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Development of Enzyme-Linked Immunosorbent Assays for Toxic Larkspur (*Delphinium* spp.) Alkaloids

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Larkspur (*Delphinium* spp.) poisons thousands of cattle on western rangelands each year. Because poisoning does not cause specific lesions, and poisoned animals are rarely found before they die, definitively identifying poisoned animals is difficult. Additionally, toxin concentrations in larkspur plants vary with environment, plant, and location. Rapid, sensitive, and specific diagnostic techniques are needed to identify poisoned animals and to determine when and what plants are likely to poison livestock. In this study, three competitive inhibition enzyme-linked immunosorbent assays (CI−ELISA) for toxic larkspur alkaloids were developed. One assay is class-specific toward the *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL) alkaloids, and two assays are specific for individual alkaloids. The assay with the lowest limit of detection had an I_{50} of 191 pg with a limit of detection of 30.5 pg for methyllycaconitine. Spike and recovery studies using bovine blood and brain tissue ranged from 52 to 89%. These findings suggest that with additional development these techniques are likely to be excellent tools for diagnosing poisoned animals and identifying highly toxic plants.

Keywords: *Larkspur; delphinium; enzyme-linked immunoassay; norditerpenoid alkaloids*

INTRODUCTION

Many of the larkspurs (*Delphinium* spp.) found on the rangelands of the western United States are poisonous to livestock, and cattle losses attributed to larkspur poisoning are valued in the millions of dollars each year (Williams and Cronin, 1996; Cronin and Nielsen, 1978; Nielsen and Ralphs, 1988; Ralphs et al., 1988). Larkspurs contain norditerpenoid alkaloids that are toxic to mammals (Pelletier and Keith, 1970; Manners et al., 1992, 1993). These compounds act as potent neuromuscular blocking agents that cause muscle weakness, paralysis, respiratory failure, and death (Olsen, 1978; Nation et al., 1982; Benn and Jacyno, 1983; Kukel and Jennings, 1994).

Over 40 norditerpenoid alkaloids have been isolated and identified in the larkspur species that grow in the western United States (Olsen et al., 1990; Manners et al., 1993). These alkaloids are divided into 3 major classes on the basis of their chemical structures and toxicity: lycoctonine, 7,8-methylenedioxylycoctonine (MDL), and *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL). Figure 1 shows structural depictions of various norditerpenoid alkaloids. Lycoctonine **(1)**, deltaline **(2)**, and methyllycaconitine **(3)** are examples of the lycoctonine, MDL, and MSAL types, respectively. The MSAL alkaloids are structurally distinguished from the lycoctonine and MDL types by an *N*-(methylsuccinimido)-anthranilic ester functional group at C-18. In general, the MSAL alkaloids are one to two orders of magnitude more toxic than the lycoctonine and MDL types (Olsen et al., 1990; Manners et al., 1993, 1995). LD_{50} values ranging from 2.7 mg/kg to 20.8 mg/kg have been measured for some of the larkspur MSAL alkaloids using mice (Manners et al., 1995). Methyllycaconitine, with an LD_{50} of 7.5 mg/kg, is the most prevalent MSAL alkaloid of the tall larkspurs in the western United States.

Because of the wide variations in structure and toxicity among the larkspur alkaloids it is important to develop analytical methodology capable of individual and class-specific measurements of these alkaloids in plant and animal tissues. Fourier transform infrared spectroscopy (FT-IR) is used to measure the concentration of MSAL-type alkaloids in plant extracts (Gardner et al., 1997). High performance liquid chromatography (HPLC) (Turek et al., 1995; Manners and Pfister, 1993), and more recently mass spectroscopy (MS) (Gardner et al., 1999), techniques have been used to measure individual larkspur alkaloids in extracts of plant and animal tissues. Although HPLC, FT-IR, and MS have proven to be rugged and reliable techniques for the identification and measurement of larkspur alkaloids, these techniques require expensive instrumentation and sample clean up procedures, and in the case of HPLC analysis, extended analysis times.

Enzyme-linked immunosorbent assays have been developed as alternatives to traditional analytical techniques for detection of natural products (Bober et al., 1989; Greirson et al., 1991; Roseman et al., 1992, 1996; Xiao et al., 1995; Langer et al., 1996; Garthwaite et al., 1998; Skerritt et al., 2000). Immunoassays can be designed to be either compound or class specific. Im-

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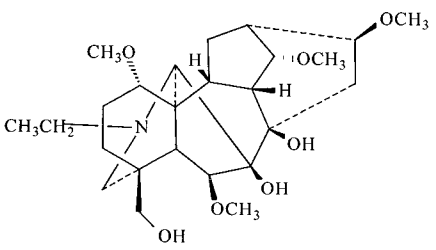
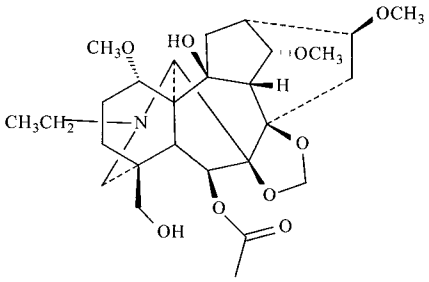
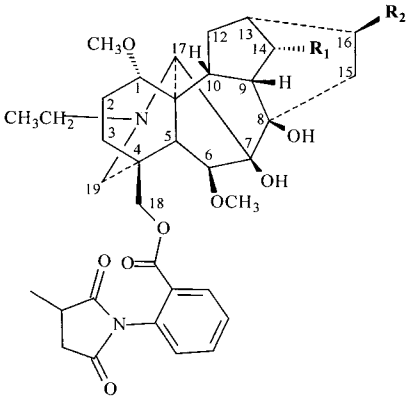


Lycoctonine (1)



Deltaline (2)



	R_1	R_2
Methyllycaconitine (3)	OCH_3	OCH_3
14-Deacetylnudicauline (4)	OH	OCH_3
Bearline (5)	OH	OAc

Figure 1. Chemical structures of lycoctonine **(1)**, deltaline **(2)**, methyllycaconitine **(3)**, 14-deacetylnudicauline **(4)**, and bearline **(5)**.

munoassays are performed in aqueous media and are uniquely suited for the analysis of toxins in biological samples. In contrast to HPLC, FT-IR, and MS, immunoassays require minimal sample preparation and offer high sample throughput.

This paper describes the preparation of six different alkaloid−protein conjugates and the generation of polyclonal antibodies to the conjugates. The antibodies are characterized by cross-reactivity studies, and three competitive inhibition enzyme-linked immunosorbent assays (CI-ELISA) are described, including one which is class specific toward the MSAL alkaloid type. Finally, we demonstrate the applicability of these assays for the detection and quantification of toxic alkaloids in bovine blood and brain tissue.

MATERIALS AND METHODS

Chemicals. Fetuin from fetal calf serum, succinic anhydride, 3,3′,5,5′-tetramethylbenzidine, *N*,*N*-carbonyldiimidazole, thimerosal, and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-hydroxysuccinimide and 1,3-dicyclohexylcarbodiimide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pyridine and dimethyl sulfoxide were purchased from Mallinckrodt Specialty Chemical Co. (Paris, KY). Chloroform, tetrahydrofuran, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Bovine albumin fraction V reagent grade and horseradish peroxidase (HRP) conjugated donkey anti-sheep IgG were 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). Silica gel 60-Å TLC plates, fluorescent at 254 nm, 500-μm layer were obtained from Whatman (Clifton, NJ).

The norditerpenoid alkaloids, lycoctonine, deltaline, and methyllycaconitine were a gift from Gary D. Manners (Western Regional Research Center, ARS, USDA, Albany, CA). 14-Deacetylnudicauline and bearline were extracted from plant material using methods previously described (Gardner et al., 2000).

Alkaloid−protein conjugates were filtered and concentrated using an ultrafiltration cell from Amicon, Inc. (Beverly, MA) and 30 000 MW cellulose ultra-filtration membranes purchased from Millipore Corporation (Bedford, MA). Enzyme-linked immunosorbent assays (ELISAs) were performed on 96-well NUNC F96 Maxisorp polystyrene microtiter plates purchased from VWR Scientific Products (Denver, CO). Microtiter plates were read at 450 nm with a BIO-RAD model 3550-UV microplate reader from Bio Rad Laboratories (Hercules, CA). Electrospray mass spectral data were acquired on a Finnigan LCQ Mass Spectrometer from Finnigan Corporation (San Jose, CA). Samples were loop injected into the electrospray source using a 50:50 methanol:1% acetic acid solution at a flow rate of 0.5 mL/min. Brain tissue was homogenized using an Omni 2000 Homogenizer purchased from Omni International Inc. (Gainsville, VA). Nonlinear curve fitting was performed using SigmaPlot software purchased from Jandel Scientific (Sausalito, CA).

Lycoctonine−*N*-Alkyl Carbamate−Protein Conjugates. Lycoctonine (3.1 mg, 6.6×10^{-3} mmol) and *N*,*N*-carbonyldiimidazole (10.9 mg, 6.7×10^{-2} mmol) were added to a 1-mL reactivial containing a triangular stir bar. Tetrahydrofuran (250 μL) was added and the reaction stirred continuously (2.5 h, 45 °C). The tetrahydrofuran was removed in vacuo.

The lycoctonine 18−imidazole carbamate residue was dissolved in tetrahydrofuran (300 μL) and added slowly to a continuously stirred solution of fetuin (15 mg) dissolved in 1 m*M* sodium borate (4 mL, pH 8.5). The reaction was stirred (16 h, RT) and then filtered (0.45 μm syringe filter). The solution was then filtered (5×) against a 30 000-MW cutoff filter with deionized distilled water (50 mL). The filtrate was then rinsed from the filter, diluted to a concentration of ~1 mg/mL in deionized distilled water, and stored (−120 °C) as 1-mL aliquots. This conjugate is designated as LYC−CDI−FET throughout this paper.

The above procedure was repeated with lycoctonine (2.4 mg, 5.1×10^{-3} mmol) and *N*,*N*-carbonyldiimidazole (8.1 mg, 5.0×10^{-2} mmol) and bovine serum albumin (BSA) (15 mg). The

targeted conjugation ratios of these reactions were ~20:1 hapten to fetuin and ~23:1 hapten to BSA. This conjugate is designated as LYC−CDI−BSA throughout this paper.

Lycoctonine 18−Succinate Protein Conjugates. Lycoctonine (20.1 mg, 0.0430 mmol) and succinic anhydride (10.0 mg, 0.10 mmol) were added to a 1-mL reactivial containing a triangular stir bar. Tetrahydrofuran (500 μL) was added and the reaction stirred continuously (16 h, 80 °C) The reaction was sampled by electrospray mass spectroscopy (ESMS). The mass spectra indicated that all of the lycoctonine (MH^+ = 468) had been consumed to form lycoctonine 18−succinate (MH^+ = 568). The tetrahydrofuran was removed (in vacuo), and the remaining white residue was washed (dry Et_2O) and dried, yielding lycoctonine 18−succinate (13.0 mg).

Lycoctonine 18−succinate (13.0 mg, 0.0229 mmol), *N*-hydroxysuccinimide (3.0 mg, 0.026 mmol), and dicyclohexylcarbodiimide (5.0 mg, 0.024 mmol) were added to anhydrous chloroform (10 mL). The lycoctonine 18−succinate and dicyclohexylcarbodiimide were dissolved, but the *N*-hydroxysuccinimide (NHS) was only partially dissolved. The reaction was stirred (16 h, RT), filtered through a fine fritted glass filter and transferred to a 35-mL round-bottomed flask. The chloroform was removed (in vacuo) and the transparent white residue was stored (−20 °C) until conjugation with the protein.

The NHS-activated lycoctonine 18−succinate was dissolved in dimethyl sulfoxide (500 μL). The dimethyl sulfoxide solution (200 μL) was added slowly to a continuously stirred solution of fetuin (15 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The remaining dimethyl sulfoxide solution (300 μL) was added slowly to a continuously stirred solution of BSA (15 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The targeted conjugation ratios of these reactions were ~30:1 hapten to fetuin and ~60:1 hapten to BSA. These reactions were stirred (16 h, RT) and and the products were then filtered (0.45 μm syringe filter). The solutions were then filtered against a 30 000-MW cutoff filter (2×) with deionized distilled water (50 mL). The filtrate was then rinsed from the filter, diluted to a concentration of ~1 mg/mL in deionized distilled water, and stored (−120 °C) as 1-mL aliquots. These fetuin and BSA conjugates are designated as LYC−SA−FET and LYC−SA−BSA, respectively, throughout this paper.

14-Deacetylnudicauline−*N*-Alkyl Carbamate−Protein Conjugates. 14-Deacetylnudicauline (5.3 mg, 7.9×10^{-3} mmol) and *N*,*N*-carbonyldiimidazole (4.6 mg, 2.8×10^{-2} mmol) were added to a 1-mL reactivial containing a triangular stir bar. Tetrahydrofuran (150 μL) was added and the reaction stirred continuously (6 h, 45 °C). ESMS indicated that all of the 14-deacetylnudicauline (MH^+ = 669) had been consumed to form 14-deacetylnudicauline−14-imidazole carbamate (MH^+ = 763). The reaction mixture was transferred to a flat-bottomed vial and the solvent was evaporated on a heating block at 65 °C under a stream of nitrogen to yield 8.8 mg of reaction residue. This procedure was repeated again for a total of 19.5 mg of reaction residue. It was estimated that ~10 mg of the reaction residue was the 14-deacetylnudicauline−14-imidazole carbamate based on the combined mass of the 14-deacetylnudicauline starting material used in the two reactions.

The 14-deacetylnudicauline−14-imidazole carbamate residue was dissolved in tetrahydrofuran (1.0 mL). The tetrahydrofuran solution (847 μL) was added slowly to a continuously stirred solution of fetuin (27.1 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The remaining tetrahydrofuran solution (153 μL) was added slowly to a continuously stirred solution of BSA (26.9 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The targeted conjugation ratios of these reactions were ~20:1 hapten to fetuin and ~5:1 hapten to BSA. These reactions were stirred (16 h, RT) and then the products were filtered (0.45 μm syringe filter). The solutions were then filtered against a 30 000-MW cutoff filter (5×) with deionized distilled water (50 mL). The filtrate was then rinsed from the filter, diluted to a concentration of ~1 mg/mL in deionized distilled water, and stored (−120 °C) as 1-mL aliquots. These fetuin and BSA conjugates are designated as 14-DAN−CDI−FET and 14-DAN−CDI−BSA, respectively, throughout this paper.

14-Deacetylnudicauline−14-Succinate−Protein Conjugates. 14-Deacetylnudicauline (15.8 mg, 0.0237 mmol) and succinic anhydride (15.5 mg, 0.155 mmol) were added to a 1-mL reactivial containing a triangular stir bar. Pyridine (120 μL) was added and the reaction stirred continuously (7 h, 60 °C). ESMS indicated that approximately $^2/_3$ of the 14-deacetylnudicauline (MH^+ = 669) had been consumed to form 14-deacetylnudicauline−14-succinate (MH^+ = 769). The reaction mixture was transferred to a flat-bottomed vial with methanol. The pyridine and methanol were evaporated off on a heating block at 65 °C under a stream of nitrogen.

The reaction mixture was then solubilized in chloroform (1.5 mL) and applied in a line to the bottom of a 20 cm × 10 cm silica TLC plate. After the mixture was air-dried, the TLC plate was developed using a mobile-phase composition of chloroform/methanol/ammonium hydroxide (70:30:1). 14-Deacetylnudicauline−14-succinate was isolated by removing the band (R_f = 0.5) on the silica plate and eluting the product with methanol. The identity of the TLC band was confirmed to be 14-deacetylnudicauline−14-succinate (MH^+ = 769) via ESMS. The methanol was evaporated on a heating block at 65 °C under a stream of nitrogen. The reaction product was then solubilized in chloroform and filtered through a cellulose filter to remove binding material soluble in methanol. The chloroform was evaporated on a heating block at 65 °C under a stream of nitrogen to yield 4.9 mg of 14-deacetylnudicauline−14-succinate.

14-Deacetylnudicauline−14-succinate (4.9 mg, 0.0064 mmol), N-hydroxysuccinimide (2.2 mg, 0.019 mmol), and dicyclohexylcarbodiimide (2.8 mg, 0.014 mmol) were added to anhydrous chloroform (2 mL). The 14-deacetylnudicauline−14-succinate and dicyclohexylcarbodiimide were dissolved, but the *N*-hydroxysuccinimide was only partially dissolved. The reaction was stirred (16 h, RT). The reaction solution was filtered through a fine fritted glass filter and transferred to a 35-mL round-bottomed flask. The chloroform was removed by rotary evaporation at ambient temperature. The transparent white residue was stored in a desiccator until conjugation with the protein.

The NHS-activated 14-deacetylnudicauline−14-succinate was dissolved in dimethyl sulfoxide (650 μL). The dimethyl sulfoxide solution (550 μL) was added slowly to a continuously stirred solution of fetuin (12.6 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The remaining dimethyl sulfoxide solution (100 μL) was added slowly to a continuously stirred solution of BSA (10.4 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The targeted conjugation ratios of these reactions were ~20:1 hapten to fetuin and ~6:1 hapten to BSA. The mixtures were stirred (16 h, RT) and then filtered (0.45-μm syringe filter). The eluent was then filtered against a 30 000-MW cutoff filter (5×) with deionized distilled water (50 mL). The product was diluted to a concentration of ~1 mg/mL in deionized distilled water and stored (−120 °C) as 1-mL aliquots. These fetuin and BSA conjugates are designated as 14-DAN−SA−FET and 14-DAN−SA−BSA, respectively, throughout this paper.

Methyllycaconitine Succinate−Protein Conjugates. Methyllycaconitine−succinate−fetuin and methyllycaconitine−succinate−BSA conjugates were prepared in a procedure similar to that for preparation of the 14-deacetylnudicauline−14-succinate−fetuin conjugate and 14-deacetylnudicauline−14-succinate−BSA conjugates. Methyllycaconitine (22.4 mg, 0.0328 mmol) and succinic anhydride (54.0 mg, 0.54 mmol) were added to a 1-mL reactivial containing a triangular stir bar. Pyridine (220 μL) was added and the reaction stirred continuously (15 h, 60 °C). The reaction was sampled by electrospray MS. The mass spectra indicated that approximately $^1/_4$ of the methyllycaconitine (MH^+ = 683) had been consumed to form methyllycaconitine−succinate (MH^+ = 783). The pyridine could not be evaporated from the MLA−succinic acid reaction; any attempt to evaporate the reaction to dryness resulted in the MLA−succinate reacting to form the epoxide of the adjacent C7 and C8 carbons. Therefore, the reaction mixture was diluted in chloroform (1.5 mL) and applied in a line to the bottom of a 20 cm × 20 cm silica TLC plate in a

hood. The TLC plate was then developed as in the 14-deacetylnudicauline−14-succinate procedure above.

Methyllycaconitine−succinate (3.4 mg) was recovered from the TLC plate ($R_f = 0.49$). This procedure was repeated to yield a total of 6.9 mg methyllycaconitine−succinate.

The NHS-activated methyllycaconitine−succinate was obtained using the same procedure to prepare the NHS-activated 14-deacetylnudicauline−14-succinate compound.

The NHS-activated methyllycaconitine−succinate was dissolved in dimethyl sulfoxide (570 μL). The dimethyl sulfoxide solution (480 μL) was added slowly to a continuously stirred solution of fetuin (17.4 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The remaining dimethyl sulfoxide solution (90 μL) was added slowly to a continuously stirred solution of BSA (18.1 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The targeted conjugation ratios of these reactions were ~20:1 hapten to fetuin and ~5:1 hapten to BSA. These reactions were stirred (16 h, RT) and then the products were filtered (0.45-μm syringe filter). The eluent was then filtered (5x) with deionized distilled water (50 mL) against a 30 000-MW cutoff filter. The product was diluted to a concentration of ~1 mg/mL in deionized distilled water and stored (−120 °C) as 1-mL aliquots. These fetuin and BSA conjugates are designated as MLA−SA−FET and MLA−SA−BSA, respectively, throughout this paper.

Immunizations. The primary immunogen injection solution was prepared by adding a 1 mg/mL hapten−fetuin conjugate and 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 crossbred ewes were initially injected subcutaneously with the primary injection solution (2 mL) or 0.4 mg of hapten−fetuin conjugate. Booster injections with $^1/_2$ the concentration of hapten−fetuin conjugate (0.2 mg) in the above injection solution were given after two six-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. Alkaloid−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 at RT and then 16 h at 4 °C). The plates were then inverted to remove the 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 (3×) 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% skim milk powder) was added and the plates were incubated (1 h, RT). The assay buffer acted as a blocking buffer to reduce nonspecific binding of the antisera. The plates were washed (3×) again and blotted dry. A 50-μL aliquot of sample or standard, diluted in the assay buffer, was added to each of the wells followed by 50 μL of antiserum diluted in the assay buffer. The plates were then incubated (2 h, RT). The plates were washed (4×), and HRP conjugated donkey anti-sheep IgG (100 μL), diluted 1:10 000 in assay buffer, was added to all wells, and the plates were incubated (1 h, RT). The plates were then washed (4×) and 100 μL of tetramethylbenzidine/H_2O_2 substrate, pH 5.5, warmed to 30 °C (Bos et al., 1981), 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 UV absorbances were measured at 450 nm (OD_{450}).

Plasma Preparation. Blood was drawn from a cow into a Na Citrate tube to prevent clotting. Aliquots of blood were spiked with methyllycaconitine 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., RT). The plasma was pulled off the samples with a pipet. An aliquot of plasma (20 μL) was diluted into 980 μL of assay buffer, and 50 μL was applied to the microtiter plates.

Brain Tissue Preparation. Frozen cow brain (1 g) was cut into ~250 mg pieces and 0.5 mL of PBS buffer was added to aid in tissue homogenization. The tissue was then homogenized for 2 min. with an Omni 2000 homogenizer. The diluted brain tissue was spiked with methyllycaconitine to result in concentrations of 25, 100, 500, 1000, and 6000 ppb methyllycaconitine in undiluted brain tissue. The brain tissue was vortexed and then refrigerated for a minimum of 16 h. The brain tissue was diluted to 5 mL with PBS buffer and vortexed. The samples were then centrifuged (15 000 rpm, 30 min., 10 °C). The supernatant was drawn off the samples with a pipet and 20 μL was diluted with 980 μL of assay buffer; a 50 μL-aliquot was applied to the microtiter plates.

RESULTS AND DISCUSSION

Hapten Synthesis. The norditerpenoid alkaloids (lycoctonine, 14-deacetylnudicauline, and methyllycaconitine) are not large enough molecules to be immunogenic. These alkaloids were therefore conjugated to a high molecular weight protein to elicit an immune response. Two methods were used to conjugate the alkaloids to the carrier and coating protein. The first method was direct activation of the hydroxyl group on the molecule with carbonyldiimidazole and reaction with the protein to form a zero-length carbamate linker. The second method involved reaction of the hydroxyl group on the molecule with succinic anhydride to form a hemisuccinate, followed by activation of the carboxylic acid moiety with NHS and reaction with the protein to form a 5-atom-length succinate linker.

Lycoctonine was the first norditerpenoid alkaloid to be conjugated to carrier and coating proteins in this study for two reasons: (1) lycoctonine has the same norditerpenoid backbone structure as methyllycaconitine and we hypothesized that antibodies raised to a protein conjugate of lycoctonine may cross-react with methyllycaconitine and other MSAL alkaloids; and, (2) the primary hydroxyl group on the C-18 carbon of lycoctonine was a highly reactive target for the formation of carbamate and hemisuccinate derivatives for protein conjugation.

14-Deacetylnudicauline is structurally similar to methyllycaconitine, the only difference being that 14-deacetylnudicauline has a hydroxyl group at the C-14 carbon whereas methyllycaconitine has a methoxy group. Conjugation through the secondary C-14 hydroxyl group of 14-deacetylnudicauline could then produce an immunoconjugate for both 14-deacetylnudicauline and methyllycaconitine. Although the reaction to form 14-deacetylnudicauline 14-carbamate was straightforward, the hemisuccinate conjugation proved more difficult. Higher yields of 14-deacetylnudicauline 14-succinate were obtained when the reaction solvent and volume was changed from 500 μL of tetrahydrofuran to 120 μL of pyridine with a concurrent increase in the ratio of succinic anhydride from 2.3:1 to 6.5:1.

Conjugation of methyllycaconitine proved to be more difficult because this compound contains only two possible reactive sites: both being tertiary hydroxyls at C-7 and C-8. Carbamate conjugates could not be prepared even when the reaction temperature was increased from 45 °C to 60 °C, the mole ratio of *N*,*N*-carbonyldiimidazole was increased to ~27 times that of methyllycaconitine, and the reaction time was extended to over 100 hours. Hemisuccinate conjugates of methyllycaconitine were prepared in low yield when methyllycaconitine was reacted with a 16.5 mole ratio of succinic anhydride in a concentrated solution of pyridine over 16 h.

Antisera. Antibody titers were determined by titration of serial dilutions (1:500−1:256 000) of sheep antisera raised against all five fetuin injection conjugates

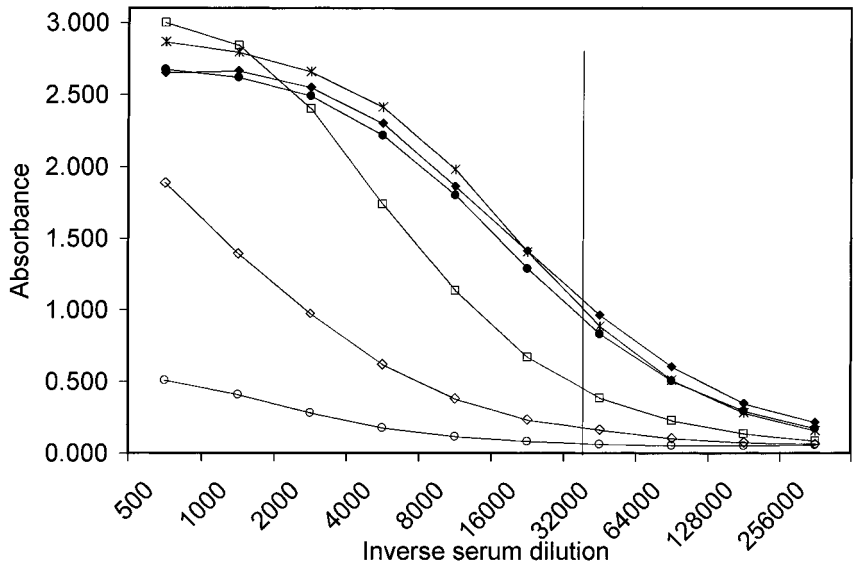


Figure 2. Titration curves for antisera−coating conjugate combinations. Antisera from sheep immunized with LYC−CDI−FET (∗), LYC−SA−FET (□), 14-DAN−SA−FET (◆), and MLA−SA−FET (●), 14-DAN−CDI−FET (○) with their corresponding BSA coatings. Antisera from sheep immunized with LYC−CDI−FET and with the LYC−SA−BSA coating (◊).

Table 1. Analytical Figures of Merit for Assays 1, 2 and 3

	assay 1				assay 2				assay 3			
compound	I_{50} (pg)	slope	LOD (pg)	sensitivity (pg)	I_{50} (pg)	slope	LOD (pg)	sensitivity (pg)	I_{50} (pg)	slope	LOD (pg)	sensitivity (pg)
lycoctonine **(1)**	80.1	3.06	14.3	15.2	*a*	*b*	*b*	*b*	1.24×10^5	*b*	*b*	*b*
deltaline **(2)**	3.57×10^5	*b*	*b*	*b*	*a*	*b*	*b*	*b*	*a*	*b*	*b*	*b*
methyllycaconitine **(3)**	191	4.00	30.5	8.93	7.37×10^3	4.44	409.8	11.9	3.99×10^3	5.30	1.09×10^3	39.8
14-deacetylnudicauline **(4)**	8.19×10^4	*b*	*b*	*b*	3.05×10^4	3.87	217.1	15.9	*a*	*b*	*b*	*b*
bearline **(5)**	1.25×10^5	*b*	*b*	*b*	1.09×10^5	3.20	581.6	12.1	*a*	*b*	*b*	*b*

[a] The I_{50} of the alkaloid is not reported if the maximum amount tested was not ≥ 50% inhibition of the maximum absorbance. [b] The slope, LOD, and sensitivity of the alkaloid are not reported when less than 3 replicates were performed.

with 250 ng/well of corresponding BSA conjugates used as well coatings. All sheep injected with conjugates produced antibodies. The sera from the sheep that resulted in the highest titers for each conjugate was selected for further ELISA development. Figure 2 shows the titration curves for six different antibody−coating conjugate combinations. Antisera from sheep immunized with LYC−CDI−FET, 14-DAN−SA−FET, and MLA−SA−FET produced the highest titers with optical densities at 450 nm (OD_{450}s) of ∼1.0 at dilutions of 1:32 000 with their corresponding BSA coatings. Antisera from sheep injected with 14-DAN−CDI−FET resulted in low titer values when run with its corresponding BSA coating. A titer curve for this sera was also run with the 14-DAN−SA−BSA coating to determine if the antibodies produced against 14-DAN−CDI−FET or if the 14-DAN−CDI−BSA coating conjugate were inferior. The antisera raised against 14-DAN−CDI−FET had increased titers when run with the 14-DAN−SA−BSA coating, however, the titers were not as high as the antisera raised against 14-DAN−SA−FET and run against its corresponding BSA coating. Sera from sheep injected with LYC−SA−FET resulted in an intermediate titer curve when run against the LYC−SA−BSA coating, but the antibodies produced against LYC−SA−FET did not compete with free lycoctonine.

Three independent competitive inhibition ELISA assays were developed with the antisera raised against LYC−CDI−FET (Assay 1), 14-DAN−SA−FET (Assay 2), and MLA−SA−FET (Assay 3) with their corresponding BSA conjugates used as coating conjugates. The optimum dilutions for both the coating conjugates and antiserum were determined by checkerboard assays. Coating conjugates and antiserum dilutions giving OD_{450} values of between 0.9 and 1.3 in the absence of free norditerpenoid alkaloids were selected.

Cross-Reactivity. Five norditerpenoid alkaloids were selected for cross-reactivity studies: lycoctonine **(1)**, deltaline **(2)**, methyllycaconitine **(3)**, 14-deacetylnudicauline **(4)**, and bearline **(5)**. These alkaloids were tested for cross-reactivity over the range of 1.95−1.024 $\times 10^6$ pg/well for each assay. Within each assay, data for each alkaloid were fit to the four parameter equation

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d \qquad (1)$$

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 alkaloid concentration required to reduce the maximum OD values to 50%), the limit of detection, 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-ELISA assays (Roseman et al., 1992). However, we chose to report the absolute I_{50} values as calculated from the equation of the curve. The limit of detection is defined in this study as the

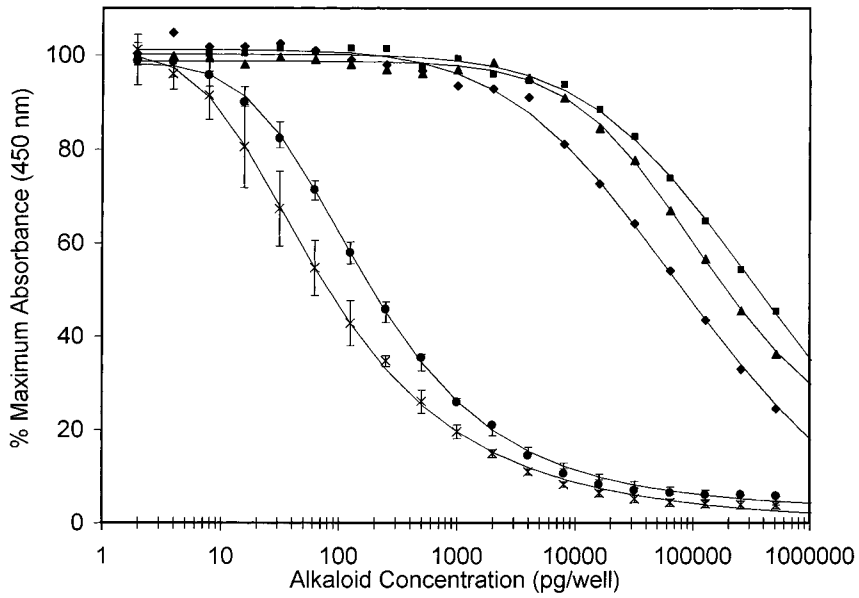


Figure 3. Inhibition curves for Assay 1. Lycoctonine (×), deltaline (■), methyllycaconitine (●), 14-deacetylnudicauline (◆), and bearline (▲). For lycoctonine and methyllycaconitine each point is the average of 8 and 6 replicates, respectively. The 14-deacetylnudicauline, bearline, and deltaline points are the average of 2 replicates.

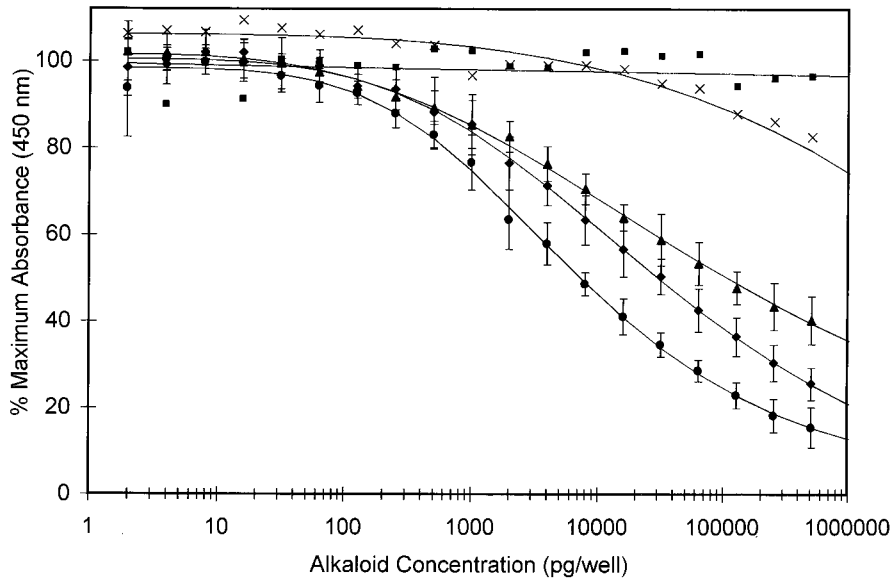


Figure 4. Inhibition curves for Assay 2. Lycoctonine (×), deltaline (■), methyllycaconitine (●), 14-deacetylnudicauline (◆), and bearline (▲). For 14-deacetylnudicauline, methyllycaconitine, and bearline each point is the average of 9, 8, and 6 replicates, respectively. The lycoctonine and deltaline points are the average of 2 replicates.

lowest concentration of free alkaloid with an absorbance reading less than the blank by three times the standard deviation the of the blank. 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 LYC−CDI−FET immunogen with LYC−CDI−BSA coating conjugate resulted in an optimum dilution of LYC−CDI−FET antisera of 1:12 800 with the LYC−CDI−BSA coating conjugate at 250 ng/well for a competitive inhibition ELISA assay. Inhibition curves for lycoctonine, deltaline, methyllycaconitine, 14-deacetylnudicauline, and bearline are shown in Figure 3.

The inhibition curves in Figure 3 and the I_{50} values reported in Table 1 show that methyllycaconitine and lycoctonine cross-react and are both recognized by the antibodies raised against the LYC−CDI−FET immunogen. Figure 3 and Table 1 also show the antibodies in this assay have reduced affinities to deltaline,14-deacetylnudicauline, and bearline. From these data we conclude that the norditerpenoid backbone structure of lycoctonine is the most likely primary antigenic site for these antibodies. As mentioned previously the only difference between methyllycaconitine and 14-deacetylnudicauline is that 14-deacetylnudicauline has a hydroxyl group at the C-14 carbon where methyllycaconitine has a methoxy group (see Figure 1). The decreased affinity of the antibodies to 14-deacetylnudicauline as compared to methyllycaconitine shows the discriminating selectivity of the antibodies.

The limits of detection (LOD) for lycoctonine and methyllycaconitine are 14.3 pg and 30.5 pg, repectively, in this assay. These limits of detection are 1−2 orders

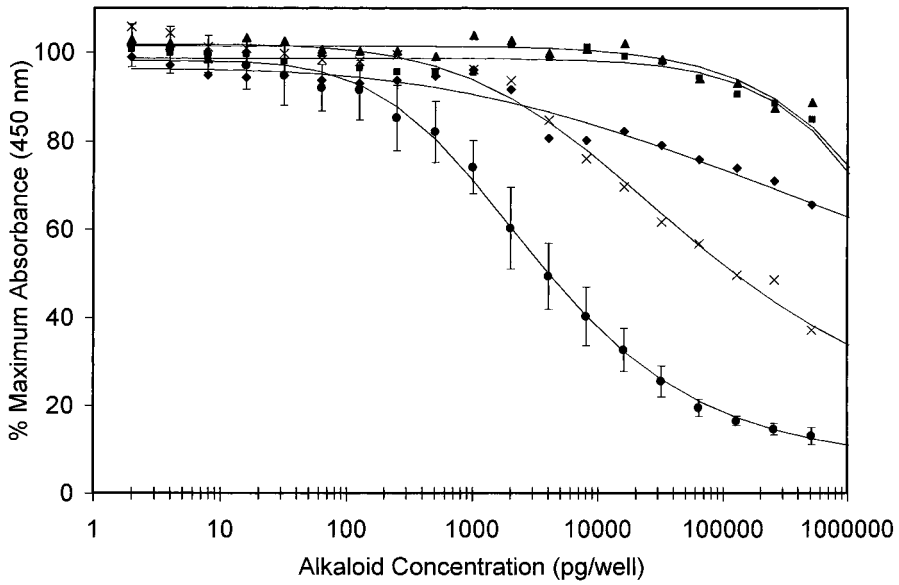


Figure 5. Inhibition curves for Assay 3. Lycoctonine (×), deltaline (■), methyllycaconitine (●), 14-deacetylnudicauline (◆), and bearline (▲). For methyllycaconitine and 14-deacetylnudicauline each point is the average of 12 and 6 replicates, respectively. The lycocotonine, deltaline, and bearline points are the average of 2 replicates.

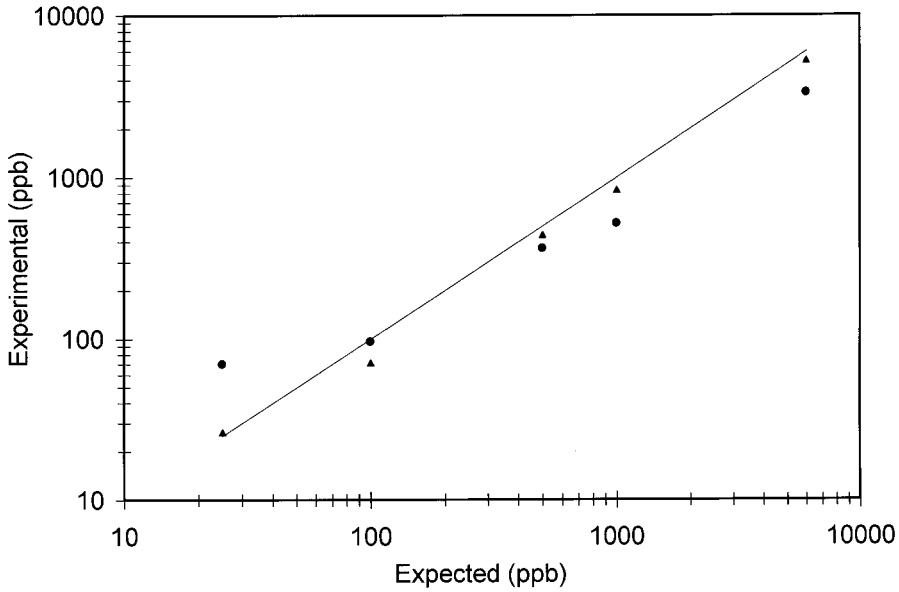


Figure 6. Correlation between spiked levels of methyllycaconitine and the level detected by Assay 1 for blood (▲) and brain tissue (●).

of magnitude lower than the limits of detection for the same or similar alkaloids in Assays 2 and 3 (Table 1) and compare favorably with those obtained using traditional analytical techniques. Reported limits of detection are 25 μg for the FT-IR method (Gardner et al., 1997), 100 pg for flow injection electrospray ionization mass spectrometry (Gardner et al., 1999), and 30 ng and 15 pg for high performance liquid chomatography with UV−Vis (Manners and Pfister, 1993) and electrochemical detection (Turek et al., 1995), respectively.

Assay 2. The optimum dilution of 14-DAN−SA−FET antisera and 14-DAN−SA−BSA coating conjugate was determined to be 1:20 000 and 100 ng/well.

On the basis of the inhibition curves in Figure 4 and the I_{50} values reported in Table 1, methyllycaconitine, 14-deacetylnudicauline, and bearline cross-react and are recognized by the antibodies raised against the 14-DAN−SA−FET immunogen. Figure 4 and Table 1 also show the antibodies in this assay have reduced affinities to lycoctonine and deltaline. From these data we conclude that the primary antigenic site for these antibodies is the common *N*-(methylsuccinimido)anthranilic ester group at the C-18 of these alkaloids. 14-Deacetylnudicauline is conjugated to the fetuin injection protein through the C-14 hydroxyl group on the norditerpenoid backbone of the alkaloid (see Figure 1). Three-dimensional models show this conjugation leaves the *N*-(methylsuccinimido)anthranilic ester group of 14-deacetylnudicauline exposed far from the C-14 site through which the carrier protein is linked. This conjugation strategy allows the *N*-(methylsuccinimido)anthranilic ester group to promote an immunologic response while the norditerpenoid backbone of the molecule appears to be shielded and is not recognized immunologically. These results show that it is possible to develop a class-specific CI-ELISA assay based on the antigenic recognition of the *N*-(methylsuccinimido)anthranilic ester portion of the alkaloid.

The limits of detection (LOD) for methyllycaconitine, 14-deacetylnudicauline, and bearline are 409.8 pg, 217.1 pg, and 581.6 pg, respectively, in this assay. The limit of detection for methyllycaconitine is about an order of magnitude higher in this assay than in Assay 1 (Table 1).

Assay 3. The optimum dilution of MLA−SA−FET antisera and MLA−SA−BSA coating conjugate was determined to be1:10 800 and 75 ng/well for this assay.

The inhibition curves in Figure 5 and the I_{50} values reported in Table 1 show antibody specificity for methyllycaconitine with some cross-reactivity with lycoctonine. The antibodies have reduced affinities to 14-deacetylnudicauline, bearline, and deltaline. These data suggest the antibodies are specific to methyllycaconitine, however, the cross-reactivity to lycoctonine also indicates the common lycoctonine norditerpenoid structure is one of the antigenic sites. The selectivity shown in this assay also reveals the effect of conjugation site. The MLA−SA−FET immunogen is linked through the C-7 or C-8 carbon of methyllycaconitine. Conjugation through the C-7 and C-8 carbons on the norditerpenoid structure leave the functional groups on the C-14, C-16, and C-10 exposed for immunologic recognition. The structural differences of 14-deacetylnudicauline, bearline, and deltaline from methyllycaconitine at the C-14 and C-16 and C-10 positions, respectively, are recognized and discriminated against by the antibodies generated against the MLA−SA−FET conjugate.

Determination of Methyllycaconitine in Bovine Blood and Brain Tissue. Assay 1 has the lowest limit of detection and is the most sensitive of the three assays described in this study. This assay was used to demonstrate the applicability of the developed ELISA for the detection and quantification of methyllycaconitine in bovine blood and brain tissue. Samples were quantified against a 10-point standard curve over the range of 15.6 pg to 8000 pg. The standard curve was determined by fitting the standards to eq 1. The r^2 values for the standard curves were >0.9962 in all cases. Figure 6 shows the spike and recovery results for five different levels of methyllycaconitine in blood and brain tissue. The lowest spike (25 ppb) is just below the limit of detection (30 pg/well) for this assay. Average recoveries for the 100, 500, 1000, and 6000 ppb methyllycaconitine in blood ranged from 71 to 89% and relative standard deviations were 26−49%. Average recoveries for the 100, 500, 1000, and 6000 ppb methyllycaconitine in brain tissue ranged from 52 to 96% and relative standard deviations were 16−71%.

Three competitive inhibition enzyme-linked immunosorbent assays (CI-ELISA) for toxic larkspur alkaloids were developed. Assay 1 was used for the detection and quantification of methyllycaconitine spiked into bovine blood and brain tissue. Assay 2 was class specific for the *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL) alkaloids. A class-specific assay for these alkaloids is of particular interest because the MSAL alkaloids are associated with the majority of larkspur poisoning. The limits of detection of all three assays were excellent and compare favorably with traditional analytical techniques. In addition, competitive inhibition enzyme-linked immunosorbent assays offer the advantages of minimal sample preparation and the ability to run many samples simultaneously. The ability to rapidly screen numerous biological samples for the presence and level of larkspur toxins will be beneficial for diagnosis of animal poisoning, pharmacological studies, and identification of toxic plants.

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