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Short communication

High-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy Application to the ecdysteroids of *Silene otites*

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Abstract

High-performance liquid chromatography (HPLC) coupled to nuclear magnetic resonance (NMR) spectroscopy has been used to obtain ^{1}H NMR spectra of ecdysteroids present in an extract of the plant *Silene otites*. Reversed-phase gradient chromatography was performed using a $^{2}H_2O$–acetonitrile–trifluoroacetic acid-based solvent system. Diagnostic NMR spectra were obtained for 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone using the stopped-flow HPLC–NMR technique. The compounds were present in the extracts in amounts between ca. 12 and 135 μg on-column (as determined using HPLC–UV). © 1998 Elsevier Science B.V.

Keywords: Nuclear magnetic resonance spectroscopy; *Silene otitis*; Ecdysteroids

1. Introduction

The newly introduced technique of high-performance liquid chromatography coupled to high field nuclear magnetic resonance spectroscopy (HPLC–NMR) has the potential to reduce greatly the time required for structure identification by eliminating the need for isolation prior to NMR identification. A number of applications of the technique in various fields have been recorded, e.g. pharmaceuticals, drug metabolism studies and polymer additives (reviewed in [1–3]). However, an obvious area that has so far received little attention is the use of HPLC–NMR in the screening of plants for useful products (for an application to crude extracts of *Gentianaceae* sp., see [4]).

Such an application of HPLC–NMR to plant extracts, namely the separation and identification of the ecdysteroids of *Silene otites*, is described here. The ecdysteroids form a family of relatively polar, polyhydroxy steroids, best known as the moulting hormones of insects and crustaceans. They are, however, widely distributed in plants, where they probably function as a defence against phytophagous insects. In excess of 250 ecdysteroids, or closely related compounds, have thus far been identified [5]. Plants which contain the highest levels of ecdysteroids are very valuable since these compounds command a high price. *Silene otites* was chosen for this study on the basis of previous work in which this

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species has been shown to contain large amounts of a number of ecdysteroids [6,7].

2. Experimental

The ecdysone and 20-hydroxyecdysone standards were gifts from various sources and were dissolved in methanol at a concentration of ca. 10 mg/ml. Aliquots (10 μl) of this solution were used for HPLC–NMR. The extract of *Silene otites* was prepared as described elsewhere [6,7] and dissolved in 150 μl of methanol. For HPLC–NMR, 20 μl volumes of this solution were taken and diluted with 60 μl of ^2H_2O prior to injection.

The quantity of the individual ecdysteroids present in the extract was determined using normal-phase HPLC as described previously [8].

Chromatography for NMR was performed using a 250×4.6 mm I.D. Spherisorb S5ODS2 C_{18} bonded HPLC column (Hichrom, Reading, UK). Gradient chromatography was performed using mobile phases composed of acetonitrile (Pestanal-grade, Riedel-de Häen, Germany) and ^2H_2O (99.9 atom %, Fluorochem, Glossop, UK) containing 1% trifluoroacetic acid (Fisons, Loughborough, UK).

The mobile phase was delivered at a rate of 1 ml/min by a Bruker LC22 pump (Bruker, Coventry, UK). Typically, the gradient employed used a linear increase in acetonitrile concentration from 20 to 25% over 20 min, followed by a rapid increase to 90% acetonitrile from 20 to 30 min, in order to elute strongly retained contaminants from the column. The gradient then returned to 20% acetonitrile for the period 25 to 30 min in order to allow the column to re-equilibrate before injection of the next sample. The eluent from the column was monitored at 254 nm.

NMR was performed in the stopped-flow mode on selected peaks using a Bruker DRX500 NMR spectrometer (Bruker) equipped with a dedicated $^1H/^{19}F$-flow probe with a cell volume of 120 μl. Stopped-flow experiments were carried out using a 1D-NOESY pulse sequence for double solvent suppression. Data were generally acquired with 64 to 1009 transients into 16K data points, with a pulse repetition time of 3.1 s. Chemical shifts were referenced to acetonitrile at δ1.93.

3. Results and discussion

3.1. HPLC–NMR of a standard mixture of ecdysone and 20-hydroxyecdysone

The chromatography of a standard mixture of ecdysone and 20-hydroxyecdysone (for structures, see insets to Fig. 1) under the gradient conditions employed here was used to determine the suitability of HPLC–NMR for the characterisation of this class of compounds. The resulting NMR spectra, corresponding to approximately 100 μg of each compound on column, are shown in Fig. 1. This quantity of material in a single peak enabled good ^1H NMR spectra to be obtained in ca. 10 min. The HPLC–

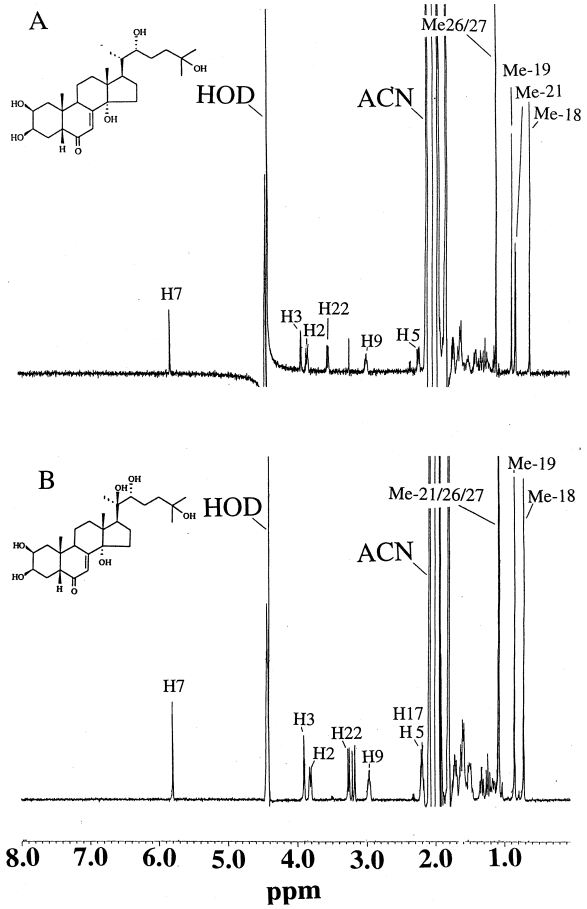

Fig. 1. Stopped-flow HPLC–NMR spectra of a standard mixture of 20-hydroxyecdysone (A) and ecdysone (B). Signal assignments are as indicated.

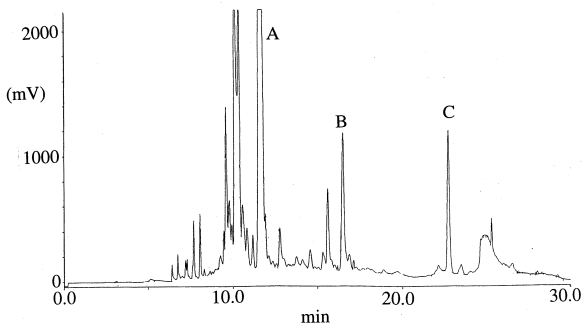

Fig. 2. HPLC–UV trace of the extract of *Silene otites*.

NMR spectra of these standards (Fig. 1A,B) clearly show resonances that are typical of the ecdysteroids. These signals were not subject to significant interference in regions critical for the identification of these compounds by the resonances due to acetonitrile. Thus, the signals for the methyls at C18, C19, C21, C26 and C27 of ecdysone were clearly visible (see Fig. 1A), as were the equivalent resonances for 20-hydroxyecdysone (Fig. 1B). In addition, a number of useful diagnostic signals for the protons at H22, H17, H9, H7, H5, H3 and H2 were also readily detectable (see Fig. 1).

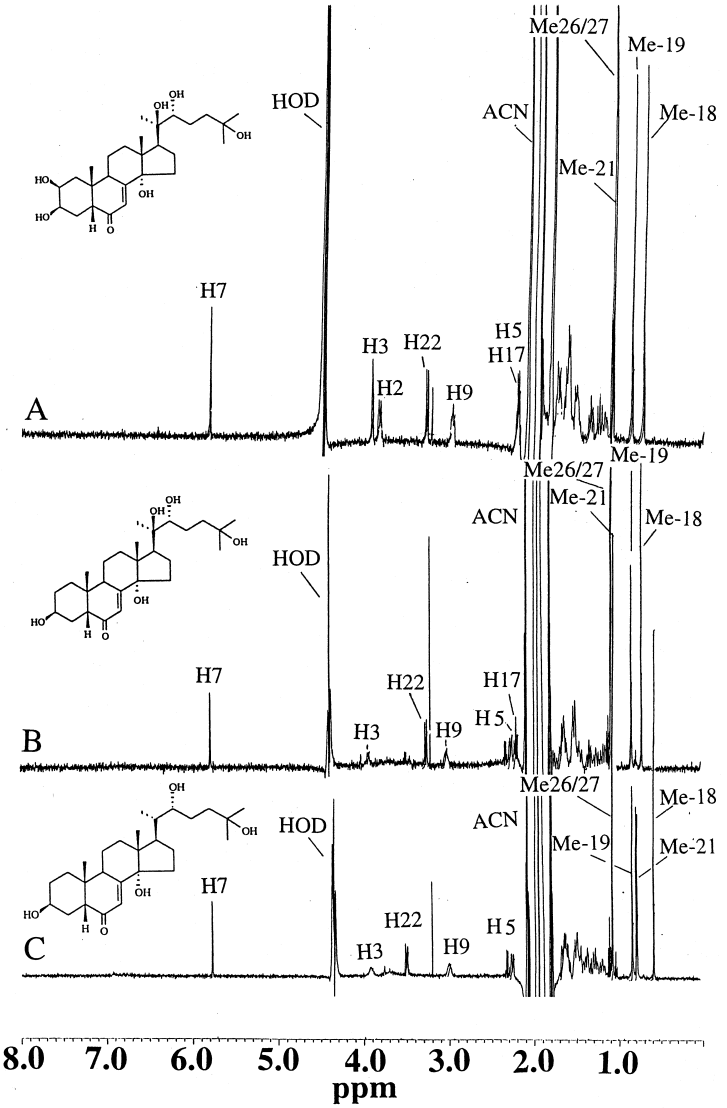

Fig. 3. Stopped-flow HPLC–NMR spectra of (A) 20-hydroxyecdysone, (B) 2-deoxy-20-hydroxyecdysone and (C) 2-deoxyecdysone present in the *Silene otites* extract shown in Figure 2.

3.2. Ecdysteroids present in an extract of Silene otites

A typical chromatogram for the *Silene* extract is given in Fig. 2. As can be seen, there are a significant number of UV-absorbing peaks, with the major peak at 11.7 min corresponding in retention time to 20-hydroxyecdysone. The stopped-flow NMR spectrum of this component is shown in Fig. 3A and clearly contains diagnostic resonances for this ecdysteroid. Quantitative analysis of the extract indicates that this peak of 20-hydroxyecdysone corresponds to ca. 135 μg of material on column. This spectrum was obtained with only 64 scans, requiring an acquisition time of approximately 3 min.

Stopped-flow NMR spectra of the peaks eluting at 16.6 and 24.9 min were also acquired, yielding the spectra shown in Fig. 3B,C, respectively. Based on previous chromatographic analysis of this sample, these peaks were expected to correspond to 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone, and the NMR spectra obtained were consistent with this identification. The quantitative analysis of the sample revealed that these peaks corresponded to ca. 12–13 μg on column for both ecdysteroids. The spectrum for 2-deoxy-20-hydroxyecdysone (Fig. 3B) required 828 scans (ca. 43 min) and that for 2-deoxyecdysone (Fig. 3C) required 1009 scans (ca. 52 min).

The use of HPLC–NMR in this way demonstrated the effectiveness of the stopped-flow HPLC–NMR technique at confirming the presence of compounds expected to be present in the extract based on previous studies. The NMR spectra of a number of other peaks were also investigated in order to determine whether or not they contained ecdysteroids. Thus, the NMR spectra of the major UV-absorbing components, eluting immediately before the 20-hydroxyecdysone peak, contained ''sugar-like'' compounds that were clearly unrelated to the ecdysteroids. The small peak at 15.6 min, eluting immediately prior to 2-deoxy-20-hydroxyecdysone, was found to contain aromatic resonances but no ecdysteroid-related compounds.

4. Conclusions

These results clearly demonstrate that HPLC–NMR is readily applicable to the identification of the ecdysteroids present in this partially purified extract of *Silene otites*. This technique was much more efficient than traditional methods requiring isolation prior to NMR, and is analogous to the use of HPLC–mass spectrometry (MS). Applications to the identification of natural products in plant extracts, of varying degrees of purity, can be expected to increase rapidly as HPLC–NMR facilities become more widely available.

Experiments are currently in progress to determine the usefulness of combined HPLC–NMR–MS systems on extracts of this type.

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