Determination of Zygacine in *Zigadenus venenosus* (Death Camas) by Image Analysis on Thin Layer Chromatography

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A new method, utilizing image analysis on TLC, was developed for the determination of zygacine, the neurotoxic steroidal alkaloid in death camas (*Zigadenus venenosus*). The quantitative detection level of zygacine on TLC was 1 μg. Diurnal changes in zygacine levels were not evident, nor were there differences in zygacine levels in death camas growing at high- or low-elevation habitats. The image analysis method was also applied to the determination of vanillylveracevine in *Zigadenus elegans*.

Keywords: *Zigadenus venenosus; death camas; steroid alkaloids; zygacine; image analysis*

INTRODUCTION

The genus *Zigadenus* (Liliaceae) in the Pacific Northwest is currently considered to be comprised of three species: *Z. elegans* Pursh., *Z. paniculatus* (Nutt.) Wats., and *Z. venenosus* Wats., which is further split into two varieties, *venenosus* and *gramineus* (Hitchcock and Cronquist, 1990). Elegant death camas (*Z. elegans*) is considered to be the least toxic of the species, whereas meadow death camas [*Z. venenosus* (*Z. gramineus*)] is the most toxic to livestock, especially sheep (Kingsbury, 1964; Collett et al., 1996). Human poisoning by *Zigadenus* species has also been reported (Spoerke and Spoerke, 1979; Wagstaff and Case, 1987).

The toxicity of *Zigadenus* species has been attributed to a group of zygadenine esters (Figure 1) that are steroid alkaloids with chemical and pharmacological properties resembling those of veratridine, a hypotensive agent (Kupchan and Deliwala, 1953). The esters include the acetyl (Figure 1), angelyl, veratryl, and vanillyl derivatives of zygadenine (Kupchan, 1961).

The quantitative analysis of veratridine (the veratryl ester of veracevine) and cevadine (the angelyl ester of veracevine) from *Schoenocaulon officinale* by high-performance liquid chromatography(HPLC) using ultraviolet (UV) detection was recently reported (Hare, 1996). However, the completely saturated veracevine showed little UV absorption. Similarly, our attempts to measure zygacine by HPLC using UV detection were unsuccessful due to the UV transparency of the acetyl ester. To quantify zygacine in *Z. venenosus*, we developed a thin layer chromatography (TLC) scanning method that measured zygacine fluorescence after treatment of silica gel TLC plates with methanolic sulfuric acid (Majak et al., 1992), a common spray reagent for visualizing the color or fluorescence of steroids on TLC (Stahl, 1969). The color is formed after the TLC plate is heated, and then there is an inherent decline in the intensity of the color of the spot. This decline can be significant during the scanning time of the plate. To circumvent the problem, image analysis of TLC was attempted to obtain an instantaneous record of the acid-treated plate with a video camera. The TLC spots could then be quantified with an interfaced computer. The image analysis method was used to examine diurnal changes in zygacine levels in *Z. venenosus* var. *gramineus*. Zygacine levels were also compared in plants collected from subalpine areas and grassland sites, which represent the higher and lower range of the plant in the interior of British Columbia. The method was also used to determine vanillylveracevine levels in *Z. elegans*.

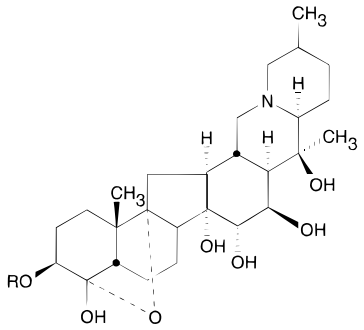

Figure 1. Structure of zygadenine (R = H) and zygacine (R = acetyl).

EXPERIMENTAL PROCEDURES

Plant Material. Plant samples (aerial portions) of *Z. venenosus* var. *gramineus* were collected during 1992−1994 from three upper grassland sites (n = 18) and four subalpine sites (n = 19) near Kamloops, BC. The elevations at the subalpine sites were 400−800 m higher than at the grassland sites (Majak, 1993). Composite samples (50−200 g fresh weight) at the vegetative, bud, bloom, and pod stages of growth were frozen, freeze-dried, and ground to pass through a 2 mm screen. Each sample (1 g) was extracted for free bases as described previously (Majak et al., 1987, 1992), and the final volume of the extract was adjusted to 1.5−2.0 mL with chloroform. Diurnal levels of zygacine in death camas were examined at two upper grassland sites near Kamloops, BC, during June 10 and 11, 1996.

TLC Development. The zygacine standard, 50 mg, isolated from death camas as described previously (Majak et al., 1992) was dissolved in a minimum volume of methanol, and the volume was adjusted with acetonitrile to afford a 5 mg/mL stock solution. Storage in acetonitrile minimized the hydrolysis of zygacine to zygadenine. Standards for TLC, 1−5 mg/mL, were prepared from the stock, and these were applied to the silica gel plate (EM Science, silica gel 60, No. 5721-7, 20

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× 20 cm) with 1 μL micropipets (Drumond Microcaps) as were the samples. Each sample application was replicated on a second plate as were the set of standards, and the duplicate plates were developed in chloroform/methanol/aqueous 35% NH_4OH, 90:10:1. After drying, the plates were sprayed with 5% sulfuric acid in methanol. It was necessary to spray the plate three or four times to completely saturate the spots with acid and thus to obtain a uniform red spot after the plate was heated at 85−100 °C for 20 min in a forced-air convection oven. The efficacy of the multiple spray was deduced from preliminary charring experiments with sucrose on TLC. The charring was complete if the acid was applied three times, yielding an acceptable standard curve for sucrose ($r = 0.99$) in the 2−8 mg/mL range.

Image Analysis. The image analysis system consists of computer, video camera, frame grabber board, and software package. We used an IBM 486 compatible computer equipped with MS DOS 5.0, 64K cache memory, ATI XL VGA Wonder card, a Microsoft compatible mouse, a Tatung video display monitor, and Vision plus AT overlay frame grabber. The camera was a COHU Model 4915-2001 0.5 in. monochrome CDD camera with COSMICAR 16 mm television lens. Software was Image-Pro Plus version 2.0 from Media Cybernetics Inc.

The visible spots on TLC were photographed promptly before they had time to fade, and the image was stored for later analysis. The best lighting conditions for photography were obtained by minimizing direct external light sources and using incandescent lamps powered by variable transformers on either side of the picture board. The incident lighting was adjusted for homogeneity across the plate. To represent each spot as a real value, we used a cone-shaped model (Calter, 1986) to calculate a volume for the TLC spot from the area (number of pixels) and intensity (log inverse gray value) data obtained from the area of interest histogram (Media Cybernetics, 1990; Bioscan, 1991). The volume of each cone section was calculated as follows, and the volumes were summed over all levels of gray.

$$\text{volume (pixel}^3) = (h/3)(A_1 + A_2 + \sqrt{A_1 A_2}) \times \ln(255/l)$$

l is the gray level [1 (black) to 255 (white)]; A_1, A_2 are the horizontal areas of cone section in number of pixels; and h is one gray level increment.

Estimation of Vanillylveracevine in *Z. elegans*. The ester was initially isolated from air-dried aerial portions of *Z. elegans* (137 g) collected near Cochrane, AB, at the bloom stage of growth. The material was extracted with methanol, and free base extracts were prepared at pH 4, 6, and 8 as described previously (Majak et al., 1992). The pH 6 extract was separated by centrifugally accelerated TLC (Chromatotron) on a 2 mm rotor prepared from silica gel 60 PF_{254} (Merck No. 7749) using chloroform/methanol/aqueous 35% NH_4OH, 50: 1.25:0.5, as the eluting solvent (7 × 20 mL). The second and third fractions yielded pure vanillylveracevine (15 mg) as shown by ^1H and ^{13}C NMR spectroscopy (Bruker AM400 spectrometer). The spectra were virtually identical to those reported for veratridine (Agrawal et al., 1991), except for the resonances of the aryl group that showed only one methoxyl function. The isolate was used as the TLC standard for *Z. elegans*, which was then extracted for free bases by the procedure developed for *Z. venenosus* and vanillylveracevine (R_f 0.30) was estimated by image analysis on TLC using the TLC solvent system developed for zygacine (R_f 0.36) determination.

RESULTS AND DISCUSSION

Typically, linear correlation coefficients (r) exceeded 0.99 for zygacine standard curves (1, 2, 3, 4, and 5 μg) on TLC. A significant variation was detected when three standard curves (3 × 5 spots) were spotted on the left, center, and right side of a plate. To minimize this inherent variation within a plate, standards were spotted across the plate rather than in one area of the plate.

Table 1. Diurnal Levels of Zygacine in Death Camas

site	date of collection	time of collection	zygacine levels (%) plate 1	plate 2	av	SD
A	June 10	2:20 p.m.	0.26	0.23	0.25	0.02
		6:35 p.m.	0.37	0.37	0.37	
	June 11	7:15 a.m.	0.31	0.29	0.30	0.01
		11:20 a.m.	0.29	0.23	0.26	0.04
		3:40 p.m.	0.23	0.22	0.23	0.01
B	June 10	2:25 p.m.	0.42	0.44	0.43	0.01
		6:45 p.m.	0.39	0.42	0.41	0.02
	June 11	7:25 a.m.	0.47	0.36	0.42	0.08
		11:15 a.m.	0.41	0.38	0.40	0.02
		3:45 p.m.	0.54	0.51	0.53	0.02

Table 2. Zygacine Levels in Death Camas Growing at Grassland and Subalpine Sites

site	stage of growth	*N*	zygacine levels (%) av	SD
grassland	vegetative	3	0.43	0.03
	bud	3	0.46	0.25
	bloom	5	0.34	0.08
	pod	7	0.39	0.12
subalpine	vegetative	5	0.51	0.11
	bud	3	0.53	0.17
	bloom	8	0.47	0.15
	pod	3	0.32	0.20

It should also be reiterated that duplicate plates (each with a set of standards) were always used for zygacine determination in death camas. When zygacine was added to crude methanolic extracts of death camas, it was recovered in 95−100% yield ($n = 4$).

Diurnal Levels of Zygacine. A significant diurnal fluctuation in zygacine levels in death camas was not evident (Table 1). This contrasts with the marked diurnal fluctuation in the alkaloids of *Lupinus albus* (Wink and Witte, 1984). Increases of 2- and 3-fold during the day were reported for the quinolizidine alkaloid lupanine, and even greater increases were observed during the day with cell suspension cultures of *Lupinus* spp. and other legumes (Wink and Hartmann, 1982). The absence of a diurnal change in zygacine levels in death camas suggests that the biosynthesis of the alkaloid is not light-dependent. The data in Table 1 indicate that the zygacine levels were higher at site B than at site A. The major difference between the sites was the southern exposure at B as compared to the northern exposure at A.

The data in Table 1 indicate that the reproducibility of the TLC method for zygacine determination is acceptable in most cases. A third plate can be used if there is poor agreement between the initial two plates. Table 1 also shows that alkaloid levels at site B were consistently higher than at site A.

Zygacine Levels at Subalpine and Grassland Sites. The results of the survey of zygacine levels at high- and low-elevation sites are shown in Table 2. When the data in Table 2 were subjected to analysis of variance, differences were not detected between sites, among stages of growth, or in the site × stage interaction ($P > 0.2$). The hypothesis that zygacine levels might differ at the extremes of the growing range of death camas was not substantiated. Similarly, methyllycaconitine, the major diterpenoid alkaloid in low larkspur (*Delphinium nuttallianum*), did not show exceptional levels when it was collected at high elevations (Majak, 1993). Low larkspur and death camas often grow together in similar habitats. It is well-

documented that alkaloid levels vary with the stage of growth of the plant (Pfister et al., 1994; Majak, 1993; Majak et al., 1992). In *Delphinium* spp. there is usually a decline in alkaloid levels with advancing stages of growth, and the opposite appears to be true for *Z. venenosus*. These phenological trends are usually established by systematic collections of plants within a site of a climatic zone, such as the upper grassland zone of British Columbia. The absence of a phenological trend in Table 2 could be attributed to the different method of plant collection in the present study. Plants from different sites in different zones were compared each year, and phenological trends could be obscured. This may have also contributed to the considerable variability in zygacine levels in Table 2. The objective was mainly to compare death camas at high- and low-elevation sites. Elevation does not appear to be a factor affecting alkaloid accumulation in death camas.

Other Esters in *Z. venenosus* and Vanillylveracevine in *Z. elegans*. In addition to zygacine, the acetyl ester of zygadenine, death camas also contains the angelyl and veratryl esters of zygadenine, which become prominent constituents at mature stages of death camas growth, particularly when approaching senescence (Majak et al., 1992). These esters are poorly resolved in the present solvent system, but an image analysis estimate of their total level can be made by developing the TLC plate to one-fourth of its length and treating the two esters as one spot. In zygacine equivalents, the ester levels in death camas ranged from 0.24 to 1.14% [mean 0.69% ± 0.28 (SD), $N = 12$] on a dry matter basis. The mean value exceeds the values shown for zygacine (Tables 1 and 2) but it should be reiterated that these esters only become major components at later stages of pod development.

In addition to *Z. venenosus* var. *gramineus*, we also examined a sample of *Z. venenosus* var. *venenosus*. Zygacine was also the major alkaloid in this variety of death camas. Zygacine did not occur in *Z. elegans*, but we identified vanillylveracevine as a major component in this species in addition to other veracevine and zygadenine alkaloid bases (W. Majak, unpublished data). Using vanillylveracevine as a TLC standard, we estimated this alkaloid by image analysis to be present at 0.05% of the dry matter in aerial portions of the plant.

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