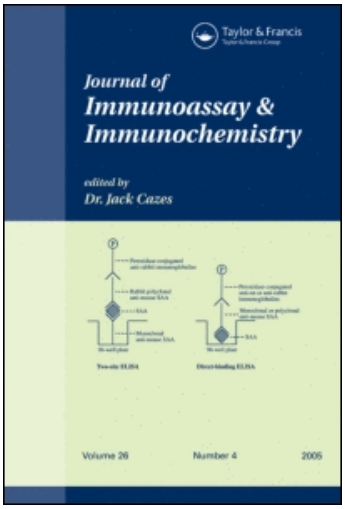

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AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE ANTINEOPLASTIC AGENT VINCRISTINE

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ABSTRACT

An enzyme-linked immunosorbent assay for vincristine was developed, based on a new procedure for synthesizing the hapten-protein conjugate. In both the immunogen and the enzyme tracer a spacer group was introduced between the hapten and protein, and the vincristine was coupled at a site far from its functional groups. The antibody produced proved to be exceptionally specific as compared with previous immunoassays for bis-indole alkaloids. Thousandfold antibody dilutions could be used and samples at the femtomole range were assayable. Applications of the method to patient plasma samples and to plant material are described. (Key Words: Catharanthus alkaloids; immunoassay; protein conjugates; vincristine; cancer chemotherapy).

INTRODUCTION

The bis-indole alkaloids vincristine (VCR) and vinblastine (VBL) isolated from Catharanthus roseus (L.) G. Don. and the related semisynthetic product vindesine (Fig. 1) are used for the chemotherapy of different malignant tumors (1,2).

Since the first immunoassay for VCR and VBL (3) a number of steps have been taken to improve the sensitivity and specificity

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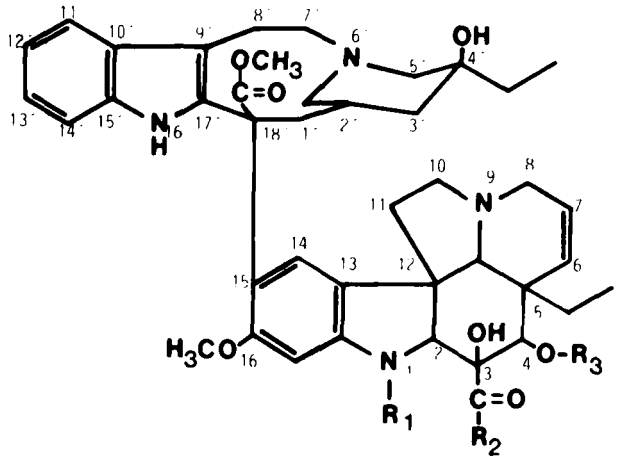
of the assay (4-10). Most (6-10) of these represent modifications of the original procedure, being based on an immunogenic conjugate prepared by the conversion of VBL to 4-deacetyl-3-hydrazide and its subsequent coupling to protein through the 3-carbon. Unfortunately, this approach results in the concealment of the characteristic structural features of the alkaloid, which have proved important in physiological interactions (2). Consequently the assays (6-10) have comparable affinities for all compounds that have differences only in this area. Some improvement in selectivity was achieved in the recent investigation (10) where monoclonal antibodies were derived against the 4-deacetyl VBL conjugate.

Teale et al. (4) and Langone et al. (5) prepared immunogens in which the vindoline moiety, i.e. the lower half of the dimer in Fig. 1, is more exposed. Though this improved the specificity, titers of the antisera remained low and probably there were other problems as well, since the methods appear not to have been adopted in wider practice.

Besides the use in clinical monitoring of drug levels, immunoassays of Catharanthus alkaloids have potential applications in pharmacological studies and the screening of alkaloid levels in C. roseus for the breeding of better producer plants. In all these instances the specificity of the assay is essential since closely related metabolites of the molecule to be determined will be present in crude samples and may interfere with an unspecific assay. We have been exploring specific procedures for the above



	R_1	R_2	R_3
VINCRISTINE:	-CHO	$-OCH_3$	$-COCH_3$
VINBLASTINE:	$-CH_3$	$-OCH_3$	$-COCH_3$
VINDESINE:	$-CH_3$	$-NH_2$	-H

FIGURE 1. Structures of antineoplastic bis-indole alkaloids presently in clinical practice.

purposes and have now developed an enzyme-linked immunosorbent assay for VCR based on a new type of VCR-protein conjugate.

MATERIALS AND METHODS

Drugs and Reagents

Catharanthine hydrochloride, vindoline sulfate and vindesine sulfate were generously provided by Lilly Research Center, Indianapolis, IN, USA. Ajmalicine hydrochloride and loganin were purchased from Carl Roth GmbH, Karlsruhe, FRG, and tryptamine and 3-indoleacetic acid from Fluka AG, Buchs, Switzerland. VCR

sulfate, VBL sulfate and the other compounds used in cross-reaction tests, and also alkaline phosphatase (from bovine intestinal mucosa, Type VII-NL) and phosphatase substrate were from Sigma Chemical Co., St. Louis, MO, USA. [G-^{3}H] Vincristine sulfate (sp. act. 218 GBq/mmol) was from Amersham International, Amersham, Bucks, U.K. All other chemicals were of reagent grade and from normal commercial sources.

Preparation of VCR-Protein Conjugates

The drug was conjugated to bovine serum albumin and alkaline phosphatase by binding a spacer molecule, either p-aminophenylalanine or p-aminobenzoic acid, between the 12'-carbon (corresponding to the C-10' in the biogenetic numbering (12)) of the catharanthine moiety and the amino groups of the protein. Diazo coupling was used to bind VCR to the spacer, which in turn was carbodiimide conjugated to the protein. The molar VCR albumin ratio was 245:1, 22:1, and 7.5:1 for preparation of the immunogens 1, 2, and 3, respectively, and 150:1 for preparation of the alkaline phosphatase conjugate. p-Aminobenzoic acid was used as the spacer for immunogen 1 and alkaline phosphatase, and DL-p-aminophenylalanine for the other immunogens. Conjugates were separated from reaction mixtures by ultrafiltration with Amicon PM 30 membrane. Formation of the desired conjugates was verified by absorbance spectrometry. Details of the chemistry of the conjugation reactions will be published elsewhere (11, 12). The conjugates used for immunization were lyophilized for storage

and the enzyme conjugates were stored at $4^{o}C$ in the assay buffer specified below.

Antibody Production

The lyophilized immunogens were emulsified in Freund's complete adjuvant (13) and were given as multisite subcutaneous injections (14) to four New Zealand albino rabbits. Rabbits 1, 2, and 3 received the immunogens 1, 2, and 3 respectively, while Rabbit 4 also was injected with immunogen 2. The individual immunization schedules are summarized in Table 1. One week after the last booster all the animals were bled by cardiac puncture. Antibody production was screened by assaying the binding of [^{3}H]VCR by serum samples essentially as described in (15). Immunoglobulin G (IgG) was purified from the antisera by batch preparation with DEAE-cellulose (16).

Assay Procedure

The assay system, which was adapted from Reference (9), consisted of the following components: microtitet plates (Dynatech, FRG, or Eflab, Finland); 1:2,000 diluted IgG from Rabbit 2 in carbonate buffer (15 mM Na_2CO_3, 40 mM $NaHCO_3$, pH 9.6); VCR-alkaline phosphatase conjugate (about 1 µg/ml) and standards or samples in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 15 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 % gelatin (w/v), pH 7.4); PBS-Tween (as above but 0.05 % Tween 20 instead of gelatin); 3.8 µmol of p-nitrophenylphosphate dissolved before use in 1 ml of 1.25 M diethanolamine buffer (pH 9.8); 2 M NaOH.

TABLE 1

Immunization Schedules of the Rabbits Used for Antibody Production

Time of injection (weeks)	Dose of antigen (mg)			
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
0	0.75	1.00	0.40	0.60
4	0.75	-	-	-
5	-	0.80	0.50	0.45
9	0.75	-	-	-
12	-	0.25	0.25	0.30
13	0.15	-	-	-
18	0.30	-	-	-
19	-	0.30	0.30	0.20
22	0.15	0.20	0.20	0.10
27	-	0.10	-	-
31	-	0.20	-	0.27
32	-	0.50	-	-

The microtiter plates were coated with IgG by incubating 100 µl of the dilution per well for 2 h at 35°C, whereafter the plates were washed thrice with PBS-Tween. Then 75 µl of VCR-alkaline phosphatase and 25 µl of standard or sample were added and the incubation and washings were repeated. Finally 100 µl of the p-nitrophenylphosphate solution was added per well, on ice; the plates were incubated at 35°C for 2 h, the reactions were stopped with 50 µl of 2 M NaOH and absorbances at 405 nm were recorded with a Multiscan photometer (Labsystems Oy, Finland). VCR-containing components of the system were protected from light throughout storage and the assay procedure.

Preparation of Biological Samples

Heparinized blood samples from a patient were stored for 1 to 5 days at 4°C and plasma for the assay was separated by centrifuging at 3,000 g.

Leaf discs 6 mm in diameter were cut from C. roseus and extracted in 80 % methanol for 15 h at 60°C.

Both plasma and leaf samples were diluted with PBS when necessary, and the standards used with them contained the respective medium in like proportions.

RESULTS

As seen in Fig. 2 our conjugation procedure yields macromolecules with characteristic absorbances for both VCR and protein, and a typical absorbance of the intermediate azo link is prominent at 350 nm. No significant loss of alkaline phosphatase activity occurred during the coupling procedure.

Figure 3 describes the production of VCR antiserum during immunization. In spite of the rising titers the treatments were terminated at the times indicated, since the immunogens were synthesized in batches designed for immunization periods of about 6 months. Comparison of the results (Fig. 3) with the immunisation schedule (Table 1) indicates that higher immunogen doses resulted in higher titers.

Trials where unpurified serum from the rabbits was used for coating microtiter plates showed the ratio of VCR-specific antibodies to total proteins bound to the wells to be too low to catch

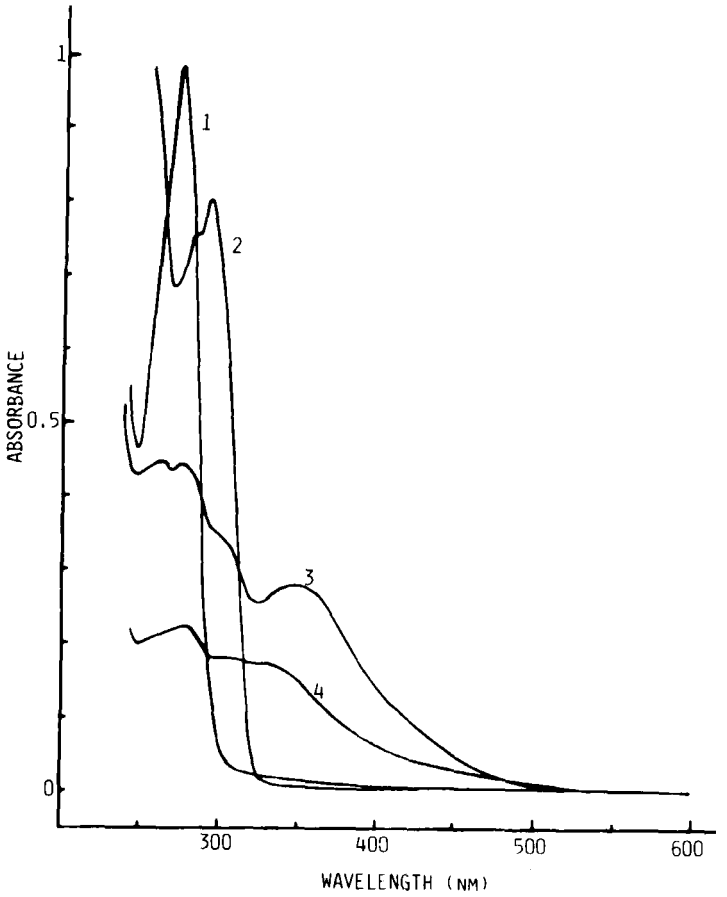


FIGURE 2. Absorbance spectra of (1) albumin (1.5 mg/ml), (2) VCR sulfate (50 μg/ml), (3) VCR-alkaline phosphatase conjugate (150 μg protein per ml if no loss during conjugation process is presumed), and (4) VCR-albumin conjugate (~150 μg/ml). The three first were measured in PBS containing 20 % (v/v) glycerol and the fourth in pure water.

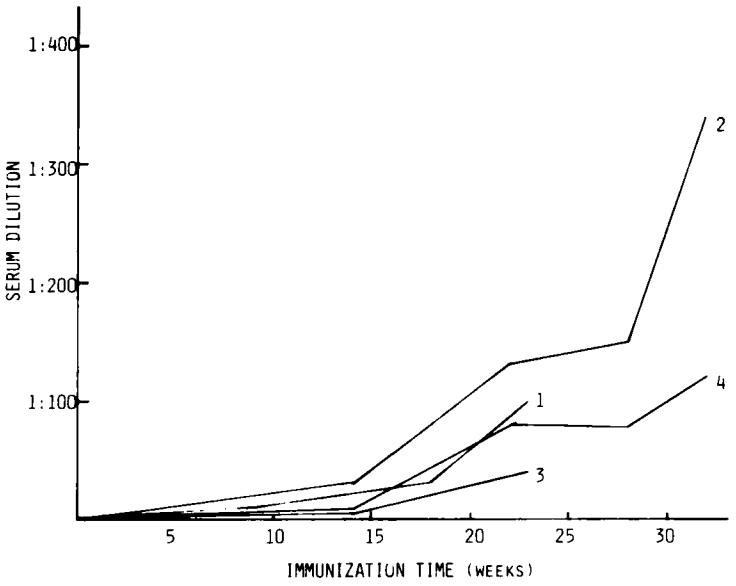


FIGURE 3. Titers of the antisera in Rabbits 1 to 4 during the immunizations outlined in Table 1. Serum dilutions indicated are those binding 50 % of the [^{3}H]VCR tracer.

enough of the enzyme-linked tracer for a feasible measurement. But when the separated IgG fraction was used for coating, the tracer binding became readily measurable. An IgG dilution of 1:2,000 (from Rabbit 2) was sufficient to saturate the wells within 2 h at 35^{o}C. Fifty percent tracer binding to the IgG was achieved within 2 h at 35^{o}C when about 1 µg/ml of the enzyme-hapten conjugate was used. In this system, which was the one routinely used the bound enzyme activity in zero samples produced a net absorbance of about 0.5 in 3 h at 35^{o}C; during this time the reaction rate was confirmed to be steady. Thus the whole assay can be carried out within one day.

The curve in Fig. 4A showing the displacement of tracer by standard VCR samples indicates that amounts between 5 and 500 pg, i.e. about 5 and 500 fmol, are measurable by the procedure. The repeatability below 25 pg was somewhat low, but could be improved by using double volumes of IgG and the tracer, and an incubation time of 4 h for the enzyme reaction. IgG from rabbits 1, 3, and 4 could also be used for assays, at dilutions of 1:200, 1:800, and 1:1.000, respectively, with comparable dispacement curves to the one in Fig. 4A.

Studies were made on the cross-reactivities of the antibody from Rabbit 2 with VBL, vindesine (Fig. 1) and eleven other compounds structurally related (sensu lato) to the immunogen: ajmalicine, p-aminophenylalanine, 3-indoleacetic acid, catharanthine, loganin, reserpine, strychnine, stryptamine, tryptophan, vincamine, and vindoline. Displacement curves could be determined for VBL and vindoline (Fig. 4B), and some displacement of the tracer by vindesine became detectable at about 1 μg; the other compounds had no effect even at 25 μg. Cross-reactivities of the other IgGs with VCR and vindoline were comparable to those of the IgG from Rabbit 2.

Applicability of the assay was tested with plasma samples from a patient undergoing VCR treatment and with samples from C. roseus. Figure 5 summarizes the measured drug levels in plasma after a single injection. Assays from the leaf extracts suggested concentrations of about 0.2 mg per gram fresh weight.

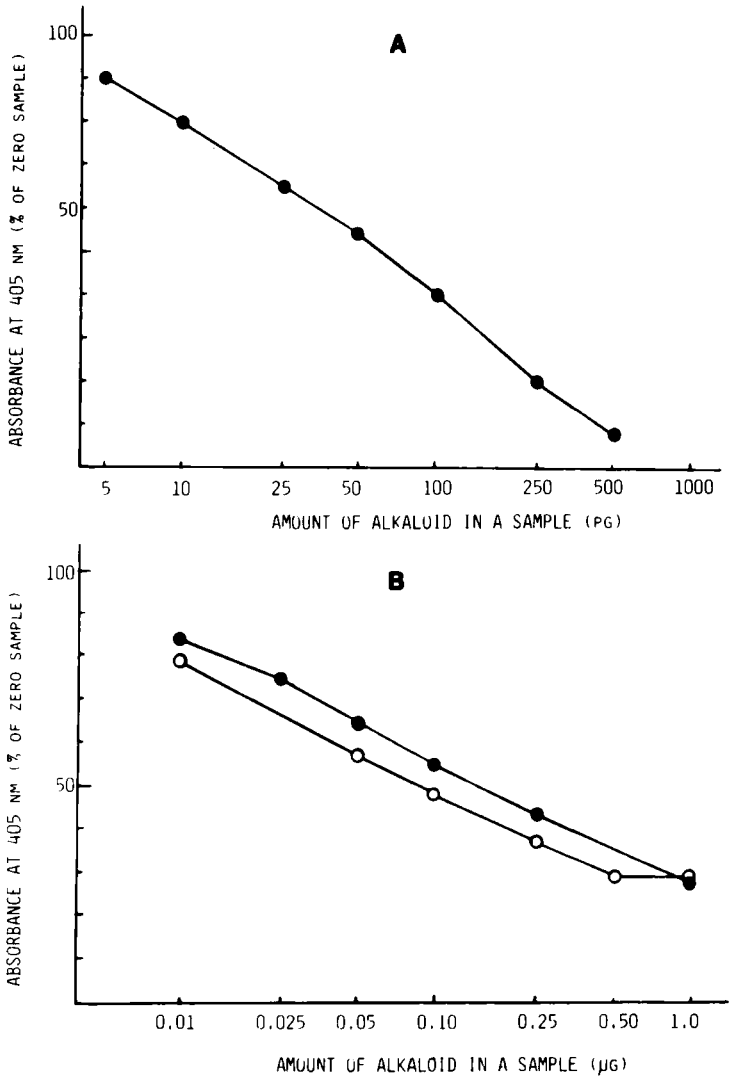


FIGURE 4. A. Displacement of the VCR-alkaline phosphatase tracer by VCR, and B. by VBL (●) and vindoline (o) under the standard assay conditions using IgG from Rabbit 2. The points represent means of 4 determinations.

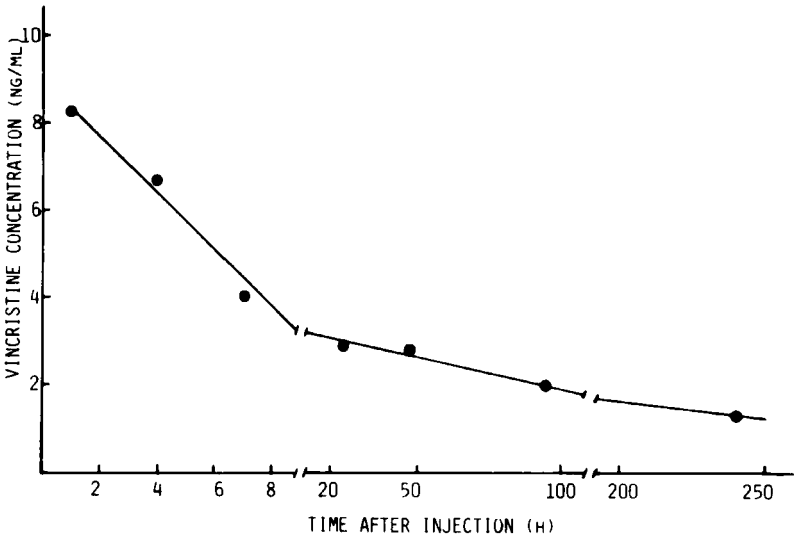


FIGURE 5. VCR concentrations in human plasma after an intravenous injection of 2 mg per 60 kg body weight. The points represent means of 4 determinations.

The unpurified serum and purified IgG retain their properties for at least 9 months at -20°C and plates precoated with diluted IgG could be used for assays after storage for 7 months at -20°C. The alkaline phosphatase-VCR tracer is destroyed if frozen in the gelatin-containing PBS, but may be stored as such at 4°C for several months or at -20°C if 20 % of glycerol is added. After longer storage periods the conjugate must be repurified in a Sephadex G 25 M column (PD-10) to assure the original sensitivity of the assay.

DISCUSSION

The immunizations were started with Rabbit 1, but when lesions suspected of being due to the toxicity of the VCR-albumin

complex developed at the injection sites we decided to use immunogens prepared with non-saturating amounts of VCR in the coupling reaction and to decrease the doses more rapidly. The anticancer bis-indole alkaloids have been shown to retain their cytotoxicity after conjugation to protein (17), and this toxicity may be the reason for the unusually slow antibody production (Fig. 2). Production was evidently slow in earlier work (4), too, where an immunogen with exposed functional groups was used. However, the present immunization results suggest that titers may be improved by further modification of the immunogen treatment.

Sensitivity of the present method (Fig. 4A) is comparable with the enzyme-linked immunoassay of Hacker et al.(9), while the sensitivity of radioimmunoassays (4-8) is about a thousandfold lower. The cross-reaction tests (Fig. 4B) showed that VBL and vindoline displace the tracer only if present in about 1,500-fold excess as compared with VCR, and demonstrate a great increase in specificity relative to earlier work. The best antiserum reported thus far (5) had about 200-fold sensitivity difference between VCR and VBL, and other assays had only some (4) or practically no selectivity (6-9). Thus the present results emphasize the view (18) that the choice of conjugation site is of great importance in preparing immunogens from haptens.

The VCR level of about 0.02 % determined for C. roseus leaves is far higher than the yield of 0.0002 % of dry weight reported for the drug (19). The most obvious source of the discrepancy is the cross-reactivity with vindoline, which is

present at average concentrations of 0.4 % dry weight in the plants (20). Thus even the slight cross-reactivity observed in our work is sufficient to distort the result. By contrast, the plasma concentrations determined (Fig. 5) are in good accord with other reports (4,8), and indicate that our method is suitable for assaying VCR for clinical or pharmacological purposes. In these applications the potential cross-reacting compounds are the known (c.f. 21) and probable other metabolites of VCR, and an evaluation of these on the basis of their structures suggest that the cross-reactivities would be in the range of or below those obtained for VBL and vindoline. In conclusion, the new conjugate resulted in an assay that has the best combination of high titer, sensitivity, and specificity published so far for a bis-indole Catharanthus alkaloid; and considering the good storage properties of the components the method should prove suitable for routine clinical use.

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