Purification and Quantitative Analysis of Veratridine and Cevadine by HPLC

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As part of a study to determine the variation in the effectiveness of commercial pesticide formulations of ester steroidal alkaloids from sabadilla (*Schoenocaulon officinale* Grey) seeds in the integrated pest management of citrus, a method to isolate and measure large quantities of alkaloids from natural starting materials was needed. The addition of the solvent modifier sodium dodecyl sulfate (SDS) to a previously published HPLC method greatly improved peak resolution by minimizing peak tailing. The use of the non-ester alkaloid papaverine as an internal standard permitted quantification of sabadilla alkaloids by HPLC, and this modified method was then used to monitor the breakdown of cevadine and veratridine, the two most abundant sabadilla alkaloids, in aqueous solutions as a function of time and pH.

Keywords: *Veratridine; cevadine; veratrine; HPLC*

INTRODUCTION

Steroidal alkaloids of the veratrum group are found in plants of the family Liliaceae and have a relatively rich pharmacological history. These alkaloids have been used as hypotensive agents, although the natural products now have been superseded by synthetic analogues. However, veratridine, one of the alkaloids of this group, still has a role in physiological investigations on the structure and function of ion channels [e.g., Wang et al. (1990)]. In addition to their role in medicine, crude mixtures of veratrum alkaloids from seeds of "sabadilla," *Schoenocaulon officinale* Grey, have been used since prehistoric times as a natural insecticide (Crosby, 1971). Formulations of sabadilla seeds once again are receiving considerable attention in pest management because of their low toxicity to natural enemies (Bellows et al., 1985; Morse and Bellows, 1986; Bellows and Morse, 1993).

The alkaloid fraction of sabadilla seeds is known to contain a mixture of several compounds. Two esters of veracevine, veratridine and cevadine (Figure 1), are known to comprise more than 90% of the alkaloid fraction of sabadilla (Holan et al., 1984), but relatively little attention has been focused on their separation and quantification. Two reversed phase HPLC methods for the separation of cevadine and veratridine have been published (Holan et al., 1984; Reed et al., 1986), but both methods suffer from poor resolution and excessive peak tailing.

As part of a study to better determine the relative toxicity of these alkaloids to citrus thrips, *Scirtothrips citri* (Moulton) (Thysanoptera: Thripidae), and the range of natural variation in their concentration in commercial sabadilla formulations, a method was needed to isolate relatively large quantities of these alkaloids for bioassay. Because commercial formulations of sabadilla seeds are applied for pest control in aqueous solutions, part of the variation in the effectiveness of commercial sabadilla might be due to partial base hydrolysis of the ester alkaloids in alkaline well waters prior to application. Thus, to test this hypothesis, a method also was needed to monitor the breakdown of the esters to the less toxic alkanolamine in the environment.

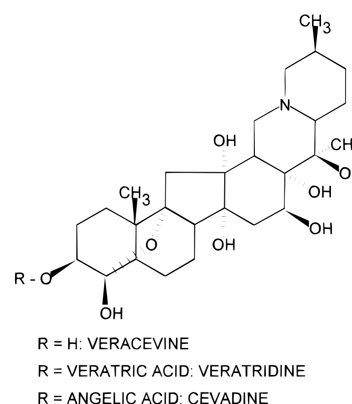

Figure 1. Structures of veratridine and cevadine.

This study reports the improvement of a reversed phase HPLC method to separate and quantify veratridine and cevadine by the addition of a solvent modifier. This general method allowed preparative-scale purification of veratridine and cevadine from both veratrine and sabadilla seeds. In addition, with the inclusion of a non-ester alkaloid as an internal standard, the method also allowed the monitoring of the breakdown of veratridine and cevadine in aqueous solutions over time as a function of pH.

MATERIALS AND METHODS

Sample Preparation. Veratrine and veratridine (Sigma Chemical Co.) were dissolved in methanol (Fisher Optima grade) at 5 mg/mL and analyzed by HPLC without any additional treatment because all material dissolved in the methanol. Alkaloids were extracted from Veratran D (Dunhill Chemical Corp., Rosemead, CA), a formulation of sabadilla seeds, using "classical" procedures for alkaloid extraction (Hartmann, 1991). Briefly, 50 g of Veratran D was extracted in 500 mL of 5% (v:v) acetic acid in water at room temperature with stirring for 1 h. The solution was vacuum-filtered to remove solid debris. The filtrate was basified with 4 N NaOH to pH >10.0, and the alkaloids were extracted with three 500 mL portions of CH_2Cl_2. The CH_2Cl_2 solution was concentrated to dryness by rotary evaporation, and the alkaloids were redissolved in methanol for HPLC analysis or preparative-scale HPLC. Veracevine was prepared from purified veratridine by methanolic base hydrolysis following the procedures of Pelletier and Jacobs (1953).

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Analytical HPLC. Chromatography was carried out with a Beckman Model 332 chromatograph and an ISCO Model V4 variable-wavelength UV detector. Initial HPLC conditions were those of Reed et al. (1986), using various modifications to their isocratic mobile phase of methanol/0.1 M ammonium acetate, pH 5.5 (60:40). The mobile phase was filtered (0.22 μm) and degassed with helium before use. Analytical HPLC was carried out using a 4.6 mm × 250 mm Beckman Ultrasphere C_{18} column, 5 μm particle size, with a flow rate of 1.5 mL/min and effluent monitoring at 245 nm. The injection volume was 20 μL. Eluates were collected with an automated fraction collector. The identities of veracevine, veratridine, and cevadine were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy in $CDCl_3$. Identifications were made on the basis of the presence or absence of observed spectral peaks in each sample compared to previously published spectral assignments (Krishnamurthy and Casida, 1988).

Solvent Modifiers for Improved Separation. The addition of a basic modifier, such as triethylamine, to the aqueous phases of HPLC solvents often improves the separation of basic compounds, such as amines, on silica-based columns. The addition of an ion-pairing agent to the mobile phase also has been reported to improve the separation of ionic compounds such as alkanolamines (Stadalius et al., 1988; Szepesi, 1992). The addition of triethylamine to the mobile phase in concentrations up to 50 mM was investigated, as was the effect of adding sodium dodecyl sulfate (SDS) at 0.5 mM concentration to the aqueous mobile phase. Various modifications to the ionic strength of the buffer and pH were made, but none improved the separation, and they will not be reported in detail. For this particular column, a pH of 4.5 for the ammonium acetate buffer was optimal.

Preparative-Scale HPLC. Preparative-scale separations of veratridine and cevadine from either veratrine or an extract of Veratran D were carried out on a Phenomenex Particil C_8 column, 9.4 mm × 500 mm, 10 μm particle size. The mobile phase was a 70:30 mixture of methanol/0.1 M ammonium acetate in water to which 0.5 mM SDS (see Results and Discussion) was added and the pH of which had been adjusted to 4.5 with acetic acid. The flow rate was 5 mL/min, the injection volume was 500 μL, and the effluent was monitored by UV detection at 245 nm.

Fractions were collected and concentrated by rotary evaporation to remove the methanol, and then the fractions were basified with 4 N NaOH to pH >10.0. The purified alkaloids were then separated from the residual SDS and ammonium acetate by extracting with three 250 mL portions of CH_2Cl_2. The CH_2Cl_2 fractions were reduced in volume by rotary evaporation, and then the fractions were transferred to individual vials and taken to dryness by evaporation under a stream of N_2. The dried alkaloids were stored at −20 °C until needed.

Evaluation of Potential Internal Standards. The nonester alkaloids papaverine and berberine were evaluated for use as internal standards. An appropriate internal standard would be (1) soluble in dilute acid but extractable into organic solvents under basic conditions (as are veratridine and cevadine), (2) chromatographable and detectable under our analytical conditions but have a retention time different from those of veracevine, veratridine, cevadine, or any impurities in sabadilla extracts, and (3) unaffected by prolonged exposure to moderately basic pH. Both papaverine and berberine were extractable and chromatographable under the same conditions used for veratridine and cevadine. Berberine, however, showed a degree of peak tailing that would interfere with the measurement of veratridine and was not investigated further.

Papaverine showed more promise in that its retention time differed from all other compounds of interest (see Results and Discussion). Three replicates of four papaverine standards ranging in concentration from 0.00195 to 0.0313 mg/mL were prepared in CH_3OH. Standards were analyzed by HPLC as described above to determine the linearity of response of papaverine and to develop a calibration curve.

The stability of papaverine under exposure to alkaline conditions was determined as follows. Duplicate 50 mL solutions of 0.0083 mg/mL of papaverine in ammonium acetate (0.1 M, pH 4.5) were prepared. The pH of each was then adjusted to 5.5 with NH_4OH. The two solutions then were divided into six pairs of aliquots of 8 mL each. No pH adjustment was made to the pair of solutions assigned to the pH 5.5 treatment. The pH of the remaining five pairs was brought to 7.0, 8.5, 10.0, 11.5, or 13.0 with either NH_4OH (pH 7−10) or NaOH (pH 11.5 and 13). All samples were kept at a constant temperature of 25 °C. The samples were placed on a shaker and removed 24 h later.

Sample workup was conducted as follows: For the treatments at pH ≤10, the pH was raised to >10 with NH_4OH. Each of the 12 samples was extracted with two 10 mL portions of CH_2Cl_2. The two CH_2Cl_2 extracts per sample were combined and concentrated by rotary evaporation followed by removal of traces of solvent under a stream of N_2. The residue was redissolved in 2 mL of CH_3OH and subjected to HPLC analysis as described above.

The effluent was monitored at 245 nm, and the quantity of papaverine in each sample was determined from the calibration curve (above). HPLC analyses were run in duplicate for each sample. The 24 values (6 pH treatments × 2 replicates per treatment × 2 analysis per replicate) were subjected to analysis of variance to determine if the quantities of papaverine recovered from the samples after 24 h differed significantly from the initial quantity.

Quantification and Analyses of Breakdown of Cevadine and Veratridine. An internal standard calibration table was generated by performing HPLC analysis on three replicate calibration mixtures of papaverine at 0.024 mg/mL and one of four concentrations each of veratridine and cevadine in 5% acetic acid and subjected to all extraction and workup steps described below. The range of concentrations for veratridine was from 0.03 to 1.00 mg/mL, and that for cevadine was from 0.06 to 1.00 mg/mL.

For the breakdown studies, duplicate mixtures of 30 mg of veratridine and cevadine each were dissolved in 5 mL of 5% acetic acid. Fifty-five milliliters of ammonium acetate (0.1 M, pH 4.5) were then added to each mixture. A 1.5 mL portion of a 1 mg/mL solution of papaverine in 5% acetic acid also was added as an internal standard to provide a uniform initial concentration of 0.024 mg/mL of papaverine in all samples. The pH of both mixtures was then adjusted to 5.5 with NH_4OH. Each mixture then was divided into six equal portions, yielding two mixtures for each pH treatment. No pH adjustment was made to the first pair. The pH of the remaining five pairs was brought to 7.0, 8.5, 10.0, 11.5, or 13.0 with either NH_4OH or NaOH as above. This caused the veratridine and cevadine to precipitate and form a cloudy suspension in the pH ≥10 treatments. Each of these six pairs of mixtures was divided into five 2 mL portions, one pair for each sampling time, and placed in a polyethylene centrifuge tube. Care was taken to ensure that all solid material remained in suspension during transfer by vortexing. The samples for time 0 were immediately extracted as described below. The remaining pairs were placed on a shaker and removed 1, 2, 4, or 24 h later. All samples were kept at a constant temperature of 25 °C.

Sample workup was conducted as follows: For the samples at pH ≤10, the pH was raised to >10 with NH_4OH. Alkaloids were then extracted with six 10 mL portions of CH_2Cl_2, and the CH_2Cl_2 extracts per sample were combined and concentrated and then dried under N_2 as described above. The alkaloids were redissolved in 2 mL of CH_3OH and subjected to HPLC analysis. Quantities of veratridine and cevadine recovered were calculated relative to the internal standard, and the percent loss of each alkaloid over time was then calculated as a function of pH. The mean percent recoveries (± standard errors of the mean) of veratridine and cevadine are reported.

RESULTS AND DISCUSSION

Absorbance of UV light in the 240−260 nm region is due to the presence of veratric acid or angelic acid esterified with veracevine. Veracevine itself has com-

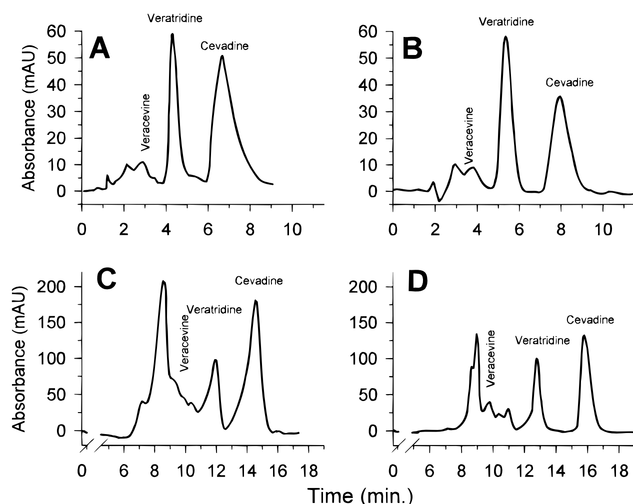

Figure 2. HPLC chromatograms of veratrine alkaloids: (A and B) veratrine without and with a solvent modifer on a 4.6 mm × 250 mm Beckman Ultrasphere C_{18} column, 5 μm particle size, flow rate 1.5 mL/min; (C and D) Veratran D without and with a solvent modifier on a 9.4 mm × 500 mm Phenomenex Particil C_8 column, 10 μm particle size, flow rate 5 mL/min. Eluents: (A) 60% methanol, 40% 0.1 M ammonium acetate, pH 4.5; (B) 60% methanol, 40% 0.1 M ammonium acetate + 0.5 mM SDS, pH 4.5; (C) 70% methanol, 30% 0.1 M ammonium acetate, pH 4.5; (D) 70% methanol, 30% 0.1 M ammonium acetate + 0.5 mM SDS, pH 4.5. Detection for all was by UV at 245 nm.

paratively little UV absorbance in this region. The use of 245 nm for detection followed the methods of Holan et al. (1984) and was a compromise between optimal detectability of all compounds and optimal signal to noise ratio in our system. Refractive index detection has also been used with these compounds (Holan et al., 1984). However, in my study, there was a considerable loss of sensitivity and an increase in the baseline noise when refractive index detection was evaluated in tandem with UV detection. Refractive index detection therefore was deemed to be unsuitable for quantification of veratrum alkaloids in this study.

Solvent Modifiers for Improved Separation. Without any solvent modifiers, cevadine, especially, exhibited substantial peak tailing on both the C_{18} analytical column (Figure 2A) and the C_8 preparative column (Figure 2C). Triethylamine at concentrations up to 50 mM offered no improvement in peak separation or resolution. The addition of SDS at 0.5 mM to the aqueous mobile phase reduced substantially the tailing of cevadine (Figure 2B) and allowed for baseline separation of veratridine from cevadine on the preparative column (Figure 2D). SDS at 0.5 mM therefore was added routinely to the ammonium acetate mobile phase in all subsequent studies.

Evaluation of Potential Internal Standards. The retention time of papaverine fell conveniently between those of veracevine and veratridine (Figure 3). Peak areas of papaverine increased linearly with concentration over the range between 0.00195 and 0.0313 mg/mL (r^2 = 0.996, four concentrations analyzed in triplicate). The limit of detection (three times signal noise) was 0.00025 mg/mL. No significant breakdown of papaverine was observed after exposure to any pH from 5.5 to 13 for 24 h (Table 1).

Quantification and Analyses of Breakdown of Cevadine and Veratridine. The peak area of veratridine increased linearly with concentration between 0.03 and 1.00 mg/mL (r^2 = 0.994), and the peak area of cevadine increased linearly with concentration between 0.06 and 1.00 mg/mL (r^2 = 0.992). Limits of detection (three times signal noise) were 0.0076 mg/mL for veratridine and 0.018 mg/mL for cevadine.

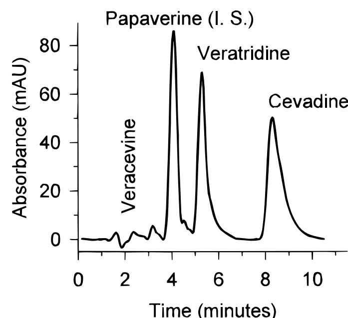

Figure 3. Chromatography of veracevine, veratridine, and cevadine with SDS and with papaverine as an internal standard for quantitation. Conditions were the same as in Figure 2B.

Table 1. Recovery of Papaverine after 24 h at Different pH Levels

pH	recovery of papaverine[a] (%)	pH	recovery of papaverine[a] (%)
5.5	98.6 ± 0.03	10.0	96.9 ± 0.89
7.0	103.8 ± 3.64	11.5	100.7 ± 0.50
8.5	99.9 ± 0.99	13.0	99.0 ± 0.05

[a] Mean ± standard error of the mean for duplicate samples.

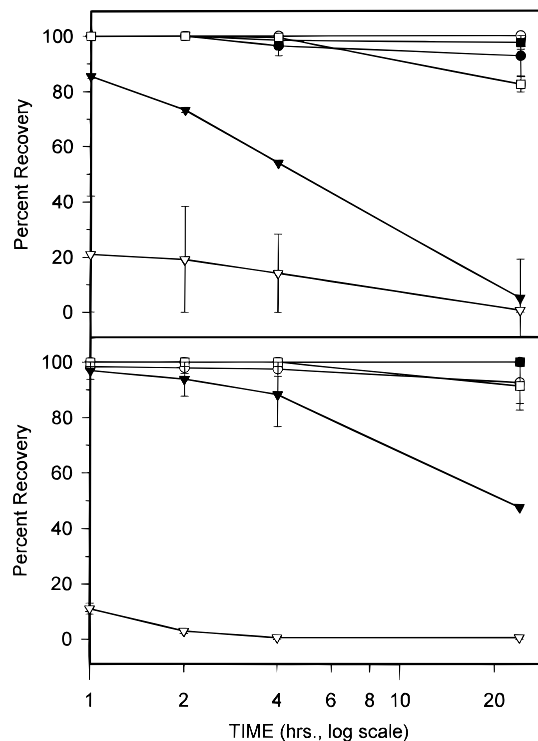

Figure 4. Mean (± standard error) percent recovery of veratridine (top) and cevadine (bottom) in aqueous solutions at 25 °C as a function of pH and time: (●) pH 5.5; (○) pH 7.0; (■) pH 8.5; (□) pH 10.0; (▼) pH 11.5; (▽) pH 13.0.

No significant breakdown of either cevadine or veratridine was observed over 24 h at pH ≤10 (Figure 4). Because it is unlikely either that the water used for pesticide application is as basic as this or that the pesticide is left in the sprayer for more than 1 day, commercial pesticide formulations of sabadilla alkaloids are unlikely to be hydrolyzed prior to application. Higher, albeit environmentally unrealistic, pH values were included in this experiment simply to better

understand the sensitivity of cevadine and veratridine to hydrolysis under basic conditions. At a pH of 11.5, 45.9% of the veratridine was lost after 4 h but 47.6% of the cevadine remained after 24 h. Both alkaloids were 80−90% degraded within 1 h at pH 13 and completely degraded after 24 h. Thus, while both veratridine and cevadine can be degraded by high pH, such high pH values are unlikely to be encountered during the process of mixing and applying commercial sabadilla pesticide formulations to crops. This HPLC method also may provide the basis for techniques to monitor the breakdown of veratridine and cevadine to their relatively less toxic precursors (Allen et al.; 1945, Ikawa et al., 1945; Bergmann et al., 1958) in the environment after application.

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