludes the prediction of which conjugate or which species would consistently produce antibodies of higher titers, greater specificity, or greater sensitivity for the RIA.

OCTOBER 1974

Radioimmunoassays for Adriamycin and Daunomycin

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 1974 American Association for Cancer Research.
The goat produced antibodies that permitted us to develop the most sensitive assay for adriamycin and daunomycin. The inhibition of the binding of the adriamycin,¹²⁵I derivative to goat anti-adriamycin by adriamycin and structurally related substances is shown in Chart 1. Adriamycin, daunomycin, and 2 derivatives of daunomycin, N-acetyldaunomycin and daunomycin benzoylhydrazone, compete equally well for the receptor sites of the antibodies. The goat antibodies recognize the amino sugar, since adriamycin aglycone reacts only about 25% as effectively as adriamycin. Alternatively, the absence of the amino sugar may affect the conformation of the remainder of the molecule. The serological activity is not altered by lack of a hydroxyl group on C-14 (daunomycin) or the presence of the relatively bulky benzoylhydrazone on the C-13 keto group of daunomycin. These data suggest that the area of the drug complementary to the antibody receptor site does not encompass the C-13 and C-14 positions.

As expected, the specificities of the antibodies vary among the individual animals, the species, and even during the course of immunization. The data in Table 1 show the levels of adriamycin and related compounds required for 50% inhibition of immune binding with antisera from 2 rabbits and the goat. These animals were immunized with conjugates in which adriamycin was linked through the amino group of the sugar to carboxyl groups of the protein by amide bonds. Not only do these antibodies recognize the amino sugar as part of the antigenic determinant (the aglycone inhibits about 25% as effectively as adriamycin), but they also recognize the amide bond (N-acetyldaunomycin inhibits 9 times more effectively than daunomycin). The amide bond also appears to be part of the antigenic determinant for the antibodies produced in Rabbit 880C-6. Compared to daunomycin, N-acetyldaunomycin inhibits 7 times more effectively.

The 2 monkey antisera and the 3 rabbit antisera obtained after injection with the HSA:adriamycin conjugates prepared with glutaraldehyde do not distinguish among these inhibitors. They all inhibit 50% at about the 20-pmole level. With all these antisera, vitamin K₁, or chemotherapeutic agents, e.g., tetracycline, vinblastine, vincristine, and methotrexate, that may be used in conjunction with the anthracycline drugs, did not inhibit even at levels 10³ to 5 X 10⁴ higher than the homologous antigen.

**Analyses of Physiological Fluids and Tissues.** The 3 antisera (Table 1) were used to determine the fate of adriamycin in rabbits given injections of the drug. For these studies, 5 mg of adriamycin-HCl were administered by rapid injection into the marginal ear vein of 3 rabbits weighing 3.5 kg. Blood was obtained before administration of the drug and at intervals thereafter from the other marginal ear vein. The blood was collected in a heparinized tube, the red cells were removed by centrifugation, and the plasma was analyzed for adriamycin by RIA. With antibodies from Rabbts 873 and 880, the volume of rabbit serum that could be assayed is limited to 10 µl, since the 2nd antibody system, i.e., the goat anti-rabbit γ-globulin, used in this procedure would not precipitate the adriamycin,¹²⁵I-bound rabbit antibody quantitatively if more than 10 µl of rabbit plasma were present (19). With the goat anti-adriamycin, however, 100 µl of rabbit serum could be analyzed. At times when adriamycin levels in the blood are low, i.e., 5 hr after the administration of the drug, 100 µl of rabbit plasma were required for analysis. The presence of 100 µl of undiluted rabbit serum (or monkey or human serum or plasma) amplified the inhibition of adriamycin. Thus, instead of obtaining 50% inhibition with 3.3 pmole of adriamycin, 1.2 pmoles were required. This enhancement is reproducible with individual sera; 100 µl of 8 individual human sera, 4 individual rabbit sera, and 4 monkey sera enhanced the inhibition of adriamycin to the same extent. This amplified calibration curve (i.e., in the presence of 100 µl of sera) is used to quantitate adriamycin levels only in those samples in which 100 µl of plasma are analyzed. The enhancement is not seen with 100 µl of undiluted urine, nor is it seen with 10 µl of undiluted serum or plasma. Therefore, to quantitate adriamycin levels in these samples a calibration curve obtained with adriamycin in EDTA gel:Tris buffer is used. Several dilutions of unknown were analyzed and the dose-response curve

### Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>RA 880C-6</th>
<th>RA 873C-4</th>
<th>Goat 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>40.0</td>
<td>13.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>54.0</td>
<td>16.0</td>
<td>3.3</td>
</tr>
<tr>
<td>N-Acetyldaunomycin</td>
<td>7.4</td>
<td>1.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Daunomycin benzoylhydrazone</td>
<td>92.0</td>
<td>18.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Adriamycinone</td>
<td>80.0</td>
<td>56.0</td>
<td>13.2</td>
</tr>
</tbody>
</table>

The carrier molecules in the conjugate used for immunization were hemocyanin for Ra 880 and Goat 4 and HSA for Ra 873. The details of the RIA procedure are given in "Materials and Methods."
paralleled that of the standard. Clearance of adriamycin from rabbit plasma was very rapid for 2 or 3 hr after the drug was administered (Chart 2). From the 5th to the 30th hr, clearance was markedly decreased. Essentially the same profile of disappearance was found when adriamycin (1 mg/kg) was administered to a monkey (Chart 3). The small number of animals used precludes assigning half-lives to various stages of disappearance, but it is quite clear that the rapid extravascular distribution and more prolonged disappearance of adriamycin from rabbit and monkey plasma can be detected by the RIA.

Although the individual antisera have specificities that are too broad to distinguish among the drugs and their metabolites (Table 1), analysis of individual samples with all 3 antisera permits us to draw certain conclusions. For example, if at 4 hr after administration of adriamycin to the monkey an appreciable percentage of adriamycin had been metabolized to adriamycinone, the results of analyses based on the adriamycin inhibition curve with each antiserum would be different. At 4 hr the analysis for adriamycin with each antiserum was similar. Thus, we conclude that at this time adriamycin and/or metabolite(s) that cross-react proportionally with the 2 rabbit and 1 goat immune system are being measured.

Rabbit urine also was collected and assayed for adriamycin by RIA. The presence of serologically reactive compounds was detected in the urine even 3 to 4 days after the drug was administered. Efforts are being made to identify the serologically reactive molecule(s). The RIA's of the 0- to 24-hr urine sample with 3 antisera (Table 1) indicate that the majority of the serologically reactive compounds are adriamycin or metabolites, which are recognized by each antiserum proportionally.

The distribution of adriamycin in various tissues of a rabbit that had received adriamycin 24 hr before it was sacrificed is shown in Table 2. The 24-hr urine sample of this animal contained 10.2 µg of adriamycin equivalents per ml and extracts of the feces contained 0.4 µg/g, wet weight, of material.

In the monkey, we also determined the distribution of the drug in the formed elements of the blood (Table 3). The cellular fraction of the blood contained amounts of adriamycin that equaled or exceeded those found in plasma. A percentage of the adriamycin is easily removed by washing the cells with EDTA gel:Tris buffer, but a considerable and possibly constant percentage of adriamycin appears to be tightly bound. The cell type, i.e., red blood cells, white cells, or various types of platelets, which may be involved in adriamycin binding, has not yet been identified.

If a correlation between plasma levels and toxicity of adriamycin or daunomycin is found to exist, then a rapid assay such as the one in which nitrocellulose membrane filtration is used would be advantageous (11). The ability of nitrocellulose membrane filters (0.45-µm pore size, 25-mm diameter from Carl Schleicher and Schuell, Keene, N. H.) to retain proteins while permitting the hapten to pass through formed the basis for separation of free antigen from antibody-bound antigen in the RIA's developed for prostaglandins, angiotensin, digoxin, morphine, and cyclic 3', 5'-AMP (11). In the present study, however, the adriamycin-125I derivative has physical proper-

Chart 2. Plasma adriamycin concentrations in rabbits after a single i.v. injection of adriamycin. Three female albino New Zealand rabbits, each weighing approximately 3.5 kg, were given injections of 5 mg of the drug. At each time interval, approximately 3.0 ml of blood were collected into a heparinized tube and, after centrifugation, the plasma was removed and stored at —20° until analyzed.

Chart 3. Plasma adriamycin concentrations in a rhesus monkey after a single i.v. injection of adriamycin. (Details of the experiment are the same as given in Table 3.)

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Adriamycin equivalents (µg/g, wet wt, tissue)</th>
<th>Sample</th>
<th>Adriamycin equivalents (µg/g, wet wt tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>8.9</td>
<td>Skeletal</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>7.0</td>
<td>muscle</td>
<td>0.7</td>
</tr>
<tr>
<td>Lung</td>
<td>1.5</td>
<td>Liver</td>
<td>0.3</td>
</tr>
<tr>
<td>Uterus</td>
<td>1.2</td>
<td>Brain</td>
<td>0</td>
</tr>
<tr>
<td>Heart</td>
<td>0.9</td>
<td>Plasma</td>
<td>0.0174 µg/ml</td>
</tr>
</tbody>
</table>

OCTOBER 1974

2549
ties such that it is retained on the filter even when the capacity of the filter for protein is exceeded. Under these conditions, only the antibody bound adriamycin$^{125}$I derivative passes through the filter.

The protocol and the results of such a RIA for adriamycin in sera obtained from a patient 24 and 72 hr after administration of adriamycin are shown in Table 4. Even in the presence of 100 μl of normal human serum, about 3,800 cpm out of 17,000 cpm added were bound to the nitrocellulose membrane filter. (In the absence of 100 μl of normal human serum, approximately 6,000 cpm out of 17,000 cpm added are bound to the filter.) After reaction with 0.1 μl of the goat immune serum (added as 0.1 ml of a 1:1000 dilution) about 1,100 cpm remained on the filter; 2,700 cpm had been bound to the antibodies which passed through the filter. (One μl of goat immune serum binds all of the adriamycin$^{125}$I so that only around 100 cpm are on the filter.)

Since adriamycin competes with the radioactive antigen for the antibody receptor sites, addition of increasing amounts of the drug diminishes the amount of antibody available to bind with the labeled derivative leaving more radioactive material on the filter. If all of the antibody binds unlabeled antigen, the counts on the filter approach those in the absence of the immune serum. When the adriamycin present in 100 μl of human serum obtained 24 and 72 hr after administration of the drug was allowed to compete for the limited number of antibody sites, 41 and 33% of the binding was inhibited, respectively. This corresponded to 2.2 and 1.6 ng of adriamycin per 100 μl of serum or 39 and 28 pmoles per ml of serum, respectively. Although the experiment shown in Table 4 took about 1 hr to complete, the procedure can be shortened considerably. In Chart 4 is shown the inhibition by a constant quantity of adriamycin (5 ng) of the binding of adriamycin$^{125}$I and 0.1 μl of goat anti-adriamycin as a function of time at 25°. The reaction between adriamycin and adriamycin$^{125}$I with the anti-adriamycin has reached equilibrium in about 10 min at 25°.

### Table 3

**Distribution of adriamycin in monkey blood (plasma and cells)**

A female Rhesus monkey was given an i.v. injection of adriamycin (1 mg/kg) through the femoral vein. At the times indicated, blood was collected in a heparinized syringe and centrifuged to separate plasma and cells. The cells were washed with a volume of buffer (EDTA gel-Tris) equal to that of the plasma. After the 4th washing, the cells were lysed by sonication in a Raytheon Model DI-101 Sonic Oscillator, the samples were analyzed as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Plasma</th>
<th>RBC (washed 4 times and lysed)</th>
<th>1st wash</th>
<th>2nd wash</th>
<th>3rd wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>65</td>
<td>61</td>
<td>43</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>1.0</td>
<td>43</td>
<td>30</td>
<td>18</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>23</td>
<td>18</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>18</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4

**Inhibition of adriamycin$^{125}$I anti-adriamycin binding as measured by nitrocellulose membrane filtration**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Buffer$^a$ (ml)</th>
<th>Normal human serum, undiluted (ml)</th>
<th>Adriamycin in normal human serum, undiluted (ml)</th>
<th>Adriamycin (ng added)</th>
<th>Goat antiserum, diluted 1:1000 (ml)</th>
<th>Adriamycin-125I derivative, 17,000 cpm/0.1 ml</th>
<th>cpm on filter after 30 min incubation at 37° and filtration</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1$c$</td>
<td>0.1</td>
<td>3392, 3861, 3894</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.1</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>3677, 3664, 3868</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>1095, 1104, 1254</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0.1</td>
<td>0.1</td>
<td>10.0</td>
<td>0.1</td>
<td>0.1</td>
<td>2767, 2855, 3314</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>0.1</td>
<td>0.1</td>
<td>2.0</td>
<td>0.1</td>
<td>0.1</td>
<td>2129, 2082, 2243</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>1551, 1422, 1343</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1$^d$</td>
<td>0.1</td>
<td>2141, 2195, 2271</td>
<td>41</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1$^e$</td>
<td>0.1</td>
<td>1940, 1981, 1901</td>
<td>33</td>
</tr>
</tbody>
</table>

$^a$ 0.01 M Tris, 0.14 M NaCl, 0.01 M EDTA, pH 7.5, containing 0.1% gelatin.

$^b$ The analyses were carried out in triplicate. The mean percentage of error is 4.7%.

$^c$ Normal goat serum diluted 1:1000.

$^d$ Serum from patient 24 hr after administration of adriamycin.

$^e$ Serum from same patient 72 hr after administration of adriamycin.
DISCUSSION

In addition to being rapid, sensitive, and specific, the RIA for adriamycin can be used to analyze plasma and urine without prior extraction. With our reagents, the limit of sensitivity is 2 pmoles/ml. Below this level, the biological fluid for adriamycin can be used to analyze plasma and urine.

Evidence indicates that binding of adriamycin to plasma proteins does not interfere with its determination in undiluted serum or plasma. First, addition of adriamycin or daunomycin to undiluted normal serum or plasma and subsequent RIA in the presence of normal serum yields essentially the same or even more effective inhibition of the binding of the adriamycin-\textsuperscript{125}I derivative to the antibody than that obtained in the absence of serum. Secondly, analyses of sera for adriamycin before and after extraction by ethanol:HCl (6) yield essentially the same values. If adriamycin is bound to plasma proteins, its determinants are still available for reaction with the antibodies. Alternatively, the binding of adriamycin to antibody is much tighter than the binding of adriamycin to plasma proteins.

It is known that daunomycin and adriamycin bind to DNA (8,10). Radioimmunoanalysis of adriamycin in the presence of native or denatured DNA (with GC contents of 32 and 63\%) was performed with the 2 rabbit antisera and the goat antiserum (Table 1). With the antiserum from Rabbit 880, only a fraction of the adriamycin was available for reaction. Addition of increasing amounts of DNA (0.3 to 300 nmoles) to a constant amount of adriamycin (0.86 nmole) steadily decreased the amount of drug that could be determined by RIA. In a mixture containing 100 ng of DNA and 500 ng of adriamycin in 1.0 ml of 0.14 M NaCl:0.01 M Tris buffer, pH 7.4, less than 1\% of the drug was found to be available for reaction with the antibody. Daunomycin, daunomycin benzo-ylhydrazone, and adriamycin were masked to an identical extent, while adriamycin aglycone and N-acetyldaunomycin were fully recoverable in the presence of DNA. These findings are consistent with the observations of Calendi et al. (8) that the amino group of the sugar is necessary for the tight binding of daunomycin to DNA. With the Rabbit 873 and the goat antiserum the serological activity was not masked by DNA. Differences in the affinities of the antibodies for the drug may account for these observations. Since additional 1:50 and 1:450 dilutions of the assay mixture are necessary for analysis with the Rabbit 873 and goat antiserum, respectively, the difference in availability may also be due to the disruption of the equilibrium between drug and DNA. There was no loss in serological activity when adriamycin was added to yeast RNA, polynositate, polyuridylate, or polycytidylate and tested with any of the 3 antisera.

The pharmacokinetics of adriamycin in man (6, 9, 10, 15) and in other species (5, 21) has been studied by spectrofluorometric and isotopic techniques. Qualitatively, all of these studies show a rapid disappearance into the extravascular compartment followed by a relatively long period of elimination from the blood. The results obtained by RIA with 3 rabbits given injections of 5 mg of adriamycin fit this general pattern. At the end of 24 hr, approximately 0.01 nmole of adriamycin is present per ml of plasma. The excretion of adriamycin equivalents in the urine (based on total dose administered) was 12.7, 14.6, and 8.1\%, respectively. In the tissues of a rabbit sacrificed 24 hr after the drug was administered (Table 2), adriamycin was found in the kidney, spleen, lung, uterus, heart, skeletal muscle, and liver of the animal. None was found in the brain. A statistically significant quantitative comparison of this distribution with that of other species requires analysis of additional animals. Adriamycin levels in the blood cellular fraction of the monkey was found to be similar to the corresponding plasma levels even after the cells were washed 4 times with buffer. Rosso et al. (15) found a similar distribution of the drug in man.

The data for these studies have been expressed as adriamycin equivalents. From the specificity of the antisera, certain general conclusions about the parent compound and the aglycone have already been made. We have not administered daunomycin to either rabbits or monkeys, but it is unlikely that the antisera would differentiate between daunomycin and the major metabolite daunorubicinol in which a hydroxyl group rather than a keto group is present on C-12 (3). However, introduction of bulky groups on the A, B, or C ring of the anthracycline moiety or destruction of the anthracycline structure would, in all likelihood, yield metabolites that escape detection by the antisera. As metabolites of the drugs are identified, they will be tested serologically for their inhibitory capacities.

REFERENCES

11. Gershman, H., Powers, E., Levine, L., and Van Vunakis, H. Radioimmunoassay of Prostaglandins, Angiotensin, Digoxin, Mor-


Radioimmunoassays for Adriamycin and Daunomycin


Cancer Res 1974;34:2546-2552.

Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/34/10/2546

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.