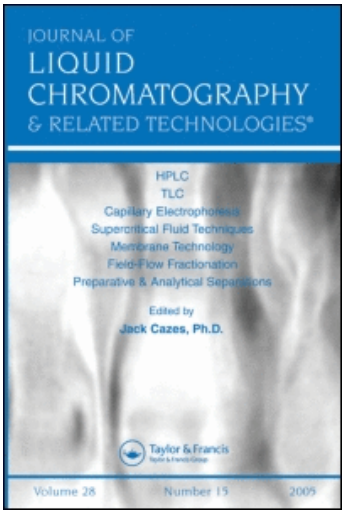

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Comparison of High Performance Liquid Chromatography with ^1H Nuclear Magnetic Resonance Spectrometry for the Quantitative Analysis of Pyrrolizidine Alkaloids from *Senecio Vulgaris*

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COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ^1H NUCLEAR MAGNETIC RESONANCE SPECTROMETRY FOR THE QUANTITATIVE ANALYSIS OF PYRROLIZIDINE ALKALOIDS FROM *SENECIO VULGARIS*

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ABSTRACT

A high-performance liquid chromatographic method for the analysis of pyrrolizidine alkaloids from Senecio vulgaris has been developed using narceine-HCl as an internal standard. A reversed phase procedure with a methanol/.01 M KH_2PO_4 30/70 solvent system is described, and compared with a quantitative ^1H Nuclear Magnetic Resonance spectrometric method. Some advantages and limitations are discussed.

INTRODUCTION

Pyrrolizidine containing plants are found all over the world. These alkaloids show a remarkable toxicity, which is

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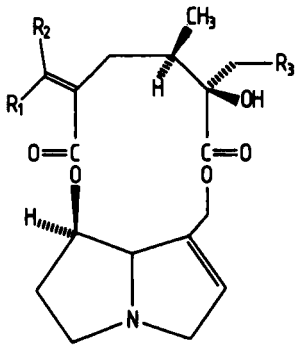
due to metabolic activation of the free alkaloid base or its N-oxide to the corresponding pyrrole derivate. Being alkylating agents, these metabolites are able to interfere with mitosis. The pyrrolizidines should therefore also be considered as potential antitumoral products (1). Nevertheless the analytical tools for the determination of the individual pyrrolizidine alkaloids in mixtures are very limited. Recently we have described a method to analyze pyrrolizidine mixtures using ^{1}H Fourier Transform Nuclear Magnetic Resonance Spectroscopy (^{1}H FT NMR) in a quantitative way. Senecio vulgaris L. (Compositae), known as common groundsel, was chosen as pyrrolizidine containing plant (2). Because most laboratories don't have the disposal of a FT NMR instrument for routine analyses, we wanted also to develop a High-Performance Liquid Chromatographic (HPLC) method, in order to compare it with the ^{1}H FT NMR procedure. At the moment some procedures for the isolation and identification of pyrrolizidine alkaloids by HPLC have been published (3,4,5). However a quantitative HPLC method has been described for Senecio jacobaea only (5). Therefore we have chosen a HPLC system, which has already been established, and which is generally applicable for a wide range of pyrrolizidines (4), in order to adapt it as a quantitative method, using an internal standard, and to examine if this technique is a good alternative for quantitative ^{1}H NMR spectroscopy, which is often employed for the routine analysis of samples containing pyrrolizidine alkaloids at the moment.

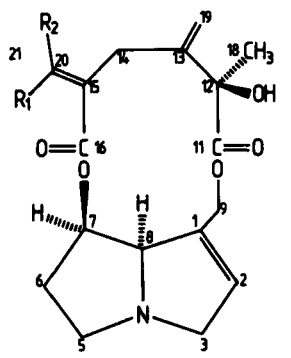
MATERIALS AND METHODS

HPLC conditions. The modular HPLC apparatus consisted of a glass solvent reservoir, equipped with a low pressure mobile phase filter with a pore size of 2 μm, a diaphragm pump (Orlita, Germany), equipped with a pulse dampener, a Model CV-6UHPa-N60 7000 psi manual sample injection valve (Valco Instruments Co., USA), and a variable wavelength UV detector (Model 450, Waters Associates, USA). A 10 μ C18 Bondapak™ reversed phase column (30 cm x 3.9 mm) (Waters Associates, USA) was used. The chromatograms were recorded on a Hewlett Packard Model 3380 A integrator. The mobile phase was a degassed methanol - .01 KH_2PO_4 (pH 6.3) 30/70 v/v mixture. The samples were dissolved in methanol for injection. After study of the UV spectrum of seneciphylline (sph), senecionine (sn) and retrorsine (rs), which showed an UV absorbance peak at 218,8 nm (Perkin-Elmer Lamba 5 UV/VIS Spectrophotometer), a detector wavelength of 219 nm was chosen. All chemicals were analytical grade. The structures of the alkaloids are shown in fig. 1.

Calibration A mixture of pure sph, sn and rs was used for calibration. Five reference samples were prepared, with different concentrations of these pyrrolizidine alkaloids, and a fixed amount of narceine-HCl as an internal standard. Narceine-HCl was purchased from the Aldrich Chemical Company. This product showed a considerable UV absorbance at 219 nm. The concentration range of rs was 0,03-0,25 mg/ml, of sph 0,08-0,64 mg/ml, and of sn 0,05-0,38 mg/ml. The concentration of



R_1=-CH_3, R_2=-H, R_3=H SENECIONINE
R_1=-H, R_2=-CH_3, R_3=H INTEGERRIMINE
R_1=-CH_3, R_2=-H, R_3=-OH RETRORSINE
R_1=-H, R_2=-CH_3, R_3=-OH USARAMINE



R_1=-CH_3, R_2=-H SENECIPHYLLINE
R_1=-H, R_2=CH_3 SPARTIOIDINE

Figure 1.

The pyrrolizidine alkaloids from Senecio vulgaris.

narceine-HCl was 0,057 mg/ml. The references were chromatographed, (injection volume 4-8 µl, flow rate 2 ml/min) and after integration three relative calibration curves were calculated by a least squares method.

Procedure Sample preparation and quantitative ^1H FT NMR analysis of the pyrrolizidine alkaloids from S.vulgaris followed standard prccedures (2). 141 Grams of finely ground, dried plant material were extracted. All the N-oxides were reduced. About 5 mg of the ultimate pyrrolizidine sample was dissolved in methanol (20 ml), containing 0,038 mg/ml narceine-HCl as an internal standard and chromatographed several times. The injection volume was 6 µl, and the flow rate va-

ried between 1,4-1,8 ml/min. The other part was analyzed by quantitative ^{1}H FT NMR spectroscopy.

RESULTS

Fig. 2 shows the separation of a reference mixture of pure sph, sn, rs and narceine-HCl. Coefficients of the calibration curves and retention times, relative to narceine-HCl, are listed in table 1.

Fig. 3 shows the separation of an unknown pyrrolizidine containing sample from S.vulgaris. The relative retention times of sph, sn and rs correspond with three peaks in this chromatogram. Because of the presence of the geometrical isomers spartioidine (sp), integerrimine (int) and usaramine (us), established by ^{1}H NMR spectrometry (2), the peaks are broadened or even split up. The presence of these alkaloids in the sample was confirmed by ^{13}C NMR spectrometry.

The chromatograms were quantified by measuring the ratio of drug to internal standard, based on peak area. Results of

TABLE 1

Relative retention times and calibration curves for rs, sph and sn

	peak no.	relative retention time	a*	b*	linear correlation
Rs	1	0,30	0,39911	-0,01336	0,99942
Sph	2	0,43	0,37392	0,02892	0,99566
Sn	3	0,68	0,31964	-0,00604	0,99923
Narceine-HCl	4	1,00			

* Relative calibration curves $y = ax + b$ with $y = \text{Area}\left(\frac{\text{component}}{\text{standard}}\right)$ and $x = \text{Weight}\left(\frac{\text{component}}{\text{standard}}\right)$

$$\text{Weight (component)} = \left[\text{Area}\left(\frac{\text{component}}{\text{standard}}\right) - b\right] \frac{\text{Weight(standard)}}{a}$$

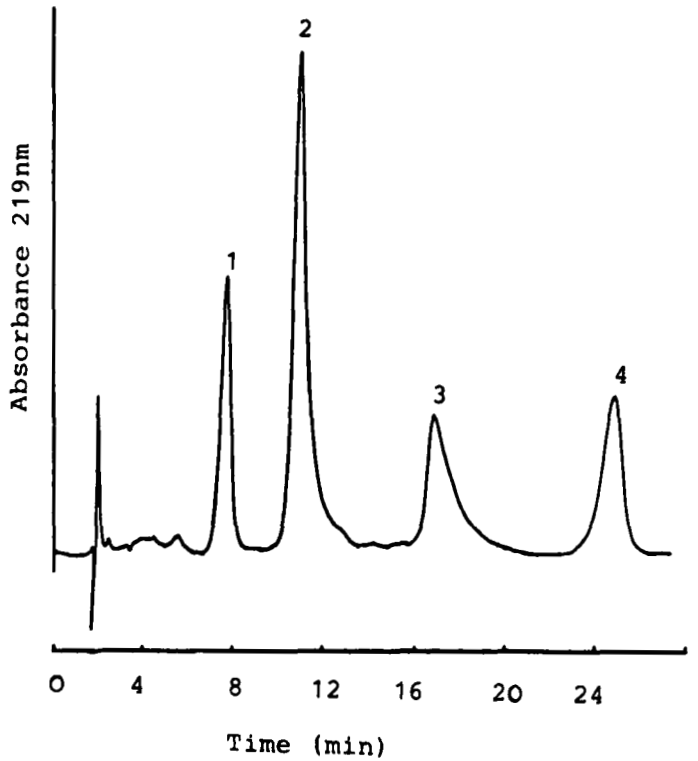


Figure 2.

Chromatogram of a reference solution, containing retrorsine (0,125 mg/ml), seneciphylline (0,322 mg/ml), senecionine (0,193 mg/ml) and narceine-HCl (0,057 mg/ml) in methanol. Peak 1 = rs, Peak 2 = sph, Peak 3 = sn, Peak 4 = narceine-HCl. HPLC conditions : experimental section.

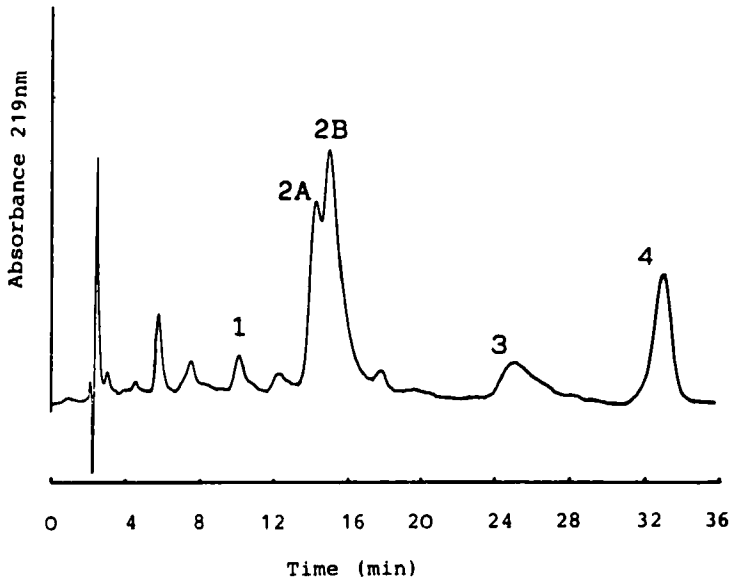


Figure 3.

Chromatogram cf a pyrrolizidine alkaloid sample from S.vulgaris containing about 0,26 mg/ml pyrrolizidines and 0,038 mg/ml narceine-HCl in methanol. Peak 1 = rs/us, Peak 2A = sp, Peak 2B = sph, Peak 3 = sn/int, Peak 4 = narceine-HCl. HPLC conditions : experimental section.

both HPLC and ^{1}H NMR spectroscopic analyses, calculated for the total extract, are given in table 2. To allow quantitation of the chromatograms the UV absorbance of the Z- and E-isomers was supposed to be completely similar. In view of some reference data, this generalisation seems to hold for a methanolic-aqueous solvent system (6).

The HPLC results are "day to day" values. The difference in

TABLE 2

Comparison of the two methods of analysis. Results and faults (standard deviation of the mean)

		HPLC-method			^{1}H FT NMR-method *	
	peak no.	n	weight(mg)	%	weight(mg)	%
Rs/Us	1	6	9,0±1,5	8,6±1,5	10,3±0,3	9,6±0,3
Sph/Sp	2	6	73,1±3,2	69,5±4,4	72,4±0,2	67,5±0,4
Sn/Int	3	6	23,1±3,2	22,0±3,2	24,6±0,1	22,9±0,2
			105,2 mg		107,3 mg	

* The % of Z - isomers (Rs, Sph and Sn), calculated by ^{1}H FT NMR, was 76,8 %

precision between "day to day" and "within day" measurements was not significant.

DISCUSSION

This HPLC method appears to be an excellent procedure for resolving closely related pyrrolizidine alkaloids. Different methanol-water proportions were tested, going from 60% to 20% v/v methanol. The pyrrolizidines were always resolved very well, but the separation of the impurities from the alkaloids in the extract was best achieved by using a methanol-.01 M KH_2PO_4 30/70 v/v system. The solvent systems containing the highest amount of water produced the longest retention times.

Narceine-HCl was most convenient as an internal standard. It can also be employed for the analysis of other pyrrolizidine alkaloid mixtures. Internal standards are used in order to compensate for a number of variables, most criti-

cally the injection volume. They are almost essential for precise and credible results with syringe injection. A lot of alkaloids were tested as an internal standard, but the only product having a satisfactory retention time in our HPLC system was narceine-HCl. Because of its solubility it was not possible to used the same compound as an internal standard to compensate for recovery factors during the extraction. The narceine base is nearly insoluble in chloroform, which is used for the extraction of the pyrrolizidines from an alkaline phase, and in addition it has a carboxylic acid function (pk_a = 9,3). Therefore it is added to the final extract, just before the quantitative analysis.

Because of the benefits of using this product as a standard for the HPLC analysis, and because a standard procedure for the extraction of alkaloids, which has been optimized for pyrrolizidines by many authors, was used, this is only a minor disadvantage.

The data presented show that HPLC, with an internal standard, is a fast and efficient method for the analysis of the pyrrolizidine alkaloids containing extracts. Quantitative ^1H FT NMR spectroscopy, however, gives more precise and reliable results than HPLC.

The separation of pyrrolizidines from impurities is very important for HPLC, because peak overlapping can lead to systematic errors. For ^1H NMR the presence of impurities seems to be less critical. With our HPLC system it is impossible to distinguish between Z and E geometrical isomers,

TABLE 3

Comparison of the HPLC and ^{1}H FT NMR techniques for the determination of pyrrolizidine alkaloids

characteristic	HPLC	^{1}H FT NMR
reliability	good	good
precision	acceptable	good
sensitivity	high	low
interferences (impurities)	fairly good	good
manipulation time	long	short
blocking of apparatus	important	slight
adaption to other assays	complicated	easy
apparatus cost price	low	high
reagent cost price	low	high
personnel qualifications	normal	high

althcugh the retention times are slightly different. The Z/E proportion can be calculated by means of quantitative ^{1}H NMR spectroscopy.

The advantages and disadvantages of both methods are shown in table 3. This HPLC procedure prcduces reliable results, but as an analytical method quantitative ^{1}H FT NMR spectrometry is superior, especially for samples containing a considerable amount of impurities. Nevertheless HPLC, which is commonly used in most analytical laboratories, can be applied for the analysis of relatively pure pyrrolizidine alkaloids containing extracts.

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