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A Sensitive Radioimmunoassay for Vincristine and Vinblastine

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Summary. *A rapid, sensitive and highly reproducible radioimmunoassay (RIA) has been devised for vincristine and vinblastine. These alkaloids bind to the antiserum very tightly with an association constant (K_a) of 5 × 10^9 M^{-1} as determined by Scatchard analysis. The kinetic association constant of the alkaloids, as determined by the 'on' and 'off' rates at 4° C, is 30-fold higher than the Scatchard analysis value. At 4° C, 80% of the alkaloid is bound to the antiserum within 20 min, and the remaining 20% reaches an equilibrium within 48–72 h. In the RIA procedure the amount of the alkaloid (1–2 ng) needed to compete for 50% binding remains unchanged when the reaction mixtures are incubated for 2, 4, or 20 h. By a modification of the RIA procedure, where the antiserum is first allowed to react with the unlabeled alkaloid followed by addition of the radiolabeled alkaloid, sensitivity of the assay has been increased five- to tenfold. Other drugs, such as adriamycin, CCNU, methyl CCNU, cyclophosphamide, 5-fluorouracil, heparin, hydrocortisone, prednisone, and nitrogen mustard do not interfere with the assay. This assay can thus be used to determine vincristine or vinblastine levels in 5 × 10^{-10}–10^{-9} M concentrations in biological fluids.*

Introduction

Catharanthus alkaloids vincristine and vinblastine are widely used as antitumor agents, singly or in combination with other chemotherapeutic agents, in a variety of neoplastic diseases. The use of these drugs is clinically limited due to myelosuppression of vinblastine and neurotoxicity of vincristine. Pharmacology of these alkaloids has been studied in experimental animals [3–5, 7], and in man [2, 6, 8] with radioactively labeled drugs. Radioimmunoassay (RIA) has been developed to investigate the pharmacokinetics of vincristine, vinblastine, and vindesine [9, 10]. The latter procedure involved a 4-day incubation period and the assay was sensitive up to 2–5 ng drug concentrations. In this study we have determined the binding association constant of these alkaloids with the antiserum, and describe a modified RIA procedure which is rapid, highly reproducible, and five- to tenfold more sensitive.

Materials and Methods

Drugs. Vincristine sulfate (NSC 67574) and vinblastine sulfate (NSC 49842) were kindly provided by Dr. John Douros of National Cancer Institute, Bethesda, Maryland. Radioactive (G-^{3}H) vinblastine sulfate (specific activity 10.4 Ci/mmol) and (G-^{3}H) vincristine sulfate (specific activity 3.2 Ci/mmol) were purchased from Amersham Corporation, Arlington Heights, Illinois. Radioactive ^{3}H-vincristine sulfate (specific activity 7 Ci/mmol), prepared by Moravek Biochemicals, California, and distributed by Research Triangle Institute, North Carolina, was also used in some experiments, and was a generous gift from Dr. R. Bender, National Cancer Institute. Adriamycin HCl (Adria Laboratories); bleomycin, lomustine (CCNU), methyl CCNU (Bristol); cyclophosphamide (Mead-Johnson); 5-fluorouracil (5-FU) (Hoffman-LaRoche); heparin, hydrocortisone, prednisone (Upjohn), and nitrogen mustard (Merck, Sharp and Dohme) were obtained from the indicated pharmaceutical companies.

Vinblastine Antiserum. Vinblastine was converted to 4-deacetyl vinblastine C-3 carboxyhydrazide on treatment with hydrazine in methanol [1]. By treatment of the hydrazide with nitrous acid, it was converted to 4-deacetyl vinblastine C-3 carboxazide [1] which was covalently coupled to bovine serum albumin (BSA) in dioxane. 4-Deacetyl vinblastine-BSA was injected into rabbits to raise antiserum [11]. The unfractionated vinblastine antisera in a lyophilized form was generously provided by Dr. Mary Root, Lilly Research Laboratories, Indianapolis, Indiana. Antiserum lot 24-245-2-R was used. Various dilutions of the lyophilized antiserum were made in the glycine buffer (0.2 *M* glycine HCl, pH 8.8; 0.25% crystalline human albumin, ICN Pharmaceuticals, Cleveland, Ohio; 1% normal lamb serum, North American Biologicals, Miami, Florida; 242 mg merthiolate/l) to find the appropriate concentration of the antiserum which would bind 40–50% of the input ^{3}H-vinblastine. The diluted antiserum samples were allowed to react with 1 pmol of ^{3}H-vinblastine for 2 h at 4° C, and

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the free alkaloid was separated by dextran-coated charcoal (DCC) method as described below. The amounts of radioactivity bound to the antiserum at 1 : 1,000, 1 : 2,500, 1 : 5,000, and 1 : 10,000 dilutions of the antiserum were 66, 55, 21, and 12%, respectively, of the total input radioactivity. Binding of the alkaloid to the antiserum was linear between 1 : 1,000- to 1 : 5,000-fold antiserum dilutions. Each new antiserum lot was analyzed for appropriate dilution before use.

Radioimmunoassay. In a total volume of 400 µl in glass test tubes (12 × 75 mm) the reaction mixture contained 100 µl ^{3}H-vinblastine (dilution 1 : 2,400) or ^{3}H-vincristine (dilution 1 : 1,000), 100 µl unlabeled alkaloid in the glycine buffer, 100 µl diluted antiserum or normal rabbit serum, and the glycine buffer. The reaction mixtures were incubated at 4° C in enclosed plastic containers for the appropriate length of time. At the end of incubation period, 400 µl well-stirred DCC suspension (1% carbon decolorizing, Fisher Scientific, Fairlawn, New Jersey, and 0.5% dextran 70, Pharmacia Fine Chemicals, Uppsala, Sweden, suspended in the glycine buffer) were added to each tube, and the tubes were mixed gently, kept at room temperature for 20 min, and centrifuged at 2,700 rpm for 10 min. Five-hundred µl of the clear supernatant was withdrawn carefully, mixed thoroughly with 4 ml toluene-PPO-POPOP : Triton X-100 (2 : 1) scintillation fluid, and the radioactivity was counted. Radioactivity from each sample was converted into disintegerations per minute (DPM) from a standard quench curve. From the total amount of input radioactivity, percent labeled alkaloid bound was calculated after substracting the nonspecific radioactivity. The nonspecific binding of ^{3}H-vinblastine or ^{3}H-vincristine was between 1–3% of the total input radioactivity.

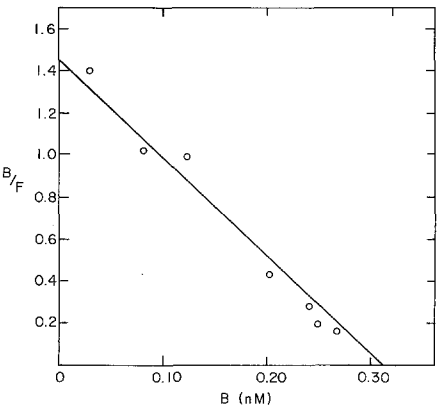

Fig. 1. Scatchard analysis of vinblastine binding to the antiserum. Increasing amounts (435, 992, 1,250, 1,482, 4,242, 6,695, and 11,490 cpm) of radioactive vinblastine (specific activity 1,200 cpm/pmol) were allowed to react at 4° C for 2 h with 50 µl 1 : 3,000 diluted antiserum, in duplicate test tubes, according to conditions described in text. Diluted normal rabbit serum was used for nonspecific binding. From the amount of bound (B) radioactivity, free (F) vinblastine was determined in each case. These results were plotted according to Scatchard [12], i.e., B versus B/F. Linear regression analysis were performed on these data points. A correlation coefficient, r^2 of 0.95, were obtained. From these data association constant, K_a of 4.96 × 10^9 M^{-1} and the antiserum binding capacity of 88 pmol vinblastine/mg protein was calculated

Results

Binding of Catharanthus Alkaloid to the Antiserum. In preliminary experiments, a rapid rate of the labeled alkaloid binding to the vinblastine antiserum was observed within the first 30 min of the reaction, followed by a slower rate, with achievement of a plateau or equilibrium at 24–48 h. Eighty percent of the alkaloid bound at a fast rate and the remaining 20% bound at a slower rate. The rates of vincristine and vinblastine binding to the antiserum seemed to be similar.

Binding of the alkaloid to the antiserum can be described according to the law of mass action as:

$$A + B \underset{k-l}{\overset{kl}{\rightleftharpoons}} AB,$$

where A is the alkaloid, B is the antiserum, AB is the alkaloid-antiserum complex, and kl and k-l are the respective 'on' and 'off' rate constants of the complex formation and its dissociation. At equilibrium the association constant, K_a can be written as: $K_a = (kl/k\text{-}l) = ([AB]/[A][B])$. Scatchard analysis were performed to determine K_a and the capacity of antiserum for its binding to the alkaloid (Fig. 1). With radioactive vinblastine a K_a of 4.96 × 10^9 M^{-1} and antiserum binding capacity of 88 pmol vinblastine/mg protein were obtained. With ^{3}H-vincristine K_a of 5.0 × 10^9 M^{-1} and a binding capacity of 60 pmol vincristine/mg antiserum were obtained (data not shown). The initial rates of the alkaloid binding of the antiserum at 4° C were linear up to 6–9 min. These rates were 0.0776 nM vincristine min^{-1} and 0.033 nM vinblastine min^{-1} at 7.41 nM total vincristine and 1.83 nM total vinblastine concentrations, respectively. Assuming that the antiserum bound to the alkaloid in 1 : 1 molar ratios, the amounts of the antiserum in the reaction mixtures were calculated from the binding capacity of the antiserum as 0.375 nM and 0.55 nM, respectively, for vincristine and vinblastine. From these data kl for vincristine and vinblastine were calculated to be 2.80 × 10^7 M^{-1} min^{-1} and 3.28 × 10^7 M^{-1} min^{-1}, respectively. Under similar experimental conditions the half-life ($T_{1/2}$) of dissociation of the alkaloid-antiserum complex were determined in the presence of 13.55 µM alkaloid, and the $T_{1/2}$ were 55 h and 62 h respectively, for vincristine and vinblastine. There was practically no decay of the alkaloid-antiserum complex in the absence of the unlabeled alkaloid over a period of 96 h. From these data the k-l of 2.1 × 10^{-4} min^{-1} and 1.86 × 10^{-4} min^{-1} were calculated for vincristine and vinblastine. The K_a values from these kinetic experiments for both alkaloids are in the range of 1.33–1.76 × 10^{11} M^{-1} (Table 1). These values are about 30-fold higher than those obtained by Scatchard analysis.

Radioimmunoassay of Vincristine. In the original procedure [10, 11], the reaction mixtures were incubated for 4 days at 4° C. The kinetics of the binding between the alkaloid and the antiserum indicated that shorter incubation

Table 1. Association and dissociation rate constants of the antiserum and Catharanthus alkaloids at 4° C

Alkaloid	kl (M^{-1} min^{-1} × 10^7)	$T^1/_2$ (h)	$k-l$ (min^{-1} × 10^{-4})	$K_a = kl/k-l$ (× 10^{11} M^{-1})
Vincristine	2.80	55	2.10	1.33
Vinblastine	3.28	62	1.86	1.76

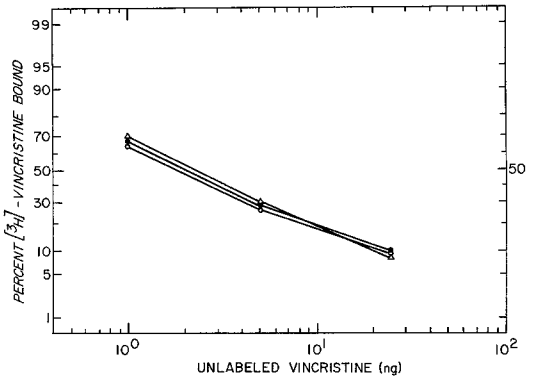

Fig. 2. Influence of incubation time on the radioimmunoassay of vincristine. In duplicate reaction tubes with an assay volume of 500 µl, containing 100 µl 1 : 1,600 diluted normal rabbit serum or the antiserum, 100 µl 1 : 1,000 diluted ^{3}H-vincristine, increasing amounts (1–25 ng) of unlabeled vincristine, and glycine buffer were incubated for 2 h (○ – ○), 4 h (● – ●), and 20 h (△ – △) at 4° C. At the end of incubation, 500 µl of either glycine buffer, or dextran-coated charcoal suspension were added to each tube, and these were processed according to the procedures described in text. From these results, percent of ^{3}H-vincristine bound at different concentrations of unlabeled vincristine were determined and plotted on a log-logit scale

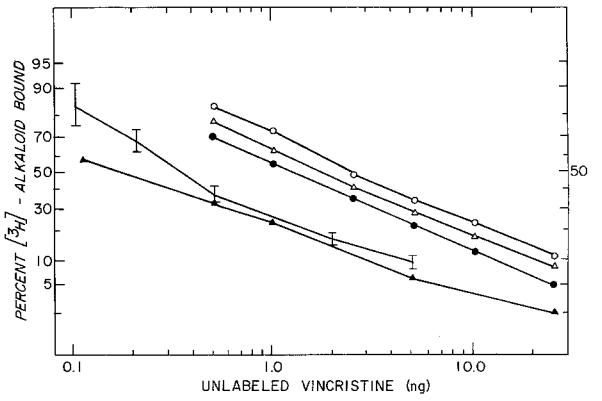

Fig. 3. Comparison of the standard radioimmunoassay (RIA) and the sequential saturation RIA for the estimation of vincristine. In the standard RIA, different amounts of unlabeled vincristine were mixed either with labeled vincristine (○ – ○) or labeled vinblastine (△ – △), and the diluted antiserum was added and incubated for 2 h at 4° C according to the conditions described in Figure 2. In the sequential saturation RIA, the unlabeled vincristine reaction tubes were first incubated for 60 min with the diluted antiserum, and thereafter the labeled vincristine (● – ●) or vinblastine (▲ – ▲) were added, and incubated further for 60 min at 4° C. The amounts of bound radioactivity were determined by dextran-coated charcoal procedure. Appropriate controls were performed in each experiment. A standard curve for vincristine estimation by sequential saturation RIA (⊢–⊣) from 20 different experiments, when labeled vinblastine was used, is also plotted

periods should suffice for the RIA of vincristine. Experiments were designed to assay vincristine at 2, 4, and 20-h incubation periods (Fig. 2). The competition curves for vincristine during different incubation times were very similar and almost superimposable. The level of unlabeled vincristine needed to compete for 50% bound radioactivity in these experiments was approximately 2.1 ng.

To increase the sensitivity of the standard RIA procedure, a sequential saturation RIA method [14] was investigated. In this procedure the unlabeled vincristine was first incubated for 60 min with the antiserum. Thereafter, the labeled vinblastine was added and the reaction mixture was incubated for an additional 60 min, followed by DCC treatment. Results of these experiments by using ^{3}H-vinblastine or ^{3}H-vincristine, as compared with the standard RIA, are depicted in Fig. 3. In the standard RIA procedure the levels of unlabeled vincristine needed to compete for 50% of bound radioactivity in the ^{3}H-vincristine-antiserum and ^{3}H-vinblastine-antiserum complexes were 2.5 and 1.8 ng, respectively, whereas in the sequential saturation RIA the respective amounts of vincristine were 1.2 and 0.17 ng. These data indicate that when the ^{3}H-vinblastine-antiserum complex is used in the RIA, a tenfold increase in sensitivity is obtained by this procedure. A standard curve for vincristine determined by the sequential saturation RIA method from 20 different experiments gave a 50% competition level at 0.33 ng vincristine (Fig. 3).

Influence of Other Drugs on the Radioimmunoassay. Drugs which are commonly given in combination with vincristine or vinblastine were tested for their interference with the RIA. These data are summarized in Table 2. Adriamycin, CCNU, methyl CCNU, cyclophosphamide, 5-FU, hydrocortisone, prednisone, and nitrogen mustard do not interfere with the RIA at 10 and 100 µg drug

Table 2. Influence of other drugs on the radioimmunoassay

Drugs	Percent control[a] at 100 μg drug concentration	Percent control[a] at 10 μg drug concentration
Adriamycin HCl[b]	112	91
CCNU	87	86
Methyl CCNU	83	86
Cyclophosphamide	75	74
5-Fluorouracil	87	89
Hydrocortisone	92	88
Prednisone[b]	89	88
Mechlorethamine HCl (nitrogen mustard)	90	79
	Percent control[a]	
Bleomycin		
10^{-1} U/assay	172	
10^{-2} U/assay	93	
Heparin		
10^{2} U/assay	85	
10 U/assay	97	
1 U/assay	91	

[a] Control, ^{3}H-vinblastine bound to the antiserum, 4,966 DPM (100%)

[b] These drugs remained in suspension at 1 mg/ml concentration in the glycine buffer, pH 8.8

concentrations. Similarly, heparin at 1, 10, and 100 units does not interfere with the assay. Bleomycin at 0.01 units does not show any interference, but at 0.1 units indicates a 72% increased binding of the labeled vinblastine to the antiserum.

Discussion

The antiserum produced in rabbits by injecting covalent conjugate of deacetyl vinblastine with BSA do not show any differences in recognition of minor structural differences in vincristine and vinblastine. The antiserum has similar binding kinetics, dissociation rates, capacities, and association constants for these alkaloids. The alkaloids bind to the antiserum very tightly with a K_a of $5 \times 10^9\ M^{-1}$ as determined by Scatchard analysis. However, a 30-fold higher K_a value is obtained by a different technique of kinetic association and dissociation rate constants. A similar range of K_a value has been obtained from rabbit antiserum which was injected with a BSA-vinblastine conjugate synthesized by Mannich reaction [13]. The antiserum also shows a slightly higher capacity for its binding to vinblastine (88 pmol/mg) as compared to vincristine (60 pmol/mg). These differences could be due to the purity of radioactive alkaloids and/or their variation in the specific activity determinations. It is known that vincristine is more labile than vinblastine. We have recently found by fluorography of the alkaloids, on thin-layer chromatography on silica gel in diethylether : n-propanol : triethylamine (72 : 48 : 6) solvent system, that the radioactive vinblastine (Amersham, 6 months old) was 95% pure, whereas ^{3}H-vincristine from Amersham (3 months old) and Moravek Biochemicals (18 months old), were 75% and 50% pure, respectively (Sethi, unpublished data). Differences in the purity of the radioactive alkaloids could, therefore, be responsible for variable binding capacites.

The crude antiserum used in our experiments shows biphasic binding kinetics with the alkaloids. Eighty percent of the alkaloid is bound rapidly within 30 min at 4° C, whereas the remaining 20% reaches plateau between 24–72 h. These results could be explained by assuming either two different types of proteins in the antiserum or two kinetically different binding sites on the same antibody molecule. Our results cannot distinguish between these two possibilities.

The RIA procedure described here is rapid and sensitive. In contrast to the 4-day incubation period [10, 11] our data clearly demonstrate that a 2–4 h incubation period is sufficient to achieve comparable results. In the sequential saturation RIA where the unlabeled alkaloid is allowed to react first with the antiserum for 60 min, followed by addition of the labeled alkaloid to bind to the unreacted sites on the antibody molecules for another 60 min, the sensitivity of the assay is increased five- to tenfold. This method seems to be particularly advantageous in measuring low levels (10^{-10}–$10^{-9}M$) of these alkaloids.

Drugs (adriamycin, CCNU, methyl CCNU, 5-FU, heparin, hydrocortisone, prednisone, and nitrogen mustard) which are commonly used in combination chemotherapy with vincristine or vinblastine do not interfere with the assay at 100 μg drug concentrations. Bleomycin at very high concentrations of 0.1 units shows 72% increased binding, which could possibly be due to binding of the alkaloid to the tripeptide bleomycin which is not adsorbed by DCC. These results suggest that the RIA method could be successfully used in monitoring alkaloid levels in patients receiving other antitumor drugs. By giving radioactive vinblastine or vincristine by bolus IV injection to man, three phases of the exponential decay (α, β, and γ) of the alkaloid in the blood serum have been described [9, 11]. Levels of $10^{-9}\ M$ alkaloid concentrations in the γ-phase have been difficult to measure accurately with the result that microscopic kinetic rate constants in various compartments have not been determined. By the modified RIA procedure it should be possible to better investigate the pharmacokinetics of these alkaloids.

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