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RADIOIMMUNOASSAY DETERMINATION OF VINDOLINE

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Key Word Index—Vindoline; alkaloid; radioimmunoassay; tritium labelling; vindoline–bovine serum albumin conjugate.

Abstract—A radioimmunoassay (RIA) for the Aspidosperma alkaloid vindoline has been developed. Antibody production was achieved by injection of vindoline–bovine serum albumin conjugate into rabbits. The RIA method is specific and sensitive with detection limits of 5 ng of alkaloid.

INTRODUCTION

The use of radioimmunoassay (RIA) as an analytical tool for quantitative detection of various types of molecules in biological media is well established [1]. The more recent studies on the application of this technique to research with plant material or plant tissue cultures [2] have been encouraging and have prompted various investigators to apply the RIA method. Here we report experiments on the development of RIA for the alkaloid vindoline (**1**), one of the major components of *Catharanthus roseus* and the dihydroindole unit of the clinically important anti-tumor alkaloids vinblastine (**2a**) and vincristine (**2b**).

RESULTS AND DISCUSSION

Preparation of hapten–protein conjugate

It is well known that under ordinary circumstances low MW compounds (below 1000) are generally not immunogenic unless bound as haptens to an immunogenic carrier, such as a protein, to form a conjugate [3]. Specific antibodies against these haptens may then be obtained from animals immunized with the conjugate. For this purpose we chose to react vindoline (hapten) with bovine serum albumin (BSA) to provide the required hapten–protein conjugate. The site of the bond between the hapten and the protein is one of the most important factors influencing antibody affinity and specificity and our choice was to couple vindoline at the C-4 position. The successful sequence for the conjugation is illustrated in Scheme 1.

Vindoline (**1**) via its known derivative, desacetylvindoline (**3**) [4], was converted, in 82% yield, to the hemisuccinate (**4**) by reaction with succinic anhydride at room temperature. The corresponding succinate derivative involving both carboxyl functions was not detected and thus specific coupling of **4** to the protein was possible. *In situ* generation of the mixed anhydride (**5**) by reaction of **4** with isobutylchloroformate and subsequent amide formation with free amino groups in BSA afforded a direct route to the conjugate (**6**).

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Of the various methods which could be employed to determine the number of vindoline molecules per unit of BSA, UV spectroscopy proved to be ideal. It could be estimated (see Experimental) that 18 molecules of vindoline had been incorporated into each protein unit.

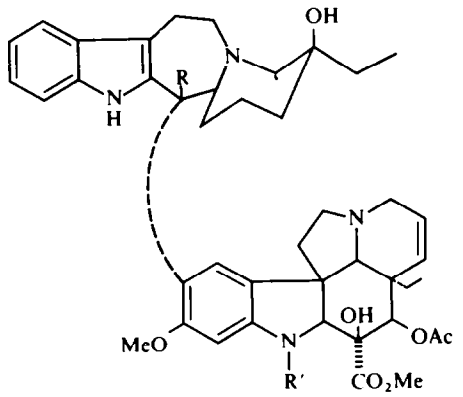
Preparation of radioactive hapten

Efforts in these laboratories [5] resulted in the development of a general method for introduction of tritium into the aromatic ring of acid-sensitive alkaloids. This technique has been employed extensively in our numerous investigations in the biosynthesis of indole alkaloids (for a general review see ref. [6]) and subsequently elsewhere [7].

Here a slight modification in using just sufficient trifluoroacetic anhydride to prepare tritiated anhydrous trifluoroacetic acid was necessary in order to avoid undesirable acylation of the aromatic ring in the alkaloid by excess anhydride. Tritiation in the presence of a platinum-on-charcoal catalyst under strictly anhydrous conditions provided [^{3}H]-vindoline with a specific activity of 2.042×10^{11} dpm/mmol. This sample was then utilized as the labelled antigen in the radioimmunoassay.

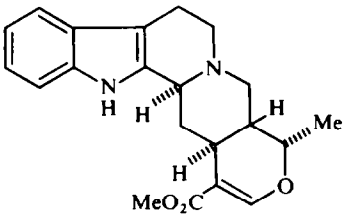
Production and evaluation of antiserum

Three female white rabbits were immunized subcutaneously with either 1, 5 or 20 mg of the vindoline–BSA conjugate emulsified in Freund's adjuvant. Booster injections of the same dosage were administered at 2 week intervals. Blood was collected via the ear veins 10 days after inoculations and the antigen-binding capacity of the serum was determined by serial dilution. After the fourth booster, significant antibody against vindoline was detected in the sera from all three rabbits. The antibody affinity was found to increase following each of the three subsequent booster injections with the highest antigen (vindoline) binding provided by the antisera from the rabbit immunized with 1 mg doses of conjugate. All suitable antisera were pooled and diluted to give a final working titre of 1:80 under constant assay conditions and so as to bind 45% of a fixed mass of radioactive hapten (1.14×10^{-7} mmol *ca* 8500 cpm [^{3}H]-vindoline). The total assay volume was 500 μl and the antibody-bound



2a R = CO_2Me; R′ = Me

2b R = CO_2Me; R′ = CHO



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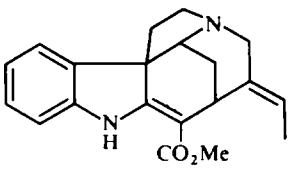

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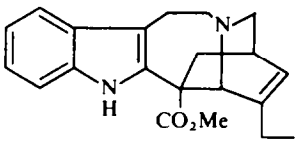

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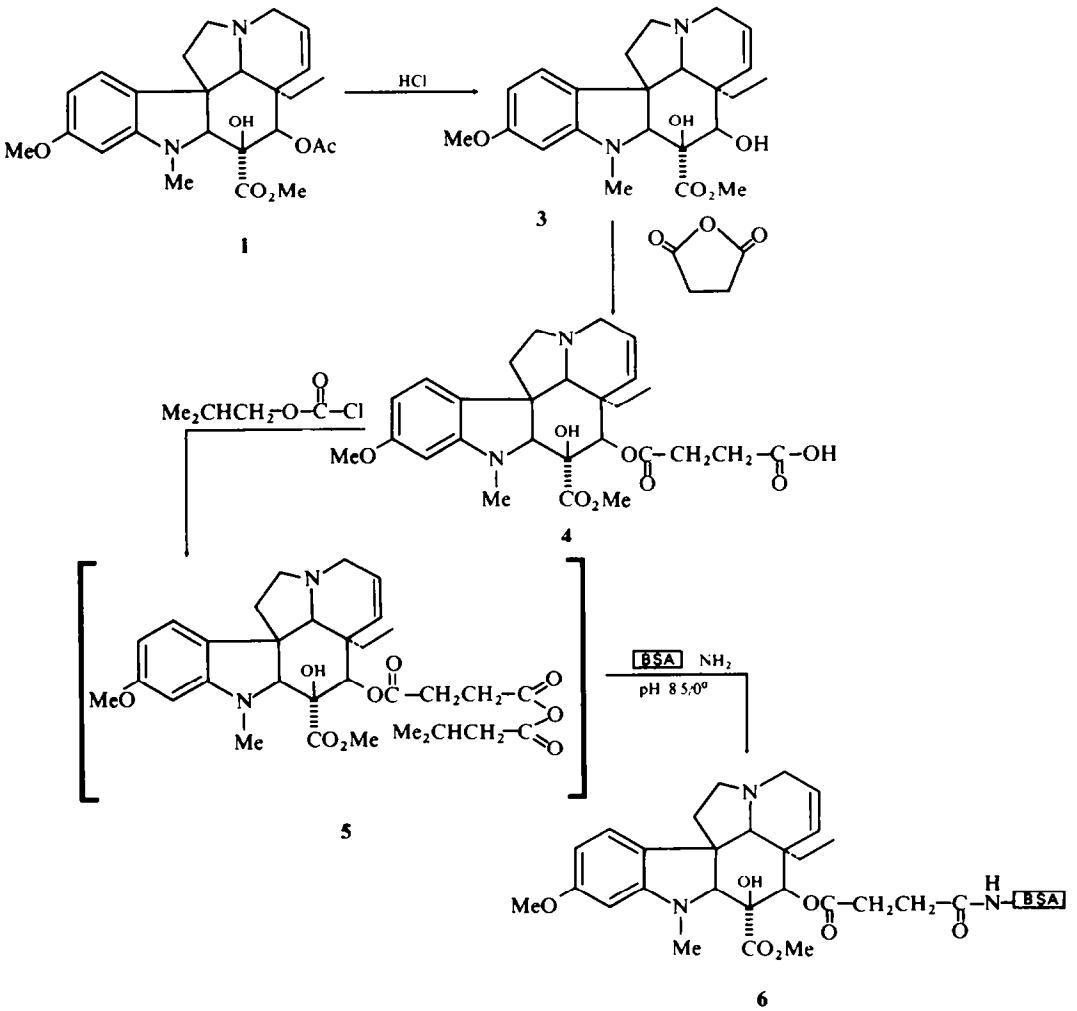

1 **3** **4** **5** **6**

Scheme 1.

[^{3}H]-vindoline was separated from the free vindoline by ammonium sulfate precipitation. With this procedure non-specific binding of less than 4% was attained. The frozen antiserum revealed no deterioration in activity even upon long standing (more than 9 months).

The standard curve for the radioimmunoassay over a range of unlabelled vindoline concentrations is shown in Fig. 1, and expressed in two possible plots. The sensitivity of the assay, defined as the minimum amount of antigen that may be detectable, is in the region of 5–8 ng of vindoline. The usable range of the standard curve is extended to 1 μg of vindoline.

From a Scatchard plot [9], a maximum affinity constant of $K_a = 0.3 \times 10^8$l/mol and the corresponding value for the total number of antibody binding sites $A_0 = 0.4 \times 10^{-12}$mol/l were determined. The occurrence of several sets of antibody populations in the antiserum used, each with a different K_a value, was reflected by the nonlinear Scatchard plot. However, for the present study this antibody heterogeneity does not adversely affect the assay.

Since the plant and tissue culture extracts used for the radioimmunoassay will contain some methanol (up to a maximum of 50 μl, 10% of total assay volume) the effect of methanol on the binding between antibody and hapten was also studied. No deviation in the assay results was detected up to 15% methanol.

Specificity of radioimmunoassay

A number of cross-reactivity experiments were carried out to determine the specificity of the antiserum. For this purpose, a spectrum of known *Catharanthus roseus* alkaloids was selected for analysis. These include ajmalicine (**7**) (Corynanthé), akuammicine (**8**) (Strychnos), catharanthine (**9**) (Iboga) and the 'dimeric' alkaloid vinblastine (**2a**). Competitive binding assays were performed with various concentrations of each of the above alkaloids. No inhibition of the binding of [^{3}H]-vindoline with the antiserum was observed with any of the four alkaloids examined, even at levels of 100 μg. We have also studied the inhibition effects of desacetylvindoline (**3**) and the hemisuccinate (**4**). The results are shown in Fig. 2.

It is clear that the hemisuccinate (**4**) competes as effectively as does vindoline for the active sites of the antibody while a lesser degree of affinity is directed towards desacetylvindoline (**3**). This may be explicable in light of the greater structural similarity of vindoline to the immunizing hemisuccinate group than exists with desacetylvindoline. This supports the premise that the nature of the linkage between hapten and carrier protein is important in determining the affinity and specificity of the antibody produced. Similar observations have been reported in cross-reactivity studies with the RIA method in the steroid field [10] and with other drugs, such as morphine [11].

In conclusion we have demonstrated the preparation of antibodies against the alkaloid vindoline by immune response to a conjugate with bovine serum albumin. The radioimmunoassay developed with this antiserum is of high specificity and good sensitivity. This favorable combination should provide a powerful method for the analytical determination of vindoline. Work is now underway to determine the applicability of this radioimmunoassay (as well as others being developed in these laboratories) to the screening of secondary plant products in various plant tissue cultures.

EXPERIMENTAL

General. Mps were determined on a Kofler block and are uncorr. UV spectra were recorded in MeOH or H_2O soln; wavelengths of absorption maxima are reported in nm with ε values in parentheses. IR spectra were measured in $CHCl_3$ soln; absorption maxima are reported in wavenumbers (cm^{-1}), calibrated with respect to the absorption band of polystyrene at 1601 cm^{-1}. ^{1}H NMR were measured in $CDCl_3$ soln at ambient temp. at 100 MHz; chemical shift values are given in δ (ppm) relative to TMS used as standard. The integrated peak areas, signal multiplicities and proton assignments are given in parentheses. Microanalyses were carried out by Mr. P. Borda of

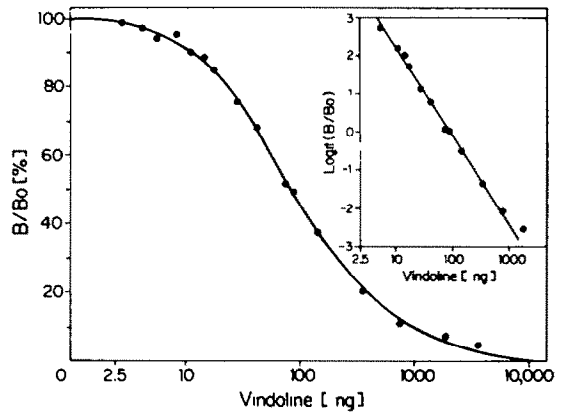

Fig. 1. Vindoline standard curve which is obtained by plotting B/Bo vs mass of unlabelled vindoline on logarithmic scale. The insert shows the linearized logit–log plot [8] using the same experimental data over the range useful for the analysis of vindoline. B = % binding in the presence of unlabelled vindoline; Bo = % binding in the absence of unlabelled vindoline; logit $(B/Bo) = \ln \frac{(B/Bo)}{(1 - B/Bo)}$. Non-specific binding = 2.9%.

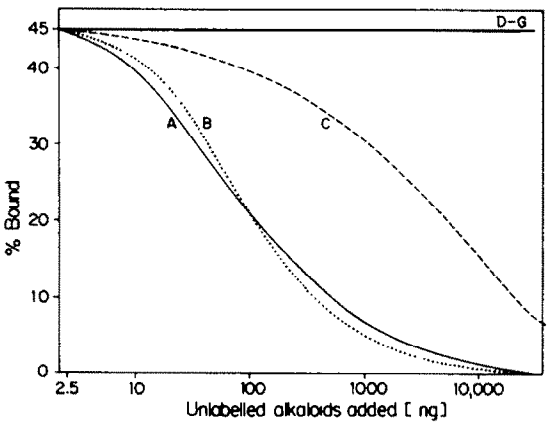

Fig. 2. Cross reactivities of the anti-vindoline antibody against *C. roseus* alkaloids. The curves are obtained by plotting the % bound [^{3}H]-vindoline vs the mass of unlabelled alkaloids added on logarithmic scale. Curve A (———): Vindoline (**1**) standard curve. Curve B (·····): desacetylvindoline-4-hemisuccinate (**4**). Curve C (– – –): desacetylvindoline (**3**). Curves D–G (– – –): ajmalicine (**7**), akuammicine (**8**), catharanthine (**9**), vinblastine (**2a**). Non-specific binding = 2.9%.

the Microanalytical Laboratory, University of British Columbia. T_2O (sp. act. 5 Ci/g) was obtained from New England Nuclear, Canada. Radioactivity measurements were made by liquid scintillation counting. BSA was obtained from Behringwerke AG, W. Germany and Freund's complete and incomplete adjuvants from Miles Laboratories, U.S.A.

Desacetylvindoline-4-hemisuccinate (**4**). Desacetylvindoline (**3**) (415 mg), succinic anhydride (210 mg) and dry pyridine (1 ml) were stirred at 25° for 48 hr when TLC showed complete conversion of starting material to a more polar compound. Pyridine was evapd under red. pres. and the residue triturated with cold MeOH to remove excess anhydride and coloured impurities. The residual white solid was recrystallized from EtOAc–MeOH to afford **4** (422 mg, 82%), mp 114°; ν_{max} ($CHCl_3$) 3640 *w*, 3050 *s*, 2965 *b*, 1740 *s*, 1720 *s*, 1620 *s*, 1600 *m*, and 1500 *s* cm^{-1}; λ_{max} (MeOH) 250 and 303 nm (ε 5900 and 3980); $\delta(CDCl_3)$ 0.46 (3 H, *t*, J = 7Hz, $-CH_2-CH_3$) 2.58 (4 H, *m*, $-CH_2-CH_2-CO_2H$), 2.62 (3 H, *s*, N–CH_3), 3.71, 3.73 (6 H, 2*s*, OCH_3 and CO_2CH_3), 5.21 (1 H, *d*, J = 10 Hz, C6-H), 5.42 (1 H, *s*, *C4-H*), 5.79 (1 H, *dd*, J = 10 Hz, 4 Hz, C7-H), 6.03 (1 H, *d*, J = 2 Hz, C17-H), 6.25 (1 H, *dd*, J = 2 and 8 Hz, C15-H), 6.86 (1 H, *d*, J = 8 Hz, C14-H) and 8.1 (2 H, *br s*, ex. D_2O, OH and CO_2H). mS, *m/e* 514 (M^+), 496, 414. (Found: C, 61.50; H, 6.74; N, 5.30. $C_{27}H_{34}N_2O_8$.MeOH requires C, 61.47; H, 6.96; N, 5.12%).

BSA-desacetylvindoline-4-hemisuccinate conjugate (**6**). Desacetylvindoline-4-hemisuccinate (**4**) (258 mg, 0.5 mmol) in dry *p*-dioxane (22 ml) and tri-*n*-butylamine (93 mg, 0.12 ml) was treated with isobutylchloroformate (68.3 mg, 0.5 mmol) at 10° with stirring for 1 hr to generate the mixed anhydride (**5**). This was added to a soln of BSA (1.45 g, 0.02 mmol) in H_2O (100 ml) and dioxane (100 ml), and stirred at *ca* 5°. The pH of the soln was raised to 8.5 by appropriate additions of N NaOH. An additional vol. of H_2O (22 ml) was added and stirring continued for 4 hr. The reaction mixture was lyophilized and then triturated with dry Me_2CO to remove most of the unreacted **4** and other impurities. The residue was further purified on a column of Sephadex G-25 (Coarse) with H_2O as eluant to give the conjugate (**6**) (1.45 g). The TLC behavior of **6** was similar to BSA except that it showed a red-purple colour on ceric sulfate spraying, characteristic of vindoline, **3** and **4**. The number of molecules of **4** conjugated per molecule of BSA was determined by UV. For BSA: λ_{max} (H_2O) 276 (ε 46000), 281 *sh* nm (pH 5.6). For the conjugate (**6**); λ_{max} (H_2O) 303 (A = 1.04), 283 (A = 0.96), 275 sh and 250 nm. Concentration of soln is 29.4 mg of conjugate in 25 ml H_2O (pH 5.7)

$$= \frac{29.4 \times 10^{-3}}{M} \times \frac{1000}{25} \text{ mol/l}$$

where M = MW of conjugate

$$= 70000 + (D \times 500)$$

where D = the number of hemisuccinate (**4**) molecules conjugated and the MW of BSA is taken as 70000. Assuming all A at 303 nm is due to **4** incorporated, its concn in the above conjugate soln $= \frac{1.04}{3980}$, since $C = \frac{A}{\varepsilon l}$ and ε = 3980 for **4** at this wavelength.

$$\text{Therefore } D = \frac{1.04}{3980} \div \frac{29.4 \times 10^{-3} \times 1000}{(70000 + 500D)25},$$

therefore D = 18.

Preparation of [^{3}H]-*vindoline*. Reaction conditions were first established with nonradioactive compounds. Conditions used for [^{3}H]-3′,4′-dehydrovinblastine proved to be unsuitable, as excess TFAA used as solvent in the exchange reaction, led to acylation of the aromatic ring of vindoline resulting in a more polar product. Therefore 1 equivalent of TFAA was used to ensure only CF_3CO_2T and the catalyst mixture were present. Thus, T_2O (0.2 ml, 1 Ci) was dist. under vacuum onto frozen TFAA (1.25 ml, 1 eq.) and then warmed to 20° for 30 min to generate CF_3CO_2T. This was then distilled under vacuum onto a frozen mixture of vindoline (200 mg), Pt–C catalyst suspension (1.2 ml in CCl_4) and CCl_4 (1 ml). The mixture was stirred at 20° for 20 hr and then treated with MeOH to remove exchangeable T. The residue was extracted with CH_2Cl_2 (3 × 15 ml). The combined CH_2Cl_2 extract was washed with 10% NH_4OH soln, H_2O and dried (Na_2SO_4). The solvent was evapd to give a solid which had TLC behavior identical to that of vindoline. Recrystallization (from Et_2O) to constant sp. act. yielded [^{3}H]-vindoline, 2.042 × 10^{11} dpm/mmol ≡ 91 mCi/mmol.

Immunization with conjugate (**6**) *and radioimmunoassay*. Three female white rabbits were bled for normal serum and then inoculated subcutaneously, at multiple sites, with the emulsified doses shown in Table 1.

Booster injections of the same dosage (except that the conjugate was emulsified in Freund's incomplete adjuvant) were administered at 2 week intervals. Serum was obtained 10–12 days after each booster injection and analysed for antibody specific to vindoline. After the 4th booster, antibody was detected in the serum of each rabbit. The antigen-binding capacity of the sera was determined by a modification of the usual $(NH_4)_2SO_4$ pptn method. Thus the following reagents were pipetted into a test-tube in an icebath: 0.3 ml PBS (0.04% BSA added), 0.1 ml [^{3}H]-vindoline in PBS (1.14 × 10^{-7} mmol, *ca* 8500 cpm) and 0.1 ml serially diluted antibody soln. Blanks for determination of non-specific binding contained an additional 0.1 ml PBS instead of the

Table 1

Rabbit	A	B	C
BSA-DAV-4-HS conjugate (**6**) (mg)	1	5	20
PBS*(ml)	1	1	1
Freund's Complete Adjuvant (ml)	1	1	1

*PBS, phosphate-buffered saline (0.9% NaCl 0.01 M PO_4^{3-}, pH 6.8).

Table 2

Standard vindoline solution in PBS*	PBS* (μl)	MeOH (μl)	Ab (μl)	[^{3}H]-vindoline (μl) in PBS* (*ca* 8500 cpm)
NSB	350	50	--	100
Bo —	250	50	100	100
100 μl	150	50	100	100

*0.04% BSA added.

antibody soln. Total assay vol. was 0.5 ml and each assay was performed in duplicate. The samples were incubated at 4° for 20 hr. Normal rabbit serum (50 μl) was then added to each sample followed by satd $(NH_4)_2SO_4$ (0.5 ml) to ppt the antibody-tracer complex. The ppt was separated by centrifugation and washed with 50% $(NH_4)_2SO_4$ (1 ml). The residual Ab–Ag* complex was dissolved in H_2O (1 ml) and counted for radioactivity by liquid scintillation. Non-specific binding of less than 4% was attained by this procedure.

All suitable antisera were pooled together and a standard curve calibrated at $\frac{1}{80}$ antiserum dilution with a series of standard vindoline solns (24.4 μg/100 μl PBS–2.852 ng/100 μl PBS). The protocol in Table 2 was used.

The assay was carried out at 0°. To increase the sensitivity of the assay the standard vindoline solns were incubated with the antiserum soln for 1 hr before addition of [^{3}H]-vindoline soln. Each sample was then incubated at 4° for 20 hr and the antibody bound [^{3}H]-vindoline separated by the $(NH_4)_2SO_4$ sulfate method described above. The results are shown in Fig. 1.

Cross reactivity determination. Standard solns of ajmalicine (**7**), akuammicine (**8**), catharanthine (**9**), vinblastine (**2a**), desacetylvindoline-4-hemisuccinate (**4**) and desacetylvindoline (**3**) were parepared in MeOH. The assay procedure was similar to that described for the standard curve calibration except for the substitution of the vindoline solns with the above standard solns. Ajmalicine, akuammicine, catharanthine and vinblastine did not show any cross reactivity even in amounts of 100 μg. Desacetylvindoline-4-hemisuccinate and desacetylvindoline exhibited inhibition of the binding of [^{3}H]-vindoline and these results are shown in Fig. 2.

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