Journal of Chromatography, 336 (1984) 211—220
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2297

ISOLATION OF TERATOGENIC ALKALOIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C.A. BROWNE* and F.R. SIM

Department of Physiology, Monash University, Clayton, Victoria 3168 (Australia)

I.D. RAE

Department of Chemistry, Monash University, Clayton, Victoria 3168 (Australia)

and

R.F. KEELER

U.S. Department of Agriculture, Agricultural Research Service, Poisonous Plant Research Laboratory, Logan, UT 84321 (U.S.A.)

SUMMARY

Reversed-phase high-performance liquid chromatography was used for both analytical and preparative separations of several steroidal alkaloids which occur in extracts of *Veratrum californicum*. The inclusion of 0.1% trifluoroacetic acid in the mobile phase improved the efficiency of the chromatography and the solubility of the compounds in aqueous acetonitrile. Nuclear magnetic resonance was used to assist the identification of the isolated steroidal alkaloids. The effect of the interaction of trifluoroacetic acid with the alkaloids could be clearly seen by changes in the chemical shifts in the nuclear magnetic resonance spectra.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been used for the separation of a wide range of chemical compounds. Although there have been many reports of the use of HPLC in the separation of steroids, there have been only four reports to date of the application of HPLC to the separation of steroidal alkaloids. The earliest attempt used normal-phase chromatography on Porasil A, which is a very inefficient, coarse (37–75 μm particle size) support by modern standards, to separate tomatidine, solasodine, veratramine and jervine (Fig. 1) related alkaloids [1]. Surprisingly good separations were obtained by using gradients from acetone—hexane (2:1) to 97% aqueous acetone

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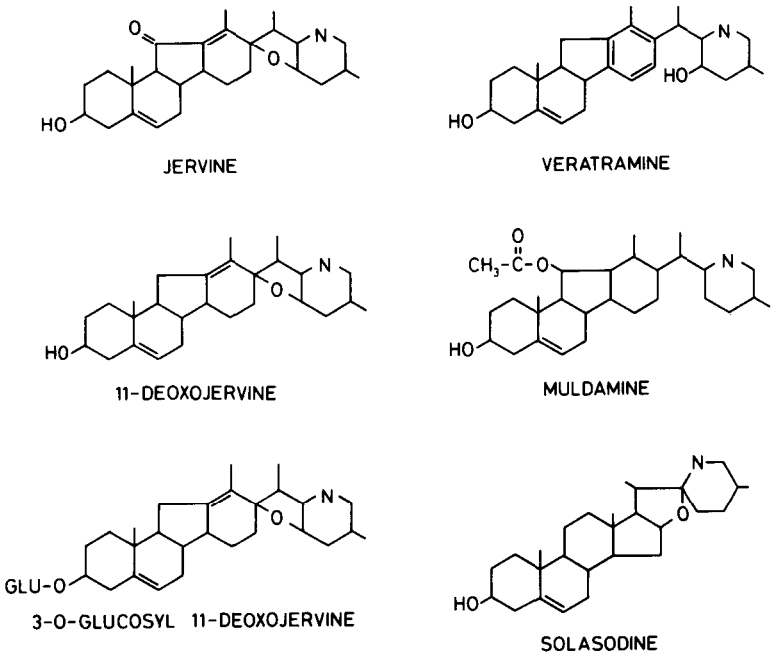


Fig. 1. Structural formulae of steroidal alkaloids.

and thin-layer chromatography as the mode of detection. Hunter et al. [1] used a 4.8 m long column of this material to prepare 1 g of tomatidine by isocratic elution. Normal-phase chromatography of these compounds was subsequently improved by Hunter et al. [2] by changing to a Zorbax-Sil (6 μm particle size) support. The first reversed-phase HPLC separation was reported for the related glycoalkaloids, α-chaconine, β-chaconine and α-solanine [3]. The alkaloids were eluted isocratically from a C_{18} μBondapak column with tetrahydrofuran—water—acetonitrile (50:30:20). A more recent report of the separation of solasodine from solasodine glycosides also used a C_{18} μBondapak column, this time with a methanol—Tris buffer, pH 7 isocratic elution system [4].

We decided to develop a method for the purification of steroidal alkaloids from extracts of the roots of the plant *Veratrum californicum*. Crude extracts of the plant roots had been demonstrated to have teratogenic activity in a number of animals, the major defect being holoprosencephaly which sometimes results in cyclopia [5]. Hence the activity was referred to as "crude cyclopamine". Three alkaloidal steroids, jervine, 11-deoxojervine and glycoside of 11-deoxojervine, all found in the crude root extract, were found to have teratogenic activity [6]. We wish to obtain the main component, 11-deoxojervine in a pure form to study its mechanism of action [7].

EXPERIMENTAL

The chromatography equipment consisted of two Waters Assoc. Model 6000A solvent delivery systems controlled by a Model 660 solvent programmer and a Waters U6J injector. Peak detection was achieved by ultraviolet (UV)

absorbance at 254 nm using a 441 nm fixed-wavelength detector or at lower wavelengths with a 481-nm variable-wavelength detector (Waters Assoc.). Flow-rate was 1.0 ml/min throughout and all separations were performed at 22°C. The following columns were tested: three 30 cm × 3.9 mm C_{18} μBondapak (10 μm particle size) from Waters Associates, Carlton, Australia; two 25 cm × 4.6 mm ODS Ultrasphere (5 μm particle size) from Beckman, Notting Hill, Australia; one 7.5 cm × 4.6 mm RPSC column (5 μm particle size) from Beckman. Acetonitrile and methanol were both HPLC grade (Waters Assoc.), trifluoroacetic acid (TFA) was purchased from BDH and was purified prior to use by making up a 1% (v/v) aqueous solution and passing it through a C_{18} Sep-Pak (Waters Assoc.) [8]. Glass-distilled water was further purified by a Milli-Q system (Millipore, Carlton, Australia) consisting of one charcoal, two ion-exchange and one Organics cartridge and a 0.45-μm Millistack filter. Precise chromatographic conditions are given in the relevant figure legends and in the text. The measured pH of the mobile phase was between 1.79 for 20% acetonitrile—0.1% TFA to 1.92 for 60% acetonitrile—0.1% TFA. Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker 90 90-MHz spectrometer (Selby Scientific, Glen Waverley, Australia). Proton magnetic resonance data were obtained in the pulse/Fourier transform mode. NMR spectra were obtained at 300°K on steroidal alkaloids dissolved in C^2HCl_3 containing tetramethylsilane (TMS) as internal standard. Sweep width was 1202 Hz, with either 4 K or 8 K real-data points collected to give a digital resolution of 0.34 or 0.17 Hz per point. Crude cyclopamine was prepared by benzene extraction of the roots of *Veratrum californicum* which were harvested in Idaho, U.S.A. The crude extract was partially purified by liquid partition through aqueous acid and chloroform and fractional crystallization as described previously [6, 9]. All other steroidal alkaloids were prepared as described previously [5, 6, 9].

RESULTS

Initial attempts to chromatograph veratramine, jervine and 11-deoxojervine by reversed-phase HPLC were hampered by two major problems, low solubility of 11-deoxojervine and jervine in water—methanol or water—acetonitrile or water—methanol—acetonitrile mixtures, and the peaks obtained for the three compounds on all three columns were asymmetric, with severe tailing. Crabbe and Fryer [4] had tried to overcome a similar problem using a methanol—Tris buffer, pH 7.0–7.5 mobile phase. This is a non-volatile solvent system, which still does not give efficient chromatography and narrow symmetrical peaks for the non-conjugated steroidal alkaloids. We found that a water—acetonitrile—0.1% TFA mobile phase solved both problems. All three alkaloids chromatographed on all three reversed-phase columns with greatly improved peak shapes. The C_{18} μBondapak column proved to be the most efficient, typically showing 8000–10,000 plates per metre for jervine and 11-deoxojervine (Fig. 2). The ODS Ultrasphere columns were less efficient, (2000–3000 plates per metre) and the peak shapes for all three test compounds showed significant tailing. This result was found on two different ODS Ultrasphere columns and three different C_{18} μBondapak columns. All columns were

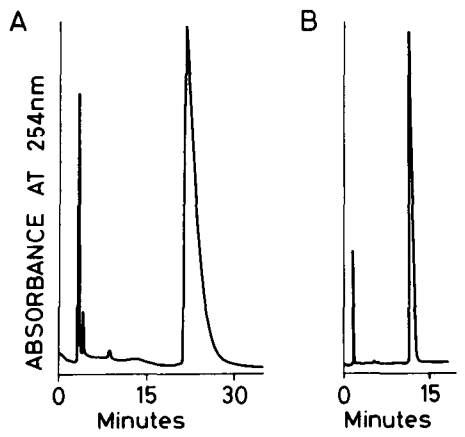


Fig. 2. Isocratic reversed-phase HPLC separation of jervine (A) without and (B) with 0.1% TFA in the mobile phase. A C_{18} μBondapak column was used for both chromatograms. (A) The mobile phase was acetonitrile—water (36:64); 20 μg of jervine were injected in 50 μl of the mobile phase; detector sensitivity was 0.05 a.u.f.s. (B) The mobile phase was acetonitrile—water—TFA (40:59.9:0.1); 20 μg of jervine were injected in 50 μl of the mobile phase; detector sensitivity was 0.1 a.u.f.s.

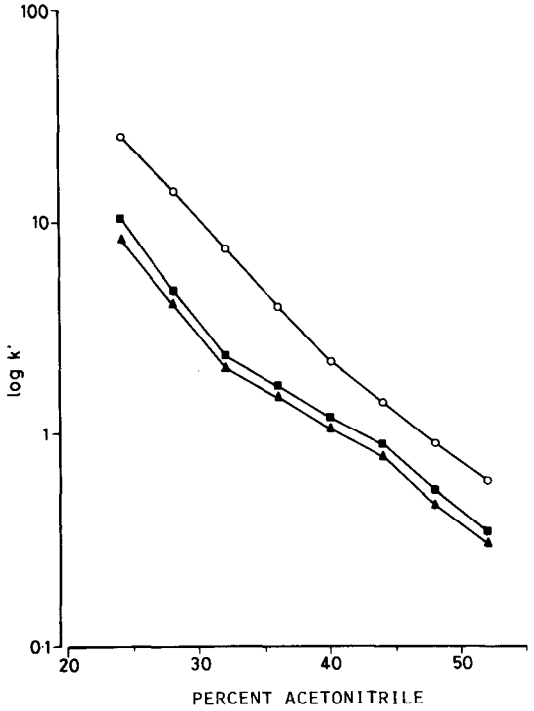
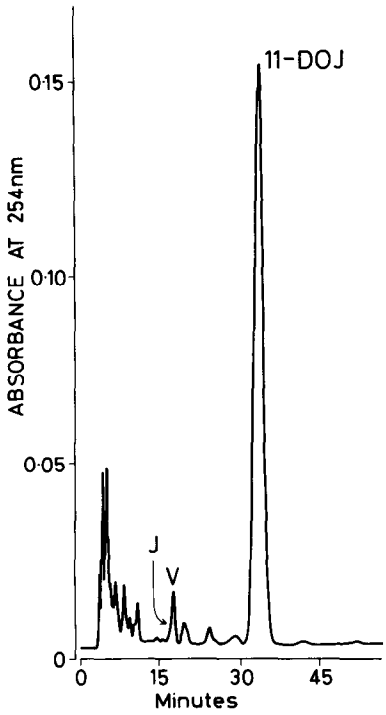


Fig. 3. Plot of log k'—acetonitrile concentration for jervine (▲), 11-deoxojervine (○) and veratramine (■). All data were obtained using the chromatographic conditions described for Figs. 4 and 5. TFA concentration was 0.1% throughout.

tested for efficiency using a standard uracil–acenaphthalene mixture, supplied with one of the C_{18} μBondapak columns by Waters Assoc. and an acetonitrile–water elution system (60:40, v/v, for the C_{18} μBondapak and 50:50, v/v, for the ODS Ultrasphere). Average efficiency for acenaphthalene on the C_{18} μBondapak columns was 16,500 plates per metre and on the ODS Ultrasphere columns was 33,800 plates per metre, under comparable conditions (flow-rate 1.0 ml/min, sample volume 10 μl, k' between 3 and 6). Isocratic elution of the steroidal alkaloids with mixtures of between acetonitrile–water–TFA (28:71.9:0.1) and acetonitrile–water–TFA (40:59.9:0.1) gave good resolution of veratramine and jervine from 11-deoxojervine (Fig. 3). Although 11-deoxojervine was only sparingly soluble (<0.1 mg/ml) in acetonitrile–water–TFA (80:19.9:0.1) and in water–TFA (99.9:0.1), it was found to be much more soluble (5–10 mg/ml) in the range acetonitrile–water–TFA (20:79.9:0.1) to acetonitrile–water–TFA (65:34.9:0.1). The reason for this effect is probably due in part to the TFA forming an ion pair with the cyclic amine function.

Two different batches of crude cyclopamine were chromatographed isocratically in the acetonitrile–water–TFA system (Figs. 4 and 5). Although



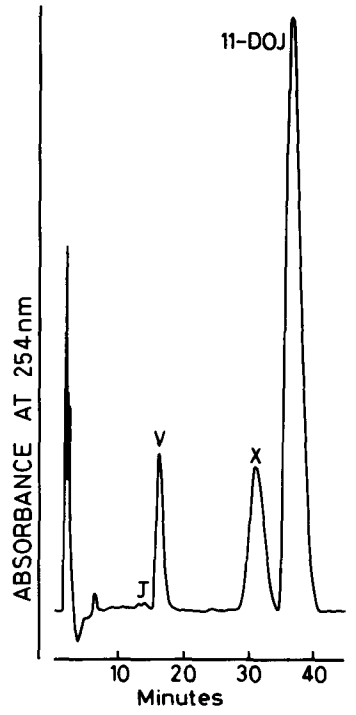


Fig. 4. Isocratic reversed-phase HPLC separation of crude cyclopamine "1981 batch". Crude cyclopamine (100 μg) was injected in 50 μl of the mobile phase, acetonitrile–water–TFA (32:67.9:0.1) onto a C_{18} μBondapak column. Detector sensitivity was 0.2 a.u.f.s. Peaks: J = jervine; V = veratramine; 11-DOJ = 11-deoxojervine.

Fig. 5. Isocratic reversed-phase HPLC separation of crude cyclopamine "1982 batch". All conditions were as described for Fig. 4, except that the mobile phase was acetonitrile–water–TFA (28:71.9:0.1). Peaks: J = jervine; V = veratramine; X = unknown; 11-DOJ = 11-deoxojervine.

the major peak of the UV-absorbing material corresponded in elution position to 11-deoxojervine in both preparations, it was clear that the pattern of the minor peaks in the two extracts was different. Each pattern was always highly reproducible and there was no evidence for degradation. The reason for this is not clear, but it may be due to seasonal variation in the composition of the roots, as one extract was made twelve months before the other. The nature of the contaminant peak X (Fig. 5) is not known. It is important to note that different isocratic conditions were used for each extract. This was to produce the optimal resolution of the 11-deoxojervine from the various minor peaks. The glucoside of jervine was not retained on the column under either of the chromatographic conditions (Figs. 4 and 5). Muldamine and solasodine were both greatly retained on the column under these conditions ($k' > 100$) and were recovered by elution with 100% methanol or 100% acetonitrile. Large-scale (50--100 mg) preparations were successfully completed by trace enrichment of a dilute solution (1--2 mg/ml) of the crude cyclopamine in acetonitrile—water—TFA (20:79.9:0.1) onto a C_{18} μBondapak column, followed by isocratic elution at acetonitrile--water—TFA (32:67.9:0.1). Gradient elution of the steroidal alkaloids was also examined. An example is shown in Fig. 6 where 3-O-acetyl jervine, which was prepared from jervine by chemical synthesis [10], was eluted in a pure form by a acetonitrile gradient. One of the contaminants

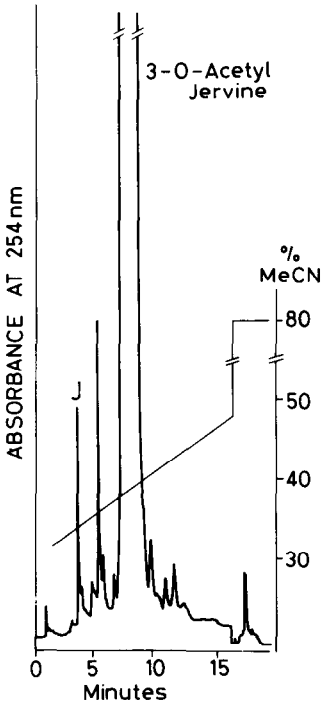


Fig. 6. Gradient chromatography of 3-O-acetyl jervine. A 450-μg amount of 3-O-acetyl jervine was injected in 450 μl of acetonitrile—water—TFA (32:67.9:0.1) onto a C_{18} μBondapak column equilibrated in the same mobile phase. The chromatogram was developed by a linear gradient from 32% to 48% acetonitrile containing 0.1% TFA throughout, over 15 min at a flow-rate of 1 ml/min. Detector sensitivity was 0.1 a.u.f.s. Peak: J = jervine.

was clearly free jervine. The identity of the 3-O-acetyl jervine was confirmed by NMR. Fig. 6 also demonstrates the efficiency of the chromatography at a moderate (450 μg) sample loading. 3-O-Acetyl jervine is of importance as it represents the only radiolabelled form of jervine (3-O[^{14}C]acetyl jervine) currently available.

In order to ensure that the material contained in the peaks obtained from the chromatograph was the desired compound, infrared and NMR spectra were run on the crude and purified alkaloids. The NMR spectra of crude cyclopamine, veratramine, and purified 11-deoxojervine are shown in Fig. 7A, B and C. The crude cyclopamine clearly had typical aromatic C—H resonances (Fig. 7A), around 7 ppm, which were similar to those seen in the NMR spectrum of

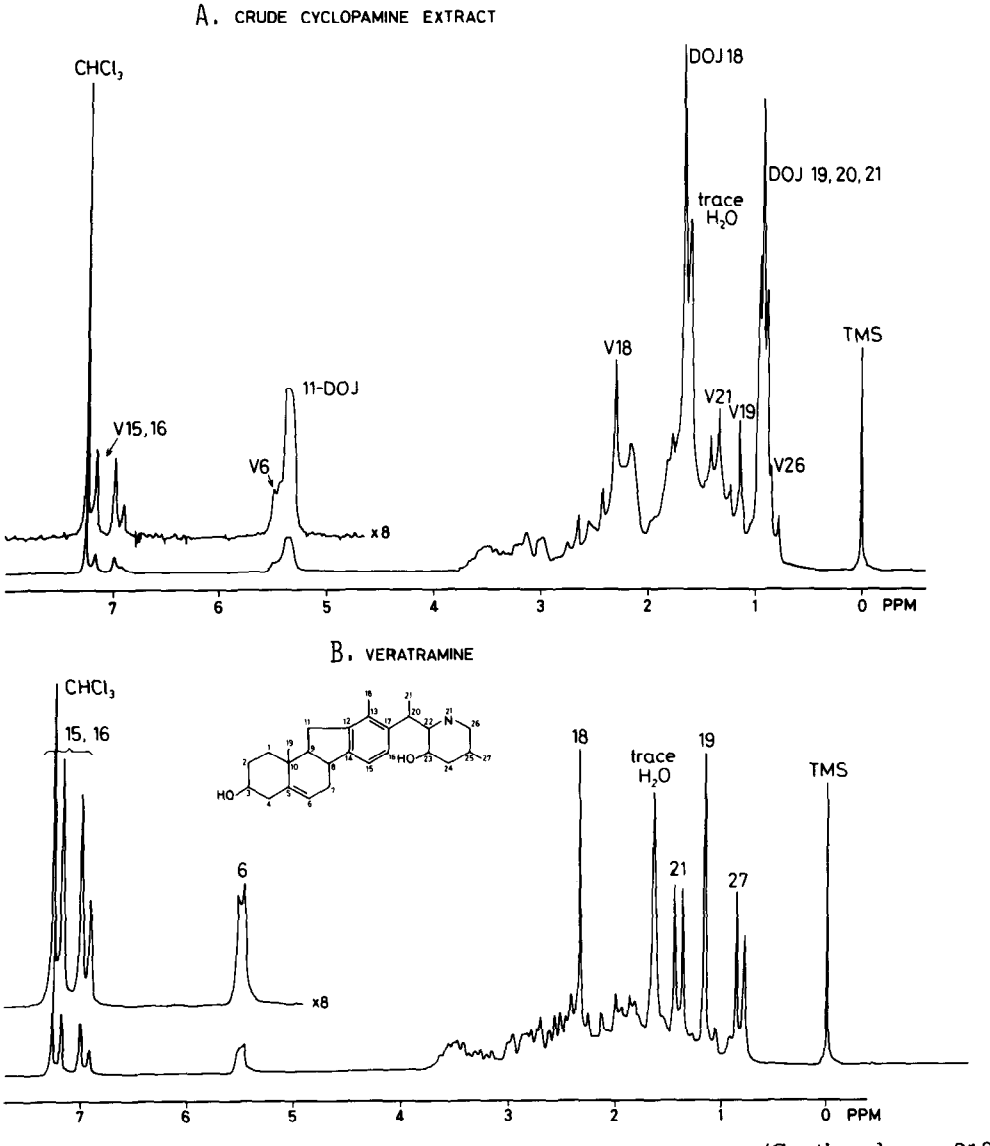


Fig. 7.

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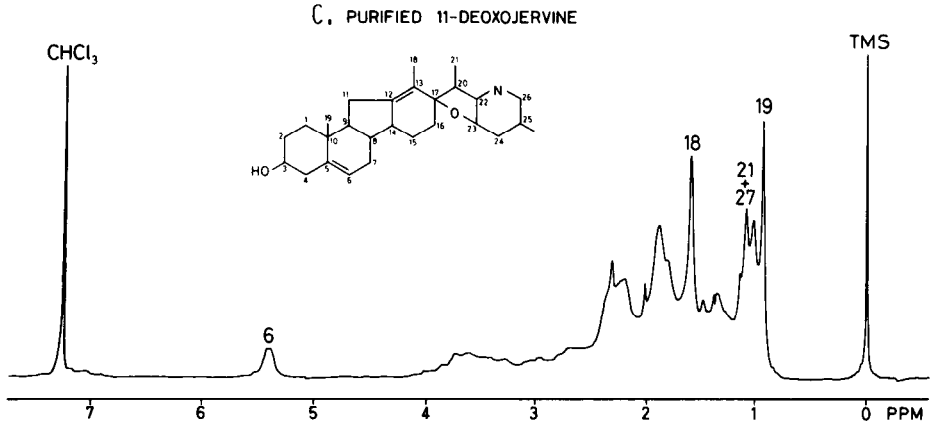

Fig. 7. Proton NMR spectra of (A) crude cyclopamine (B) veratramine and (C) reversed-phase HPLC purified 11-deoxojervine. Resonances in A which are marked with a V are due to contamination by veratramine or a closely related compound. The jervine numbering system of Brown [11] has been adopted here. Tentative assignments of the major resonances have been indicated.

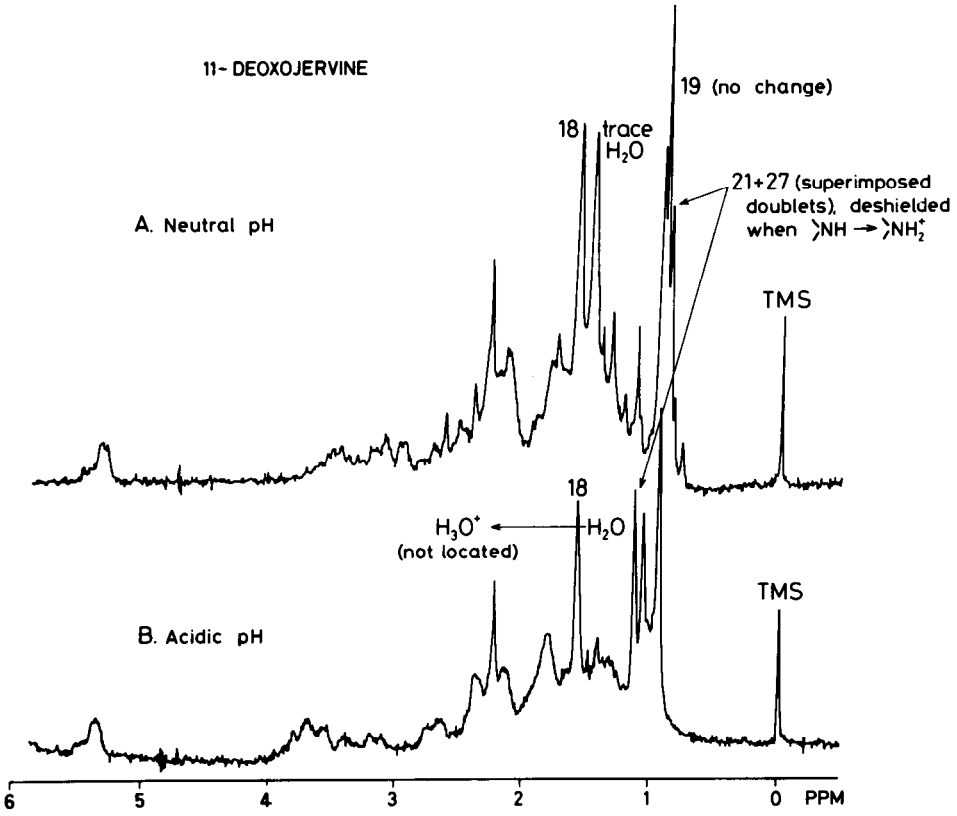

Fig. 8. Proton magnetic resonance spectra of crude cyclopamine (A) at neutral pH and (B) after acidification with 0.1% TFA. Note the pair of doublets at about 1 ppm which are dishielded by 0.176 ppm after acidification. The disappearance of the "trace H_2O" resonance in (A) is due to protonation of the H_2O, which results in a broader H_3O^+ resonance at lower field.

veratramine (Fig. 7B). The purified 11-deoxojervine no longer had the aromatic resonances. The major resonances for the veratramine and 11-deoxojervine NMR spectra were tentatively assigned from the chemical shifts and J constants. The jervine numbering system of Brown [11] has been used. There was an obvious loss of the veratramine resonances (labelled V) when the crude cyclopamine was purified to yield pure 11-deoxojervine (Fig. 7). Another change was noted in the 11-deoxojervine spectra at around 1 ppm (see Fig. 7A and C). The pair of doublet resonances owing to the C_{21} and C_{27} methyl groups had shifted downfield in the purified 11-deoxojervine compared to the crude cyclopamine. This change could be easily explained when the NMR spectra of cyclopamine before and after acidification with TFA were examined (Fig. 8). Clearly acidification had caused the shift in C_{21} and C_{27} methyl resonances. We also examined the NMR spectra of jervine, 3-O-acetyl jervine, solasodine and muldamine [12] to assist with the assignments shown in Figs. 7 and 8.

DISCUSSION

We have successfully used reversed-phase HPLC to separate steroidal alkaloids with reasonable efficiency. The inclusion of 0.1% TFA in the mobile phase gave improved peak shapes and excellent recovery of material. We now routinely use isocratic reversed-phase HPLC (Figs. 4 and 5) to prepare pure 11-deoxojervine for our biological experiments, although it is clear that gradient chromatography can also be useful in the isolation of jervine and jervine derivatives (Fig. 6). One extremely useful result of our approach has been to dramatically improve the solubility of the crude cyclopamine. Traditionally this has always been a problem in any attempt to use crude cyclopamine in aqueous physiological environment. It has routinely been administered to experimental animals as a suspension with a solid carrier such as carboxymethyl cellulose [13]. After purification of the 11-deoxojervine by reversed-phase HPLC, we concluded that the basic nitrogen atom has a TFA anion complexed to it. This is supported by the chemical shift changes shown for the C_{21} and C_{27} methyl resonances (Fig. 8). We routinely exchange this TFA ion for chloride by diluting the eluate containing 11-deoxojervine 1:1 with 0.2% (v/v) hydrochloric acid and loading the purified 11-deoxojervine into a C_{18} Sep-Pak [8]. After washing with 20 ml of aqueous 0.2% hydrochloric acid, we elute the alkaloid with 5 ml of 60% acetonitrile containing 0.2% (v/v) hydrochloric acid. This solution is diluted 1:1 with 0.2% (v/v) hydrochloric acid and is lyophilized. By this means, we obtain the pure 11-deoxojervine as a dry chloride salt which is then readily soluble in water (up to 2 mg/ml). Previous difficulties in solubility of the crude cyclopamine preparation must be put down in part to the presence of insoluble impurities, and in part to difficulty in forming the salt. In our hands, prior treatment with 0.1% TFA increased the solubility of 11-deoxojervine in 0.2% hydrochloric acid.

NMR spectroscopy was a very useful tool for the identification of the eluted alkaloids. As a general rule in HPLC, an alternative method of peak identification, other than elution volume, is mandatory. In the absence of a specific biological or immunological assay, spectroscopy seemed a reasonable

approach. Infrared spectra were generally found to be of a limited use (data not shown) although the presence of the carbonyl group in jervine gave a peak in the infrared spectrum at 1700 cm^{-1} which clearly distinguished jervine from 11-deoxojervine. We were able to obtain recognisable NMR spectra with amounts as low as 1.5 mg of steroidal alkaloid, although the spectra were much improved if 10 mg were used. The availability of a more powerful spectrometer (300 MHz) will improve the sensitivity of "detection" of the steroidal alkaloids eluted from reversed-phase HPLC by at least five-fold.

It can be seen that complete resolution was obtained between jervine and 11-deoxojervine, but that rather incomplete resolution could be obtained between jervine and veratramine (Figs. 3–5). We have found that a methanol–water–TFA mobile phase in the range 40–50% methanol will give complete resolution of jervine and veratramine [14] using isocratic elution and a C_{18} μBondapak column. The glucoside of jervine elutes just after the injection artefact in both the acetonitrile–water–TFA system and the methanol–water–TFA system. A ternary elution system comprising of aqueous TFA—methanol–acetonitrile may turn out to be the optimal isocratic system. Further studies will pursue this possibility.

ACKNOWLEDGEMENTS

The authors wish to thank Professor G.D. Thorburn for his encouragement and support. This work was supported by a Monash University Special Research Grant.

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