Development of an Antibody to Actinomycin D and Its Application for the Detection of Serum Levels by Radioimmunoassay

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ABSTRACT

An antibody specific for actinomycin D (Act D) has been developed and used in a rapid, sensitive radioimmunoassay for detection of this antitumor drug in serum. The 2-amino group of the heterocyclic chromophore of Act D was covalently coupled to available free carboxyl groups of bovine serum albumin with carbodiimide. The resulting complex was then used for the production of a specific antibody to Act D in two male New Zealand rabbits. Antibody production was of sufficient titer in both rabbits to allow the development of a radioimmunoassay for the free drug which is rapid and sensitive enough to accurately measure 0.1 pmol of Act D. The antibody produced was characterized to be immunoglobulin G by virtue of its ability to bind to Protein A-Sepharose columns. With the use of Act-D analog, actinomine, the antibody was characterized to be specific for the pentapeptide portion of the molecule. Pharmacokinetic analysis of serial serum samples obtained from a patient who received the drug i.v. revealed a biphasic response with an α-serum half-life of 1.78 min and a β serum half-life of 34 min. An i.v. injection of Act D into a dog and assay of serum concentration revealed a similar biphasic response with an α serum half-life of 0.78 min and a β-serum half-life of 208 min.

INTRODUCTION

The naturally occurring antibiotic Act D is proposed to intercalate into the helix of DNA. Intercalation of Act D into DNA prevents the synthesis of RNA by interfering with transcription by RNA polymerases. In eukaryotic cells, Act D in low concentrations (0.001 to 0.1 μg/ml) is a potent and selective inhibitor of rRNA synthesis. While it has been proposed that the drug inhibits rRNA synthesis at rDNA within the nucleus, more recent studies reveal that its action may be more complex.

As an antitumor agent, Act D is clinically effective in the treatment of certain human cancers. Dosing of the drug for treatment of certain human cancers (1) is aimed at achieving those concentrations of the drug within body fluids which are similar to those which are effective in the body fluids and tissues. Knowledge of the pharmacokinetic distribution of Act D in the body fluids and tissues may aid in increasing the effective use of the drug for treatment. Previous pharmacokinetic studies of Act D required the use of tritiated drug, thus using radioactivity as an indirect measure of the drug itself. Because selectivity, specificity, and enhanced drug detection are necessary for good pharmacokinetic elucidation, an antibody to the drug was prepared, and a radioimmunoassay was developed to measure levels of the drug. This paper describes the methodology for the antibody production, the initial characterization of antibody specificity, and the technique developed for the measurement of Act D by radioimmunoassay. Initial application of the assay to measure Act D levels in a patient and a dog demonstrates its utility in the assessment of basic pharmacokinetic distribution.

MATERIALS AND METHODS

Coupling Reaction for Hapten. Seventy-five mg Act D (Merck, Sharpe, and Dohme, Rahway, N. J.) was combined with tracer amounts (1.8 μCi) of [3H]Act D (12.7 Ci/mmol, Batch 33; Amersham/Searle Corp., Arlington Heights, Ill.) and 25 mg BSA (Sigma Chemical Co., St. Louis, Mo.) in a final volume of 1 ml and was maintained at 0°C in ice slush with constant stirring. This temperature was necessary to maintain Act D in solution. At 1-hr intervals for 8 hr, 10 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Bio-Rad, Richmond, Calif.) was added as a coupling reagent. The pH of the solution was maintained at 4.5 by the addition of 0.1 N HCl as needed. Several trials revealed that the optimum coupling ratio was Act D:BSA, 3:1 (w/w). The mixture was stirred for an additional 10 hr at 4°C. The reaction mixture was then transferred to a dialysis bag and dialyzed against 500-mI volumes of 1 M NaCl containing 1 mM β-mercaptoethanol (Sigma) until the deep yellow color of Act D was no longer seen in the dialysate. The insoluble protein in the dialysis bag was yellow-orange, and the additional removal of the noncovalently bound drug was achieved by extensive dialysis against 8 M urea (Schwarz/Mann, Orangeburg, N. Y.) containing 1 mM β-mercaptoethanol. When the contents of the bag were no longer yellow, stepwise dialysis against lower concentrations of urea were performed with a final dialysis against a 0.9% NaCl solution. Remaining material in the dialysis bag was white and only partially soluble. The tritium tracer was used to quantitate the amount of Act D bound. The amount of BSA in the final complex was determined according to the assay method of Lowry et al. (5) with BSA as a standard.

Antibody Production in Rabbits. Aliquots containing either 100 or 250 μg of the Act D:BSA complex (4.4 and 11 μg Act-D, respectively) were emulsified with an equal volume of Freund’s complete adjuvant (Difco Laboratories, Detroit, Mich.), and 2 male New Zealand rabbits were given 10 s.c. injections over sites along both sides of their back. A control animal received BSA in Freund’s complete adjuvant. After 3 weeks, the rabbits were boosted with the same amount of emulsified complex in complete adjuvant. One week later, each rabbit was bled from the ear artery, and serum was separated by centrifugation at 500 g and analyzed as described in the following section. Blood was collected from the rabbits for the following 2 weeks until potency of the antibody began to decrease. A boosting schedule with Act D:BSA in incomplete adjuvant (Difco) was used for continued antisera collection.

1 This work was supported by Grant GM-22897.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: Act D, actinomycin D; BSA, bovine serum albumin.
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Antibody production peaked approximately 2 weeks after each boost. Serum samples containing antibody were stored at −20°C until use.

**Antibody Detection by Gel Filtration.** The rabbit serum (25 µl) was mixed with an equal volume of [3H]Act D (0.1 µg/ml) and allowed to incubate on ice for 1 hr. The Act D and serum were then passed over a Bio-Gel P-10 column (15 × 1.5 cm) (BioRad Laboratories, Richmond, Calif.), and protein content (absorbance at 280 nm) and Act D content (dpm) were determined for each fraction.

**Radioimmunoassay for Act D.** It has been observed in our laboratory that Act D in aqueous solution binds to plastics and glassware with a significant loss of the drug. Therefore, all material which would come into contact with Act D was first treated with silicone (Sigma). To perform the radioimmunoassay for Act D, undiluted serum (25 µl) was mixed with an equal volume of [3H]Act D (0.1 µg/ml; 0.025 µCi) in a 1.5-ml microfuge tube and incubated on ice. After 1 hr, 100 µl of 0.01 M Tris buffer, pH 7.3, were added to increase the working volume. One 2.0-mg dextran-coated charcoal pellet (Westchem Products, San Diego, Calif.) was added to the tube and mixed. After 5 min, samples were centrifuged at 12,000 × g, and 75 µl of the supernatant were transferred to 3MM filters (Whatman Ltd., Maidstone, Kent, U.K.), dried, and counted in a toluene-based fluor containing 2.5% NCS (Amersham, Chicago, II) and Omnifluor (New England Nuclear, Boston, Mass.) in a Searle Analytic 81 liquid scintillation counter. To develop a standard curve, increasing amounts of unlabeled Act D were added to 25 µl of [3H]Act D (0.1 µg/ml) in the same volume. The mixture was combined with antiserum and assayed as described previously. Triplicate samples were analyzed at each concentration of Act D, resulting in a standard radioimmunoassay curve. Characterization of antibody specificity was performed by addition of actinomycin, actinomycin V (Sigma), or doxorubicin (Adria Laboratories, Wilmington, Del.).

**Pharmacokinetic Analysis of Act D.** The human subject studied was a 59-year-old male patient receiving treatment for metastatic melanoma at the University of Arizona Health Sciences Center. The patient received Act D (Cosmegen; Merck, Sharpe, and Dohme) by i.v. push at 1.50 mg Act D per sq m. Serial blood samples were removed via an i.v. catheter from the opposite arm from which the drug was administered. Ten ml of blood drawn prior to dosing (Time 0) and 3 ml each at 1, 5, 10, 30, 45, 60 min were immediately transferred into silicone-coated tubes (Sherwood Medical, St. Louis, Mo.) After the blood samples clotted, each sample was centrifuged at 550 × g for 15 min, and the serum was separated and stored in siliconized tubes at −20°C. An analysis of blood levels of Act D was performed by adding a constant amount of [3H]Act D (0.10 µCi) to 100 µl of serum sample. Aliquots of 10 µl were taken from each sample for determination of input tritium counts so that all samples could be compared accurately with each other. A standard aliquot of 25 µl of rabbit anti-Act-D was then added to an equal volume of sample serum, allowed to incubate on ice for 1 hr, and assayed as described above in "Radioimmunoassay for Act D." The unknown amount of Act D present in the serum was quantitated by interpolation on the linearized standard curve described below.

A female mongrel dog received Act D (Cosmegen; Merck, Sharpe, and Dohme) by i.v. push at 2.0 mg Act D per sq m. Serial blood samples were removed via an i.v. catheter from the opposite leg from which the drug was administered. Blood collection and serum analysis were the same as described previously for the human subject.

For both the patient and the dog, standard curves were performed with serum obtained prior to drug administration by the addition of known amounts of Act D. Half-lives were calculated using nonlinear regression analysis with a computer program.

**RESULTS**

Act D was coupled to BSA with the intention of producing a specific anti-Act D antibody. The 2-amino group of the heterocyclic chromophore of Act D (Chart 1) provides a suitable means of coupling the drug to free carboxyl groups of proteins. After the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-catalyzed coupling reaction reached completion and following extensive dialysis, it was determined that 2.4 mol Act D bound each mol of BSA.

Four weeks after the initial inoculation with the Act D:BSA complex, antibodies were detected in 2 of 2 rabbits. Initial demonstration of Act D bound to antisera was obtained by reacting tritiated drug with antisera and performing gel filtration. It can be seen in Chart 2 that bound and free drug are well separated. Antiserum from a control rabbit presented with BSA in adjuvant revealed no Act D binding to the void volume protein (data not shown), indicating that Act D does not bind to serum.
proteins in a nonspecific fashion. With the use of gel filtration as a criterion for binding, antiserum obtained from the rabbit initially given an injection of the highest amount of Act D:BSA complex (250 μg) consistently demonstrated higher Act D binding than did the antiserum from the rabbit that received the smaller initial amount of antigen (100 μg).

Due to the time-consuming nature of the gel filtration procedure, a more rapid assay procedure was investigated. It has been demonstrated previously in this laboratory that charcoal is effective for removal of free Act D from solution. No significant loss of Act D antibody due to the presence of charcoal in serum has been observed. The charcoal assay was highly sensitive, it allowed for the analysis of small volumes of serum and drug, it exhibited low background due to nonspecific binding, and it required a minimum amount of time. Serum taken from rabbits at various times after boosting revealed different amounts of Act D binding, and this method proved to be an excellent technique for determining antibody titer.

The charcoal assay was then used in the development of a radioimmunoassay for Act D. It can be seen in Chart 3 that increasing concentrations of unlabeled Act D produced a characteristic standard curve indicative of the competition of unlabeled drug with labeled drug, as classically demonstrated in other radioimmunoassays. When plotted on logit-log paper, a linearized standard curve was obtained with a correlation coefficient of -0.980. This linear curve permitted quantitation of Act D accurately down to 0.1 pmoI (0.005 μg/ml).

Since a large number of Act D analogs have been identified (6, 7), we sought to characterize the specific part of the molecule which contains the antigenic determinant. Since we anticipated that the pentapeptide portion of the drug might be antigenic, we tested an analog with a substantial synthetic modification at that part of the molecule. Actinomine contains the same chromophore as Act D but lacks the pentapeptide lactone ring (Chart 1). When actinomine was substituted for unlabeled Act D in the radioimmunoassay, it can be seen from Chart 3 that very little competition with Act D was observed. Actinomycin V, which has oxoproline substituted for proline in the pentapeptide loop of the molecule, produced identical results to that of Act D. Doxorubicin, which also has a heterocyclic ring system and intercalates into DNA, produced no displacement in this assay (data not included).

The utility of the radioimmunoassay can be seen in Chart 4 in which actual serum levels were quantitated after Act D administration to a human and a dog. Since the human subject had received previously one identical course of therapy 3 weeks prior to this dose, it was necessary to determine if antibodies to Act D were present in the serum. This was done by comparison of the subject’s zero time serum with that of the nonimmune rabbit control. No Act D antibody was detected. Reproducibility (i.e., percentage of coefficient of variation) of the assay for both human and dog samples were determined to be 9.4 and 5.1%, respectively. Serum levels revealed a biphasic pharmacokinetic pattern within 1 hr after administration. The human α-phase serum half-life was 1.78 min, whereas the β-phase was 34 min. The α-serum half-life in the dog was 0.78 min, and the β-phase was 208 min. As the sensitivity of the assay was known to be at least 0.005 μg/ml, it would appear that additional values beyond the 1-hr time point are possible to obtain.

DISCUSSION

A specific antibody to Act D has been developed and used in a rapid and sensitive radioimmunoassay for the detection of Act D in serum. The Act D radioimmunoassay described is a simple procedure requiring few manipulations and has excellent reproducibility. When antibody specificity was first detected by gel filtration (Chart 2), we realized that a quicker, more efficient assay was necessary for multiple sample analysis. The double antibody technique using goat anti-rabbit IgG

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6 J. J. Duffy and T. J. Lindell, manuscript in preparation.
7 Preliminary studies with Protein A-purified IgG indicate that protein levels must be increased to approximate serum concentrations to prevent extensive loss of Act D IgG when the charcoal assay is used.

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Chart 3. Competition of Act D and actinomine for specific Act D antibody. C, Act D; O, actinomine. Increasing amounts of the respective compounds were added to [3H]Act D, the mixture was combined with antibody and assayed as described in Materials and Methods. The percentage [3H]Act D bound to the antibody at the various concentrations was calculated: 100% = 20,000 dpm; background = 500 dpm. The abscissa range is 10^-12 to 10^-10 M for Act D and 10^-11 to 10^-8 M for actinomine. Data represent the average of triplicate samples with the maximum S.E. 2.7%.

Chart 4. Serum concentrations of Act D with time after the initial dose. Serum samples obtained at various times after Act D injection were assayed using the radioimmunoassay described in Materials and Methods. The percentage [3H]Act D bound to the antibody at the various concentrations was calculated: 100% = 20,000 dpm; background = 500 dpm. The abscissa range is 10^-12 to 10^-10 M for Act D and 10^-11 to 10^-8 M for actinomine. Data represent the average of triplicate samples with the maximum S.E. 2.7%.
(9) was attempted and proved useful in antibody detection but was unacceptable because of high nonspecific background binding. The charcoal assay, on the other hand, proved to be extremely efficient in removing free Act D and in reducing the background. In the 5-min incubation period with charcoal, greater than 99% of the free Act D was bound to the charcoal, leaving only Act D-antibody complex present in solution; hence, labeled drug in the supernatant after centrifugation was a good measure of $[^3]H$Act D bound to antibody. The charcoal assay is rapid, with an initial Act D antiserum incubation period of only 1 hr. This initial incubation is the rate-limiting factor of the assay but is far superior to many radioimmunoassays.

The utility of the Act D antibody has been demonstrated with the development of a radioimmunoassay, which was used for the quantitation of serum levels of the drug in a patient and a dog. The differences in serum half-lives for the human and the dog (Chart 4) are not unexpected. Galbraith and Mellett (2) show species differences in Act D serum clearance. More complete pharmacokinetic studies are necessary before any conclusions can be drawn about actual half-lives and species variation. Due to the simplicity of the assay described, complete pharmacokinetic analysis could be easily performed.

Pharmacokinetic studies of Act D in humans and animals have been reported previously (2, 14) using labeled drug. The use of radioimmunoassay eliminates the introduction of unnecessary radioactivity into the patient. Furthermore, since tritiated drug was used in the previous study, the possibility exists that some tritium may exchange with body water, and the amount of drug detected in later time samples could be due to $^3$H$O$ since actual drug was not measured. The use of a radioimmunoassay specific for the drug eliminates this possible artifact.

While 2 phases of serum levels of Act D have been observed previously in humans (14), the half-life of the $\alpha$-phase has not been reported accurately. By examining early time points, we have determined this value to be 1.78 min, which indicates the rapid redistribution of the drug from serum. It is probable that the half-life of the $\beta$-phase will be determined more accurately upon examination of serum samples beyond 1 hr. We anticipate extended studies to obtain this information in the near future.

A preliminary characterization of an antibody to Act D was presented previously by Raso (13) with sensitivity reported to 1 pmol. The work described in this paper details the methodology for Act D antibody preparation and presents a simplified radioimmunoassay procedure which has a 10-fold increase in assay sensitivity as compared to the Raso procedure. Our study also demonstrates a clinical application of this radioimmunoassay by detection of Act D serum levels. From preliminary results, we are confident that sensitivity can be increased by varying volumes of antiserum used and amounts of $[^3]H$Act D in the assay. Sensitivity of the assay might also be increased with Protein A-purified IgG instead of whole serum in the radioimmunoassay. However, if this is done, the protein content must be increased with BSA to a concentration similar to that of serum to prevent nonspecific binding of the IgG.

Chemotherapeutic use of Act D is known to suppress the immune system (12). However, the present study demonstrated that Act D can elicit an antibody response when the drug is bound to BSA. Apparently, the drug-BSA complex did not inhibit an immune response. Further, our data suggest that the antigenic determinant resides in the pentapeptide lactone portion of the molecule (Chart 3). Because Act D binds to DNA by intercalation of the heterocyclic chromophore moiety, it should be possible to ultimately examine drug binding to DNA at the molecular level at concentrations which are known to inhibit rRNA synthesis in vivo. Since a unique binding isotherm for Act D has been found at this concentration range, the antibody preparation described may be useful in further defining this site.

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