Development of Enzyme-Linked Immunosorbent Assays for the Hepatotoxic Alkaloids Riddelliine and Riddelliine *N*-Oxide

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Pyrrolizidine alkaloid-containing plants are widely distributed throughout the world and are particularly common in the genus *Senecio*. The structural types and concentrations of the alkaloids vary among plant species. In addition, within a species of plant, concentrations vary with environment and location. Many pyrrolizidine alkaloids are toxic and cause poisoning in livestock and in humans. Rapid, sensitive, and specific diagnostic techniques are needed to identify poisoned animals and to determine the particular plants and conditions under which livestock are likely to be poisoned. In this study, two competitive inhibition enzyme-linked immunosorbent assays for riddelliine, riddelliine *N*-oxide, and other closely related pyrrolizidine alkaloids were developed using polyclonal antibodies. One assay is class specific toward the free base forms of the pyrrolizidine alkaloids; the other assay showed cross-reactivity to both the free base and *N*-oxide forms of the alkaloids. The assay with the lowest limit of detection had an I_{50} of 803.9 pg with a limit of detection of 47.5 pg for riddelliine. Spike and recovery studies for riddelliine in bovine blood ranged from 45 to 74%. The assay that showed cross-reactivity between the *N*-oxide and free base forms of the pyrrolizidine alkaloids allowed estimation of the total pyrrolizidine alkaloid content in *Senecio riddellii* in admixture with alfalfa. These findings suggest that these techniques will be excellent tools to diagnose poisoned animals and identify highly toxic plants.

Keywords: *Enzyme-linked immunoassay; pyrrolizidine alkaloids; riddelliine; riddelliine N-oxide; plant toxicity; Senecio*

INTRODUCTION

Plants containing pyrrolizidine alkaloids are widely distributed throughout the world. More than 350 pyrrolizidine alkaloids have been identified in over 6000 plants in the Asteraceae, Boraginaceae, Compositae, and Leguminosae plant families (*1*). Many pyrrolizidine alkaloids are toxic and cause poisoning in livestock and in humans (*1*−*5*). Poisoning is generally manifested by hepatotoxicity, which may not present any overt signs until the animal is stressed by other factors. Pyrrolizidine alkaloidosis or seneciosis is therefore often not detected until irreversible damage has occurred. Most pyrrolizidine alkaloid-containing plants produce mixtures of the free bases and their corresponding *N*-oxides in varying concentrations. In general, the free base and *N*-oxide forms are similar in toxicity if absorbed via the gut (*4*, *6*).

Plants of the *Senecio* genus (Asteraceae family) comprise the majority of the pyrrolizidine alkaloid-containing plants in the United States. Three species are of concern to livestock owners in the western United States: *S. douglasii* var. *longilobus* (threadleaf groundsel) and *S. riddellii* (Riddell's groundsel) in the mid- and southwestern United States and *S. jacobaea* (tansy ragwort), an introduced species, in the Pacific northwest. Threadleaf groundsel contains four primary pyrrolizidine alkaloids: senecionine (**7**), seneciphylline (**3**), retrorsine (**5**), and riddelliine (**1**) (*4*). These four alkaloids have relatively similar chemical structures, all being 12-membered macrocyclic esters of the necine base, retronecine (**8**) (see Figure 1). The alkaloid content in threadleaf groundsel varies from 0.63 to 2.02% of dry plant weight (*7*). Riddell's groundsel is unique among such plants in that it contains one primary alkaloid, riddelliine (**1**) (*8*). The riddelliine concentration in Riddell's groundsel can vary greatly from 0.2 to 18% of dry plant weight (*9*). Nine pyrrolizidine alkaloids have been identified in tansy ragwort with a total concentration of ∼0.2% of dry weight (*1*, *4*). Three of the alkaloids that have been detected in tansy ragwort are senecionine (**7**), seneciphylline (**3**), and retrorsine (**5**) (*4*). It has been determined that tansy ragwort contains 75% free base and 25% *N*-oxide, whereas threadleaf grounsel and Riddell's grounsel contain 10−20% free base and 80−90% *N*-oxide (*10*). Many other pyrrolizidine alkaloid-containing plants contain senecionine (**7**), seneciphylline (**3**), retrorsine (**5**), and riddelliine (**1**) and their corresponding *N*-oxides. In addition, there are many pyrrolizidine alkaloids that are structurally similar to senecionine (**7**), seneciphylline (**3**), retrorsine (**5**), and riddelliine (**1**).

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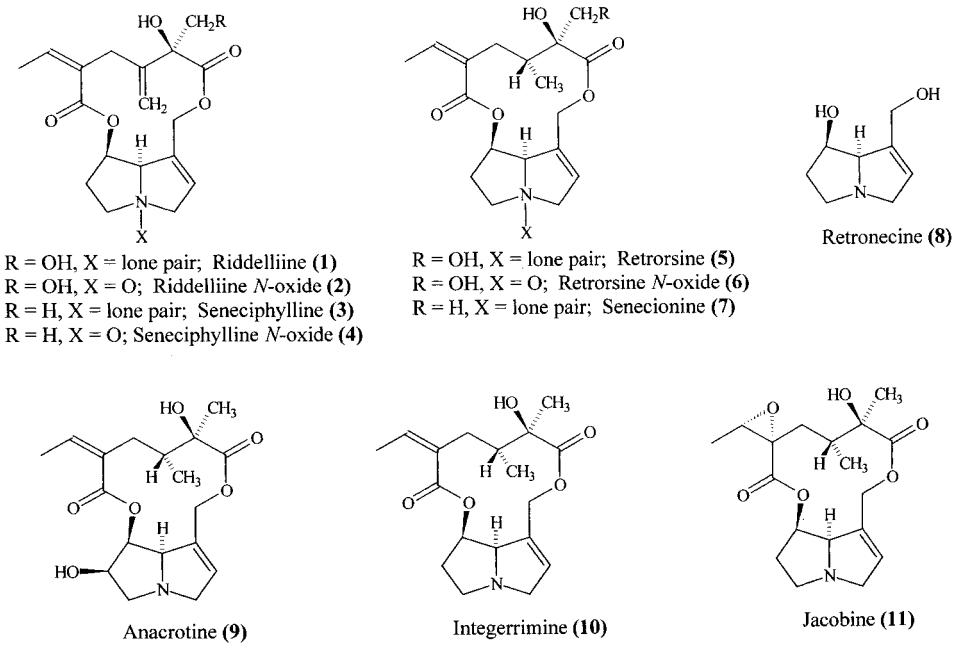

Figure 1. Chemical structures of necine free base, retronecine (**8**), and 12-membered macrocylic pyrrolizidine alkaloids.

Because of the wide number of naturally occurring pyrrolizidine alkaloids and because of the variation in toxicity and concentration that can occur in plants and animal tissues, it is important to develop rapid and sensitive methods to predict toxicity levels in plants and to identify animals poisoned by pyrrolizidine alkaloids. It is also important to develop analyses to monitor feed and food supplies that are intended for livestock or human consumption for pyrrolizidine alkaloid contamination. Enzyme-linked immunosorbent assays (ELISAs) have been developed as alternatives to traditional analytical techniques for detection of pyrrolizidine alkaloids (*11*−*16*). ELISAs offer the advantages of minimal sample preparation and the ability to run many samples simultaneously. Immunoassays can be designed to be either compound or class specific. Immunoassays are performed in aqueous media and are uniquely suited for the analysis of toxins in biological samples. Four of the reported immunoassays have been developed using retrorsine (**5**)−protein conjugates (*13*−*16*), three have been developed using retronecine (**8**)−protein conjugates (*11*−*13*), and one has been developed using monocrotaline (**12**) (*13*). All of these studies have reported cross-reactivity for pyrrolizidine alkaloids of similar structure. However, none of the previously reported immunoassays for pyrrolizidine alkaloids have demonstrated detection or cross-reactivity for riddelliine (**1**) or riddelliine *N*-oxide (**2**). In addition, only one immunoassay has reported detection of a pyrrolizidine in its *N*-oxide form [retrorsine *N*-oxide (**6**)] (*13*).

This paper describes the preparation of two different riddelline−protein conjugates and the generation of polyclonal antibodies to the conjugates. The antibodies are characterized by cross-reactivity studies. Two competitive inhibition ELISAs (CI-ELISAs) are described. One assay is specific to the free base form of the pyrrolizidine alkaloids of interest. The other assay demonstrates cross-reactivity to the *N*-oxide form of the pyrrolizidine alkaloids. We show the applicability of these assays for the detection and quantification of riddelliine (**1**) in bovine blood and riddelliine (**1**) and its corresponding *N*-oxide in plant samples.

MATERIALS AND METHODS

Chemicals. Fetuin from fetal calf serum, ovalbumin (OVA), succinic anhydride, 3,3′,5,5′-tetramethylbenzidine, thimerosal, polyoxyethylene sorbitan monolaurate (Tween 20), and phosphate-buffered saline (PBS) tablets were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide, and ethylene glycol dimethyl ether were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pyridine and dimethyl sulfoxide (DMSO) were purchased from Mallinckrodt Specialty Chemical Co. (Paris, KY). Chloroform, tetrahydrofuran, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Methyl 4-bromocrotonate was purchased from Avocado Research Chemicals Ltd. (Heysham, U.K.). Bovine serum albumin (BSA) fraction V reagent grade was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). DEAE-dextran was purchased from Pharmacia Biotech (Uppsala, Sweden). Quil A saponin was obtained from Superfos Biosector a/s (Frydenlundsvej, Denmark). Montanide 888 was purchased from Seppic (Paris, France). Carnation nonfat dry milk was obtained from Nestle USA, Inc. (Solon, OH).

The pyrrolizidine alkaloids anacrotine **(9)**, echinatine **(15)**, heliotrine **(16)**, jacobine **(11)**, integerrimine **(10)**, lasiocarpine **(17)**, retronecine **(8)**, and seneciphylline **(3)** were gifts from John A. Edgar (CSIRO, Livestock Industries, Geelong, VIC, Australia). Junceine **(14)**, retrorsine **(5)**, and senecionine **(7)** were obtained from Russell J. Molyneux (Western Regional Research Center, Albany, CA). Monocrotaline **(12)** was purchased from Sigma Chemical Co.. Riddelliine **(1)** was extracted from *Senecio riddellii* collected near Hobbs, NM (PPRL collection 96-3,4) and isolated from plant material using methods previously described (*10*). Monocrotaline *N*-oxide **(13)**, retrorsine *N*-oxide **(6)**, riddelliine *N*-oxide (**2**), and seneciphylline *N*-oxide **(4)** were synthesized by treatment of a solution of the alkaloid in chloroform/ethanol with 30% hydrogen peroxide (*17*).

Alkaloid−protein conjugates were filtered and concentrated using an ultrafiltration cell from Amicon, Inc. (Beverly, MA) and 30000 MW cellulose ultrafiltration membranes purchased from Millipore Corp. (Bedford, MA). ELISAs were performed on 96-well Nunc F96 Maxisorp polystyrene microtiter plates purchased from VWR Scientific Products (Denver, CO). Microtiter plates were read with a Bio-Rad model 3550-UV microplate reader from Bio-Rad Laboratories (Hercules, CA) at 450 nm. Electrospray mass spectral data were acquired on a Finnigan LCQ mass spectrometer from Finnigan Corp. (San Jose, CA). Samples were loop injected into the electrospray

source in a 50:50 methanol/1% acetic acid solution at a flow rate of 0.5 mL/min. Nuclear magnetic resonance (NMR) spectra were taken on a JEOL JNM-GSX 270 NMR spectrometer with ^{1}H spectra at 270 MHz. ^{13}C spectra were taken on a Bruker ARX400 at 100 MHz. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter, and $[\alpha]^{24}_D$ are given in 10^{-1} deg cm^2 g^{-1}. Nonlinear curve fitting was performed using SigmaPlot software purchased from Jandel Scientific (Sausalito, CA).

Riddelliine 18-Succinate—Protein Conjugates (20). Riddelliine **(1)** (982.9 mg, 2.816 mmol) and succinic anhydride (428.9 mg, 4.298 mmol) were added to an 8 mL vial containing a stir bar. Pyridine (1.5 mL) was added and the mixture stirred continuously (17 h, 60 °C). The reaction was sampled by electrospray mass spectrometry (ESMS). The mass spectrum indicated that all of the riddelliine ($MH^+ = 350$) had been consumed to form riddelliine 18-succinate **(18)** ($MH^+ = 450$). Succinylation at C-18 was evident in NMR spectra from the downfield shift of both C-18 (+0.9 ppm) and H-18 (+0.6 ppm) signals when compared to the riddelliine starting material. In addition, collision-induced dissociation (CID) fragmentation of the protonated molecular ion ($MH^+ = 450$) yielded a 320 fragment ion resulting from a loss of $-CHCO_2C_2H_4CO_2H$, which would not have been possible if succinylation were at C-12. The reaction mixture was transferred to a 20 mL scintillation vial. Distilled water (12 mL) and methanol (4 mL) were added to the reaction mixture and crystals started to form. The reaction mixture was put in the freezer (2 h, −20 °C) and then filtered (at room temperature) using a Büchner funnel. Dry weight of the crystals was 1.0201 g (2.272 mmol, 80.7% yield): mp 177−178 °C dec; $[\alpha]^{24}_D$ −13.9° (*c* 0.83, $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.85 (3H, d, $J = 7.1$ Hz, H-21), 2.29 (1H, m, H-6a), 2.40 (1H, dd, $J = 5.2$ Hz, 13.8 Hz, H-6b), 2.52 (4H, s, H-2′,3′), 2.66 (1H, m, H-5a), 2.81 (1H, d, $J = 16.4$ Hz, H-14a), 2.90 (1H, d, $J = 16.4$ Hz, H-14b), 3.48 (1H, dd, $J = 3.6$, 15.4 Hz, H-3a), 3.58 (1H, t, $J = 8.8$ Hz, H-5b), 3.88 (1H, d, $J = 12.1$ Hz, H-9a), 4.18 (1H, d, $J = 15.4$ Hz, H-3b), 4.50 (2H, s, H-18), 4.71 (1H, s br, H-8), 5.08 (3H, m, H-7,19), 5.26 (1H, d, $J = 12.1$ Hz, H-9b), 5.89 (1H, q, $J = 7.1$ Hz, H-20), 6.14 (1H, s br, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.3 (C-21), 30.0, 30.2 (C-2′ or C-3′), 34.5 (C-6), 37.4 (C-14), 52.9 (C-5), 60.4 (C-9), 60.8 (C-3), 67.1 (C-18), 74.1 (C-7), 76.6 (C-8), 77.7 (C-12), 115.0 (C-19), 130.4, 130.8 (C-1 or C-15), 134.1 (C-20), 137.6 (C-2), 142.8 (C-13), 166.3 (C-16), 172.2, 173.2, 176.7 (C-11 or C-1′ or C-4′). Anal. Calcd for $C_{22}H_{27}NO_9$: C, 58.79; H, 6.05; N, 3.11; O, 32.04. Found: C, 58.51; H, 5.90; N, 3.02; O, 33.29.

Riddelliine 18-succinate **(18)** (249 mg, 5.55×10^{-1} mmol), NHS (70.0 mg, 6.08×10^{-1} mmol), and dicyclohexylcarbodiimide (115 mg, 5.57×10^{-1} mmol) were added to anhydrous chloroform (15 mL). The riddelliine 18-succinate (18) and dicyclohexylcarbodiimide dissolved, whereas the NHS was only partially dissolved. The reaction was stirred (16 h, room temperature), the reaction flask cooled with dry ice, and the solution filtered through a Büchner funnel and transferred to a 35 mL round-bottom flask. The chloroform was removed (in vacuo) at ambient temperature. The light yellow powder was stored (−20 °C) until used in the protein conjugation reaction.

The NHS-activated riddelliine 18-succinate **(19)** (20.0 mg) was dissolved in DMSO (0.5 mL). The DMSO solution was added slowly to a continuously stirred solution of fetuin (44.0 mg) dissolved in 0.1 M $NaHCO_3$ (5 mL). The NHS-activated riddelliine 18-succinate **(19)** (3.6 mg) was dissolved in DMSO (0.5 mL) and added slowly to a continuously stirred solution of OVA (100 mg) dissolved in 0.1 M $NaHCO_3$ (6 mL). The targeted conjugation ratios of these reactions were ∼50:1 hapten/fetuin and ∼3.6:1 hapten/OVA. These reactions were stirred (16 h, room temperature) and then filtered (0.45 μm syringe filter). The reactions were then filtered (two times) with PBS (40 mL, pH 7.5) against a 30000 MW cutoff filter. The product was then diluted to a concentration of ∼1 mg/mL in PBS and stored (−120 °C) as 1 mL aliquots. These fetuin and OVA conjugates are designated RID-SA−FET **(20)** and RID-SA−OVA **(20)**, respectively, throughout this paper.

Riddelliine *N*-Crotonate—Protein Conjugates (22). 4-Bromocrotonic acid was prepared by the saponification of methyl 4-bromocrotonate using the procedure described by Braun (*18*). The NHS ester of 4-bromocrotonic acid (*N*-[(4-bromocrotonyl)oxy]succinimide) was prepared as described by Roseman et al. (*13*).

The reaction of the NHS ester of 4-bromocrotonic acid with riddelliine **(1)** to form the *N*-[[(succinimid-3-yloxycarbonyl)-ethynyl]methyl]riddelliine, bromide salt **(21)**, was also performed following the general method using alkyl halides to *N*-alkylate pyrrolizidine alkaloids described by Roseman et al. (*13*). Briefly, riddelliine **(1)** (49.0 mg, 1.4×10^{-1} mmol) was dissolved in dry ethylene glycol dimethyl ether (1.6 mL), and a solution of the NHS ester of 4-bromocrotonic acid (50.2 mg, 1.92×10^{-1} mmol) in ethylene glycol dimethyl ether (1.2 mL) was added with rapid stirring. After 2 min, the stirring was discontinued, and the solution was kept overnight. A fine white powder appeared in the bottom of the flask. The powder was collected by filtration and washed with fresh ethylene glycol dimethyl ether (3 mL). The powder was transferred to a weighed vial and residual solvent removed (in vacuo). The powder was sampled by ESMS. The mass spectra indicated that the white powder was the *N*-[[(succinimid-3-yloxycarbonyl)ethynyl]methyl]riddelliine, bromide salt **(21)**, ($MH^+ = 531$). The white powder (79.2 mg) was used without further purification and was stored (−20 °C) until used in the protein conjugation reaction.

Fetuin (30.3 mg) was dissolved in PBA (4 mL, pH 7.4). The fetuin PBS solution was cooled in an ice bath. *N*-[[(Succinimid-3-yloxycarbonyl)ethynyl]methyl]riddelliine, bromide salt **(21)** (10.3 mg), was dissolved in DMSO (900 μL). The DMSO solution (625 μL) was added slowly to the continuously stirred fetuin PBS solution. BSA (44.1 mg) was dissolved in PBS (4 mL) and cooled in an ice bath. The *N*-[[(succinimid-3-yloxycarbonyl)ethynyl]methyl]riddelliine, bromide salt (**21**), in DMSO solution (225 μL) was added slowly to the continuously stirred BSA PBS solution. The targeted conjugation ratios of these reactions were ∼22:1 hapten/fetuin and ∼7.4:1 hapten/BSA. These reactions were then stirred (16 h, 4 °C) and filtered (0.45 μm syringe filter). The reactions were then filtered (five times) with distilled deionized water (50 mL) against a 30000 MW cutoff filter. The product was diluted to a concentration of ∼1 mg/mL in PBS and stored (−120 °C) as 1 mL aliquots. These fetuin and BSA conjugates are designated RID-CROT−FET (**22**) and RID-CROT−BSA (**22**), respectively, throughout this paper.

Immunizations. The primary injection solution was prepared by adding a 1 mg/mL hapten−fetuin conjugate-distilled water solution (6 mL) to a 1.25% DEAE-dextran, 0.125% Quil A, distilled water solution (9.6 mL) and emulsified with Montanide 888 (14.4 mL) for a total volume of ∼30 mL. Two cross-bred ewes were initially injected subcutaneously with primary injection solution (2 mL) or 0.4 mg of hapten−fetuin conjugate. Booster injections with half of the concentration of hapten−fetuin conjugate (0.2 mg) in the above injection solution were given after two 6-week intervals. Blood samples were drawn immediately before the initial injection and 14 days after the second booster injection. Sera were stored at −120 °C.

ELISA Procedure. Riddelliine−protein coating conjugates were dissolved in carbonate buffer (0.05 M, pH 9.6), and 100 μL was added to each well of the microtiter plate. The microtiter plates were incubated (2 h, room temperature, and then 16 h, 4 °C). The plates were then inverted to remove excess coating solution. The plates could then be covered with an adhesive plate sealer and stored in a plastic bag (−20 °C) for up to 6 months. The plates were washed (three times) with saline−Tween buffer (0.15 M NaCl, 0.5% Tween 20) and blotted dry. Next, 150 μL of assay buffer (0.1 M Tris, pH 7.5, 0.1% Tween 20, 5% Carnation nonfat dry milk powder) was added, and the plates were incubated (1 h, room temperature). The assay buffer acted as a blocking buffer to reduce nonspecific binding of the antisera. The plates were washed (three times) and blotted dry. A 50 μL aliquot of samples or standards diluted in the assay buffer were added to the wells followed

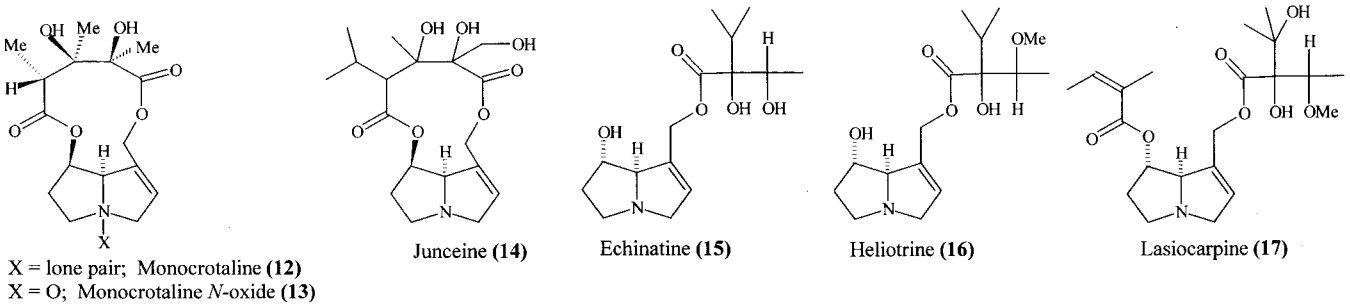

Figure 2. Chemical structures of 11-membered macrocyclic, monoester, and diester pyrrolizidine alkaloids.

by 50 μL of antiserum diluted in the assay buffer. The plates were then incubated (2 h, room temperature). After the 2 h incubation, the plates were washed (four times), horseradish peroxidase (HRP) conjugated donkey anti-sheep immunoglobulin G (IgG) (100 μL), diluted 1/10000 in assay buffer, was added to all wells, and the plates were incubated (1 h, room temperature). The plates were then washed (four times), and 100 μL of tetramethylbenzidine/H_2O_2 substrate (pH 5.5, 30 °C) (*19*) was added to each well. After 10 min, the reaction was stopped by the addition of 50 μL of 0.5 M H_2SO_4 to each well. The absorbances were measured at 450 nm (OD_{450}).

Plasma Preparation. Blood was drawn from a cow into a sodium citrate tube to prevent clotting. Aliquots of blood were spiked with riddelliine **(1)** at concentrations of 25, 100, 500, 1000, and 6000 ppb (pg/μL), vortexed, and refrigerated for a minimum of 16 h. The samples were then centrifuged (2100 rpm, 30 min, room temperature). The plasma was removed from the samples with a pipet. Twenty microliters of the plasma was diluted into 980 μL of assay buffer, and 50 μL was applied to the microtiter plates.

Plant Material Preparation. *S. riddellii* and alfalfa plant materials were air-dried and ground, and 500 mg was weighed into 50 mL extraction tubes in ratios of 0:100, 0.5:99.5, 5:95, 50:50, and, 100:0 *S. riddellii*/alfalfa. Methanol (25 mL) was added and the plant material extracted by continuously inverting the samples for 16 h. The samples were then centrifuged (25000 rpm, 10 min, room temperature). The methanol extract (10 μL) was diluted into 1990 μL of assay buffer, and 50 μL was applied to the microtiter plates.

RESULTS AND DISCUSSION

Riddelliine (**1**) and riddelliine *N*-oxide (**2**) are not large enough molecules to be immunogenic per se. Riddelliine (**1**) was therefore conjugated to a high molecular weight protein to elicit an immune response. Two methods were used to conjugate riddelliine (**1**) to the carrier and coating proteins. The first method was reaction of the C-18 primary hydroxyl group on the molecule with succinic anhydride to form a hemisuccinate (**18**), followed by activation of the carboxylic acid moiety with NHS (**19**) and reaction with the protein to form a five atom length succinate linker (**20**) (Figure 3). The second method was accomplished using the alkyl halide, *N*-[(4-bromocrotonyl)oxy]succinimide, to *N*-alkylate riddelliine (**21**), with subsequent reaction with the protein to form a four atom linker (**22**) (Figure 4).

Antisera. Antibody titers for the RID-SA−FET antisera were determined by titration of serial dilutions (1/500−1/256000) of sheep antisera against 250 ng/well of RID-SA−OVA coating conjugate. Antibody titers for the RID-CROT−FET antisera were determined by titration of serial dilutions (1/500−1 /2048000) of sheep antisera against 250 ng/well of RID-CROT−BSA coating conjugate. All sheep injected with conjugates produced antibodies. The sera from the two sheep that were injected with the same immunoconjugate were compared. The sera from the sheep that resulted in the highest titers was selected for further ELISA development.

Two CI-ELISAs were developed with the antisera raised against the RID-SA−FET and RID-CROT−FET immunoconjugates and using the RID-SA−OVA conjugate as a coating conjugate. The optimum dilutions for both the coating conjugate and antiserum were determined by checkerboard assays. The concentration of the coating conjugate and antiserum were selected on the basis of the combination at which the greatest difference in optical density was observed at 450 nm (OD_{450} values) between the wells of no free riddelliine and the presence of riddelliine at levels of 0.1−10 ng/well, and yet still giving an optical density between 0.9 and 1.3 in the absence of free riddelliine.

Cross-Reactivity. Sixteen pyrrolizidine alkaloids were selected for cross-reactivity studies: riddelliine *N*-oxide (**2**), seneciphylline (**3**), seneciphylline *N*-oxide (**4**), retrorsine (**5**), retrorsine *N*-oxide (**6**), senecionine (**7**), retronecine (**8**), anacrotine (**9**), integerrimine (**10**), jacobine (**11**), monocrotaline (**12**), monocrotaline *N*-oxide (**13**), junceine (**14**), echinatine (**15**), heliotrine (**16**), and lasiocarpine (**17**). Figures 1 and 2 show the structures of these alkaloids. The alkaloids were tested for cross-reactivity over the range of 1.95−1.024 × 10^6 pg/well. The data for each alkaloid were fit to the four parameter equation

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

to yield inhibition curves. In this equation a and d are the upper and lower asymptotes, b is the slope of the linear portion of the curve, and c is the midpoint of the linear portion of the curve. Table 1 reports the I_{50} values, the limit of detection (LOD) (I_{80}), the slope (b) of the curves, and the sensitivity of the assay for each alkaloid. The constant c in eq 1 has been reported as I_{50} values for CI-ELISAs (*13*). However, we chose to report the absolute I_{50} values as calculated from the equation of the curve. The LOD is conservatively defined in this study as the I_{80} values, as calculated from the equation of the curve. The sensitivity of the assay for each alkaloid reported in Table 1 is the product of the slope of the curve with the standard deviation of the experimental measurement closest to the I_{50} value of each alkaloid.

Assay 1. Checkerboard assays using the antiserum raised against the RID-SA−FET immunogen with RID-SA−OVA coating conjugate resulted in an optimum dilution of antisera of 1/7000 with the coating conjugate at 50 ng/well for a CI-ELISA. Inhibition curves for anacrotine (**9**), jacobine (**11**), integerrimine (**10**), retrorsine (**5**), retrorsine *N*-oxide (**6**), riddelliine (**1**), rid-

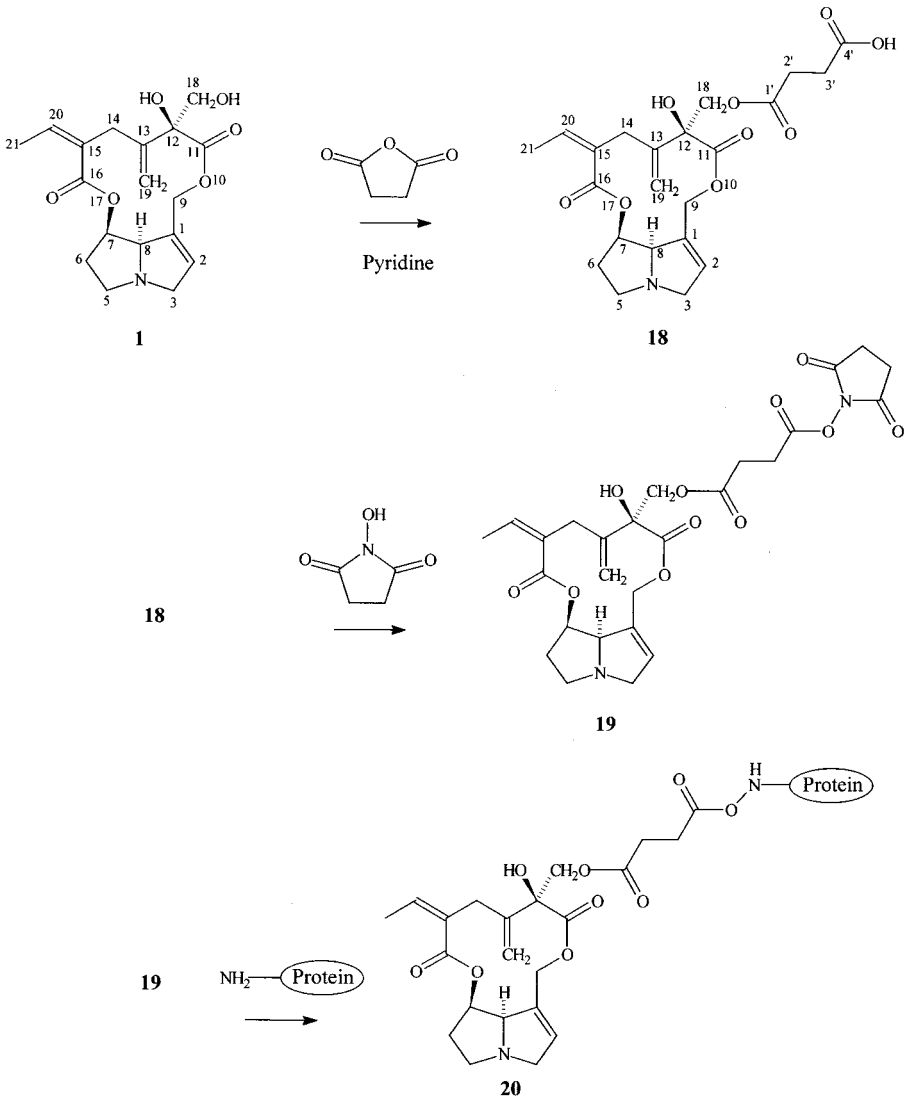

Figure 3. Synthesis of riddelliine 18-succinate−protein conjugates.

Table 1. Analytical Figures of Merit for Assays 1 and 2

	assay1				assay 2			
	I_{50} (pg)	slope	LOD (I_{80}) (pg)	sensitivity (pg)	I_{50} (pg)	slope	LOD (I_{80}) (pg)	sensitivity (pg)
riddelliine (**1**)	804	2.81	47.5	6.09	1.96×10^3	6.48	325	12.0
riddelliine *N*-oxide (**2**)	*a*	*a*	1.21×10^5	*a*	3.11×10^3	4.84	284	20.1
seneciphylline (**3**)	1.74×10^3	3.60	109	3.28	3.49×10^3	6.15	534	11.9
seneciphylline *N*-oxide (**4**)	2.93×10^4	7.00	3.20×10^3	9.80	2.11×10^3	2.97	31.3	5.02
retrorsine (**5**)	949	1.99	21.0	6.78	3.92×10^4	7.89	6.11×10^3	45.9
retrorsine *N*-oxide (**6**)	1.08×10^6	9.72	3.30×10^4	12.8	3.32×10^4	8.56	5.34×10^3	36.2
senecionine (**7**)	218	2.53	17.9	22.7	8.91×10^5	7.07	1.38×10^4	30.7
anacrotine (**8**)	8.65×10^4	8.56	7.04×10^3	26.1	1.10×10^5	7.50	7.93×10^3	33.3
intergerrimine (**9**)	2.58×10^4	3.20	274	27.3	7.29×10^4	8.43	1.28×10^4	12.8
jacobine (**10**)	4.00×10^4	3.97	621	27.7	*b*	*b*	*b*	*b*

[a] The I_{50}, slope, and sensitivity are not reported if the maximum amount of PA tested was not ≥50% inhibition of the maximum absorbance. [b] Competitive inhibition was not performed on assay 2 with jacobine.

delliine *N*-oxide (**2**), senecionine (**7**), seneciphylline (**3**), and seneciphylline *N*-oxide (**4**) are shown in Figure 5. Echinatine (**15**), heliotrine (**16**), junceine (**14**), lasiocarpine (**17**), monocrotaline (**12**), monocrotaline *N*-oxide (**13**), and retronecine (**8**) showed no cross-reactivity with the antibodies over the concentration range studied in this assay.

The inhibition curves in Figure 5 and the I_{50} values reported in Table 1 show riddelliine (**1**), retrorsine (**5**), senecionine (**7**), and seneciphylline (**3**) cross-react and are recognized by the antibodies raised against the RID-SA−FET immunogen. Figure 5 and Table 1 also show that the antibodies in this assay have some affinity to anacrotine (**9**), jacobine (**11**), intergerrimine (**10**), and seneciphylline *N*-oxide (**4**), whereas retrorsine *N*-oxide (**6**) and riddelliine *N*-oxide (**2**) are recognized to an even lesser degree. All of the pyrrolizidine alkaloids that cross-react with the antibodies in this assay are 12-membered macrocyclic diesters and are considered to be senacanine pyrrolizidine alkaloids. The antibodies in this assay do not strongly recognize the *N*-oxide forms of retrorsine, riddelliine, and seneciphylline. From these

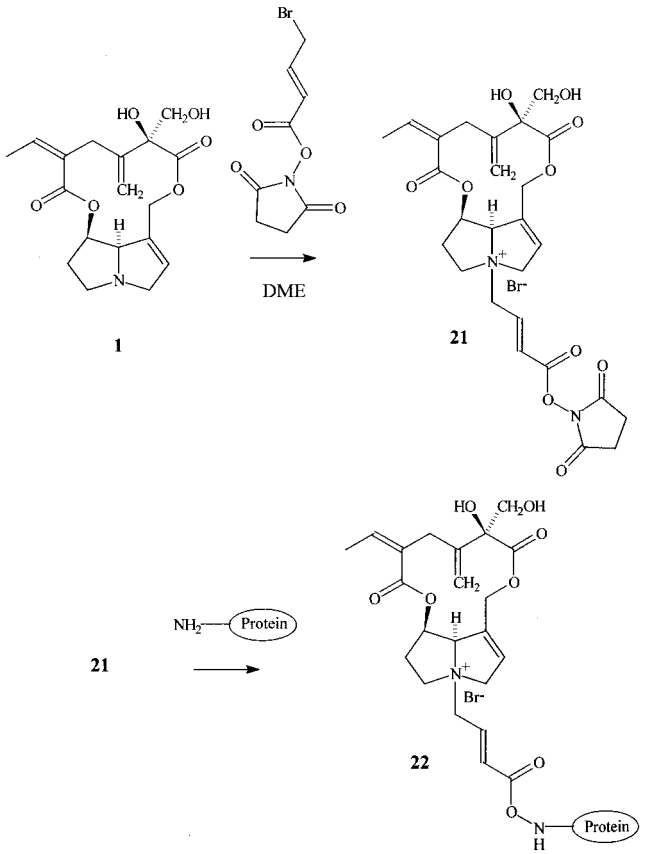

Figure 4. Synthesis of riddelliine *N*-crotonate−protein conjugates.

data we conclude that the two primary antigenic sites are the 12-membered macrocyclic diester and the necine free base moiety [retronecine **(8)**] of these alkaloids. This assay can be considered to be class specific toward the free base senacanine pyrrolizidine alkaloids. In general, within the senacanine class, those molecules with the closest structural similarity to riddelliine (**1**), such as retrorsine (**5**), senecionine (**7**), and senecipylline (**3**), have high cross-reactivity with riddelliine.

The LODs for riddelliine (**1**), retrorsine (**5**), senecionine (**7**), and seneciphylline (**3**) are 47.5, 21.0, 17.9, and 109 pg, respectively. These LODs compare favorably with the previously reported LODs using CI-ELISA for retrorsine (**5**) of 200 pg (*14*), 22.9 pg (*15*), and >1 ng (*16*).

Assay 2. This assay was first optimized and performed as a homologous CI-ELISA using RID-CROT−BSA as the coating protein. To lower the LOD and I_{50} value of the assay, RID-SA−OVA was used as the coating protein and optimized, resulting in a heterologous CI-ELISA. The optimum dilutions of RID-CROT−FET antisera and RID-SA−OVA were determined to be 1/51200 and 200 ng/well, respectively. The LOD and I_{50} for riddelliine (**1**) were decreased from 1.50×10^3 and 8.87×10^3 pg, respectively, with the homologous assay, to 325 and 1.96×10^3 pg, respectively, using the heterologous assay.

Inhibition curves for anacrotine (**9**), integerrimine (**10**), retrorsine (**5**), retrorsine *N*-oxide (**6**), riddelliine (**1**), riddelliine *N*-oxide (**2**), senecionine (**7**), seneciphylline (**3**), and seneciphylline-*N*-oxide (**4**) are shown in Figure 6. Echinatine (**15**), heliotrine (**16**), junceine (**14**), lasiocarpine (**17**), monocrotaline (**12**), monocrotaline *N*-oxide (**13**), and retronecine (**8**) showed no cross-reactivity with the antibodies over the concentration range studied in this assay.

The inhibition curves in Figure 6 and the I_{50} values reported in Table 1 show that riddelliine (**1**), riddelliine *N*-oxide (**2**), seneciphylline (**3**), and seneciphylline *N*-oxide (**4**) cross-react and are recognized by the antibodies raised against the RID-CROT−FET immunogen. Figure 6 and Table 1 also show the antibodies in this assay have some affinity to anacrotine (**9**), intergerrimine (**10**), retrorsine (**5**), retrorsine *N*-oxide (**6**), and senecionine (**7**). As in assay 1, all of the pyrrolizidine alkaloids that cross-react with the antibodies are 12-membered macrocyclic diesters and are considered to

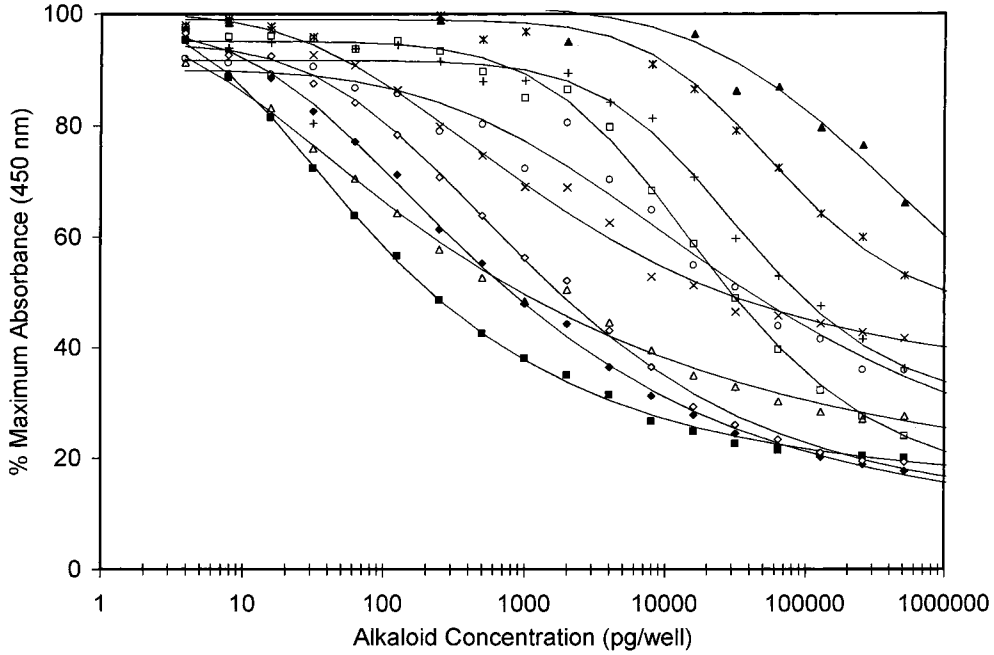

Figure 5. Inhibition curves for assay 1: riddelliine (◆), $n = 16$; riddelliine *N*-oxide (▲), $n = 3$; seneciphylline (◇), $n = 3$; seneciphylline *N*-oxide (□), $n = 3$; retrorsine (△), $n = 16$; retrorsine *N*-oxide (∗), $n = 3$; senecionine (■), $n = 4$; anacrotine (+), $n = 2$; integerrimine (×), $n = 4$; jacobine (○), $n = 8$. RSDs for graphical data points ranged from 0.08 to 40%.

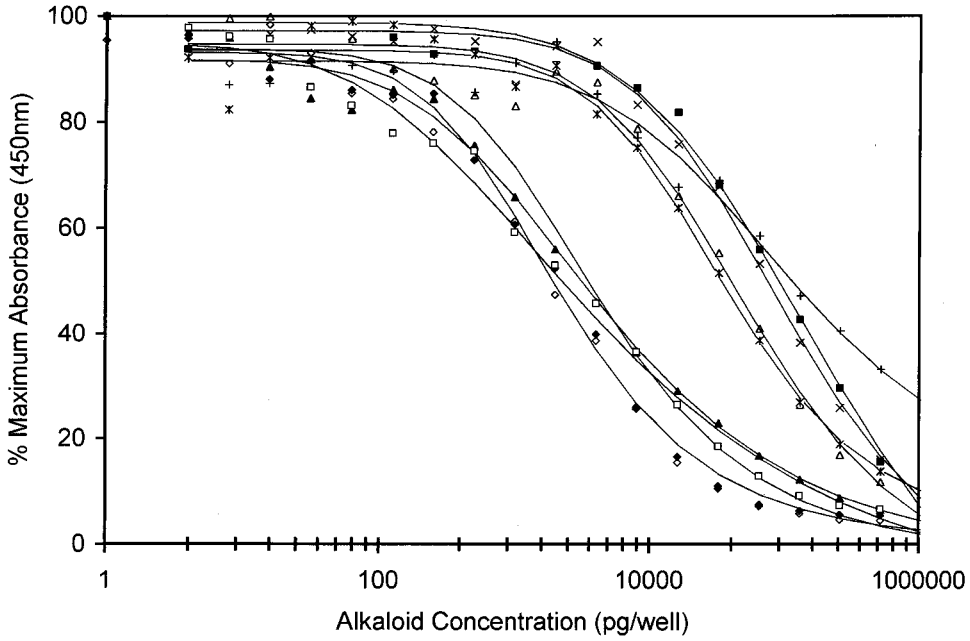

Figure 6. Inhibition curves for assay 2: riddelliine (◆); riddelliine *N*-oxide (▲); seneciphylline (◇); seneciphylline *N*-oxide (□); retrorsine (△); retrorsine *N*-oxide (∗); senecionine (■); anacrotine (+); integerrimine (×). Each point is the average of three replicates. RSDs for graphical data points ranged from 0.24 to 36%.

be senacanine pyrrolizidine alkaloids. In contrast to assay 1, the *N*-oxide forms of riddelliine (**1**), seneciphylline (**3**), and retrorsine (**5**) have cross-reactivities similar to those of their corresponding free base pyrrolizidine alkaloids. Riddelliine *N*-oxide (**2**), seneciphylline (**3**), and seneciphylline *N*-oxide (**4**) have the highest cross-reactivity with riddelliine (**1**) and have the most similar macrocyclic diester structure of the compounds examined with this assay. From these data we conclude the 12-membered macrocyclic diester and the methylene group at the C-19 carbon on these compounds are primary antigenic sites for the antibodies raised against the RID-CROT−FET antisera. Riddelliine (**1**) is conjugated to the protein through the nitrogen on the necine base of the alkaloid (see Figure 4). Three-dimensional models show that this conjugation leaves the 12-membered macrocyclic diester and the methylene group at C-19 far from the site through which the carrier protein is linked. This conjugation strategy allows the methylene group at C-19 to promote an immunologic response and be a determinant in the antisera that are produced and used in this assay.

This assay can be considered to be class specific toward senacanine pyrrolizidine alkaloids and their corresponding *N*-oxides. However, within the senacanine class, riddelliine *N*-oxide (**2**), seneciphylline (**3**), and seneciphylline *N*-oxide (**4**) have the highest cross-reactivity with riddelliine (**1**). The LODs for riddelliine (**1**), riddelliine *N*-oxide (**2**), seneciphylline (**3**), and seneciphylline *N*-oxide (**4**) are 325, 284, 534, and 31.3 pg, respectively.

Determination of Riddelliine (1) in Blood. We evaluated the applicability of assay 1 for the detection and quantification of riddelliine (**1**) in bovine blood. Samples were quantified against a 10-point riddelliine standard curve over the range of 15.6−8000 pg. The standard curve was determined by fitting the standards to eq 1. The r^2 for the standard curves was >0.9866 in all cases. Figure 7 shows the spike and recovery results for five different levels of riddelliine (**1**) in blood. The lowest spike (25 ppb) is just below the LOD (47.5 pg)

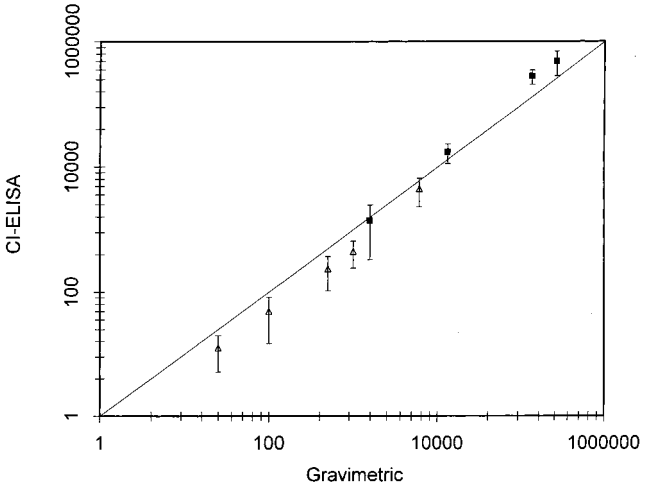

Figure 7. Correlation between spiked levels of riddelliine and the level determined by assay 1 for bovine blood (△) and correlation between expected levels based on gravimetric determination of total PAs in *S. riddellii* plant material using extraction/isolation methods described in Molyneux et al. (*10*) and the level detected by assay 2 for *S. riddellii*/alfalfa plant material (■). The line represents the theoretical correlation of riddelliine levels.

for this assay. Average recoveries for 100, 500, 1000, and 6000 ppb of riddelliine (**1**) in blood ranged from 45 to 74%, and relative standard deviations were 45−69%.

Determination of Riddelliine (1) in Plant Material. Assay 2 was evaluated for the detection and estimation of riddelliine (**1**) and riddelliine *N*-oxide (**2**) in plant material. Samples were quantified against a 10-point riddelliine *N*-oxide (**2**) standard curve over the range of 200−500000 pg. The standard curve was determined by fitting the standards to eq 1. The r^2 for the standard curves was >0.9977 in all cases. Figure 7 shows the results for the determination of riddelliine in mixed *S. riddellii*−alfalfa plant material extracts. Average recoveries for 0.263, 2.63, 26.3, and 52.66 parts per thousand of riddelliine in *S. riddellii*−alfalfa plant

material ranged from 88 to 212%, and relative standard deviations were 26−76%.

Two CI-ELISAs for riddelliine (**1**), riddelliine *N*-oxide (**2**), and other closely related pyrrolizidine alkaloids were developed. Assay 1 was class specific for the free base forms of riddelliine (**1**) and closely related alkaloids. The LODs of assay 1 were excellent and compare favorably with other CI-ELISAs for similar pyrrolizidine alkaloids. This assay was used for the detection and quantification of riddelliine (**1**) in blood. Assay 2 showed cross-reactivity to the *N*-oxide forms of riddelliine and closely related pyrrolizidine alkaloids in addition to the free base forms. The cross-reactivity between the *N*-oxide and free base forms of this assay allowed us to estimate the total pyrrolizidine alkaloid content in *S. riddellii*−alfalfa plant samples. The simple extraction−ELISA methods described in this paper demonstrate the potential of using these techniques for the rapid screening of biological samples for the presence and levels of pyrrolizidine alkaloids and will be beneficial in the diagnosis of animal poisonings, pharmacological studies, and identification of toxic plants.

ABBREVIATIONS USED

BSA, bovine serum albumin; CI-ELISA, competitive inhibition enzyme-linked immunosorbent assay; CID, collision-induced dissociation; DMSO, dimethyl sulfoxide; ESMS, electrospray mass spectrometry; LOD, limit of detection; NHS, *N*-hydroxysuccinimide; I_{50}, the mass of alkaloid at which the absorbance at 450 nm is 50% of the maximum absorbance; I_{80}, the mass of alkaloid at which the absorbance at 450 nm is 80% of the maximum absorbance; NMR, nuclear magnetic resonance spectrometry; OD_{450}, optical density at 450 nm; OVA, ovalbumin; PBS, phosphate-buffered saline solution (pH 7.4); PPRL, Poisonous Plant Research Laboratory; RID-CROT−BSA, riddelliine *N*-crotonate bovine serum albumin conjugate; RID-CROT−FET, riddelliine *N*-crotonate fetuin conjugate; RID-SA−FET, riddelliine 18-succinate fetuin conjugate; RID-SA−OVA, riddelliine 18-succinate ovalbumin conjugate; Tween 20, polyoxethylene sorbitan monolaurate.

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