Note

Comparison of capillary gas chromatography with ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy for the quantitation of pyrrolizidine alkaloids from *Senecio vernalis*

L. A. PIETERS*

Department of Pharmaceutical Sciences, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Antwerp (Belgium)

T. HARTMANN

Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, Mendelssohnstrasse 1, D-3300 Braunschweig (F.R.G.)

and

J. JANSSENS and A. J. VLIETINCK

Department of Pharmaceutical Sciences, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Antwerp (Belgium)

(First received May 11th, 1988; revised manuscript received September 27th, 1988)

Plants that contain hepatotoxic pyrrolizidine alkaloids occur all over the world. The pyrrolizidines may be of greater importance as a cause of human disease than the presently known outbreaks of poisoning would indicate. Poisoning in man can occur by the use of pyrrolizidine-containing plants as medicinal herbs, or by accidental contamination of food by such plants[1–3]. Therefore reliable and accurate methods for the quantitation of pyrrolizidine alkaloids are necessary. A titrimetric procedure[4] and a spectrophotometric method based on a colour reaction[5,6] have been described for the estimation of the total pyrrolizidine alkaloid level in biological samples. In recent years these methods have been replaced in many cases by quantitative ^{1}H nuclear magnetic resonance (NMR) spectroscopy[7–10]. Several analytical techniques have been described for the determination of individual pyrrolizidines in mixtures, including relative densitometric estimations, which are not generally applicable because the differences in R_F values may be quite small[4,11], high-performance liquid chromatography (HPLC)[12] and, more recently, quantitative ^{13}C NMR spectroscopy[9,10,13]. In this work, ^{1}H NMR was compared with gas chromatography (GC) for the determination of the total pyrrolizidine alkaloid level, and for the quantitative analysis of the individual pyrrolizidines ^{13}C NMR was compared with GC. *Senecio vernalis* Waldst. & Kit. (Asteraceae) was chosen as the pyrrolizidine-containing plant. The pyrrolizidine alkaloids seneciphylline, senecionine, integerrimine, retrorsine, senecivernine and senkirkine have been reported as constituents of *Senecio vernalis*[14–16]. With the exception of senkirkine, they are synthesized and accumulated as N-oxides[16,17]. In this study the alkaloid N-oxides were reduced during sample preparation to give the respective tertiary alkaloids.

EXPERIMENTAL

A voucher specimen of *Senecio vernalis* Waldst. & Kit. is kept at the Institut für Pharmazeutische Biologie, Braunschweig. Samples were prepared from freeze-dried flower heads of *Senecio vernalis* according to a known procedure[8].

^{1}H and ^{13}C NMR

A Jeol FX 200 Fourier transform (FT) NMR system, exhibiting standard resonance frequencies of 199.50 MHz for ^{1}H and 50.10 MHz for ^{13}C, was used. Samples were dissolved in [^{2}H] chloroform (99.8%). Chemical shift values are reported on the δ scale, relative to tetramethylsilane. ^{1}H and ^{13}C NMR recording conditions for quantitative measurements were selected[8,10,13]. To perform quantitative ^{1}H NMR experiments, a precisely weighed amount of *p*-dinitrobenzene was added to the sample as an internal standard.

Gas chromatography–mass spectroscopy (GC–MS)

All GC–MS analyses were performed on an Hewlett-Packard 5890 A GC-5970 B quadrupole mass spectrometer, equipped with an Hewlett-Packard 12 m × 0.203 mm cross-linked methyl silicone fused-silica capillary column and a split/splitless injection system, used in the split mode (20:1). The initial oven temperature was 150°C, and programmed to 275°C at 10°C/min (2-min solvent wait, isothermal). Helium was used as the carrier gas at a flow-rate of 1 ml/min. The column was inserted directly into the mass spectrometer. Data acquisition and reprocessing were carried out on an Hewlett-Packard 9816 Workstation with a 15 MB Winchester disc. Electron impact (EI) (70 eV) mass spectra were recorded, scanning continuously from m/z 50 to 500. Retention times: senecivernine, 12.3 min (m/z 335, [M]$^+$); senecionine, 12.4 min (m/z 335, [M]$^+$); seneciphylline, 12.6 min (m/z 333, [M]$^+$); integerrimine, 12.9 min (m/z 365, [M]$^+$); retrorsine, 14.3 min (m/z 351, [M]$^+$).

Quantitative analysis by capillary GC

The mixture of tertiary alkaloids prepared according to ref. 8 was dissolved in methanol and was separated and evalutated quantitatively on wall-coated open tubular fused-silica columns (15 m × 0.25 mm; DB-1, J & W Scientific). Conditions: injector, 250°C; temperature programme, 120–290°C, 6°C/min; splitting ratio 1:50; injection volume 1–2 μl; carrier gas, helium 0.7 bar; detectors, flame ionization detector, nitrogen selective detector. Senecionine or monocrotaline, obtained from Aldrich, was used as the external standard. The retention indices of the individual alkaloids are given in ref. 17.

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of a mixture of pyrrolizidine alkaloids from *Senecio vernalis* is very complex, and contains many overlapping signals. The vinylic C-2 hydrogen of macrocyclic diester pyrrolizidines, such as the alkaloids from *Senecio vernalis*, always resonates at 6.2 ppm. A known amount of *p*-dinitrobenzene is added to the sample as an internal standard, and the total pyrrolizidine alkaloid level is calculated by comparing the integration value of this signal, occurring at 8.43 ppm,

with the integration value of the signal at 6.2 ppm. The pyrrolizidine alkaloid content was calculated as senecionine (molecular weight 335), which appeared to be the principal alkaloid in our samples. The molecular weight of senecivernine and integerrimine are also 335. Because of the complexity of the ^{1}H NMR spectrum, it is not possible to estimate the individual alkaloids[7,8].

^{13}C NMR spectroscopy was used for the quantitation of the individual pyrrolizidine alkaloids. ^{13}C NMR spectral data for all the pyrrolizidines from *Senecio vernalis* have already been published[10,14]. The ^{13}C NMR signals of pyrrolizidine alkaloids are very sensitive to structural variation in both the diester moiety and the heterocyclic ring system. The usefulness of ^{13}C Fourier transform NMR as an analytical technique stems from the potentially direct relationship between the area under a NMR peak and the number of nuclei that give rise to the signal. Unfortunately, extracting the desired quantitative information from a ^{13}C NMR spectrum is hampered by several experimental and instrumental limitations, which have been discussed[10,13]. In addition, only carbon atoms having a relatively short spin–latice relaxation time, T_1, can be used. A list of ^{13}C resonance signals that are well resolved, that are specific for one individual alkaloid or for the total alkaloid content and that have a $T_1 < 1.5$ s is given in Table I. Because the minimal pulse delay for quantitative measurements using ^{13}C NMR is 6.5 T_1, a T_1 of 1.5 s corresponds to a pulse delay of 10 s, which is still an acceptable value for long accumulations.

Some specific and well resolved resonance signals, *e.g.*, the C-8 carbonyl signal of senkirkine, cannot be used for quantitative ^{13}C NMR measurements because their relaxation is very slow. More information about these relaxation times and related problems can be found in refs. 9, 10 and 13. By integrating the resonance signals listed in Table I, it is possible to obtain an integration value for each individual alkaloid and for the total alkaloid content.

TABLE I

SOME CHARACTERISTIC ^{13}C NMR SIGNALS USEFUL FOR THE QUANTITATIVE ANALYSIS OF MIXTURES OF PYRROLIZIDINE ALKALOIDS FROM *SENECIO VERNALIS*

Carbon No.*	Signal (ppm)	Alkaloid
20	120.3	Senecivernine
19	113.9	Seneciphylline
9	63.7	Senkirkine
3	58.3	Senkirkine
5	52.7–52.9	Total
13	39.3	Integerrimine
14	37.4	Senkirkine
6	36.0	Senkirkine
6	33.5	Integerrimine
14	29.4	Integerrimine
18	25.9	Senecivernine
18	25.0	Integerrimine
9	11.6	Integerrimine
19	10.8	Senecionine
19	5.4	Senecivernine

* Numbering of the carbon atoms of the pyrrolizidine alkaloids (senecane structure) according to ref. 18.

TABLE II

QUANTITATIVE EVALUATION OF THE ALKALOID CONTENT AND PATTERN OF *S. VER-NALIS* BY ¹H AND ¹³C NMR IN COMPARISON TO CAPILLARY GC

Total alkaloid level (%, dry weight)			Individual alkaloids (%)	
¹H NMR	GC		¹³C NMR	GC
0.54	0.54	Senecionine	38.0	44.0
		Integerrimine	6.2	5.9
		Senecivernine	33.3	28.2
		Senkirkine	10.6	11.3
		Seneciphylline	9.6	10.6
		Retrorsine	2.3	Traces

In order to obtain quantitative results, the same procedure as for *Senecio vulgaris* and *Senecio jacobaea*[9,10,13], was used. The results of the quantitative ¹H and ¹³C NMR analyses were compared with those obtained with GC (Table II). The results for the total alkaloid level are in good agreement with each other. Both methods produce reliable results, but the sensitivity of GC is higher. As far as the individual alkaloids are concerned, there is a difference for senecionine and senecivernine. This may be due to the fact that their retention times hardly differ, and that the signals may not be completely resolved. There is a good agreement for the sum of the results for senecionine and senecivernine. The results for the other alkaloids are in good agreement with each other.

It appears that the two methods are equivalent for the quantitative analysis of mixtures of pyrrolizidine alkaloids. Some characteristics of GC and quantitative ¹³C NMR are summarized in Table III. The main disadvantage of ¹³C NMR when compared with GC is that the sensitivity is rather low. The minimum sample size for ¹³C NMR is about 10 mg, and a long accumulation time is necessary. A quantitative GC analysis is less time-consuming, but care must be taken that no overlapping of

TABLE III

COMPARISON OF GC AND ¹³C NMR FOR THE QUANTITATION OF PYRROLIZIDINE ALKALOIDS

Characteristic	GC	¹³C NMR
Reliability	Good	Good
Precision	Good	Good
Sensitivity	High	Low
Interferences (impurities)	Good	Good
Manipulation time	Short	Short
Blocking of apparatus	Slight	Important
Adaption to other assays	Easy	Easy
Apparatus cost price	High	High
Reagent cost price	Low	High
Personnel qualifications	Normal	High

peaks occurs. In order to perform a quantitative ^{13}C NMR experiment, it is essential that the ^{13}C NMR spectral data and the most important relaxation times of all the components of the mixture are known.

The detection and identification of an unexpected or new alkaloid in a mixture by ^{1}H and ^{13}C NMR may be rather difficult, in particular if it is only a minor component. If all the signals are completely resolved, a GC–MS experiment would show a new peak providing additional information (retention time, mass spectral data) for identification purposes. The same techniques can also be applied for other plants containing pyrrolizidine alkaloids.

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