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Development of a Class-Specific Competitive Enzyme-Linked Immunosorbent Assay for the Detection of Pyrrolizidine Alkaloids in Vitro

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A two-step enzyme-linked immunosorbent assay (ELISA) was developed for the determination of the presence of retronecine in the parts per billion range. By modification of retronecine two haptens were synthesized. An I_{50} value of 11 ± 3 ppb (Figure 2a) was obtained, with detection limits of 1.0–100 ppb. Indirect detection of monocrotaline (325 g/mol), retrorsine (351 g/mol), and senecionine (352 g/mol) was achieved by hydrolytic digestion of these naturally occurring pyrrolizidine alkaloids (PAs), demonstrating the utility of the assay as a *class-specific* detection method. PAs were detected in *Senecio vulgaris* and *Crotalaria retusa* plant sample extracts. No cross-reactivity was seen with either swainsonine or lupinine, both of which are structurally similar to retronecine in that they are also fused bicyclic alkaloids.

INTRODUCTION

The pyrrolizidine alkaloids (PAs) constitute a class of secondary plant metabolites of wide geographical and botanical distribution which are toxic to both humans and animals (Smith and Culvenor, 1981; Culvenor, 1980). They occur in numerous plant families (including Boraginaceae, Compositae, Gramineae, Leguminosae, Orchidaceae, Rhizophoraceae, Santalaceae, and Saptoaceae) which are indigenous throughout the world (Smith and Culvenor, 1981). Indeed, Culvenor (1980) estimates that 3% of all flowering plants (6000 species) are PA-producing, and while many of these biosynthesize more than one PA, the structural similarities (for example, see Figure 1) in the greater than 250 known PAs are striking (Robins, 1982).

Collectively, the PAs exhibit a broad range of cytotoxic and pathological actions with hepatotoxic (Mattocks and Bird, 1983; Segall et al., 1985), pneumotoxic (Chesney and Allen, 1973), embryotoxic (Bull et al., 1968; McLean, 1970; Peterson and Culvenor, 1983; Mattocks, 1986), mutagenic (Ames et al., 1973), carcinogenic (Kuhara et al., 1980; Allen et al., 1975), and teratogenic (Green and Christie, 1961; Keller, 1983) effects having been described. Chronic gastrointestinal (Hooper, 1975), cardiopulmonary (Bull et al., 1968; McLean, 1970), and central nervous system (Hooper, 1972) disorders are further manifestations of PA poisoning. Several members of the PA class of compounds have been reported to express antiinflammatory, antispasmodic, antitumor, hypotensive, and local anesthetic properties (Atal, 1978; Gelbaum et al., 1982), but the adverse toxicity of these compounds usually precludes therapeutic application. Although the toxic response depends both on the structure of the ingested PA and on the animal species involved, the development of liver disease (veno-occlusive) is a common occurrence in laboratory and farm animals, particularly horses, cattle, sheep, goats, pigs, chickens, and turkeys (Bull et al., 1968; McLean, 1970; Peterson and Culvenor, 1983; Mattocks, 1986).

The health and economic implications of PA contamination in products for human consumption are enormous.

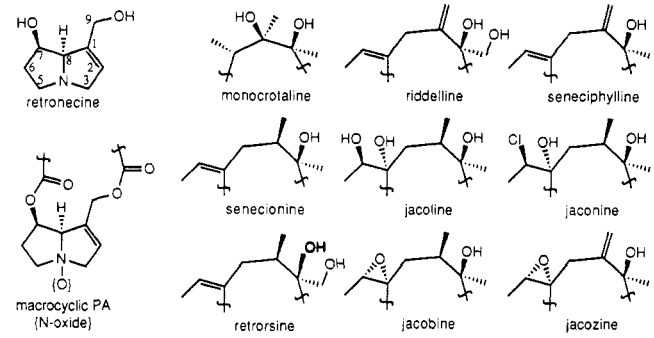

Figure 1. Structures of some common pyrrolizidine alkaloids.

To date, the list of products in which PAs have been detected include folk medicines and herbal teas (Huxtable, 1979), cereal grains (Mohabbat et al., 1976; Tandon et al., 1976), milk (Dickinson et al., 1976), and honey (Deinzer et al., 1977); there is a growing concern for the processing of asymptomatic animals for human consumption. Moreover, PA poisoning in humans is not uncommon, and there are documented reports from many countries. For example, mass poisoning has occurred in Africa and Asia when cereal grains contaminated with PA-producing plants were used in bread making (Mohabbat et al., 1976; Tandon et al., 1976). Likewise, PA-induced veno-occlusive disease resulting from folk medicine practices is also prevalent in Africa, South America, and island cultures, such as in Jamaica (Bras et al., 1957, 1961). PA poisonings have also been recorded in the United States (Fox et al., 1978). Thus, while the agricultural ramifications are apparent and continue to escalate, the implications of widespread PA contamination of agricultural products for human consumption are of even more concern.

The scientific community lacks sensitive and accurate methods to measure PAs and PA metabolites in biological samples—a fact with three important consequences. First, no reliable diagnostic methods are available for early detection of PA exposure, and the medical professional must rely on indicative liver symptoms or autopsy/biopsy pathogenic liver changes for diagnosis of PA poisoning. Unfortunately, the severe chronic hepatotoxicity of PAs means that irreversible damage has almost certainly occurred by the time diagnosis is made. Second, available

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analytical methods for PA detection are complicated and require sophisticated instrumentation. As a result, they are not generally available and "preventive" PA analysis of foodstuffs has not been practical. Third, and perhaps most importantly, the evidence implicating PAs as a causative agent in disease is circumstantial since methods are not available for low-level detection of PAs and PA metabolites in biological samples. Because of these public health, medical, and scientific issues, there is an urgent need for a reliable, rapid, and sensitive method for identification/quantification of PAs and PA metabolites in biological/environmental samples. The ubiquitous nature of the PAs makes them a worldwide concern and encourages development of immunoassay techniques which are amenable to the large-scale, rapid screening of biological samples. Since enzyme-linked immunosorbent assays (ELISAs) have demonstrated efficacy in the detection/quantification of small-molecule toxins, they seem ideally suited for this screening.

Several workers (Mattocks, 1972; Stillman et al., 1977; Huxtable, 1980; Fox et al., 1978) have defined the minimum PA structural features required for the hepatotoxicity as (i) an unsaturated 3-pyrroline ring, (ii) one or more oxy substituents on the pyrroline ring, (iii) esterification of at least one of these oxy groups (diesters are the most toxic), and (iv) the presence of bulky Cα substituents in the esterifying acid. The most toxic PAs incorporate all of these features within a macrocyclic system (Mattocks, 1981). Thus, while structural variations are myriad among the macrocyclic PAs, the common feature is an amino alcohol (necine base) combined through diester linkages to a dicarboxyl (necic acid) moiety (Schoental, 1968).

Early diagnosis and prevention of PA poisoning are clearly urgent priorities, and the establishment of rapid, reliable, sensitive, specific, and inexpensive methods for the detection of PAs and/or PA metabolites in biological and environmental samples would accelerate progress in these areas. As detailed herein, our approach to meeting these objectives has involved the development and refinement of immunoassays for the detection of retronecine, the most commonly encountered necine base occurring naturally in 6 botanical families and 26 genera (Robins, 1982; Bull et al., 1968; McLean, 1970).

EXPERIMENTAL DESIGN AND METHODS

Chemicals. Senicionine, *Senecio vulgaris*, and *Crotalaria retusa* samples were a gift from Dr. H. J. Segall (Veterinary Medicine Pharmacology and Toxicology, UCD). *Lupinus* spp. seeds were purchased from Charles H. Lilly Co. (−)-Lupinine hydrochloride was purchased from Research Plus. Monocrotaline was purchased from Trans World Chemicals. Adipic acid, succinic anhydride, solvents, and inorganic reagents were purchased from Fisher Scientific. Swainsonine, phosphate-buffered saline (PBS), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC), Tween 20, tris(hydroxymethyl)aminomethane (Tris) base, citric acid, biotinylated goat anti-rabbit antibody, avidin–horseradish peroxidase conjugate type VI, *o*-phenylenediamine, Fruend's complete and incomplete adjuvants, bovine serum albumin, and chicken egg ovalbumin were purchased from Sigma Chemical Co. Retrorsine, retrorsine *N*-oxide (isatidine), dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich Chemical Co. Dialysis tubing was purchased from Spectrum Medical Industries. Diethyl ether (DME) was distilled under a nitrogen atmosphere from sodium/potassium benzophenone ketyl immediately prior to use. Chloroform was washed with water to remove ethanol, dried over $MgSO_4$, and distilled under nitrogen atmosphere immediately prior to use in reactions.

Instruments. Enzyme-linked immunosorbent assays (ELISAs) were carried out on 96-well Immulon II microtiter plates (Dynatech) and read with a UV_{max} microtiter plate reader (Molecular Devices) at 490 nm. Nuclear magnetic resonance spectra were taken on a QE-300 NMR spectrometer (General Electric) with proton spectra at 300 MHz and carbon-13 spectra at 75.6 MHz. IR, ^1H NMR, ^{13}C NMR, and mass spectral assignments for the compounds prepared in this paper are available as supplementary material.

Retronecine (2). In a 250-mL round-bottom flask equipped with a reflux condenser and magnetic stirrer, monocrotaline (1, 6.00 g, 18.4 mmol), $Ba(OH)_2{\cdot}8H_2O$ (15.00 g, 47.55 mmol), and water (125 mL) were combined and heated at reflux for 3 h while nitrogen was bubbled through continuously. The mixture was cooled to room temperature and saturated with solid carbon dioxide. The barium carbonate was removed by suction filtration through Celite, and the clear filtrate was concentrated under vacuum to 25% of its original volume. The aqueous concentrate was basified (pH 10) with Na_2CO_3, saturated with NaCl, and continuously extracted with ether for 72 h. The organic extract was concentrated under reduced pressure and evacuated at 1 Torr overnight to give a light brown solid. Decolorization with activated carbon and recrystallization from acetone gave **2** (2.05 g, 13.2 mmol, 72%) as white prisms: mp 120–121 °C.

Hydrogen [(1*R*-*trans*)-2,3,5,7a-Tetrahydro-1-hydroxy-1*H*-pyrrolizin-7-yl]methyl Butanedioate (3a). Succinic anhydride (16.4 mg, 0.164 mmol) was added to a stirred solution of (+)-retronecine (**2**, 25.0 mg, 0.161 mmol) in dry $CHCl_3$ (5.0 mL) under argon. After 24 h at room temperature, the solvent was removed under reduced pressure to give a colorless oil. Evacuation at 1 Torr overnight gave **3a** (together with some of the corresponding C_7 monosuccinate **3b**; **3a**:**3b**::5:1; 100% by ^1H NMR) as a colorless gum. The zwitterionic mixture was used without further purification in the preparation of protein conjugates.

4-BSA Antigen. To a stirred solution of hapten **3a**/**3b** (20.0 mg, 0.0783 mmol) in phosphate-buffered saline (pH 7.5–8.9 mL) was added bovine serum albumin (BSA, 44.3 mg). The solution was cooled to 0 °C, and ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC, 15.0 mg, 0.0783 mmol) was added in a single portion. The reaction mixture was stirred for 12 h and then dialyzed (MW cutoff 10 000) against deionized H_2O (2 × 2000 mL) at 0 °C overnight to remove any uncoupled hapten. The dialysate was transferred to glass vials and stored at −20 °C until required for immunochemical studies.

4-OVA Antigen. To a stirred solution of hapten **3a**/**3b** (20.0 mg, 0.0783 mmol) in phosphate-buffered saline (pH 7.5, 8.8 mL) was added ovalbumin (OA, 88.2 mg). The solution was cooled to 0 °C, and ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC, 15.0 mg, 0.0783 mmol) was added in a single portion. The reaction mixture was stirred for 12 h and then dialyzed (MW cutoff 10 000) against deionized H_2O (2 × 2000 mL) at 0 °C overnight to remove any uncoupled hapten. The dialysate was transferred to glass vials and stored at −20 °C until required for immunochemical studies.

Monochlororetronecine Hydrochloride (5). (+)-Retronecine (**2**, 1.00 g, 6.44 mmol) was added to stirred thionyl chloride (4.98 g, 3.05 mL, 41.9 mmol) under argon at −5 °C during a 15-min period. After the addition was complete, the solution was allowed to warm to 5 °C during 2 h. The thionyl chloride was removed under reduced pressure, and the residue was taken up in a minimum amount of hot (70 °C) 95% ethanol. Ether was added dropwise until the solution became slightly cloudy. Upon cooling, the solution deposited **5** (0.946 g, 4.50 mmol, 70%) as short white needles: mp 152–153 °C.

Retronazide (6). In a 10-mL round-bottom flask equipped with a reflux condenser and magnetic stirrer, monochlororetronecine hydrochloride (**5**, 0.9461 g, 4.503 mmol), sodium azide (1.26 g, 19.4 mmol), and water (6.3 mL) were combined and heated at 95 °C for 2 h. The aqueous solution was cooled to room temperature, basified (pH 9) with 50% aqueous NaOH, and extracted with chloroform (3 × 5 mL). The combined organic extracts were washed with brine (1 × 5 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give a tan solid. Purification by recrystallization from ether gave **6** (0.5771 g, 3.202 mmol, 71%) as colorless needles: mp 68.5–70 °C.

Retronamine (7). Retronazide (**6**, 381.2 mg, 2.115 mmol) was added to a stirred suspension of $LiAlH_4$ (190.2 mg, 5.013 mmol) in dry ether (15.25 mL) under argon. After 2 h at room

temperature, excess reductant was destroyed by the slow addition of cold water (4 mL). The complex lithium and aluminum salts were decomposed by acidification (pH 3) with 6 N HCl. The solution was basified (pH 10) with 50% NaOH, and the solid precipitate was removed by suction filtration. The aqueous filtrate was evaporated to dryness under high vacuum (1 Torr, 12 h), and the residue was extracted with chloroform (3 × 5 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give a viscous oil which crystallized on trituration with ether. Recrystallization from ethyl acetate gave **7** (221.0 mg, 1.434 mmol, 68%) as colorless prisms: mp 81–82 °C.

Hexanedioic Anhydride (10). Adipic acid (25.00 g, 0.1711 mol) and acetic anhydride (52.39 g, 48.42 mL, 0.5132 mol) were combined under argon in a 250-mL round-bottom flask equipped with a reflux condenser and magnetic stirrer. The mixture was heated at reflux for 2 h and then cooled to room temperature. The acetic acid and excess acetic anhydride were removed by distillation (20 Torr) to give a white polymeric solid. Distillation at 0.1 Torr provided the monomeric anhydride **10** (10.46 g, 0.08164 mol, 60%) as a colorless liquid: bp 98–100 °C (0.1 Torr).

6-[[[(1*R-trans*)-2,3,5,7a-Tetrahydro-1-hydroxy-1*H*-pyrrolizin-7-yl]methyl]amino]-6-oxohexanoic Acid (11). Hexanedioic anhydride **10** (62.7 mg, 0.489 mmol) was added to a stirred solution of retronamine (**7**, 74.7 mg, 0.484 mmol) in dry $CHCl_3$ (8.0 mL). After 24 h at room temperature, the solvent was removed under reduced pressure to give a colorless oil. Evacuation at 1 Torr overnight gave **11** (100% by ^1H NMR) as a white solid, which was used without further purification in the preparation of protein conjugates.

12-BSA Antigen. To a stirred solution of hapten **11** (30.0 mg, 0.106 mmol) in phosphate-buffered saline (pH 7.5, 12 mL) was added bovine serum albumin (BSA, 60.0 mg). The solution was cooled to 0 °C and ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC, 20.4 mg, 0.106 mmol) was added in a single portion. The reaction mixture was stirred for 12 h and then dialyzed (MW cutoff 10 000) against deionized H_2O (2 × 2000 mL) at 0 °C overnight to remove any uncoupled hapten. The dialysate was transferred to glass vials and stored at −20 °C until required for immunochemical studies.

12-OVA Antigen. To a stirred solution of hapten **11** (30.0 mg, 0.106 mmol) in phosphate-buffered saline (pH 7.5, 12 mL) was added ovalbumin (OA, 120.0 mg). The solution was cooled to 0 °C and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC, 20.4 mg, 0.106 mmol) was added in a single portion. The reaction mixture was stirred for 12 h and then dialyzed (MW cutoff 10 000) against deionized H_2O (2 × 2000 mL) at 0 °C overnight to remove any uncoupled hapten. The dialysate was transferred to glass vials and stored at −20 °C until required for immunochemical studies.

Rabbit Immunization. Three-month-old New Zealand white rabbits were immunized sc with 100 ng of immunogens **4**-BSA (rabbits 333, 352, and 353) and **12**-BSA (rabbits 1315 and 1322) emulsified in Freund's complete adjuvant (1:1). After three such inoculations at 3-week intervals, six injections of 150 ng of immunogen in Freund's incomplete adjuvant were given at 3-week intervals. Blood samples were collected 10 days following the final booster injection. Serum was stored at −70 °C.

Checkerboard Titrations. Optimum dilution factors for both the antiserum and coating antigen used in each assay were determined by checkerboard titrations. Microtiter plates were coated by overnight incubation with coating antigen solutions (100 μL/well) ranging from 0.1 to 18.85 μg/mL. Next, solutions of antisera (50 μL/mL) with dilution factors ranging from 1:250 to 1:2000 were added to each well. Coating and antisera concentrations giving OD_{490}s between 0.9 and 1.3 after 5 min were selected.

General ELISA. Basic solid-phase immunoassay principles were followed (Laurent et al., 1988) in performing enzyme-linked immunosorbent assays (ELISAs) in 96-well microtiter plates (Immulon II). Thus, microtiter plates were coated with OVA conjugates [70–100 μL/well of a 0.05 M carbonate buffer (pH 10) solution containing 1 μg/mL of **4**-OVA or **12**-OVA] by storing the plates at 4 °C overnight. The plates were washed four times with saline–Tween buffer [PBS (Sigma, containing 120 mM NaCl, 2.7 mM KCl, and 10 mM inorganic phosphate at pH 7.4) diluted 1:4, 9 g/L NaCl, and 0.5 mL/L Tween 20] and blotted dry. Each washing was accomplished by filling all wells with saline–Tween buffer, soaking for 2 min, emptying, and tapping somewhat dry. Appropriate antisera dilutions in PBS were prepared from previously frozen samples of rabbit antisera, and after preincubation with standards at 4 °C for 12 h, 50 -μL aliquots were pipetted into each well and the plates allowed to stand covered at 20 °C for 2 h. Washing four times with saline–Tween solution was followed by the addition of 50 μL/well of commercial biotinylated goat anti-rabbit IgG (1.0 mg/mL) diluted 1:2000 with PBS. The plate was then incubated for 1 h at 20 °C and washed with saline–Tween.

Fifty microliters per well of a solution consisting of avidin-conjugated horseradish peroxidase (type VI, 1 mg/mL) diluted 6:10 000 with PBS was added, and the plate was incubated for 30 min at 20 °C. Next, 50 μL/well of a solution consisting of *o*-phenylenediamine (1 mg/mL) and 30% hydrogen peroxide (1 μL/mL) in a Tris–citrate buffer (24.2 g/L Tris base and 12.6 g/L citric acid adjusted to pH 6.0) base was added to the plate. When the kinetic format was used, the rate of color change (mOD/min) at 450 nm was recorded. When the endpoint format was employed, the reaction was stopped after 5 min by the addition of 50 μL/well of 2 N H_2SO_4 and the final optical density (OD_{490}) was measured at 490 nm.

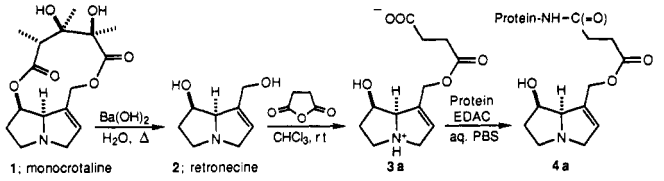
Nonspecific Binding Controls. As a control, each microtiter plate had 12 wells free of coating antigen, having contained only the coating buffer during the coating procedure. To the first three wells was added only *o*-phenylenediamine; to the second three wells were added both avidin-conjugated horseradish peroxidase and *o*-phenylenediamine; to the third three wells were added biotinylated goat anti-rabbit conjugate, avidin-conjugated horseradish peroxidase, and *o*-phenylenediamine; to the last three wells were added antisera, biotinylated goat anti-rabbit conjugate, avidin-conjugated horseradish peroxidase, and *o*-phenylenediamine.

Competitive ELISA with Retronecine Standards. For standard curves with less sensitive antisera (sera 333, 352, and 353), a primary standard of 10.00 mg/mL of retronecine was used to make a series of diluted standards in PBS buffer with concentrations ranging from 500 μg/mL (500 ppm) to 0.1 ng/mL (0.1 ppb). For more sensitive antisera (sera 1315 and 1322), PA stock solutions in 10 mM PBS were diluted from 100 000 to 0.01 ppb in 1:10 dilution steps. An antisera solution of appropriate dilution for the particular coating antigen to be used was prepared in PBS. Samples of each retronecine standard (1 part) were diluted 1:10 with antisera solution (9 parts) in test tubes and preincubated overnight at room temperature or at 37 °C for 2 h. One standard, containing only antisera and buffer (1:10), was used as the control to determine the maximum kinetic or endpoint OD reading. Another sample containing only buffer was used as a blank. Inhibition curves were analyzed by a four-parameter logarithmic curve-fitting procedure which calculated I_{50} values (inhibitor concentration giving 50% inhibition) and the slope of the linear portion of the curve (see Table I). Below 5% solvent content, methanol did not show any effect on antibody activity.

Comparative Inhibition Calculations. Two coating antigens (**4**-OVA and **12**-OVA) and antisera (anti-**4**-BSA and anti-**12**-BSA) combinations were evaluated in assays using retronecine as analyte. Initial screens were performed using retronecine standards decreasing by factors of 10 from a concentration value of 100 000 (100 ppm) to 0.1 ng/mL (0.1 ppb). For those combinations where retronecine showed a promising inhibition range, additional standards were prepared and run in the range where inhibition curves displayed nearly linear behavior. Fifty percent inhibition (I_{50}) values were determined for these systems.

Digestion of Naturally Occurring PAs. Both dilute and concentrated solutions of aqueous monocrotaline or retrorsine were deoxygenated by alternate cooling (0 °C) and heating (40 °C) while N_2 gas was bubbled through the solution. This cooling/heating sequence was repeated twice. Dilute PA samples (typically 1 mL of a 10 ppm PA solution) were basified to pH 10 by the addition of 0.2 M K_2CO_3 (giving a working K_2CO_3 concentration of 10 mM), adjusted to 10% aqueous methanol by dropwise addition of methanol, and the resulting solution was placed in a sealable tube. After being fitted with pressure-tight

Scheme I. Preparation of a Useful Coating but Labile Antigen



caps, these tubes were incubated in boiling water for 1 h. Concentrated PA samples (10 mg/mL) were basified to pH 10 with 1 M NaOH (giving 2.2 equiv of NaOH) and adjusted to 10% aqueous methanol by dropwise addition of methanol, and the resulting solution was refluxed for 2 h while N_2 gas was bubbled through the solution. When the solution had cooled to room temperature, N_2 bubbling was discontinued, and these aqueous solutions were neutralized with 1 N HCl and the volume was adjusted with distilled water to the desired analytical volume.

Competitive ELISA with Digested PA Samples. For the assay of digested PAs, an incubation buffer with greater buffering capacity containing 108 mM NaCl, 2.45 mM KCl, 45 mM inorganic phosphate adjusted to pH 7.4, and 1.4% Tween 20 was prepared. Dilute digested PA samples were diluted 1:2 with incubation buffer containing antiserum. Concentrated PA digests were diluted as required to put the final concentration within the linear range of the assay.

Detection of PAs in Digested Plant Samples. Whole *S. vulgaris* plants were ground in a blender with methanol and refluxed for 1 h with nitrogen bubbled through. *C. retusa* seeds were pulverized and refluxed with methanol as above. Solids were removed by filtration, and a portion of the filtrate was subjected to alkaline digestion as described above.

Cross-Reactivity Studies with Swainsonine and Lupinine. *Lupinus* spp. seeds were pulverized and refluxed with methanol and filtered as above. Retronecine standard curves were run in the presence of 10% v/v methanolic lupine extract. Retronecine standard curves were also run in the presence of commercially prepared swainsonine (1 μg/mL) and lupinine hydrochloride (1 μg/mL).

RESULTS AND DISCUSSION

Hapten Selection and Conjugation. Inspection of (+)-retronecine (**2**), which is readily obtained by base-catalyzed hydrolysis of commercially available monocrotaline (**1** → **2**) (Scheme I), suggested that its primary and secondary hydroxyls as well as its tertiary nitrogen would provide ample opportunity for synthetic manipulation ultimately leading to antigenic carrier protein conjugation. Indeed, acylation of (+)-retronecine with succinic anhydride produces succinate **3a** together with some of the corresponding C_7 monosuccinate (**3a**:**3b**::5:1). Subsequent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC)-mediated protein conjugation in phosphate-buffered saline (PBS) delivered antigen **4a** (+**4b** from **3b**) which, although easily formed and useful as a coating protein, proved to be too labile in vivo to serve as an immunogen. On the suspicion that the ester moiety in **4a** was hydrolytically unstable and the cause of low antibody specificity (Bober et al., 1989), an amide analogue of **3a** was targeted for preparation. Thus, (+)-retronecine was converted to retronamine (**7**; Mattocks, 1969) by a sequence consisting of allylic chlorination (→**5**), azide displacement (→**6**), and reduction (→**7**). While N-acylation with succinic anhydride smoothly produced the amide analogue of **3a**, all attempts at protein conjugation failed because ester activation of the free carboxylic acid moiety resulted in intramolecular acylation, giving succinimide derivative **8** rather than protein conjugation (Scheme II).

Fortunately, this problem was easily resolved by modifying the tether's chain length. Specifically, N-acylation of retronamine with hexanedioic anhydride **10** delivered

Scheme II. Preparation of Useful Immunizing and Coating Antigens

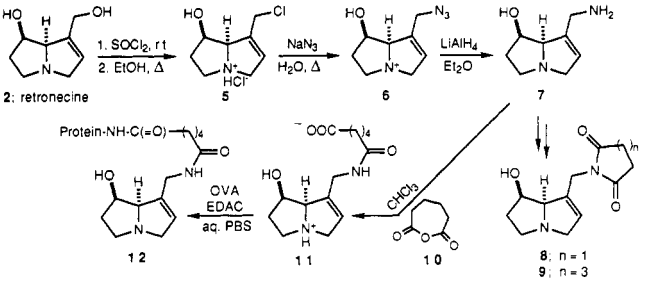


Table I. Screening of Antibodies: Titers and Competitive Binding[a]

		coating of 4-OVA			coating of 12-OVA		
serum	immunogen	titer	slope	I_{50},[b] ppb	titer	slope	I_{50}, ppb
333	4-BSA	100000	≈0	+++	100000	1.16	116000
352	4-BSA	800000	≈0	+++	80000	≈0	+++
353	4-BSA	80000	≈0	+++	80000	0.923	125000
1315	12-BSA	1000	0.912	16.5	5000	1.06	6.45
1322	12-BSA	150	0.013	+++	1000	0.387	1460

[a] Retronecine **2** was used as analyte. [b] The notation +++ indicates that meaningful I_{50}s were not available at these slopes.

amide **11** in quantitative yield. Now, with two additional methylene units in the tether, intramolecular trapping of the activated carboxylate would lead to formation of a seven-membered ring imide and EDAC-mediated acylation gave only protein conjugation (none of imide **9** was detected).

Screening of Antisera by Testing Their Titers. Antibodies were raised against immunogens **4** (protein = BSA, rabbits 333, 352, and 353; Bober et al., 1989) and **12** (protein = BSA, rabbits 1315 and 1322). Screening the antisera from 4-BSA (see Table I) against either a 4-OVA or 12-OVA coating established that rabbits inoculated with the retronecine hapten all had high titers. Unfortunately, the coating protein affinity of anti-4-BSA is so high that analyte (e.g., retronecine) cannot effectively compete. In contrast, anti-12-BSA (antiserum 1315), which is derived from the more stable amide-linked hapten, showed a reasonable titer against 12-OVA and led to an immunoassay where analyte can effectively compete for the antibody.

Assay Optimization. The antiserum raised against the 12-BSA immunogen (antiserum 1315) used against the 12-OVA coating gives excellent results in competitive assays when diluted 1:1000 as determined by checkerboard titrations. Eight standard ELISA curves for retronecine in PBS were first obtained using the optical density of oxidized *o*-phenylenediamine at 490 nm (OD_{490}) as a function of known retronecine concentrations. A best-fit four-parameter curve was calculated with Softmax software according to the equation

$$y = (a - d)/[1 + (x/c)^b] + d \quad (1)$$

where a and d are the upper and lower asymptotes, respectively; b is the slope of the linear portion of the curve; and c is the midpoint of the linear portion of curve, interpreted as the I_{50}. These data yield an average I_{50} value of 11 ± 3 ppb and an average slope of 0.73 ± 0.07.

When these data were converted to a plot of percent antibody inhibition as a function of known retronecine concentrations (Figure 2a), they revealed detection limits of 1.0–100 ppb as defined by the range within 15–80% of the maximum control. The software calculated retronecine concentration values for individual wells based on

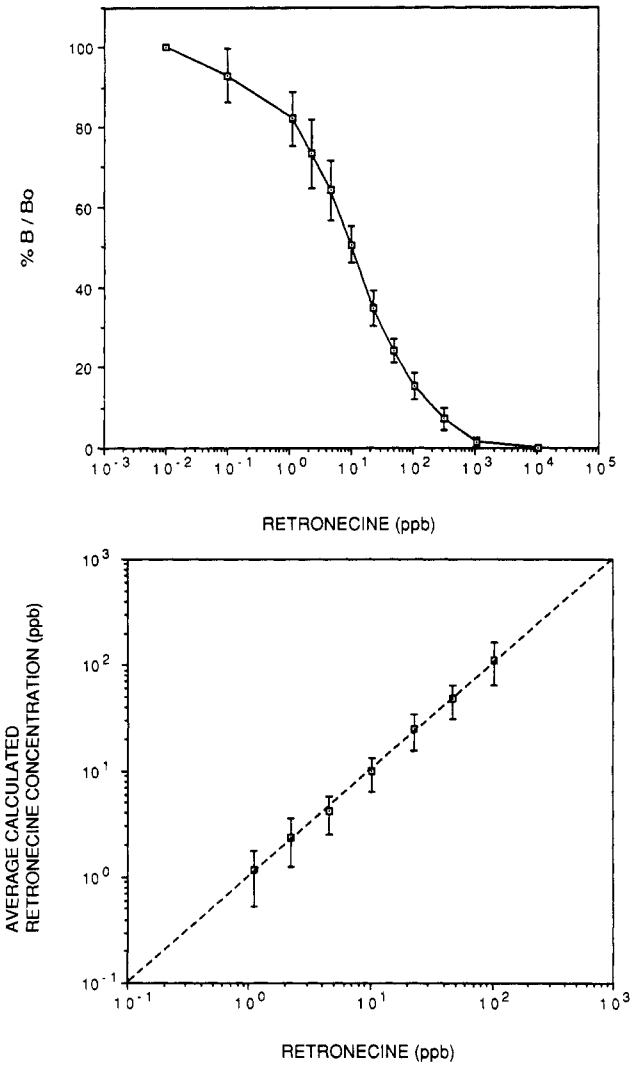


Figure 2. (Top, a) Antibody inhibition expressed as $\%B/B_0$, the percentage of bound antibody relative to the observed maximum, vs known retronecine concentration. The composite standard curve of eight runs reveals a 50% inhibition (I_{50}) of 11 ± 3 ppb in a linear range of 1.0–100 ppb. The error bars reflect the standard deviation in $\%B/B_0$ among eight runs. (Bottom, b) Representation of the standard curve as the calculated retronecine concentration vs known retronecine concentration within the linear range 1.0–100 ppb. Calculated values based on optical density were obtained by the software with reference to the four-parameter standard curve shown above. The dotted line $x = y$ is shown for comparison. The error bars reflect the standard deviation in the calculated retronecine concentration among 48 wells.

optical density with reference to the four-parameter standard curve (eq 1). Calculated retronecine concentrations plotted as a function of known values within the detection limits 1.0–100 ppb (Figure 2b) show close correlation to the theoretical ideal line $x = y$ and demonstrate that this assay detects retronecine reliably within the detection limits stated above. Hence, concentrated unknowns should be diluted so that their retronecine values fall within that range; dilute unknowns should likewise be concentrated if possible.

Immunoassays are often equally sensitive over a wide range of pH values (Kemeny and Challacombe, 1988). Retronecine, an alkaloid containing a tertiary amine, exists as a protonated ammonium salt (alkaline $pK_a \approx 10$) over the entire pH range 5.8–8.0. Consequently, within that range, the assay for retronecine is not sensitive to pH and the affinity of retronecine to antiserum 1315 is not pH dependent.

Table II. Competitive ELISA for PA Detection[a]

	parent PA[b]		digested PA[c]	
	I_{50}, ppb	slope	I_{50}, ppb	slope
monocrotaline	110000	0.627	86.2	0.795
senecionine	31200	0.658	1190	0.866
retrorsine	+++	0.070	1250	0.925
retrorsine *N*-oxide	+++	≈0	+++	≈0

[a] Using anti-12-BSA (serum 1315) with 12-OVA coating. The notation +++ indicates that meaningful I_{50}s were not available at these slopes. PAs were serially diluted and assayed; I_{50} and slope values were determined by the four-parameter fit. Retronecine liberated by hydrolysis from parent PA by alkaline digestion results in a lower PA concentration necessary for 50% antibody inhibition. [b] Data obtained using a solution of the naturally occurring, macrocyclic PA as analyte. [c] Data obtained using the alkaline digest solution as analyte.

Likewise, assay sensitivity is not highly dependent upon the incubation buffer concentration. This, plus the pH insensitivity of the assay proved to be advantageous when the PA aqueous hydrolysates were assayed (pH ≈ 10). Thus, an incubation buffer of 120 mM NaCl and 2.7 mM KCl was used with phosphate concentration increased to 45 mM to provide adequate buffering capacity for the PA hydrolysates.

Loss of Sensitivity Due to Retronecine Oxidation. Colorless PAs readily undergo oxidation to the corresponding *N*-oxide or to the corresponding pyrrole, usually brown, in the presence of air (Culvenor et al., 1970a,b). This process is accelerated at elevated temperatures. Thus, unless great care is taken to exclude oxygen from the PA digestions, one observes a significant reduction in assay sensitivity for the digested PA samples compared with the theoretical values (based upon the starting weight of undigested PA). This apparent loss of sensitivity is due to retronecine-to-pyrrole oxidation by oxygen and can be avoided by carefully purging digestion solutions with nitrogen and then completing the digestion under a nitrogen atmosphere.

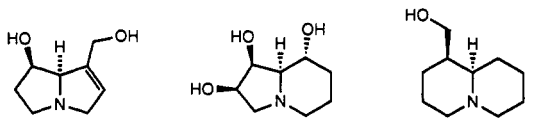
Screening of Antisera by Testing Their Recognition to Analytes. As illustrated in Figure 1, the necine based retronecine (2) is found in numerous naturally occurring PAs. Consequently, we set out to detect this substructural unit and thus develop a "class-specific PA assay". With that objective, two strategies were explored. The first and more speculative strategy involved direct detection of the retronecine substructure in intact parent PAs. Monocrotaline, retrorsine, and senecionine (see Figure 1) were selected as representative macrocyclic PA analytes to probe the feasibility of this approach to class-specific PA assay development. Unfortunately, as the "parent PA" entries in Table II demonstrate, this ELISA was able to detect these natural PAs only at high concentration levels. Apparently the unblocked secondary hydroxyl of antigen 12 is an important immunodeterminant and its acylation, as in the macrocyclic PAs of Figure 1, significantly diminishes anti-12-BSA cross-reactivity.

The second strategy involves a somewhat less direct assay but one which capitalizes on the fact that base-catalyzed hydrolysis of any of the macrocyclic diesters depicted in Figure 1 gives the necine base retronecine (2) and an accompanying necic acid (e.g., monocrotalic acid from monocrotaline). Subsequent immunochemical detection of retronecine in the aqueous hydrolysate in a buffer with sufficient buffering capacity to compensate for some alkali present (digested PA entries in Table II) completes this two-step assay and, to our knowledge, constitutes the first class-specific PA immunoassay, one with significant potential for the detection of PAs in plant, animal, and human samples.

Table III. Results of Cross-Reactivity Experiment[a]

	I_{50}, ppb	slope
retronecine standard	8.57	0.836
retronecine standard spiked to 1000 ppb of swainsonine (13)	6.67	0.694
retronecine standard spiked to 1000 ppb of lupinine (14) hydrochloride	7.83	1.14
MeOH lupine extract, 10% v/v	71.7	0.821

[a] Retronecine was serially diluted and spiked with other alkaloids; I_{50} and slope values were determined by the four-parameter fit. Retronecine (2) was detected with low I_{50}s in the presence of other alkaloids.



2 retronecine (a pyrrolizidine) 13 swainsonine (an indolizidine) 14 lupinine (a quinolizidine)

Figure 3. Structures of alkaloids tested for cross-reactivity.

Detection of PAs in Digested Plant Samples. The utility of the competitive ELISA for detecting PAs in actual plant samples was demonstrated by extracting *S. vulgaris* and *C. retusa* samples with refluxing methanol and assaying their alkaline digests [anti-12-BSA (serum 1315) with 12-OVA coating was employed]: *S. vulgaris* revealed a concentration of 870 μg/mL, *C. retusa*, 67 μg/mL (1 μg/mL equivalent weight of retronecine per volume of methanolic extract = 1 ppm). A lupine extract, by contrast, barely perturbs a retronecine standard curve (Table III). Although the PAs in *Senecio* occur largely as *N*-oxides, a sufficient quantity occur as the free base for detection with this assay. The monocrotaline in *Crotalaria* seeds occurs as the free base and so supplies sufficient retronecine for detection.

This ELISA-based PA assay has several advantages over conventional methods. Very dilute samples may be used. No column purification or two-phase extraction methods are necessary to prepare samples for this ELISA. Although the PA monoesters such as lycopsamine chromatograph poorly, both monoesters and diesters such as monocrotaline are detected with equal ease, since both are saponified to release retronecine. Lastly, many samples may be assayed on one 96-well microtiter plate for portability, rapidity, and great thrift.

Cross-Reactivity Study with Swainsonine and Lupinine. It is entirely likely that plant samples tested will contain alkaloids resembling retronecine, a pyrrolizidine, such as the indolizidines and quinolizidines, and hence cross-react with the antiserum to give a false positive. Swainsonine and lupinine (Figure 3) were selected as representative alkaloids for cross-reactivity studies. Lupine seeds which contain lupine alkaloids, including lupinine, were also tested for cross-reactivity. Reassuringly, relatively large concentrations (1000 ppb) of swainsonine and lupinine had no measurable effect on the I_{50} of the assay (Table III) and hence do not impact significantly the ability of this assay to detect PAs. Differences between the slopes are typical of the random error encountered when standard curves are run.

Conclusion. A sensitive, class-specific, and reproducible ELISA method has been developed for the detection of the pyrrolizidine alkaloids. This assay is comparable in sensitivity to GC and GC/MS methods and has the advantages of low cost, fast and easy performance, and portability. The linear ranges of inhibition curves of compounds containing retronecine using antiserum 1315 with coating 12-OVA are about 1.0–100 ppb (0.10–10.ug/well) with an I_{50} of 11 ± 3 ppb.

It is our hope that this assay can be used as a test for screening plant, herbal tea, cereal grain, milk, and honey specimens with relatively low PA concentrations. An immunogen to elicit antibodies against retronecine *N*-oxide is currently being synthesized in our laboratory with an eye toward detecting PA *N*-oxides in plant samples, foodstuffs, blood, and urine.

ABBREVIATIONS USED

Ab, antiserum/antibody; BSA, bovine serum albumin; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunosorbent assay; DCC, dicyclohexylcarbodiimide; I_{50}, the alkaloid concentration at which 50% antibody inhibition is observed; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance spectroscopy; OD_{490}, optical density at 490 nm; OVA, chicken egg ovalbumin; PA, pyrrolizidine alkaloid; PBS, phosphate-buffered saline solution (pH 7.4); PBST, phosphate-buffered saline solution containing 0.05% v/v Tween 20; ppb, w/w parts per billion; ppm, w/w parts per million; UCD, University of California at Davis.

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Supplementary Material Available: IR, ^{1}H NMR, ^{13}C NMR, and mass spectral assignments for the compounds prepared in this paper (3 pages). Ordering information is given on any current masthead page.

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