Extraction and HPLC Determination of Ranunculin in Species of the Buttercup Family

Yili Bai,[†] Michael H. Benn,[‡] Walter Majak,*[,†] and Ruth McDiarmid[†]

Range Research Station, Agriculture and Agri-Food Canada, 3015 Ord Road, Kamloops, British Columbia, Canada V2B 8A9, and Chemistry Department, University of Calgary, Calgary, Alberta, Canada T2N 1N4

New extraction procedures are described for the isolation and purification of ranunculin, the glycoside in members of the buttercup family which, upon autolysis, yield the vesicant oil, protoanemonin. The procedures permit the efficient determination of ranunculin in plant extracts by normal phase HPLC. Ranunculin levels in plant tissue ranged from 1.5 to 19.9% on a dry matter basis. Of the 11 species examined, 5 were new detections of ranunculin in the Ranunculaceae. The glycoside was rapidly hydrolyzed at room temperature in the presence of β-glucosidase as shown by HPLC of the reaction mixture. Autolysis occurred in aqueous acetone extracts at room temperature but not at −5 °C. Autolysis also occurred during cryogenic storage of plant tissue. Having extracted ranunculin with conventional solvents in the absence of acid, the glycoside can now be accepted as the natural storage form of protoanemonin and not an artifact arising from acid extraction.

Keywords: *Ranunculin; HPLC; β-glucosidase; aglycon; buttercup*

INTRODUCTION

The lactone protoanemonin (5-methylene-2-oxodihydrofuran, **1**, Figure 1) is the irritant oil responsible for the vesicant properties attributed to members of the buttercup family (Ranunculaceae) including species of *Helleborus, Anemone, Clematis, Ceratocephalus*, and most commonly *Ranunculus* (Bonora et al., 1987; Olsen et al., 1983; Shearer, 1938). The oil is derived by autolysis during maceration of fresh plant tissue and produces an intense burning sensation when chewed or erythema, dermatitis, and blistering when applied to the skin (Spoerke and Smolinske, 1990). Ingestion of the plant by all classes of livestock can cause gastric distress including irritation of the digestive tract, abdominal pain, and diarrhea (Kingsbury, 1964). Protoanemonin also has antifungal and antibiotic properties (Martin et al., 1990; Campbell et al., 1979).

Although the structure of protoanemonin was established at the beginning of the century (Asahina and Fugita, 1922), the storage or bound form of this unstable toxin has been questioned. The autolytic release of the toxin suggested that it was liberated enzymatically, possibly through the action of β-glucosidase (EC 3.2.1.21). The β-D-glucoside, ranunculin (**2**, Figure 1), was crystallized from different species of *Ranunculus* (Hill and van Heyningen, 1951), and the authors showed that treatment of the glucoside with weak alkali (sodium acetate) yielded protoanemonin. Subsequently, protoanemonin was also generated from the glucoside following its treatment with almond emulsin and steam distillation (Hellstrom, 1959). The stereochemistry of the aglycon (**3**, Figure 1) has been established (Benn and Yelland, 1968; Boll, 1968). However, Tschesche et al. (1972) have criticized the extraction method of Hill and van Heyningen that utilized 0.5 N HCl as the extracting solvent for fresh plant material. When the acid system was replaced with conventional solvents (acetone−water), ranunculin was not detected, and they concluded that it was an artifact; however, in a subsequent paper from this group (Tschesche et al., 1981) ranunculin was shown as an intermediate in a biosynthetic pathway from 5-hydroxylevulinic acid to protoanemonin.

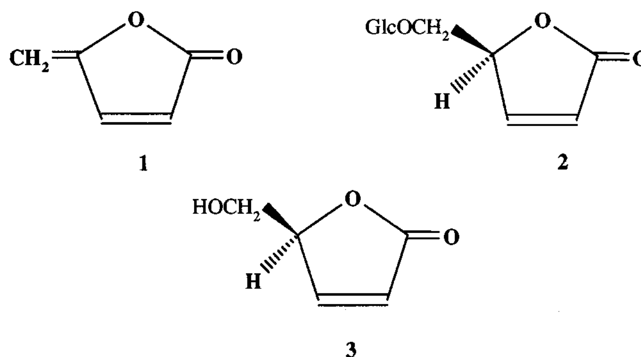

Figure 1. Structures of protoanemonin (**1**), ranunculin (**2**), and the aglycon (**3**).

With one exception that utilized NMR spectroscopy to determine ranunculin concentrations in crude extracts (Nachman and Olsen, 1983), the various other procedures that have been described to detect ranunculin in fresh plant tissues are based on the measurement of protoanemonin released upon autolysis and its analysis in steam distillates (Southwell and Tucker, 1993; Bonora et al., 1987; Ruijgrok, 1966). Reversed phase HPLC has been used for the determination of ranunculin in dried roots of *Pulsatilla chinensis*, a Chinese herbal medicine (Zhang et al., 1990). In the present study new extraction procedures are described for the isolation of ranunculin in the absence of acid, a method is described for the analysis of the glycoside by HPLC, and the combined procedures are used to determine ranunculin concentrations in 11 species of the buttercup family, of which 5 are new detections of ranunculin. The stability of ranunculin in frozen plant tissue is also examined, and its susceptibility to almond emulsin is re-examined by HPLC.

* Author to whom correspondence should be addressed.

[†] Agriculture and Agri-Food Canada.

[‡] University of Calgary.

S0021-8561(95)00626-1 CCC: $12.00 Published 1996 by the American Chemical Society

EXPERIMENTAL PROCEDURES

Plant Material. Plant samples (aerial portions) of the following species were collected between April and July 1995, from several sites near Kamloops, BC: *Actaea rubra* (Aiton) Willdenow, *Aquilegia formosa* Fischer, *Clematis ligusticifolia* Nuttall, *Clematis occidentalis* (Hornemann) DC., *Ranunculus acris* L., *Ranunculus cymbalaria* Pursh, *Ranunculus inamoenus* Greene, *Ranunculus uncinatus* D. Don, *Thalictrum occidentale* Gray, and *Trollius laxus* Salisbury. Plant samples of *Anemone multifida* Poiret (both aerial portions and roots) were collected during June 1995, near Clinton, BC. The fresh plant material was frozen, freeze-dried, and ground to pass a 2-mm sceen. When fresh material was extracted, a subsample was used for a dry matter determination. Ranunculin concentrations (percent) are expressed on a dry matter basis.

Isolation of Ranunculin. Freeze-dried plant material (*R. cymbalaria*, 10 g) was homogenized with methanol in a blender, the residue was washed with methanol, until the filtrate was clear, and the extract was evaporated to dryness under reduced pressure. The concentrate was partitioned between chloroform and water, and the aqueous phase was washed with chloroform and concentrated to 6 mL. An aliquot (1 mL) was fractionated by vacuum liquid chromatography (VLC) on silica gel 60H (Merck 7736, 45 × 65 mm) using the lower phase of chloroform−methanol−water (65:35:10) as the developing solvent. Fractions containing ranunculin were combined, concentrated, and chromatographed on a polyamide (MN polyamide CC6, 2 × 10 cm) column equilibrated in water. The clear, colorless aqueous eluate was concentrated to dryness, and ranunculin was recrystallized from methanol (white crystals, 37 mg). The physical properties were identical with those reported previously for ranunculin (Benn and Yelland, 1968). NMR spectra of ranunculin and the aglycon were obtained on a Bruker AM400 spectrometer.

HPLC System. A Varian Model Vista 5500 liquid chromatograph equipped with a UV-2050 variable-wavelength detector set at 210 nm was used with a Varian 4270 integrator and a Varian 9090 auto sampler. Separations were carried out on a Varian prepacked, normal phase NH_2-10 column protected by an NH_2-10 guard column. An isocratic mobile phase of acetonitrile−water (90:10) was used at a flow rate of 2.0 mL / min. Ranunculin concentrations were quantified by the external standard method (0.25−1.0 mg/mL) with duplicate 40-μL injections per plant sample (Figure 2). The detection limit for this method was 0.1 μg of ranunculin.

TLC Systems. Ranunculin standards and plant extracts were examined on silica gel TLC (silica gel 60 F_{254}, Merck 5714-3). The solvent systems used were chloroform−methanol−water (65:35:10) (lower phase) and acetonitrile−water (9:1). The spray reagent was anisaldehyde−sulfuric acid (1 mL of concentrated sulfuric acid was added to a solution of 0.5 mL of anisaldehyde in 50 mL of glacial acetic acid).

Acid extraction was based on the procedure of Hill and van Heyningen (1951), but the charcoal treatment step was eliminated to avoid the loss of ranunculin. In addition to the concentration suggested in the literature (0.5 N HCl), 0.05 and 0.005 N HCl were also tested.

Methanol Extraction. Pulverized freeze-dried plant material (700 mg) was packed in a small column (1.5 × 8 cm). The column was eluted with methanol until the plant material was almost colorless. Alternatively, fresh plant material was ground with methanol in a mortar and pestle, and the homogenate was filtered through a small funnel packed with a piece of cotton wool. This was repeated three times. In either procedure the combined methanol extracts were evaporated to dryness under reduced pressure (bath <35 °C), and the residue was partitioned between chloroform and water. The separation of the two layers was achieved by centrifugation, and the aqueous layer was then adjusted to 25 mL. Further dilution was achieved with HPLC mobile phase.

Acetone−water (1:1) extraction was as described by Tschesche et al. (1972). Both cold (−5 °C) and room temperature extractions were carried out according to this method.

Enzyme Activity Tests. *Experiment 1* was designed to confirm that ranunculin is a substrate for almond emulsin (β-glucosidase, EC 3.2.1.21). Ranunculin (5 mg) was dissolved in 5 mL of water, 80 μL of this solution was diluted with acetonitrile to 160 μL, and an aliquot (40 μL) was chromatographed on HPLC. β-Glucosidase (Sigma Chemical Co., G 0395, 14 units/mg) (1 mg) was then added to the ranunculin standard, and the ranunculin concentration was determined at 4, 20, 40, 70, and 120 min to monitor the enzymatic hydrolysis of ranunculin at room temperature.

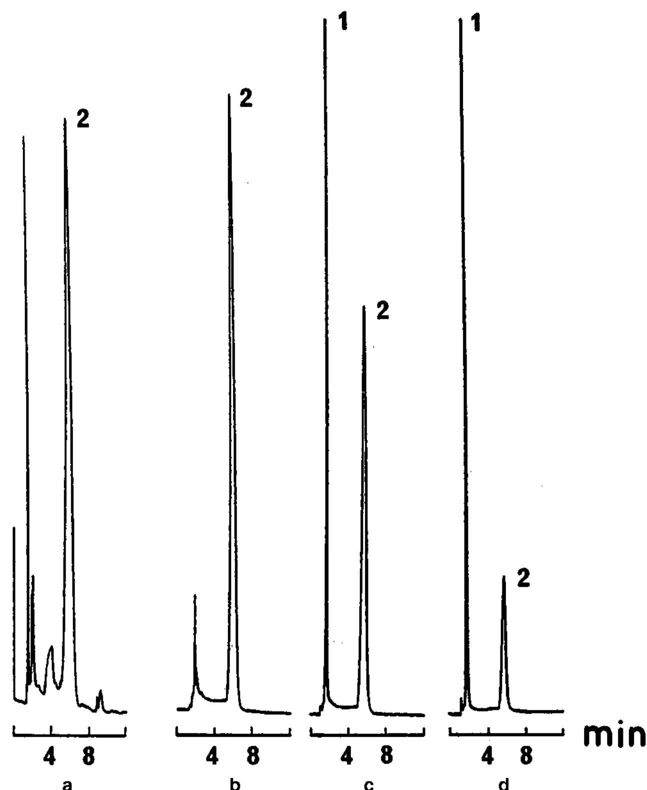

Figure 2. HPLC separation of ranunculin (**2**) and its hydrolysis product (**1**): (a) plant extract of *A. multifida*; (b) ranunculin standard; (c) hydrolysis of ranunculin at 40 min; (d) hydrolysis of ranunculin at 120 min. Column, NH_2-10 (30 cm × 4 mm); detection, 210 nm; mobile phase, acetonitrile−water (90:10).

Experiment 2 was designed to observe autolytic, endogenous enzyme activity in acetone−water extracts of plant material. Under conditions similar to those used by Tschesche et al. (1972), 4.0 g of fresh plant material (*R. cymbalaria*) in 15 mL of acetone−water (1:1) was ground in a mortar and pestle, and the solvent was removed by filtration at room temperature. This was repeated three times, and the extracts were combined in 50 mL. Five milliliters was added to 10 mg of ranunculin, of which 0.25 mL was immediately diluted with acetonitrile and chromatographed on HPLC to determine the concentration at zero time. The incubation mixture was then chromatographed at 0.7, 2, and 3.5 h.

Freezer Storage Experiment. Several preweighed fresh plant samples (*R. cymbalaria*, 5 g each) were stored in a freezer (−7 °C). At 0 (no storage), 4, 10, and 22 days, samples were removed and extracted with methanol as described above. The concentrations of ranunculin in these samples were determined by HPLC and compared to their cryogenic storage time. Linear slopes and correlation coefficients were determined for each enzymatic reaction.

RESULTS AND DISCUSSION

The NMR spectra of the ranunculin isolates were in complete agreement with reported data for the glycoside (Benn and Yelland, 1968; Nachman and Olsen, 1983). The aglycon, isolated according to the method of Hellstrom (1959) by diethyl ether extraction of ranunculin−emulsin mixtures in pH 5.9 phosphate buffer, had ^{1}H

NMR (400 MHz, $CDCl_3$) 7.48 (1H, dd, J = 1.5 and 5.8 Hz, H-3), 6.22 (1H, dd, J = 2.1 and 5.8 Hz, H-2), 5.16 (1H, m, H-4), 4.00 (1H, dd, J = 3.4 and 12.1 Hz, H5-A) and 3.80 (1H, dd, J = 5.1 and 12.1 Hz, H-5B) ppm; and ^{13}C NMR (100 MHz, $CDCl_3$) 172.7 (s, C-1) 153.1 (d, C-3), 123.2 (d, C-2), 83.8 (d, C-4), and 62.6 (t, C-5) ppm.

Selection of Extraction Procedure. The yields of ranunculin were determined by HPLC analysis of *R. cymbalaria* using different extraction procedures. The results indicated that extractions in 0.5 N HCl or methanol were both very efficient, each giving a yield of 20% ranunculin. Because no acid was involved in the methanol extraction, the latter was chosen as the method for ranunculin determination. The aqueous acid extractions also showed that the yield of ranunculin declined with decreasing acid strength in the solvent. When fresh plant samples were used and the extraction in acetone−water was carried out at room temperature, only relatively small yields of ranunculin were obtained (<5%). The yield (16%) from the low temperature (−5 °C) extraction of fresh material in acetone−water was close to the yield from methanol extraction, which indicated low-temperature inactivation of the enzymes responsible for autolysis. Cold aqueous acetone was also used to extract ranunculin from *Knowltonia capensis* (Campbell et al., 1979). When freeze-dried plant samples were extracted, similar yields (19−20%) were obtained at room temperature and at −5 °C. These results indicated that in freeze-dried plant material the endogenous enzyme was also inactivated. In fresh plant material the hydrolytic enzyme is active at room temperature even in the presence of 50% acetone, a stability which resembles that of almond emulsin, which is known to be fully active in 25% aqueous methanol (Stout et al., 1980).

Enzyme Activity Tests. *Experiment 1.* In the presence of an excess amount of β-glucosidase at room temperature, 80% of ranunculin was hydrolyzed in 2 h, in agreement with earlier work by Hellstrom (1959), who reported that ranunculin is a substrate for β-glucosidase. In the present study ranunculin was hydrolyzed at a rate of 32 μg/min (r = 0.97).

A new peak was detected on HPLC during ranunculin hydrolysis (Figure 2b−d). We speculated that it was the aglycon since it was extractable with ether. Ether extracts of emulsin-treated ranunculin contained only the aglycon by NMR spectroscopy.

Experiment 2. In the presence of endogenous enzyme in acetone−water extracts, almost half of the ranunculin was hydrolyzed in 3.5 h at room temperature and the rate of hydrolysis was 1 mg/h (r = 0.99). Therefore, the system of acetone−water in the presence of autolytic endogenous enzymes is not an appropriate solvent for the extraction of ranunculin at room temperature.

Ranunculin Concentrations Determined by HPLC. Freeze-dried plant samples of eleven species were extracted with methanol and analyzed by HPLC (Table 1). A typical normal phase HPLC separation of ranunculin from a plant extract is shown in Figure 2a. Ranunculin was detected for the first time in *R. cymbalaria*, *R. inamoenus*, *R. uncinatus*, *T. laxus*, and *C. occidentalis* as well as in *A. multifida* and *R. acris*, as previously reported by Bonora et al. (1987) and Ruijgrok (1966). Ranunculin concentrations in plant tissue ranged from 1.5 to 19.9% on a dry matter basis (Table 1). To confirm our HPLC results, the plant extracts were subjected to TLC and the identity and presence of ranunculin were confirmed by cochromatography with authentic ranunculin standards in two solvent systems.

Table 1. Ranunculin Concentrations in Freeze-Dried Species of Ranunculaceae As Determined by HPLC Using Duplicate Injections

plant tested	ranunculin concn (%)	collection date, 1995
R. cymbalaria	19.87, 19.94	April 5−May 8
A. multifida (aerial)	8.04, 8.24	June 20
A. multifida (root)	3.96, 4.00	June 20
R. uncinatus	4.32, 4.59	July 10
R. acris	2.75, 2.87	May 11
R. inamoenus	2.73, 2.80	May 30
T. laxus	1.90, 1.98	July 10
C. occidentalis	1.53, 1.59	May 13
A. rubra	−[a]	May 18, July 10
A. formosa	−	May 30, July 10
C. ligusticifolia	−	May 18
T. occidentale	−	May 18; July 10, 11

[a] Not detected by HPLC or TLC.

Freezer Storage Experiment. On the basis of the observation that ranunculin was not detected in plant samples stored in the freezer for 6 months, a series of freezer storage tests were carried out on *R. cymbalaria*. After 21 days of cryogenic storage at −7 °C, there was almost complete hydrolysis of ranunculin as the concentration decreased from 20 to 1.5%. The magnitude of the loss was approximately 0.8%/day (r = 0.99). Thus, the endogenous enzyme is active at low temperature during prolonged storage. Freeze-drying must therefore be carried out immediately after the plant material is frozen.

Conclusion. The glycoside ranunculin can now be accepted without a doubt as the bound form of protoanemonin in species of the buttercup family. As well, improved extraction and handling procedures are described for the facile determination of ranunculin by HPLC.

ACKNOWLEDGMENT

We thank Barbara Brooke, Kevin Cameron, and Reg Newman for collecting and identifying plant samples.

LITERATURE CITED

Asahina, Y.; Fujita, A. Anemonins. *Acta Phytochim. Jpn.* **1922**, *1*, 1−42.

Benn, M. H.; Yelland, L. J. Ranunculin. *Can. J. Chem.* **1968**, *46*, 729−732.

Boll, P. M. Naturally occurring lactones and lactames I. Absolute configuration of ranunculin, lichesterinic acid and some lactones related to lichesterinic acid. *Acta Chem. Scand.* **1968**, *22*, 3245−3250.

Bonora, A.; Dall'Olio, G.; Donini, A.; Bruni, A. An HPLC screening of some Italian ranunculaceae for the lactone protoanemonin. *Phytochemistry* **1987**, *26*, 2277−2279.

Campbell, W. E.; Cragg, G. M. L.; Powrie, A. H. Anemonin, protoanemonin and ranunculin from *Knowltonia capensis. Phytochemistry* **1979**, *18*, 323−324.

Hellstrom, N. Ranunculin I. The glucosidic linkage and its stability. *Lantbrukshoegsk. Ann.* **1959**, *25*, 171−184.

Hill, R.; van Heyningen, R. Ranunculin: the precursor of the vesicant substance of the buttercup. *Biochem. J.* **1951**, *49*, 332−335.

Kingsbury, J. M. *Poisonous Plants of the United States and Canada*; Prentice-Hall: Englewood Cliffs, NJ, 1964; pp 140−145.

Martin, M. L.; San Roman, L.; Dominguez, A. In vitro activity of protoanemonin, an antifungal agent. *Planta Med.* **1990**, *56*, 66–69.

Nachman, R. J.; Olsen, J. D. Ranunculin: a toxic constituent of the poisonous range plant bur buttercup (*Ceratocephalus testiculatus*). *J. Agric. Food Chem.* **1983**, *31*, 1358–1360.

Olsen, J. D.; Anderson, T. E.; Murphy, J. C.; Madsen, G. Bur buttercup poisoning of sheep. *J. Am. Vet. Med. Assoc.* **1983**, *183*, 538–543.

Ruijgrok, H. W. L. The distribution of ranunculin and cyanogenetic compounds in the Ranunculaceae. In *Comparative Phytochemistry*; Swain, T., Ed.; Academic Press: London, 1966; pp 175–186.

Shearer, G. D. Some observations on the poisonous properties of buttercups. *Vet. J.* **1938**, *94*, 22–32.

Southwell, I. A.; Tucker, D. J. Protoanemonin in Australian *Clematis. Phytochemistry* **1993**, *33*, 1099–1102.

Spoerke, Jr., D. G.; Smolinske, S. C. *Toxicity of Houseplants*; CRC Press: Boca Raton, FL, 1990; pp 37–39.

Stout, D. G.; Majak, W.; Reaney, M. In vivo detection of membrance injury at freezing temperatures. *Plant Physiol.* **1980**, *66*, 74–77.

Tschesche, R.; Welmar, K.; Wulff, G.; Snatzke, G. About subsequent products of still unknown genuine precursor of ranunculin in Ranunculaceae. *Chem. Ber.* **1972**, *105*, 290–300.

Tschesche, R.; Wirth, W.; Welmar, K. 5-Hydroxylevulinic acid, a new intermediate in the biosynthesis of protoanemonin. *Phytochemistry* **1981**, *20*, 1835–1839.

Zhang, X. Q.; Liu, A. R.; Xu, L. X. Determination of ranunculin in *Pulsatilla chinensis* and synthetic ranunculin by reversed phase HPLC. *Acta Pharma. Sin.* **1990**, *25*, 932–935.

Received for review September 19, 1995. Revised manuscript received December 19, 1995. Accepted February 8, 1996.[⊗] This project was partially funded by the British Columbia Cattlemen's Association.

JF950626M

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1996.