Journal of Chromatography, 150 (1978) 202–206
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 10,483

Note

Rapid isolation and identification of pyrrolizidine alkaloids (*Senecio vulgaris*) by use of high-performance liquid chromatography

C. W. QUALLS, Jr.*

Department of Pathology, School of Veterinary Medicine, University of California, Davis, Calif. 95616 (U.S.A.)

and

H. J. SEGALL**

Department of Physiological Sciences, School of Veterinary Medicine, University of California, Davis, Calif. 95616 (U.S.A.)

(First received May 3rd, 1977; revised manuscript received August 8th, 1977)

The pyrrolizidine alkaloids are of considerable concern to both veterinary and human medicine owing to their marked hepatotoxic[1–3], carcinogenic[4–6], teratogenic[1] and mutagenic properties[7,8]. These alkaloids are plant-derived toxins that may contaminate human and animal food sources. Plants containing these alkaloids are found world-wide and include such diverse botanical families as Compositae, Leguminosae and Boraginaceae which have little in common other than production of this characteristic group of alkaloids[1].

Pyrrolizidine alkaloid toxicity is an increasing problem in the western U.S.A. At the School of Veterinary Medicine, University of California, Davis in the Central Valley of California, a 900% increase in cases of pyrrolizidine alkaloid toxicity has occurred over the last ten years. The major cause of this has been *Senecio vulgaris*, known as common groundsel, which contaminates alfalfa hay[9]. Our laboratory, in studying this problem, found that the previous methods of purification and isolation of pyrrolizidine alkaloids were cumbersome and time consuming. In reviewing the literature we found no reference to the use of high-performance liquid chromatography (HPLC) as a method of separation. This paper is intended as an initial communication to show the applicability of HPLC to pyrrolizidine alkaloid research.

MATERIALS AND METHODS

Senecio vulgaris plants were hand picked from bales of first cutting alfalfa harvested in late March, 1975 near Turlock, Calif., U.S.A. The plants had seeded prior to harvest. The stems, leaves and seed heads were used for extraction.

Five hundred grams of finely ground plant material were refluxed with methanol

* Present address: School of Veterinary Medicine, Department of Veterinary Pathology, Louisiana State University, Baton Rouge, La. 70803, U.S.A.

** To whom reprint requests should be addressed.

for 48 h, changing the methanol every 12 h. The methanol was removed under reduced pressure, the extract solubilized with 2 *N* sulfuric acid and filtered (Whatman No. 1). The acid aqueous phase was extracted three times with mixed hexanes and three times with diethyl ether. Excess of zinc dust was added to the acid aqueous phase to reduce any N-oxides of the pyrrolizidine alkaloids and the mixture was stirred for 24 h at room temperature. The solution was filtered and ammonium hydroxide added to adjust the pH to 9. The alkaline solution was extracted five times with chloroform, the extracts combined, anhydrous sodium sulfate added and the solution filtered (Whatman No. 1). The chloroform was removed under reduced pressure, and the pyrrolizidine alkaloids were washed with cold ethanol and dried overnight over phosphorus pentoxide under high vacuum.

Isolation of individual pyrrolizidine alkaloids was accomplished by use of HPLC. All of the chemicals used were reagent grade or better. Tetrahydrofuran (THF; J. T. Baker, Phillipsburgh, N.J., U.S.A.) and chloroform (Mallinckrodt, St. Louis, Mo., U.S.A.) were distilled just prior to use and stored over molecular sieves (Linde Type 3A-PLTS). A solvent-programming system consisting of two Waters Assoc. pumps (Model 6000A), a solvent programmer (Model 600) and a Waters Assoc. 10 μm Bondapak CN column (30 cm $\times$ 4 mm) were utilized. A Schoeffel SF770 Spectroflow Monitor was used for monitoring at 235 nm and its readings were recorded on a Varian A-25 recorder.

The various pyrrolizidine alkaloids were separated as follows. The sample was dissolved in THF–0.01 *M* ammonium carbonate (Baker) (1:1) whose pH was adjusted to 7.8 with 0.1 *N* sulfuric acid. Initial solvent conditions were 13% THF and 87% 0.01 *M* ammonium carbonate (pH 7.8) with a constant flow-rate of 1.8 ml/min. The THF concentration was then increased along a linear gradient from 13 to 26% for 30 min. The individual peaks were collected and immediately lyophilized. The pyrrolizidine alkaloids were extracted with chloroform, dried over sodium sulfate, filtered, dried with nitrogen and dried overnight over sodium hydroxide pellets under a high vacuum. Excellent separation was also achieved by using isocratic conditions consisting of 16% THF and 84% 0.01 *M* ammonium carbonate (pH 7.8). The same flow-rate and UV wavelength as previously described for the gradient system were used. Samples were checked for purity by injecting isolated fractions on the high-performance liquid chromatograph using the same conditions. The samples were identified by comparison of their HPLC retention times with those of known standards* and by use of mass spectral data.

RESULTS

A 500-g sample of ground *Senecio vulgaris* yielded 0.723 g of a light tan powder which when washed with cold ethanol gave 0.583 g of a white powder. This powder, when dissolved and run on the high-performance liquid chromatograph, showed five distinct peaks (UV 235 nm). The different methods of separation are represented in Figs. 1 and 2. The initial peak was due to solvent while the remaining four peaks represent pyrrolizidine alkaloids. Pyrrolizidine alkaloid peaks 2–4 were

---

* Standards of senecionine and seneciphylline were supplied by C. C. J. Culvenor, Melbourne, Australia.

isolated and identified. The isolated fractions when run on HPLC, revealed single peaks corresponding to the original peaks. Retention times obtained for purified samples using the isocratic method were 305 sec (peak 2), 420 sec (peak 3) and 517 sec (peak 4). Standards had retention times of 420 sec for seneciphylline and 517 sec for senecionine. These correspond to peaks 3 and 4, respectively. Both standards contained one additional small peak, which we assume was due to small amounts of a pyrrolizidine alkaloid contaminant.

Peaks 2–4 were analyzed using mass spectral data. The mass spectrometer used was a Finnigan Model 3200 in conjunction with a Finnigan MS Data System

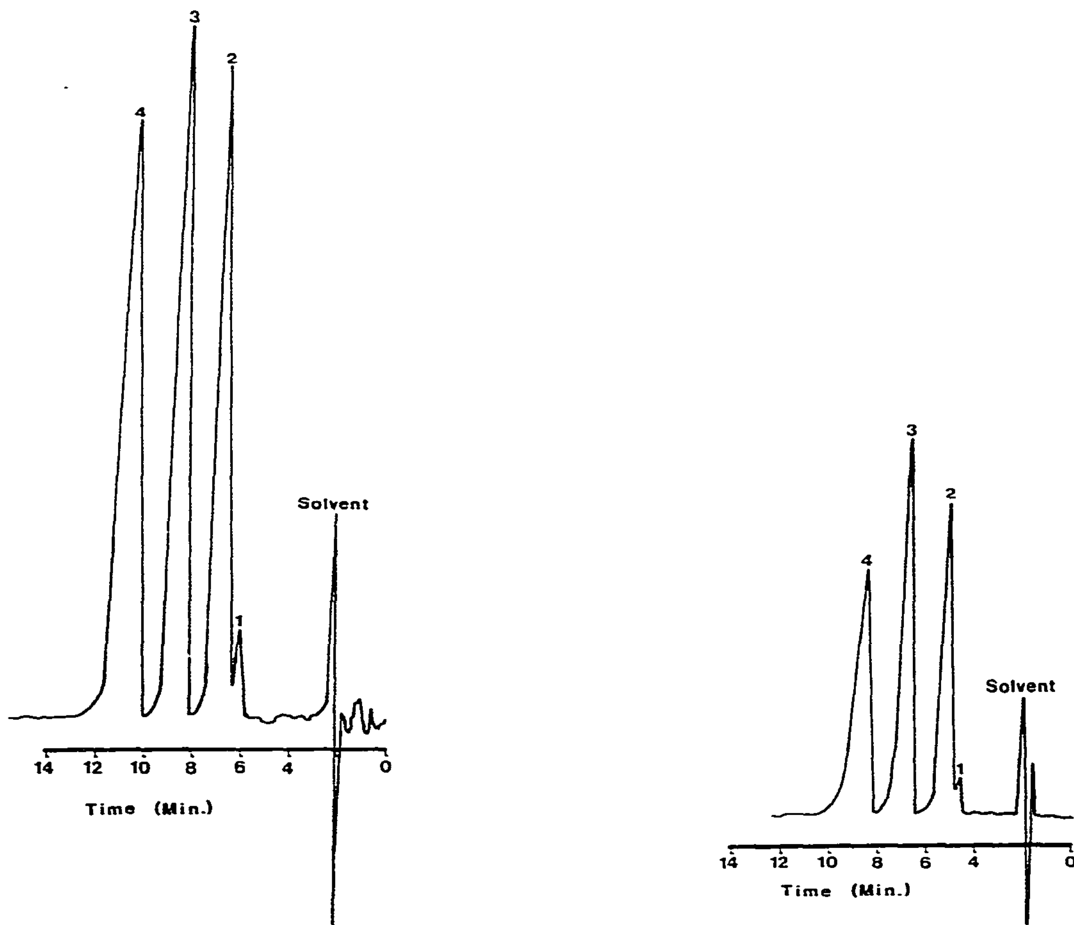

Fig. 1. Gradient analysis. Column, 300 × 4 mm I.D., packed with 10-μm C_{18} Bondapak CN (Waters). Sample, 102 μg of alkaloids in 50 μl of THF–0.01 *M* ammonium carbonate (1:1). Solvent, THF–0.01 *M* ammonium carbonate (pH 7.8) (see text); flow-rate 1.8 ml/min. Column temperature, 25°. Detector, Model SF770 Spectroflow Monitor (Schoeffel) operated at 235 nm. Peaks: 1 = unknown; 2 = retrorsine; 3 = seneciphylline; 4 = senecionine.

Fig. 2. Isocratic analysis. Sample, 41 μg of alkaloids in 50 μl of THF–0.01 *M* ammonium carbonate (1:1). Solvent, 16% THF and 84% 0.01 *M* ammonium carbonate (pH 7.8). Other conditions, and peaks, as in Fig. 1.

6000. The results indicate that peak 2 is retrorsine ($m/e = 351$), peak 3 is seneciphylline ($m/e = 333$) and peak 4 is senecionine ($m/e = 335$). Figs. 3 and 4 illustrate the mass spectra obtained for senecionine and seneciphylline. Fig. 3 shows the molecular ion of senecionine at $m/e = 335$. The loss of a COO group yields an ion at $m/e = 291$ and the eventual loss of the CO–CH_3 group leads to ions at $m/e = 248$ and 246. The ion at $m/e = 291$ has been shown to rearrange to an ion at $m/e = 220$ with loss of CH_3–CH–CO–CH_3. Seneciphylline (Fig. 4) is similar to senecionine with a parent peak at $m/e = 333$ which yields ions at $m/e = 288$ and 289. Retrorsine has a parent peak at $m/e = 351$. According to Bull *et al.*[1], all of the breakdown ions contain one hydrogen exchangeable with ^2H_2O.

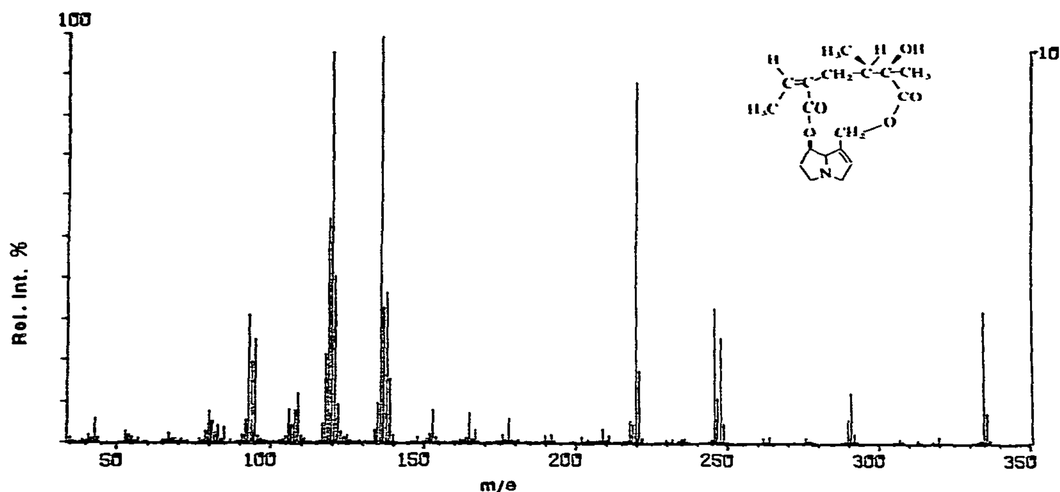

Fig. 3. Mass spectrum of senecionine (70 eV).

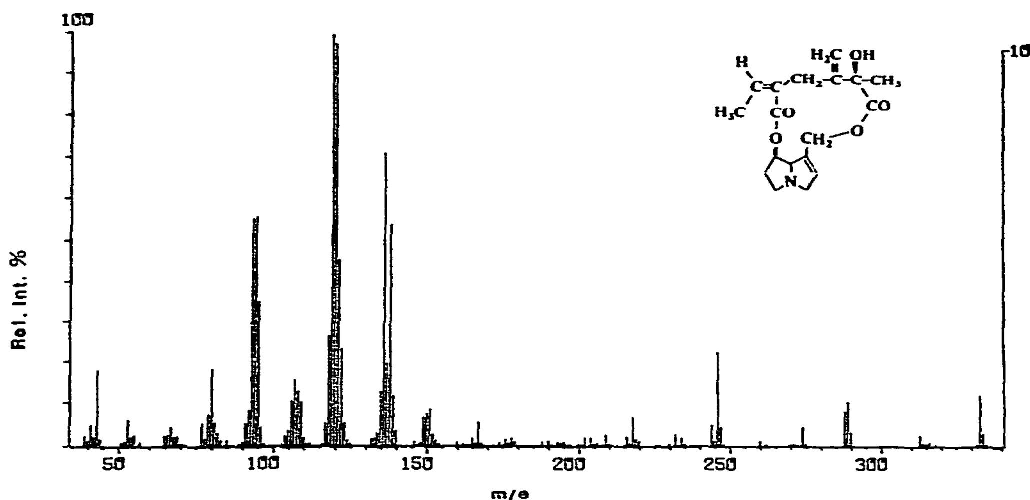

Fig. 4. Mass spectrum of seneciphylline (70 eV).

DISCUSSION

We prefer to use the isocratic method because the Bondapak CN column does not have to be reequilibrated between runs as is required with the gradient system. For alkaloid samples which give a marginal separation on HPLC, the gradient system is preferred as it yields a better separation.

The data presented show that HPLC is a fast and efficient method for the isolation and purification of the pyrrolizidine alkaloids. We feel that the speed, ease of identification and isolation of pure samples makes this method preferable to any procedure found in the literature. This method is especially significant for work requiring highly purified pyrrolizidine alkaloids. An excellent separation of seneciphylline and senecionine was achieved, although these compounds differ by only 2 mass units (333 *vs.* 335). Oddly enough, retrorsine having a molecular weight of 351 preceded both senecionine and seneciphylline.

A Whatman-Partisil-10 PAC column with a cyano-type moiety (CN) bonded to a 10-μm silica gel was utilized under identical conditions as the Waters μBondapak CN column. The Whatman CN column differed from the Waters CN column since it failed to provide adequate separation of the pyrrolizidine alkaloids. It is our opinion that the Whatman CN column is related (polarity-wise) to the Waters NH_2 column, as both are advertised for use with carbohydrates.

Only three pyrrolizidine alkaloids (retrorsine, seneciphylline and senecionine) have previously been reported in the literature[1,10,11]. Our observation that a fourth alkaloid was present resulted from the utilization of HPLC. Our goals at present are two-fold. First, we are adapting our analytical procedures for use on a preparative scale. Secondly, the isolation and identification of peak 1 is in progress. We are quite interested in this first peak as it represents a "new" alkaloid that has not been previously mentioned with *Senecio vulgaris.*

ACKNOWLEDGEMENTS

We are grateful to Dr. W. V. Ligon of General Electric Research and Development, Schenectady, New York for initial GC–MS analysis. We are indebted to Mrs. C. L. Qualls for the illustrations.

REFERENCES

1 L. B. Bull, C. C. J. Culvenor and A. T. Dick, *The Pyrrolizidine Alkaloids*, Wiley, New York, 1968.
2 E. K. McLean, *Pharmaccl. Rev.*, 22 (1970) 429.
3 K. V. F. Jubb and P. C. Kennedy, *Pathology of Domestic Animals*, Vol. 2, Academic Press, New York, 1970, p. 216.
4 J. R. Allen, I. C. Hsu and G. A. Carstens, *Cancer Res.*, 35 (1975) 997.
5 D. J. Svoboda and J. K. Reddy, *Cancer Res.*, 32 (1972) 908.
6 R. C. Shumaker, K. A. Robertson, J. C. Hsu and J. R. Allen, *J. Nat. Cancer Inst.*, 56 (1976) 787.
7 A. M. Clark, *Mutation Res.*, 32 (1976) 361.
8 C. R. Green and G. S. Christie, *Brit. J. Exp. Pathol.*, 42 (1961) 369.
9 Unpublished records of the Veterinary Medical Teaching Hospital, University of California, Davis, 1977.
10 A. Kéry, *Acta Agron. Acad. Sci. Hung.*, 24 (1975) 3.
11 T. Shun, J. Koluch and F. Šantavý, *Collect. Czech. Chem. Commun.*, 25 (1959) 934.