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Isolation and HPLC Determination of Methyllycaconitine in a Species of Low Larkspur (*Delphinium nuttallianum*)

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The neurotoxic diterpenoid alkaloid methyllycaconitine was isolated from *Delphinium nuttallianum*, and a method was developed utilizing ion-pair HPLC to determine its concentration in freeze-dried plant samples.

Larkspurs are responsible for severe livestock losses on North American rangeland (Cronin and Nielsen, 1978). One of several low-growing larkspurs in Western Canada and the United States, *Delphinium nuttallianum* Pritz., occurs from southwestern British Columbia to northern California and eastward to Colorado (Hitchcock and Cronquist, 1964). It is widely distributed on rangelands in the interior of British Columbia, where its habitat varies from sagebrush desert to upper grassland. Appearing early in spring, often before most of the grasses, *D. nuttallianum* can be palatable but poisonous to cattle (Looman et al., 1985). Methyllycaconitine was recently established as the toxic principle in a related species of low larkspur, *Delphinium bicolor* Nutt., which occurs in southeastern British Columbia and Alberta (Kulanthaivel et al., 1986). This alkaloid is a potent neuromuscular blocking agent (Benn and Jacyno, 1983; Nation et al., 1982), and recently it was shown to be a naturally occurring insecticide (Jennings et al., 1986). Since clinical signs of poisoning are similar with both species of *Delphinium*, we suspected that similar alkaloids could also be present in *D. nuttallianum* and accordingly undertook an investigation for such compounds. The absence of information on the quantitative determination of *Delphinium* alkaloids prompted us to develop an efficient method for their extraction and analysis by HPLC. The method was then applied to field samples of *D. nuttallianum*, some of which were collected from rangeland sites where cattle had been poisoned by low larkspur. Heretofore, larkspur toxicity has been mainly estimated by rat or mouse bioassays (Olsen, 1977, 1983).

EXPERIMENTAL SECTION

Plant Material. Aerial portions of *D. nuttallianum* were collected during May 1985, from the Research Station in Kamloops, BC, and from seven rangeland sites located within a 20-km radius of Kamloops. Voucher specimens were deposited in the herbarium at the Provincial Museum in Victoria, BC. The fresh material was frozen, freeze-dried, and ground to pass a 2-mm screen.

Large-Scale Isolation of Methyllycaconitine. Freeze-dried *D. nuttallianum* (960 g) was extracted by repeated maceration (Waring Blendor) in 95% ethanol (3 × 4 L). The extracts were concentrated to a dark green gum that was partitioned between 0.1 M aqueous H_2SO_4 (100 mL) and $CHCl_3$ (300 mL). The $CHCl_3$ extract was then extracted with more 0.1 M H_2SO_4 (3 × 100 mL), and the combined aqueous layers were back-washed with $CHCl_3$ (2 × 100 mL). After addition of ice, the aqueous solution was brought to pH 4.5–5.0 (external indicator paper) with saturated aqueous Na_2CO_3 and extracted with $CHCl_3$ (3 × 100 mL). This extraction was repeated at pH 8.0–8.5 and 11 (each time with 3 × 100 mL of $CHCl_3$). The $CHCl_3$ extracts were combined, dried ($MgSO_4$), and evaporated under reduced pressure to yield the crude bases. These were then redissolved in $CHCl_3$ and extracted into 0.2 M aqueous H_2SO_4 (3 × 50 mL). The combined aqueous extracts were back-washed with $CHCl_3$, basified, as before, to pH 5.0, 8.0, and 11 with Na_2CO_3, and extracted at each of these points with $CHCl_3$ (3 × 50 mL). Removal of solvents from these extracts yielded the alkaloids as off-white foams (1.0, 1.2, and 0.1 g, respectively; i.e., 2.3 g or about 0.2% of the dry weight of the plant). TLC analysis (silica gel 60; $CHCl_3$–methanol, 5:1 or 8:1, v/v) suggested that the pH 5 and 8 fractions were very similar with a single major component while the pH 11 fraction yielded more polar alkaloids.

A portion of the pH 5 mixed bases (100 mg) was fractionated by centrifugally accelerated radial TLC (Chromatotron) (1-mm silica gel 60 F254), the plate being developed with $CHCl_3$ and then $CHCl_3$–methanol (8:1). A UV-absorbing band was rapidly eluted and one fraction (40 mg) appeared to be homogeneous by TLC (system as before). The ^1H and ^{13}C NMR spectra of this material were in accord with this being methyllycaconitine (Figure 1); i.e. it was in agreement with the spectra reported for authentic specimens of this alkaloid (Pelletier et al., 1984). The MS (DCI-probe, MS-80) revealed a highest mass ion at m/z 682 as required for this alkaloid.

Quantitative Determination. A freeze-dried and ground sample of *D. nuttallianum* (1 g) was extracted with

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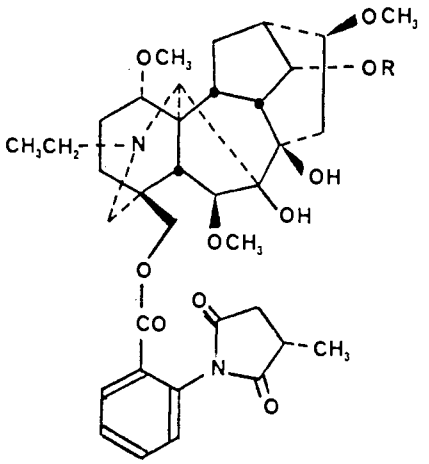

Figure 1. Structures of the diterpenoid alkaloids methyllycaconitine (R = methyl) and nudicauline (R = acetate) in *D. nuttallianum*.

methanol (200 mL) for 1 h on an orbital shaker and the extraction then continued without shaking at room temperature for approximately 16 h. The extract was filtered on a Buchner funnel, the residue was washed with an additional 100 mL of methanol, and the filtrate was concentrated to dryness in a rotary evaporator (40 °C). With the aid of glass beads, this was redissolved in 0.4 N H_2SO_4 (25 mL), and after transfer, the flask was rinsed with H_2O (25 mL). The combined aqueous extract (50 mL) was washed with $CHCl_3$ (2 × 50 mL), and the $CHCl_3$ phase was back-washed with 0.4 N H_2SO_4 (25 mL). A frothy suspension may appear at the interface, but this will collapse on standing. The acid fractions were combined and adjusted to pH 7.5 (internal electrode) with 5 and 0.1 N NaOH. This was extracted vigorously with glass-distilled $CHCl_3$ (3 × 100 mL), and the $CHCl_3$ extract was evaporated to dryness and stored at −20 °C. Before HPLC analysis, the mixture of crude bases was redissolved in 8 mL of the HPLC mobile phase: 4 mM sodium hexanesulfonate in aqueous orthophosphoric acid (0.1%)–acetonitrile (8:92).

A Varian Model 5060 liquid chromatograph equipped with a UV-100 variable-wavelength detector set at 210 nm was used with a Varian 4270 integrator. Separation was achieved with use of a Varian prepacked, reversed-phase Micropak MCH-5 (monomeric, octadecylsilane on 5-μm silica) column (30 cm × 4 mm) protected by an MCH-10 guard column. Methyllycaconitine was eluted from the column isocratically with the aforementioned solvent system. A flow rate of 0.4–0.6 mL/min was used to give a pressure of 35–50 atm. The alkaloid was quantified by the external standard method with duplicate injections per plant sample, and a 20-μL sample loop was used with each injection.

RESULTS AND DISCUSSION

Whole plants were collected at the early- to full-blossom stages of growth. Conventional extraction and processing of freeze-dried *D. nuttalianum* yielded a mixture of alkaloids that was extracted from aqueous solution with $CHCl_3$. Gravimetric determination of the isolates from preparative TLC indicated that a single alkaloid accounted for about 70% of the weight of the crude bases. Spectroscopic and chromatographic investigations (see the Experimental Section) revealed the major alkaloid as methyllycaconitine (Figure 1) and minor diterpenoid alkaloids as the closely related nudicauline (Kulanthaivel and Benn, 1985; Benn and Okanga, 1986) and hetisine 13-*O*-acetate (Benn et al., 1986). The poisonous properties

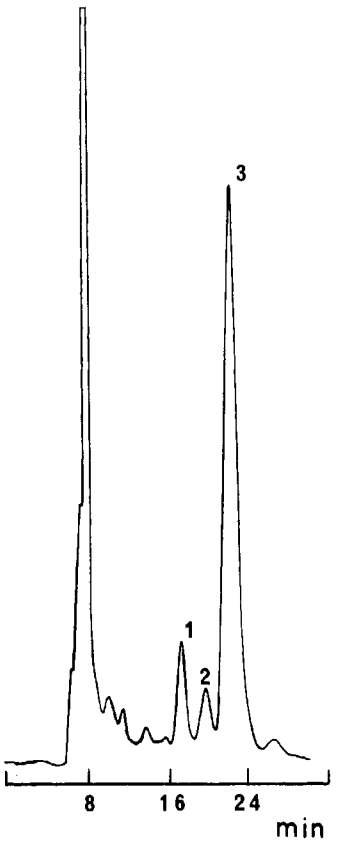

Figure 2. HPLC separation of nudicauline (1), an unknown (2) and methyllycaconitine (3) from a purified extract of freeze-dried *D. nuttallianum* (1 g). Column: Micropak MCH-5 (30 cm × 4 mm). Detection: 210 nm. Mobile phase: 4 mM sodium hexanesulfonate in aqueous orthophosphoric acid (0.1%)–acetonitrile (8:92).

of three species of *Delphinium* [including *Delphinium brownii* (Aiyar et al., 1979), a tall larkspur] that occur in Western Canada can now be largely attributed to methyllycaconitine.

The determination of methyllycaconitine by HPLC was first attempted by using the ion-pair method of Hikino et al. (1981), who resolved *Aconitum* alkaloids on an ODS chemically bonded silica gel column. Their isocratic mobile phase consisted of 0.01 m sodium hexanesulfonate in 0.05 M phosphate buffer (pH 2.7)–tetrahydrofuran (85:15). On a Varian reversed-phase MCH-5 column this system initially yielded sharp symmetrical peaks for aconitine and methyllycaconitine. However, we wanted to avoid the use of tetrahydrofuran, which can cause corrosion of seals and bushings in HPLC pumps.

The mobile system that eventually resolved the diterpenoid alkaloids of *D. nuttallianum* consisted of 4 mM sodium hexanesulfonate in a solution of aqueous phosphoric acid (0.1%)–acetonitrile (8:92). Failure to include the salt resulted in substantial peak broadening and decreased sensitivity. The detection limit for pure methyllycaconitine was <10 ppm. Figure 2 shows a typical separation of methyllycaconitine and nudicauline, whose identities in plant extracts were confirmed by spiking with authentic standards. The extraction protocol that we developed for quantitative analysis proved to be efficient as evidenced by recoveries of 92–97% for methyllycaconitine when the alkaloid (2 mg) was added to methanolic extracts of freeze-dried *D. nuttallianum* (1 g). In agreement with the results from the large-scale isolation, the alkaloid showed a broad optimum pH (4–8) for its extraction with $CHCl_3$ from basified aqueous extracts. A

Table I. Methyllycaconitine (M) and Nudicauline (N) Contents (% Dry Matter) in *D. nuttallianum* Samples Collected Near Kamloops, BC, during 1985

	May 3		May 17		May 31	
site no.	M	N	M	N	M	N
1	0.12	0.04	0.12	0.03	0.11	0.02
2			0.20	0.03	0.13	0.02
3			0.19	0.04	0.21	0.05
4					0.16	0.02
5	0.19	0.05	0.13	0.03		
6	0.20	0.03	0.13	0.01	0.06	0.02
7					0.16	0.04
8	0.15	0.03	0.15	0.02		

pH of 7.5 was chosen for extraction with $CHCl_3$. This minimized overshooting during pH adjustment and prevented the possible alkaline hydrolysis of the ester bond. Replicate extractions of subsamples of *D. nuttallianum* were in good agreement as shown by the methyllycaconitine determinations, which differed by $<\pm 8\%$ (n = 5). It was also possible to use aconitine (Sigma) as an internal HPLC standard with the following mobile phase: 6 mM sodium hexanesulfonate in a solution of aqueous phosphoric acid (0.1%)–acetonitrile–methanol (12:83:5).

Table I lists the concentrations of methyllcaconitine and nudicauline in *D. nuttallianum* at eight experimental sites during May 1985. For methyllycaconitine the average concentration was 0.15% with a range of 0.06–0.21%. Nudicauline (Figure 1) was always a minor component in these samples with an estimated concentration range of 0.01–0.05%. Sites 5 and 7, where livestock poisonings are known to have occurred, did not reveal exceptional levels of methyllycaconitine. However, a combined estimate of 0.24% was obtained for both alkaloids at site 5 on May 3. The toxicity of nudicauline has not been determined, but it contains the (methylsuccinimido)anthranoyl function that is essential for neuromuscular activity (Aiyar et al., 1979), and it only differs from methyllycaconitine at C_{14} where the latter has a methoxy group while nudicauline has an acetate function (Kulanthaivel and Benn, 1985). Studies are in progress to more fully elucidate the alkaloid levels at earlier stages of growth when the larkspur is more palatable to cattle.

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Registry No. Methyllycaconitine, 21019-30-7; nudicauline, 99815-83-5.

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