A Rapid Quantitative Method for the Analysis of Galanthamine and Other Amaryllidaceae Alkaloids by Capillary Column Gas Chromatography†

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A rapid and sensitive capillary column gas chromatographic method for the identification and quantification of Amaryllidaceae alkaloids is reported. Well-resolved, sharp and symmetrical peaks were obtained for compounds of four different chemical skeleton types of these alkaloids. A 15 m DB-1 capillary column was used for this purpose, and the time of analysis of all compounds was 15 min. This procedure required only small amounts of dried plant material and was developed with the addition of an internal standard.

Amaryllidaceae alkaloids represent a diverse class of bases occurring in different species of the family Amaryllidaceae.[1] Some of these compounds have been shown to possess a wide spectrum of useful biological activities including antiviral,[1,3] antineoplastic,[4−6] immunostimulant,[7] analgesic,[8] antimalarial,[9] and insect antifeedant.[1,10] Galanthamine, a common alkaloid in this family, has shown cholinesterase inhibitory activity and is currently undergoing clinical trials for treatment of Alzheimer's disease.[11−13] Currently, galanthamine is commercially isolated from wild *Leucojum* and *Galanthus* species. Since the supply of plant material is limited, there is an urgent need to search for new sources. As part of a project to find new natural sources for galanthamine, we have screened a large number of wild and commercially available members of the Amaryllidaceae.

Although different analytical techniques have been described to quantitate galanthamine and some other amaryllidaceae alkaloids,[14−18] a practical quantitative method for analysis of large numbers of samples from limited amounts of plant material was not available. Although methods based on high performance liquid chromatography have been described, our attempts to achieve complete separation of the standard amaryllidaceae alkaloids with desirable peak shapes using this technique were unsuccessful. However, we found capillary column gas chromatography to be a simple, rapid, sensitive, and reproducible technique for the quantitative analysis of galanthamine and other amaryllidaceae alkaloids. Using this method, we quantified alkaloids representing four different chemical skeleton types from species of Amaryllidaceae *sensu lato* (including: Agavaceae, Hypoxidaceae, and Liliaceae, of authors) including a large number of commercially available *Narcissus* cultivars. Several selected cultivars were grown under experimental conditions, and their galanthamine content was determined during their annual growth cycle. The analysis required only small samples of dried plant material (500 mg or less) and was carried out using dihydrodeoxycodeine as an internal standard. The extraction and analytical procedure we employed is presented here.

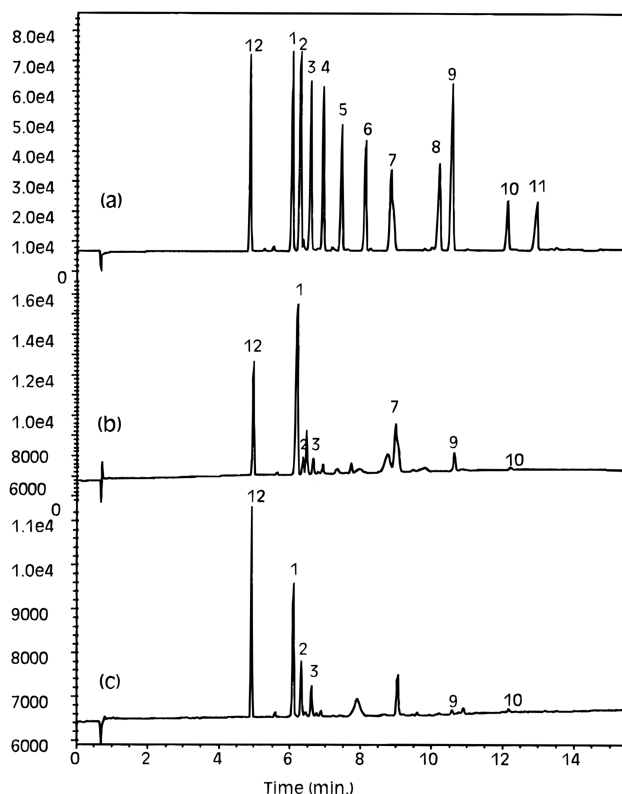

Figure 1. Capillary gas chromatogram in NPD of Amaryllidaceae alkaloids: (a) the standard mixture of compounds **1**−**12**; (b) the alkaloid fraction of the *Narcissus* cv. "Ingelscombe"; (c) the alkaloid fraction of the *Narcissus* cv. "Mount Hood".

Eleven Amaryllidaceae alkaloids, namely galanthamine (**1**), lycoramine (**2**), *N*-demethyllycoramine (**3**) *O*-methylmaritidine (**4**), caranine (**5**), pluviine (**6**), haemanthamine (**7**), lycorine (**8**), homolycorine (**9**), hippearstrine (**10**), and narcissidine (**11**), were isolated and characterized from a variety of common *Narcissus* cultivars and used as the standards in our study. Using capillary gas chromatography, we were able to achieve

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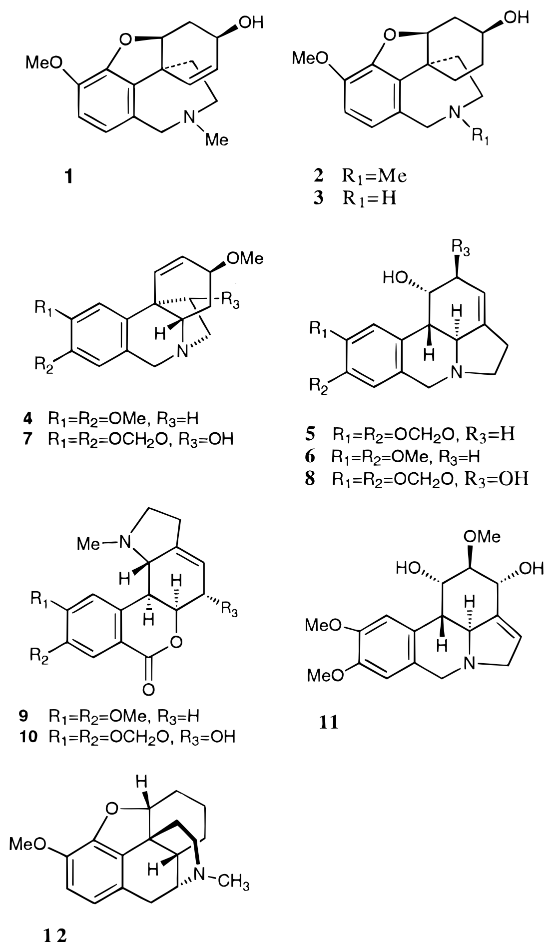
complete separation of the above compounds. The chromatogram of a 1 μL injection of a mixture of standards and internal standard deoxydihdyrocodeine (**12**) at a concentration of 1.0 mg/mL is shown in Figure 1a. The mass spectrum of each individual peak was determined by GC/MS under identical conditions, and the expected molecular weight was observed for all the compounds. The peaks for all standard compounds showed excellent concentration and detector response linearity. In our study, compounds were simultaneously detected by both flame ionization (FID) and nitrogen phosphorus (NPD) detectors. The standard compounds were detected with higher sensitivity with NPD detection, and concentrations around 10 μg/mL could be reproducibly quantitated.



A conventional alkaloid extraction process involves successive removal of nonalkaloids and alkaloids by organic solvents from acidified and basified aqueous solutions of an ethanol extract. However, application of similar methods in the extraction of alkaloids from fresh amaryllidaceae bulbs presented two major problems. The presence of large amounts of water and water-soluble polysaccharides made evaporation of the ethanol extract difficult. Additionally, water content fluctuated with season, age, tissue type, and storage conditions, making comparison of galanthamine content in different fresh plant samples difficult. To overcome this problem we dried the plant material prior to extraction and determined galanthamine content as a percentage of plant dry weight. Dried and ground plant material was directly extracted with dilute acid solution. This allowed us to avoid evaporation of the primary extract. However, on occasion, the presence of large amounts of water-soluble polysaccharides in the aqueous extract formed emulsions in subsequent steps. Centrifugation to separate phases overcame this problem. The extraction of the plant material directly by aqueous acid had an additional advantage. Aqueous acid extracts, unlike ethanol, had very small amounts of lipophilic compounds. When an acid extract was partitioned with organic solvent, amounts of nonalkaloids recovered were not significant enough to interfere with our study. Therefore, alkaloids were directly extracted after basification without prior removal of nonalkaloids. The alkaloid fractions were gas chromatographed, and compounds were simultaneously detected using FID and NPD. Interferences from nonalkaloid peaks in the region of interest of the chromatograms recorded using FID were insignificant.

Table 1. Content of Some Amaryllidaceae Alkaloids in Bulbs of *Narcissus* cv. "Inglescombe" and *Narcissus* cv. "Mount Hood" in mg/100 g of Dried Biomass

	alkaloids, mg/100 g of dry wt					
Narcissus cultivar	**1**	**2**	**3**	**7**	**9**	**10**
Inglescombe	173.7	7.4	2.5	117.5	17.4	trace
Mount Hood	53.3	18.4	4.3		trace	trace

In order to minimize personnel and equipment errors, an internal standard was added in the initial aqueous extraction step. Dried, ground plant material was extracted with dilute HCl solution containing 100 μg/mL of internal standard. Studies of the efficiency of recovery of internal standard and galanthamine were carried out in triplicate with a *Narcissus* cultivar where galanthamine was not present as a natural constituent.

The GC chromatograms (using NPD detector) of the crude alkaloid fraction from *Narcissus* cv. "Inglescombe" and *Narcissus* cv. "Mount Hood" are shown in Figure 1b,c, and the alkaloid contents are quantified in Table 1. Extractions were carried out in triplicate, and standard deviation was ±10%. The identity of the peaks was determined using retention times. The retention times of the compounds were corrected using the internal standard, deoxydihydrocodeine.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover Uni-melt capillary apparatus or a Fisher-Johns digital melting point analyzer Model 355 and were not corrected. Optical rotations were determined on a Perkin-Elmer 141 automatic polarimeter using MeOH solutions. UV spectra were taken on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer in MeOH solutions. NMR spectra were recorded at 300 MHz for proton and 75 MHz for carbon on a Varian VXR-300 instrument, and the solvent signal was used as reference. All solvents used for chromatographic purposes were AR grade.

Plant Material. Bulbs of *Narcissus* cv. "Inglescombe" and *Narcissus* cv. "Mount Hood" were purchased from Garden World Products, Inc., Cumming, GA, and Marlboro Bulb Farms, Inc., Wilmington, NC, respectively.

Standard Compounds. Standard compounds (**1**–**11**) were obtained from a variety of common *Narcissus* cultivars using standard extraction and separation methods and were identified by comparison of the

physical (mp, $[\alpha]_D$) and spectroscopic data (UV, ^{1}H and ^{13}C NMR) with literature values.[19-24] The purity of the compounds was determined by gas chromatography using FID and NPD.

Internal Standard. Internal standard deoxydihydrocodeine (**12**) was synthesized by catalytic hydrogenation of α-chlorocodeine, which was prepared from codeine (Noramco of Delaware, Inc., Wilmington, DE) using the procedure described by Stork and Clarke.[25] Catalytic hydrogenation of α-chlorocodeine (1 g) was carried out in the presence of 5% Pd on $CaCO_3$ catalyst (300 mg, Matheson Coleman and Bell, East Rutherford, NJ) in ethanol (150 mL) at 40 psi for 1 h to yield deoxydihydrocodeine in 75% yield. This was crystallized from MeOH/ethyl acetate as the HCl salt. The internal standard was added to the extracting solvent prior to extraction.

Sample Preparation and Recovery. A mixture of dried powdered bulbs (500 mg) and dilute HCl solution (6 mL of 0.05 N) containing 100 μg/mL of the internal standard was placed on a shaker at 200 rpm at 40 °C. After 2 h, the extract was centrifuged at 3500 rpm (2050*g*) for 5 min, and 3 mL of the supernatant was transferred to a 15 mL centrifuge tube. This solution was basified with NaOH (1 mL, 0.3 N), mixed well with $CHCl_3$ (3 mL), and centrifuged at 4500 rpm (3400*g*) for 5 min. The $CHCl_3$ layer was removed and dried by filtration through a Pasteur pipette containing a small amount of Na_2SO_4. The dried $CHCL_3$ extract (1.5 mL) was evaporated in a Savant Speed Vac concentrator, and the residue was dissolved in 100 μL of MeOH and analyzed by GC.

Recovery studies were carried out both for galanthamine and deoxydihydrocodeine. The observed recoveries for galanthamine and deoxydihydrocodeine were 90 and 100%, respectively.

Chromatography Conditions. Chromatographic analysis was carried out on a Hewlett-Packard 5890A gas chromatograph equipped with a FID and a NPD. The output was recorded using a Millenium 2000 data system. A DB-1 capillary column (15 m × 0.25 mm i.d. × 0.2 mm film thickness; J & W Scientific, CA) was used for this analysis. Helium at a flow rate of 1−1.5 mL/min was employed as the carrier gas, and the optimized oven temperature program was 200−250 °C, linear increase 4 °C/min, 250 °C held for 3 min. Temperatures of the injector port and detectors were held at 260 °C. Helium was used as the detector makeup gas. The injectors were operated in split mode (1/50). The NPD sensitivity was held between 28 and 33 mV. Linearity of response of the NPD was observed in a concentration range of 8.0−1000 μg/mL for compounds **1**−**7** and **10** 8.0−500 μg/mL for the compounds **8**, **9**, and **11**, and up to 2000 μg/mL for the internal standard **12**. The solutions of all standards (**1**−**11**) were prepared in MeOH to give a 1.0 mg/mL concentration of each compound. A 1.0 μL volume of all solutions was injected in all analyses.

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