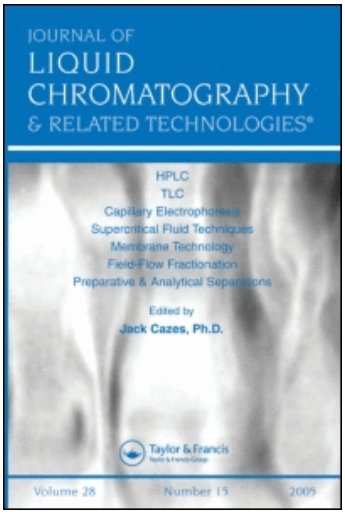

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Reverse Phase Isolation of Pyrrolizidine Alkaloids

H. J. Segall[a]
[a] Department of Physiological Sciences, School of Veterinary Medicine University of California, Davis, California

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REVERSE PHASE ISOLATION OF PYRROLIZIDINE ALKALOIDS

H. J. Segall
Department of Physiological Sciences
School of Veterinary Medicine
University of California
Davis, California 95616

ABSTRACT

A new liquid chromatography method for the isolation of pyrrolizidine alkaloids has been developed. The use of reverse phase high pressure liquid chromatography with a methanol - .01 M KH_2PO_4 (pH 6.3) solvent system is discussed. Pyrrolizidine alkaloids from Senecio vulgaris, S. longilobus, and S. jacobaea have been isolated and identified.

INTRODUCTION

During the last ten years, a growing interest has developed regarding the toxic properties of pyrrolizidine alkaloids. The toxicities which the pyrrolizidine alkaloids possess are of considerable concern to both veterinary and human medicine. These pyrrolizidine-containing plants are found worldwide, and include a variety of botanical families (1).

In a series of articles, Segall and co-workers have determined a practical high pressure liquid chromatography (HPLC) procedure to aid in the purification of pyrrolizidine alkaloids (2-4). This method was based on a THF-.01 M ammonium carbonate (pH 7.8) solvent system in conjunction with a 10 μ C18 Bondapak CN column. This article will discuss the feasibility of using reverse phase HPLC to isolate individual pyrrolizidine alkaloids

derived from Senecio vulgaris, S. longilobus and S. jacobaea. Senecio vulgaris, S. longilobus and S. jacobaea contain similar pyrrolizidine alkaloids, and are toxic to both humans and livestock in the Western U.S.A.

MATERIALS AND METHODS

Senecio vulgaris was handpicked from bales of first-cutting alfalfa near Turlock, California. Senecio longilobus was collected by Dr. A. E. Johnson (Poisonous Plant Research Laboratory, Logan, Utah) near Lovington, New Mexico, and sent to Dr. R. J. Molyneux (WRRC, ARS, Albany, California). Dr. Molyneux kindly provided the author with a white crystalline mixture of pyrrolizidine alkaloids derived from S. longilobus (4). Senecio jacobaea was collected by Dr. Peter R. Cheeke (Department of Animal Science, Oregon State University, Corvallis, Oregon) and sent to the author.

Pyrrolizidine alkaloids from S. vulgaris and S. jacobaea were obtained by refluxing dried plant material with methanol for at least 24 hours (Soxhlet). The methanol was removed under reduced pressure, the extract solublilized with 2 N H_2SO_4, and filtered (Whatman #1). The acid aqueous phase was extracted with mixed hexanes and diethylether to insure removal of chlorophyll and waxes. Excess zinc dust was added to the acid aqueous phase to reduce any N-oxides of the pyrrolizidine alkaloids, and stirred overnight at room temperature. The solution was filtered and the pH adjusted to 9 with ammonium hydroxide. The alkaline solution was extracted with chloroform, washed with water, dried over sodium sulfate and evaporated under reduced pressure. The alkaloids derived from S. vulgaris were recrystallized from ethanol while the alkaloids from S. longilobus were recrystallized from acetone-methanol (4). No attempt was made to recrystallize the pyrrolizidine alkaloids derived from S. jacobaea.

A solvent programming system (Waters Associates) consisting of 2 pumps (Model 6000 A), a solvent programmer (Model 600) and a 10 μ C18 Bondapak Reverse Phase Column (30 cm X 3.9 mm) were utilized. A Schoeffel Spectroflow Monitor (SF 770) was used to monitor at 225 nm, and recorded on a Varian A-25 recorder. Utilizing a flow rate of 1.2 ml/min, both isocratic and linear gradients were run with a methanol - .01 M potassium phosphate (MeOH/.01 M KH_2PO_4) buffer (pH 6.3).

All HPLC peaks were analyzed by Gas Chromatography Mass Spectrometry. A 3% OV-17, 80/100, 4' long column, with an oven temperature of 220°C, was used with an LKB-9000 mass spec plus computer hook-up and printout (Digital PDP-8).

RESULTS

The following separations were achieved using the mixed pyrrolizidine alkaloids of S. vulgaris. Isocratic HPLC conditions of 50% MeOH - 50% .01 M KH_2PO_4 (pH 6.3), 55% MeOH - 45% .01 M KH_2PO_4 (pH 6.3), and 60% MeOH - 40% .01 M KH_2PO_4 (pH 6.3) are illustrated in Figure 1 and Figure 2a and 2b. The initial peak was a solvent peak, while the remaining peaks represent pyrrolizidine alkaloids. The initial shoulder of Peak #1 (illustrated in Figures 1 and 2a) remains unidentified and will be discussed in a future paper. Peak 1 is retrorsine with a mass to charge ratio (m/e) of 351, Peak 2 is seneciphylline (m/e 333) and Peak 3 is senecionine (m/e 335).

The mixed pyrrolizidine alkaloids from S. longilobus were best separated under the following conditions. Utilizing a flow rate of 1.2 ml/min, a convex gradient (#7) of MeOH - .01 M KH_2PO_4 (pH 6.3) was run from 50% MeOH to 85% MeOH over a 25 minute period (Figure 3). Peak 1 is ridelline (m/e 349), Peak 2 is retrorsine (m/e 351), Peak 3 is unidentified, Peak 4 is seneciphylline (m/e 333), Peak 5 is probably senkirkine (refer to

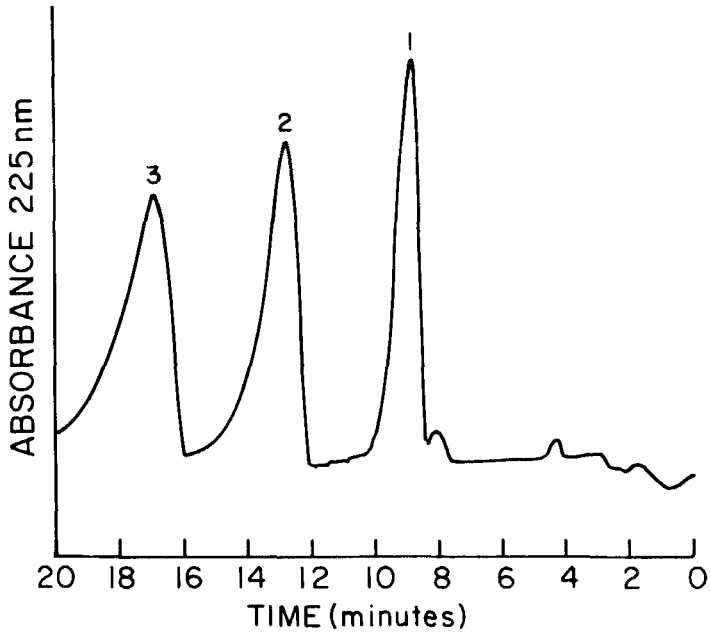

FIGURE 1

Isocratic analysis of pyrrolizidine alkaloids derived from Senecio vulgaris. A 50% MeOH - 50% KH_2PO_4 (pH 6.3) solvent system was utilized with a 300 mm X 3.9 mm 10 µ C18 Reverse Phase plus flow rate of 1.2 ml/min, injected 10 µl, stock solution - .0016 g dissolved in 200 µl methanol. Peak #1 = Retrorsine, Peak #2 = Seneciphylline, Peak #3 = Senecionine.

Discussion Section), Peak 6 is senecionine (m/e 335) and Peak 7 is unidentified.

A mixture of pyrrolizidine alkaloids from S. jacobaea was also run using the MeOH - KH_2PO_4 (pH 6.3) solvent system with a flow rate of 1.2 ml/min. A linear gradient was used which increased the MeOH from 45% to 70% over a 20 minute period. Only a few of these peaks have been identified.

DISCUSSION

The MeOH - .01 M KH_2PO_4 (pH 6.3) solvent system plus 10 µ C18 Bondapak Reverse phase column appears to be an excellent

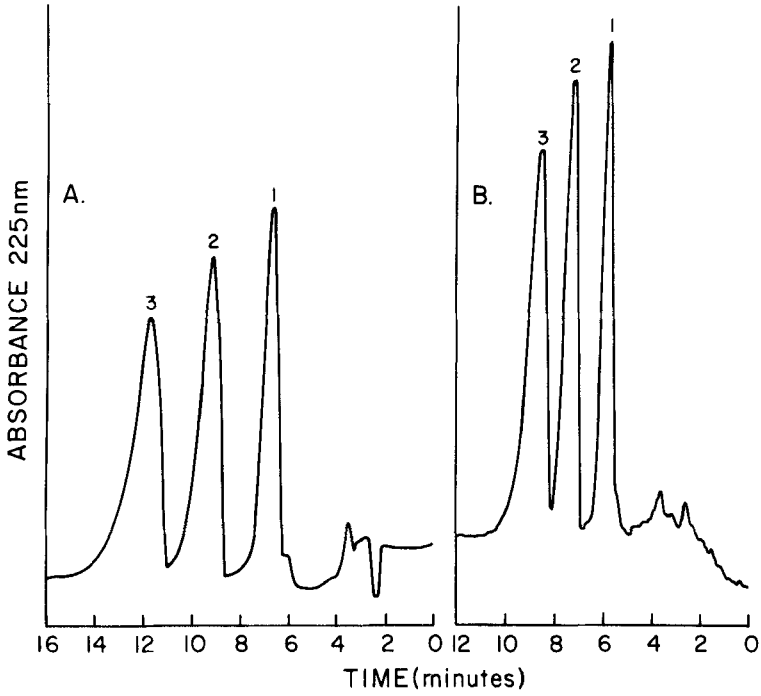

FIGURE 2a

A 55% MeOH - 45% KH_2PO_4 (pH 6.3) isocratic analysis of pyrrolizidine alkaloids derived from Senecio vulgaris. Other conditions, and peaks as in Figure 1.

FIGURE 2b

A 60% MeOH - 40% KH_2PO_4 (pH 6.3) isocratic analysis of pyrrolizidine alkaloids derived from Senecio vulgaris. Other conditions and peaks as in Figure 1.

system for resolving closely related pyrrolizidine alkaloids. In a series of experiments, the pH of the potassium phosphate buffer was tested from a high of 7.8 to 6.3. There appears to be no loss in resolution in using a .01 M KH_2PO_4 buffer at a pH of 6.3.

Any HPLC packing material which is silica based is subject to dissolution over a period of time if it is run in an aqueous solvent. Under alkaline conditions (pH $\geq$ 8), silica gel will

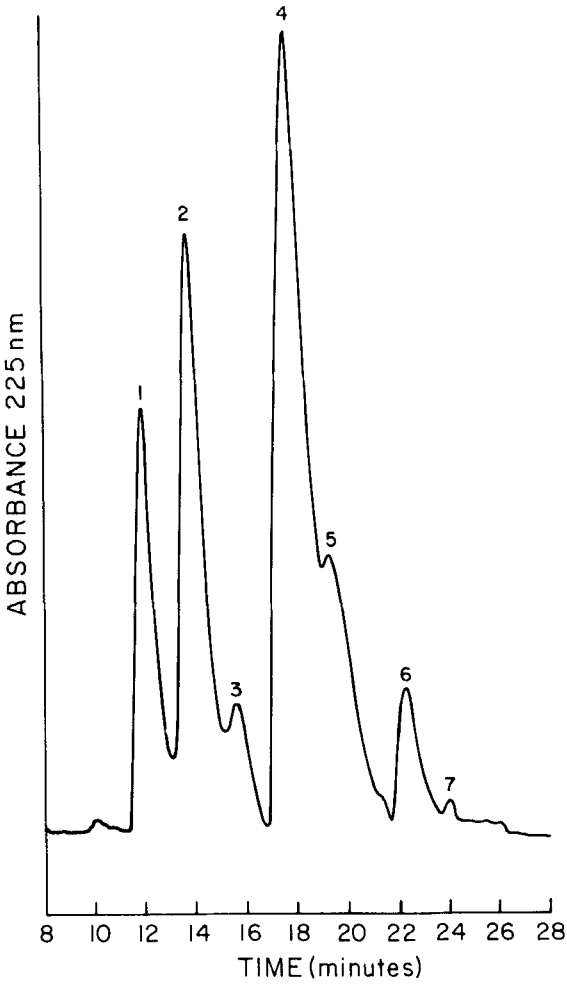

FIGURE 3

A gradient analysis of Senecio longilobus. Methanol was increased from 50-80% over 25 minutes utilizing Waters' convex program #7 (refer to Materials and Methods), injected 3 µl, stock solution - .0027 g dissolved in 200 µl methanol. Peak #1 = Ridelline, Peak #2 = Retrorsine, Peak #3 = Unidentified, Peak #4 = Seneciphylline, Peak #5 = Senkirkine (?), Peak #6 = Senecionine, Peak #7 = Unidentified.

rapidly dissolve (5). Thus, the life expectancy of a reverse phase column will be enhanced by using a buffer which is slightly acidic (.01 M KH_2PO_4 at pH 6.3).

Another advantage of the MeOH-KH_2PO_4 (pH 6.3) solvent system over the THF-.01 M ammonium carbonate solvent system is that pyrrolizidine alkaloids may be monitored at their maximum UV wavelengths. Although UV wavelengths at 225 nm were used in these studies, further trials with 218 nm and 219 nm have proven successful. The strong UV absorption by THF below 230 nm is a definite limiting factor in the identification of pyrrolizidine alkaloids.

The identification of "new" pyrrolizidine alkaloids also is increased with this system. Senecio longilobus, according to Bull et al. (1) is considered to have only 4 pyrrolizidine alkaloids (ridelline, retrorsine, seneciphylline, senecionine). In Figure 2, there are definitely 7 peaks, and it appears that Peak 5 has been identified as senkirkine (6). Future efforts will be made to isolate and identify the additional peaks.

Thus, there appears to be no loss in resolution with mixtures of pyrrolizidine alkaloids from either S. vulgaris or S. longilobus utilizing this solvent system. Identification of pyrrolizidine alkaloids from S. jacobaea with the MeOH - .01 M KH_2PO_4 (pH 6.3) system has not yielded the resolution that the THF-ammonium carbonate system provided (7).

The significance of this work is that a Reverse Phase System, especially one utilizing MeOH, is far simpler and more economical to use than a THF system. Once conditions have been determined with the analytical reverse phase column, it is possible to utilize a preparative system, thus greatly increasing the sample loading capacity. The disadvantage of the preparative unit is that it is not engineered to run gradients. Future papers from my laboratory will discuss the isolation of pyrrolizidine alkaloids utilizing a preparative system, and the characterization of "new" pyrrolizidine alkaloids.

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