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Radioimmunoassay of Bleomycin

Alan Broughton and James E. Strong

Department of Pathology, The University of Texas Medical School at Houston, Houston, Texas 77025

SUMMARY

A radioimmunoassay for bleomycin has been produced using ^{125}I-labeled bleomycin and antisera raised in rabbits against a carbodiimide-catalyzed bleomycin-bovine serum albumin conjugate. ^{125}I-Labeled bleomycin was synthesized by direct iodination of the drug using the chloramine-T technique. The standard curve of the assay was linear on a logit-log plot and the lower limit of sensitivity was 250 pg bleomycin sulfate. A mean recovery of 102.6% (±3.3% S.E.) was obtained using bleomycin added to normal sera. No significant decrease in bleomycin immunoreactivity was observed following 24 hr incubation of the drug in serum at 37°. The radioimmunoassay was also suitable for measuring bleomycin in the presence of other drugs since the assay was not significantly affected by the other antineoplastic agents tested. The sensitivity and specificity of the radioimmunoassay for bleomycin should provide a new means for pharmacokinetic and toxicity studies of bleomycin.

INTRODUCTION

Bleomycin, an important antineoplastic antibiotic isolated from *Streptomyces verticillus* (14), has been effective against a variety of neoplasms, particularly squamous cell carcinoma, lymphoma, and testicular carcinoma (1). Minor cutaneous reactions are the most common toxic effect of bleomycin. However, pulmonary toxicity, occurring as pneumonitis in about 10% of the patients, has led to pulmonary fibrosis and morbidity in approximately 1% of the patients (4).

Investigations concerning the bleomycin concentration in patients' plasma and tissues may provide a quantitative method for individualization of dosage regimens to afford maximum chemotherapeutic benefit and to avoid toxicity. The pharmacokinetics and tissue distribution of bleomycin have been examined previously using isotopically labeled drug (12) and microbiological assay (11) techniques. The use of radioactively labeled drug assays has obvious disadvantages for routine application in patient chemotherapy, whereas microbiological assays lack sensitivity and specificity and require long incubation periods. Radioimmunoassays have been shown to be sensitive, specific, rapid, and precise in estimating concentrations of drugs and other small molecules in biological fluids (3). These characteristics make radioimmunoassay an appropriate procedure for monitoring plasma bleomycin concentrations. This report describes development of a radioimmunoassay for bleomycin using ^{125}I-labeled bleomycin.

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MATERIALS AND METHODS

Preparation of Bleomycin-BSA[1] Conjugate. Bleomycin sulfate (generously supplied by Bristol Laboratories, Syracuse, N. Y.) was conjugated to BSA using 1-ethyl-3-(dimethylaminopropyl)carbodiimide to form an amide bond (5). The BSA (20 mg) was dissolved in 1.0 ml PBS containing 45 mg bleomycin. Then, 1.0 ml 1-ethyl-3-(dimethylaminopropyl) carbodiimide (800 mg/ml) was added slowly to the bleomycin:BSA mixture with constant stirring. This solution was mixed for 1 hr at room temperature followed by incubation for 3 days at 4°. The bleomycin:BSA conjugate was dialyzed for 18 hr (4°) *versus* PBS, and final purification was achieved by chromatography on Sephadex G-25. The molar ratio of bleomycin:BSA was estimated spectrophotometrically and was found to be approximately 28:1.

Immunization. The chromatographically purified bleomycin:BSA conjugate was used for production of antisera in 3 New Zealand White rabbits by the procedure of Hurn and Landon (7). Each rabbit received 0.5 mg of the conjugate, emulsified in complete Freund's adjuvant, by i.m. injection into all limbs. This was followed by monthly injections of 0.5 mg conjugate into either the fore or hind limbs. The antiserum obtained after 3 booster injections bound 50% of the labeled bleomycin (2 ng) at a final dilution of 1:10,000.

Bleomycin Iodination. A modification of the Hunter and Greenwood chloramine-T technique (6), followed by cation-exchange column chromatography, was used to obtain [^{125}I]bleomycin. The following ingredients were mixed and incubated for 1 min at room temperature: 1 mCi Na^{125}I (100 mCi/ml 0.1 N NaOH), 10 μl chloramine-T (5-mg/ml solution in 0.1 M borate buffer, pH 9.0), and 10 μl bleomycin (1-mg/ml solution in 0.1 M borate buffer, pH 9.0). Next, 10 μl of sodium metabisulfite (12-mg/ml solution in 0.1 M borate buffer, pH 9.0) were added, followed by 10 μl potassium iodide (20-mg/ml solution in 0.1 M borate buffer, pH 9.0). The iodinated bleomycin was separated from the reaction ingredients by column chromatography on Sephadex C-25 (30 x 0.9 cm). A linear gradient of ammonium formate (pH 6.4) from 0.1 to 1.0 M was used to elute the iodinated product. The specific activity was estimated to be approximately 5 μCi/μg, which gives an incorporation of 3.5×10^{-5} atom of iodine per molecule of bleomycin.

Radioimmunoassay Procedure. A competitive protein

[1] The abbreviations used are: BSA, bovine serum albumin; PBS, phosphate-buffered saline (0.01 M phosphate, pH 7.5-0.15 M NaCl-0.1% sodium azide).

binding assay between ^{125}I-labeled bleomycin and unlabeled bleomycin for antibody binding sites was used to quantitate unknown concentrations of bleomycin in serum and buffer solutions. Antibody-bound drug was isolated following dextran-coated charcoal absorption of free bleomycin. The radioimmunoassay of bleomycin was performed using the following protocol in the order given: 200 μl of 0.1% gelatin dissolved in PBS, 100 μl sample or appropriate standards, 100 μl ^{125}I-labeled bleomycin diluted to approximately 30,000 cpm in PBS, and 100 μl antiserum diluted 1:2,000 in 0.1% gelatin dissolved in PBS were thoroughly mixed and incubated (10 min at 37° and 10 min at 4°). The standard solution of bleomycin in PBS was prepared from a stock solution of bleomycin sulfate:100 μg/ml PBS to cover the range 2 to 500 ng/ml (0.2 to 50 ng/tube). Following incubation, 200 μl dextran-coated charcoal (2 g dextran T-70 and 2 g activated charcoal in 100 ml PBS) plus 400 μl PBS (4°) were added, and after a brief incubation (5 min, 4°) the mixture was centrifuged (2000 × *g*, 10 min, 4°). The supernatants were decanted and radioactivity was measured in an automatic γ counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

RESULTS

The standard curve for bleomycin was linear on a logit-log plot (Chart 1). Sensitivity of this assay, defined as the lowest amount of bleomycin that can be significantly ($p < 0.05$) distinguished from no drug, was 250 pg. Assay precision, determined from the standard curve using the index λ (9) (the ratio between the S.D. of each *Y* value and the slope of the regression line between the mean values of logit *Y* and corresponding log *X*) was 0.024 at 5 ng.

Since the microbiological assay previously used to measure bleomycin was adversely affected by serum (11), logit-log plots were obtained for standard concentrations of bleomycin prepared in either PBS or serum from normal donors. The results of this comparison (Chart 2) showed that concentrations of bleomycin prepared in serum were identical to those prepared in PBS. Recovery experiments were also performed by adding bleomycin at known concentrations to serum specimens and measuring these by radioimmunoassay. Twenty specimens were treated in this manner with bleomycin concentrations ranging from 1 to 10 μg/ml serum, and the mean recovery was 102.6% (±3.3% S.E.). Experiments were also designed to measure the change in immunoreactivity of bleomycin following incubtion in serum. Known concentrations of bleomycin were incubated in serum for up to 24 hr at 37°, and the immunoreactivity was measured at timed intervals. No significant decrease of bleomycin immunoreactivity caused by incubation in serum was observed.

The specificity of the assay was investigated for interference by other chemotherapeutic drugs with bleomycin for antibody binding sites (Chart 3). None of the drugs

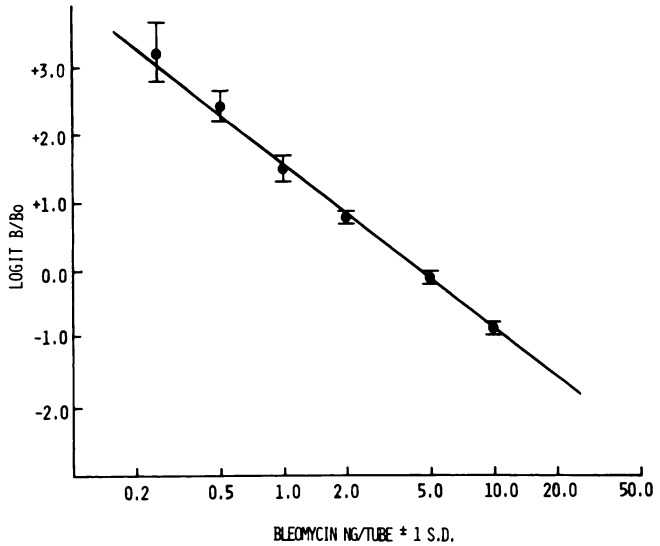

Chart 1. The standard curve for the radioimmunoassay of bleomycin. Logit B/B_0 is the logit of the ratio of antibody-bound radioactivity to radioactivity bound at zero concentration of unlabeled bleomycin. Each *point* represents the mean ± S.D. for 6 replicates.

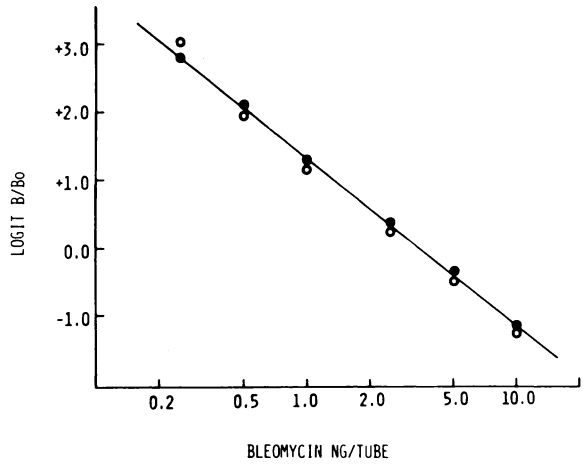

Chart 2. A comparison of standard curves for bleomycin prepared in serum or buffer. The indicated concentrations of bleomycin were prepared by adding bleomycin sulfate to the serum of a normal donor (○) or PBS buffer (●). Each *point* represents the average of duplicate samples.

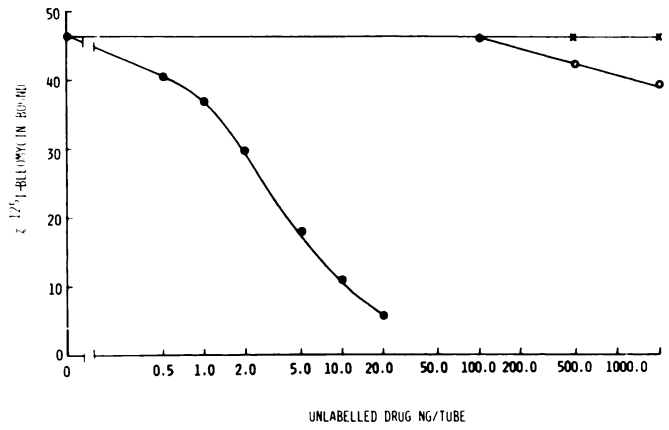

Chart 3. Specificity of the bleomycin radioimmunoassay. The proportion of antibody bound to total ^{125}I-labeled bleomycin is plotted as percentage bound: bleomycin sulfate (●); adriamycin (○); 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, 5-fluorouracil, arabinosyl cytosine, vincristine, and prednisolone (X).

examined competed significantly with ^{125}I-labeled bleomycin in the radioimmunoassay. Only adriamycin, at concentrations 2000-fold greater than bleomycin, caused reduction in ^{125}I-labeled bleomycin binding to antibody.

The affinity of antibody for labeled bleomycin was investigated by adding known quantities of either labeled or unlabeled drug to a constant amount of ^{125}I-labeled bleomycin in the radioimmunoassay (Chart 4). If the iodination of bleomycin had resulted in damage to bleomycin determinate groups, then the shape of the standard curve in Chart 4 would be different depending on whether labeled or unlabeled bleomycin was added (2). However, the 2 curves are superimposable, thus indicating little or no change in immunoreactivity caused by iodination. The affinity constant of antibody for ^{125}I-labeled bleomycin was determined by an equilibrium technique (10) and was found to be 1.3×10^9 liters mole^{-1}.

DISCUSSION

The understanding and prediction of bleomycin toxicity has been hampered by the lack of a rapid, sensitive, and precise quantitative assay. The current procedures involve either administering radioactively labeled bleomycin to patients or use of microbiological assay systems (11, 12). The radioimmunoassay described here fulfills the above criteria and is approximately 100-fold more sensitive than the microbiological assays. This sensitivity will allow the estimation of bleomycin in biological fluids and tissue extracts hitherto unavailable to the clinical investigator, and thus the toxicity and the pharmacokinetics of bleomycin may be investigated further.

The ^{125}I label used in the radioimmunoassay of bleomycin avoids the expense and technical problems of liquid scintillation counting needed for the tritiated labels commonly used in the radioimmunoassay of small molecules. The iodination was achieved by adjusting the pH of the reactants to facilitate nucleophilic substitution of iodine into the imidazole ring (13) of the bleomycin molecule. This substitution did not appear to alter significantly the antibody binding of the bleomycin molecule as indicated by the high-affinity constant for ^{125}I-labeled bleomycin (1.3×10^9 liters mole^{-1}), and the identical standard curves produced using either labeled or unlabeled bleomycin (Chart 4).

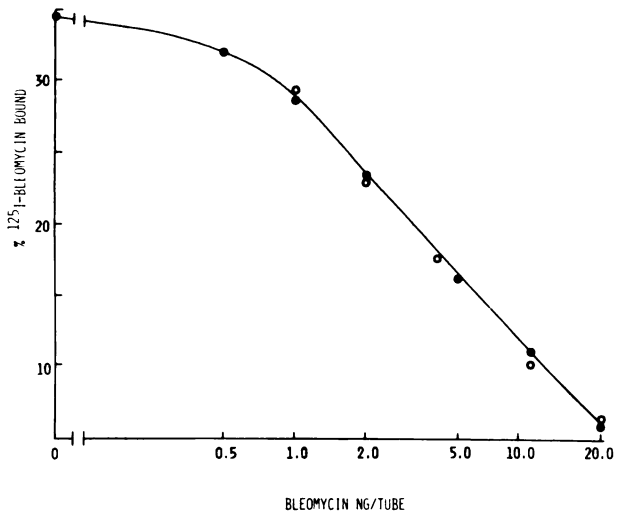

Chart 4. Comparison of the immunoreactivity of ^{125}I-labeled and unlabeled bleomycin. ^{125}I-labeled bleomycin was used in all tubes as tracer and the indicated amounts of either labeled (○) or unlabeled (●) bleomycin were added. The percentage of ^{125}I-labeled bleomycin bound was the percentage of total labeled bleomycin bound to antibody.

A recent paper (8) comparing the radioiodination of bleomycin by 3 methods, the chloramine-T technique, lactoperoxidase, and iodine monochloride, showed that the chloramine-T and iodine monochloride methods initially gave equally high yields but the latter technique produced a more stable product. The labeled material produced by our method has a lower specific activity and yield, but it is more stable (5% dissociation of ^{125}I in 1 half-life) than the chloramine-T-labeled bleomycin produced by Myers *et al.* (8). The different stabilities and yields of the 2 labeled compounds may be caused by variations in chloramine-T labeling techniques. Myers *et al.* (8) incubated chloramine-T, bleomycin, and ^{125}I at pH 7.5 for 5 min whereas our procedure included incubation for only 1 min at pH 9.0. Although their iodine monochloride labeling technique was designed for *in vivo* studies, it should be suitable for and may improve the radioimmunoassay of bleomycin.

Since bleomycin is used often in combination with other chemotherapeutic agents, the radioimmunoassay should be sufficiently specific to avoid cross-reactivity with these other drugs. Therefore, many commonly used antineoplastic agents were examined for their interference with the bleomycin radioimmunoassay. It was found that no significant cross-reactivity occurred with any of the drugs tested (Chart 3), thus indicating that the radioimmunoassay for bleomycin is appropriate for use in combination chemotherapy. The binding of bleomycin to plasma proteins also does not seem to affect the radioimmunoassay since standards prepared in either buffer or serum yield essentially the same values (Chart 2).

The inactivation of bleomycin incubated in serum was reported by Ohnuma *et al.* (11) using a microbiological assay. Using the radioimmunoassay, no significant decrease in immunoreactivity of bleomycin was observed following incubation in normal sera for 24 hr at 37°. However, the determinate groups of the bleomycin molecule recognized in the immunoassay may not be identical with that part of the molecule active in a biological assay system. Despite that possibility, the radioimmunoassay of bleomycin should provide both the researcher and clinical investigator with a new means for investigating the tissue distribution and pharmacokinetics of bleomycin.

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