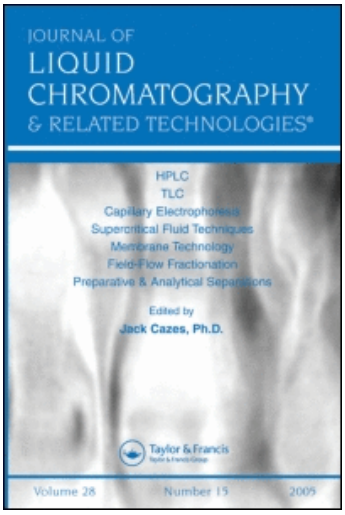

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ISOLATION OF ALKALOIDS FROM GOLDENSEAL (*HYDRASTIS CANADENSIS* RHIZOMES) USING pH-ZONE REFINING COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Goldenseal (the rhizomes of *Hydrastis canadensis*) has a long history of use in North American folk medicine and today is one of the top-selling herbal dietary supplements in the United States. The alkaloids present in the plant have been shown to be responsible for a broad range of biological activities, and the purpose of this work was to isolate preparative quantities of alkaloids present in Goldenseal for subsequent biological evaluation. Berberine chloride, canadaline, canadine, β-hydrastine, and isocorypalmine were separated from a methanolic extract of Goldenseal by a combination of solvent/solvent partition, pH-zone refining countercurrent chromatographic, and recrystallization techniques.

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INTRODUCTION

Goldenseal, the rhizomes of *Hydrastis canadensis* L. (Ranunculaceae), has been used extensively in North American folk medicine from very early times. It received widespread use by Native Americans and was quickly incorporated into the pharmacopoeia of early American pioneers.(1,2) Goldenseal is most commonly used as an antimicrobial agent, astringent, and as an immunomodifier. (3-5)

Among the 14 secondary metabolites known from this plant (12 alkaloids and two quinic acid esters),(6-8) the benzylisoquinoline alkaloids berberine (**1**) and β-hydrastine (**4**) are the most abundant bioactive constituents. Berberine (present at 1-6% w/w) possesses a broad spectrum of antimicrobial activity and berberine-containing plants have been used traditionally worldwide in the treatment of diarrhea and dysentery, and as both a treatment and prophylactic for gastrointestinal disturbances. β-Hydrastine (2-5% w/w) was formerly used as an astringent, vasoconstrictor, and uterine stimulant. Canadine (**3**, 0.1-1% w/w) has been reported to act as a specific dopamine receptor antagonist,(9) but its pharmacological contribution as a component of Goldenseal has not been thoroughly investigated. The remaining alkaloids are present in trace quantities and little biological data are available for them.(10,11)

As part of an effort to evaluate biological activities of major and minor components in this traditional herbal remedy, in particular relating to its current use in oral hygiene products, pH-zone refining countercurrent chromatography(12) was chosen to isolate preparative quantities of pure compounds from Goldenseal for further biological evaluation.

EXPERIMENTAL

Apparatus

An Ito Multilayer Separatory Extractor (Model #1, P.C., Inc., Potomac, MD, USA) containing a 160 m, 1.6 mm I.D. teflon column (total volume 380 mL) was used in this investigation. The apparatus was equipped with a Waters 6000A solvent pump, a Shimadzu SPD-6AV UV detector, and a Gilson FC205 fraction collector. Samples were introduced via a Rheodyne 7725 injector equipped with a 5 mL sample loop. Analog data were processed with a D1000 analog/digital converter from DGH Corp. (Manchester, NH, USA) and UV chromatograms were recorded on a Gateway 286-DX computer. The speed of revolution of the apparatus was maintained at 50% maximum speed (approximately 800 rpm) for all experiments. NMR spectra were recorded on a Bruker DPX-300 NMR spectrometer operated at 300 MHz and 75 MHz for ^{1}H and ^{13}C spectra,

respectively. Mass spectra were recorded with a Micromass Q-TOF-2 mass spectrometer.

Reagents and Other Materials

Petroleum ether, ethyl acetate, methanol, hydrocholoric acid, tartaric acid, and triethylamine (TEA) were all ACS grade from Fisher Scientific Co., Pittsburgh, PA, USA. Chloroform was reagent grade from Tab Chemical Co., Chicago, IL, USA. All non-aqueous solvents were distilled before use with the CCC apparatus. Deionized water was passed through a Nanopure ultrapure water system (Barnstead-Thermoline; Dubuque, IA, USA) prior to use. Silica gel Si F254 aluminum backed plates were used for TLC, and iodoplatinate and Dragendorff reagents were used to detect alkaloids. TLC solvent systems: A: $CHCl_3$-MeOH = 95:5; B: $CHCl_3$-MeOH-TEA = 95:5:0.05; C: cyclohexane-$CHCl_3$-glacial HOAc 45:45:10; D: *n*-propanol-formic acid-water 90:1:9.

Plant Extraction

Plant material [*Hydrastis canadensis* L. (Ranunculaceae), finely powdered rhizomes, 5 kg] was provided by Nature's Sunshine Products, Spanish Fork, UT, USA. A portion of the plant material (4.7 kg) was macerated with MeOH (15 l, 100%) for 24 h, followed by maceration with 95% MeOH (aqueous, 15 l, 24 h x 2). The methanolic extracts were combined, filtered, and evaporated under reduced pressure at 38°C to a thick, black syrup. The syrup was diluted with MeOH to 800 mL and gravity-filtered into a separatory funnel containing 900 mL H_2O and 1.5 l petroleum ether. Crude β-hydrastine (**4**, 22 g, 0.5% w/w) precipitated from the mother liquor. The filtrate was extracted three times with petroleum ether (D01, 32.4 g), then three times with $CHCl_3$ (D02, 74.92 g).

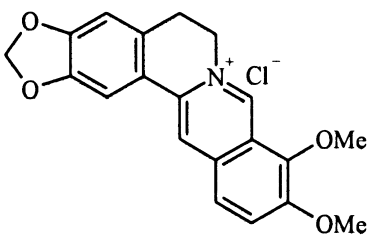

Figure 1. Structure of berberine chloride (1).

A portion of the remaining aqueous phase (5 mL) was acidified to pH 2 with 2 N HCl and crude berberine chloride (**1**, 35 mg, ~0.2% w/w) precipitated upon cooling to 4°C and standing overnight.(18) A portion of this crude berberine was purified by elution through a Sephadex column with 100% MeOH as the eluting solvent. Fraction D02 was evaporated to dryness, powdered, and dissolved in H_2O-saturated ethyl acetate (EtOAc), and extracted with tartaric acid (2% aqueous w/v, pH ~3). The tartaric acid solution was subsequently extracted with $CHCl_3$ (D02-1, 43.35 g). The remaining aqueous solution was basified to pH 8 with $NaHCO_3$ and extracted again with $CHCl_3$ (D02-2, 5.98 g).

Preparation of Solvent Systems and Sample

CCC solvents ($CHCl_3$ and H_2O) were placed together in a separatory funnel and, once equilibrated, were separated just prior to the CCC run. To the upper aqueous phase, HCl was added as a retainer acid (6-25 mmol, pH 2.6-1.7), and to the lower phase was added triethylamine (TEA) as a displacer (0.05-0.3%). The sample was dissolved in 5.1 mL solvent (either $CHCl_3$ or H_2O-saturated $CHCl_3$, with or without TEA) for subsequent injection.

CCC Separation Method and Analysis of Fractions

When the CCC column was loaded with aqueous stationary phase, the apparatus was spun 800 rpm and mobile phase was introduced (2.0-4.5 mL/min). After 35-50 mL of aqueous phase had eluted, the mobile phase began to elute. Once a steady flow of mobile phase was observed, the sample was injected. For consistency among the experiments presented here, the sample was injected after 35 mL of mobile phase had eluted. Stationary phase retention was consistently between 89-93%. For all CCC experiments presented, the separation was carried out in reverse displacement mode; HCl was added to the aqueous stationary phase (12-24 mmol) as a retainer acid, and triethylamine (TEA) was added to the $CHCl_3$ mobile phase as a displacer.

The effluent from the CCC was monitored at 290 nm and fractions were combined based on TLC with solvent systems A and/or D (see Reagents and Other Materials section). The purity of the isolated compounds was verified by TLC in four solvent systems, and the alkaloids berberine chloride (**1**), (+)-canadaline (**2**), (-)-canadine (**3**), (-)-β-hydrastine (**4**), and (-)-isocorypalmine (**5**) were identified by comparison of their mp, $[\alpha]_D$, and spectral data (UV, ^{1}H NMR, ^{13}C NMR, and MS) to literature values.(6,17,18)

RESULTS AND DISCUSSION

Fraction D02 was highly active when tested for its *in vitro* antimicrobial activity using microbial assays described previously.(15,16) It suppressed growth of the cariogenic bacteria *Streptococcus mutans*, and periodontal pathogens, such as *Porphyromonas gingivalis* and *Prevotella intermedia,* and was subsequently partitioned as outlined in the Plant Extraction section. Fraction D02-1 contained the majority of the β-hydrastine (**4**) along with all of the canadine (**3**) that was present in the original $CHCl_3$ extract (D02). Upon basification of the aqueous extract to pH 8 with $NaHCO_3$, the remainder of the β-hydrastine and all of the remaining trace alkaloids partitioned into $CHCl_3$ (D02-2). Berberine (**1**) partitioned to some degree in every fraction, but largely remained in the aqueous phase.

Two separate portions of D02-1 were subjected to pH-zone refining CCC. In the first experiment (Figure 2a), 18 mmol HCl was added to the aqueous stationary phase and 0.1% TEA added to the mobile phase with a flow rate of 3.0 mL/min. The sample (2.0 g of D02-1) was dissolved in 5 mL mobile phase, *including* TEA. Once the sample was injected, four-min (12 mL) fractions were collected and canadine (**3**) eluted approximately 32-40 min after injection, while β-hydrastine (**4**) was present in fractions eluting between 32 and 180 min. The trace amount of berberine chloride (**1**) present was retained in the aqueous stationary phase and approximately 2 mg were purified from residual tartaric acid that partitioned into the $CHCl_3$ extract by elution through a Sephadex column with MeOH.

In the second experiment (Figure 2b), all conditions were the same as that of the first experiment, with the only differences being that 24 mmol, rather than 18 mmol, of HCl was added to the stationary phase, and the flow rate of the mobile phase was reduced to 2.0 mL/min. After 2.2 g of D02-1 were injected, canadine (**3**) eluted from approximately 40 to 76 min and β-hydrastine (**4**) eluted from 48 to 195 min. Poor separation was obtained for canadine (**3**) in these first two experiments, so the canadine-containing fractions were combined for an additional CCC separation.

In this third experiment (Figure 2c), all conditions were identical to those used for the second experiment, with the only difference being that the sample (canadine containing-fractions from the first and second experiments, 1.084 g) was dissolved in H_2O-saturated $CHCl_3$, *without* the addition of the TEA displacer. Canadine (**3**) eluted from approximately 44 to 68 min, well separated from β-hydrastine (**4**), eluting from 78 to 210 min. After removal of solvents, 40.3 mg of canadine (**3**) and 986 mg of β-hydrastine (**4**) were obtained in pure form for a 94.6% recovery.

Fraction D02-2 (see Plant Extraction section) was subjected to a series of CCC separations. Conditions similar and identical to those presented for the

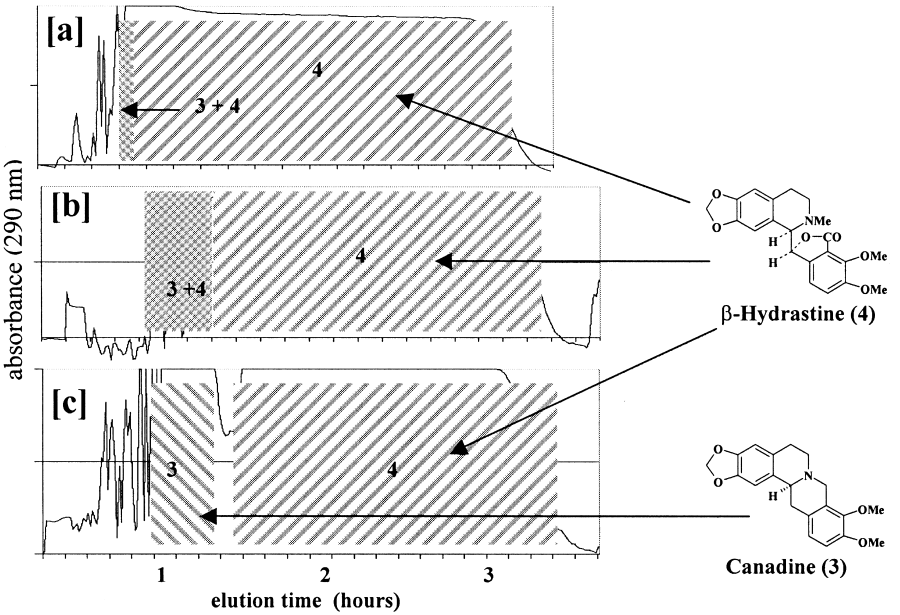

Figure 2. Separation of canadine (3) and β-hydrastine (4). [a]: Alkaloid extract D02-1 (2.0 g) was dissolved in H_2O-saturated $CHCl_3$ containing 0.1% triethylamine (TEA) for injection; stationary phase (SP) 18 mmol HCl; mobile phase (MP) 0.1% TEA in $CHCl_3$; flow rate 3.0 mL/min. [b]: D02-1 (2.2 g) was dissolved in H_2O-saturated $CHCl_3$ containing 0.1% TEA; SP 24 mmol HCl; MP 0.1% TEA in $CHCl_3$; flow rate 2.0 mL/min. [c]: All conditions identical to those in [b], except that the sample (canadine containing-fractions from [a] and [b]) was dissolved in H_2O-saturated $CHCl_3$ *without* the addition of TEA.

experiments with fraction D02-1 were effective in separating β-hydrastine (**4**) from a group of about ten more polar alkaloids (Figure 3). Pure canadaline (**2**) was obtained using the conditions presented in Figure 3, eluting between 135 and 165 min. Isocorypalmine (**5**, 10 mg) eluted in nearly pure form between 99 and 105 min after injection and was purified by recrystallization from $CHCl_3$/MeOH. Similar fractions from experiments with D02-2 were combined and subjected to further CCC experiments. Repeated injections ultimately yielded 65 mg of pure canadaline (**2**).

In summary, a suitable preparative separation of canadine (**3**) or canadaline (**2**) from β-hydrastine (**4**) was obtained using a stationary phase consisting of $CHCl_3$-saturated H_2O with 24 mmol HCl added as a retainer acid, and a mobile

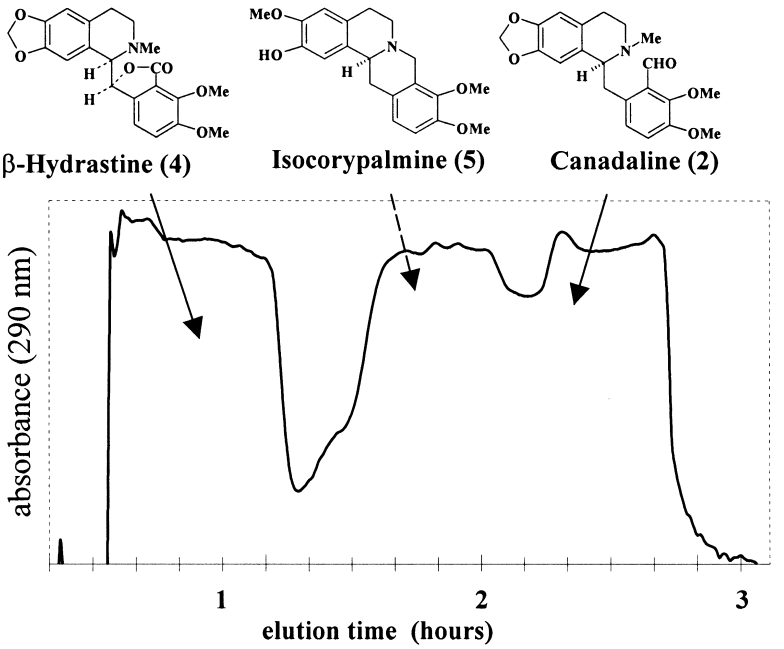

Figure 3. Purification of canadaline (2) and isocorypalmine (5) from a fraction containing β-hydrastine (4). Fraction D02-2 (1.6 g) was dissolved in H_2O-saturated $CHCl_3$ for injection; stationary phase 12.1 mmol HCl (pH 2.2); mobile phase 0.1% TEA ($CHCl_3$); flow rate 3.0 mL/min; 3-min fractions collected. Conditions identical to those used for Figure [2c] were also sufficient for the preparative isolation of canadaline (**2**), whereas isocorypalmine (**5**) was purified by recrystallization.

phase consisting of H_2O-saturated $CHCl_3$ with 0.1% triethylamine added as the displacer. Dissolving the sample in H_2O-saturated $CHCl_3$ *without* the displacer base proved essential for effective separation of these analytes under the given conditions. Isocorypalmine (**5**) did not elute in pure form under any set of conditions investigated in this work, and was obtained by recrystallization. While the trace quantities of berberine (**1**) that were present in every partition fraction could be obtained from the aqueous stationary phase at the end of each CCC experiment, facile preparative isolation of berberine was achieved through crystallization as the chloride from the acidified aqueous mother liquor.

In the three CCC experiments with fraction D02-1, altogether, 1600 mL of $CHCl_3$ were consumed. In the third experiment, which afforded 40 mg of pure canadine (**3**) and 986 mg of pure β-hydrastine (**4**), 450 mL of $CHCl_3$ were consumed. In comparison with open-column silica gel or HPLC methods, this repre-

sents very low solvent consumption for a sample of the size used in these experiments.

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