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Short communication

Observation of exchangeable protons by high-performance liquid chromatography–nuclear magnetic resonance spectroscopy and high-performance liquid chromatography–electrospray ionization mass spectrometry: a useful tool for the hyphenated analysis of natural products ☆

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Abstract

The first high-performance liquid chromatography–nuclear magnetic resonance (HPLC–NMR) investigation of exchangeable protons of low-molecular-mass natural products is reported. Model alkaloids or crude plant extracts were dissolved in $^{2}H_2O$–$^{1}H_2O$–MeCN (deuterium oxide–water–acetonitrile) or $^{2}H_2O$–MeCN and, after direct injection or chromatographic separation, examined in a 60-μl NMR flow probe. Exchangeable amino protons initially detected by HPLC–electrospray ionization mass spectrometry were subsequently identified and investigated by stop-flow ^{1}H-NMR, two-dimensional (2D) total correlation spectroscopy (TOCSY), and 2D nuclear Overhauser effect spectroscopy (NOESY). These experiments extend the applicability of HPLC–NMR for the investigation and structure elucidation of natural products.

Keywords: Proton exchange; Nuclear magnetic resonance spectrometry; Hyphenated techniques; Alkaloids

1. Introduction

Hyphenated techniques like high-performance liquid chromatography–nuclear magnetic resonance (HPLC–NMR) and HPLC–mass spectrometry (MS) play an increasingly important role in natural product analysis [2], since they permit the fast screening of crude biological extracts to deliver detailed information about metabolic profiles [3]. Furthermore, stop-flow HPLC–NMR is used to elucidate structures of new compounds prior to (or instead of) their

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– mostly time consuming – isolation [4–8]. In combination with HPLC coupled to circular dichroism (HPLC–CD), this approach does not only deliver valuable data for the dereplication of known natural products, but can even provide the absolute stereostructure of new compounds, right from crude extracts [9]. The development of HPLC–NMR was accelerated by several factors like the increasing use of high field NMR spectrometers [10], further miniaturization of NMR probeheads [11,12], the advent of powerful solvent suppression schemes, and their combination with a series of homo- and heteronuclear NMR experiments [13,14].

Despite these achievements, the elucidation of new structures from crude biological matrices with HPLC–NMR still suffers from some drawbacks. In conventional high-resolution NMR, valuable structural information can be obtained by the observation of exchangeable protons (e.g., N-H or O-H) in deuterated, aprotic solvents or in protic solvents like ^1H_2O in combination with solvent suppression schemes. The latter principle has already been utilized for the acquisition of ''fingerprint patterns'' of proteins with HPLC–NMR [15]. There are, however, no reports on the application of high-resolution HPLC–NMR with protic solvents aiming at the on-line elucidation of new structures. This would be all the more interesting, as in HPLC–MS analysis the ''reverse'' principle, the exchange of ^1H_2O against ^2H_2O, is commonly used for the determination of the total number of exchangeable protons in a molecule [16,17]. Observation of exchangeable protons with two-dimensional (2D) NMR can provide important information about the type and location of hydrogen carrying heteroatomic groups in a molecule. Therefore, we have investigated the value and limitations of the usage of protic solvents (like ^1H_2O) in high-resolution HPLC–NMR, with special respect to the applicability regarding preceding chromatographic separations and 2D-NMR spectroscopy like total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY).

2. Experimental

2.1. Chemicals

For chromatography and sample preparation, analytical-grade solvents and reagents were used. Water (^1H_2O) was purified by the Milli-Q system (Millipore, Bedford, MA, USA). Deuterium oxide (^2H_2O) (99.9 atom%) was purchased from Promochem (Wesel, Germany). Acetonitrile (MeCN) was available from Riedel-de Haën (Pestanal grade; Seelze, Germany) and trifluoroacetic acid (TFA, spectroscopic grade) was from Merck (Darmstadt, Germany). Ajmalicine hydrochloride and (−)-nicotine were purchased from Fluka (Deisenhofen, Germany). Dioncophylline A, *N*-methyldioncophylline A, and korupensamine A were isolated as described previously [18–20].

2.2. Sample preparation

All pure reference samples were dissolved in ^2H_2O–MeCN (1:1, v/v, pH 3 with TFA) and ^2H_2O–^1H_2O–MeCN (5:45:50, v/v, pH 3 with TFA). The concentrations were 0.5 mg ml^{-1} (korupensamine A), 0.2 mg ml^{-1} (ajmalicine hydrochloride, dioncophylline A, *N*-methyldioncophylline A) and 0.1 mg ml^{-1} [(−)-nicotine]. For HPLC–electrospray ionization (ESI) MS loop injections, all standards were diluted to 20 ng ml^{-1} in the respective solvent.

For extract analysis, 5 g of dried and ground twigs from *Triphyophyllum peltatum* (Dioncophyllaceae) [18] were extracted with a mixture of water (pH 2 with TFA)–MeCN (8:2, v/v) in an ultrasonic bath at room temperature for 2 h. The extract solution was filtered and lyophilized. A 4.5-mg amount of this extract was redissolved in 300 μl ^2H_2O–MeCN (8:2, v/v) and filtered through a 0.2-μm membrane filter. The resulting solution was used for HPLC–NMR analysis.

2.3. Instrumentation and analytical procedures

HPLC–NMR analyses were performed on a Bruker DMX 600 NMR spectrometer operating at 600.13 MHz ^1H frequency (Bruker, Rheinstetten, Germany) and controlled by the software system XWinNMR from Bruker. The outlet of the UV detector was connected to the flow probe by a polyether ether ketone (PEEK) capillary via a BPSU interface (Bruker), controlling the experimental

modes. The spectrometer was equipped with an inversely constructed ^{1}H, ^{13}C flow probe with a 3 mm (60 μl) detection cell (Bruker).

Chromatographic separation of extracts was performed on a Symmetry C_{18} column (250×4.6 mm I.D., 5 μm) from Waters (Eschborn, Germany). The chromatographic system consisted of a Bruker LC22 pump and a UV detector from Bischoff working at 254 nm absorption. The flow-rate was set to 1 ml min^{-1} and the injection volume was 100 μl of the extract solution. Solvent A, which was kept constant at 5% of the overall solvent mixture, was 0.01% (v/v) TFA in $^{2}H_2O$, solvent B 0.01% (v/v) TFA in $^{1}H_2O$, and solvent C MeCN, which was added to A and B to make up the full percentage of 100%. The HPLC gradient for the detection of exchanging protons was as follows: $t=0$ min 90% B, 12 min 75% B, 19 min 65% B, 25 min 55% B, 30 min 35% B. For ''conventional'' HPLC–NMR, $^{1}H_2O$ was replaced by $^{2}H_2O$.

For NMR analysis of pure samples in $^{2}H_2O$–MeCN or $^{2}H_2O$–$^{1}H_2O$–MeCN, the prepared solutions were directly injected into the flow cell by a syringe.

The one-dimensional ^{1}H-NMR spectra were obtained using the following parameters: free induction decays (FIDs) were collected into 32 K data points with a spectral width of 12 kHz. 90° pulses were used with an acquisition time of 1.36 s and the relaxation time delay was set to 1 s. Typically 32 scans were accumulated. A WET [13] solvent suppression with ^{13}C decoupling was performed on the signals of MeCN and $^{1}HO^{2}H$ (from $^{2}H_2O$) using standard Bruker pulse programs. Prior to Fourier transformation, an exponential apodization function was applied corresponding to a line broadening of 0.5 Hz. For calibration the residual signal of the solvent MeCN was set to 2.0 ppm.

Phase-sensitive TOCSY experiments were carried out using a standard Bruker pulse program with a binomial pulse solvent suppression. Typically the FIDs were collected into 2 K data points with a spectral width of 12 kHz in both dimensions. 90° pulses were used with an acquisition time of 0.122 s and the relaxation delay was set to 2 s. The MLEV spin-lock field was applied for 65 ms. The data were apodized with a squared $\pi/2$ shifted sinusoidal window function in both dimensions. For resolution enhancement in F1, a linear prediction to 1 K data points was applied.

Phase-sensitive gradient selected NOESY spectra were acquired using a modified NOESY sequence with a WET pulse sequence [13] and ^{13}C decoupling for solvent suppression during relaxation delay. Typically the FIDs were collected into 2 K data points with a spectral width of 12 kHz in both dimensions. 90° pulses were used with an acquisition time of 0.122 s and the relaxation delay was set to 2 s. The mixing time was 500 ms. The same data manipulation was performed as for the TOCSY experiment described above.

Due to the low content of $^{2}H_2O$ in exchange NMR experiments (max. 5%, v/v), the lock power should be set to the highest possible level without causing saturation.

HPLC–ESI-MS–MS analyses were performed with a triple stage quadrupole TSQ 7000 mass spectrometer equipped with an ESI interface (Finnigan MAT, Bremen, Germany), and a personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT). Nitrogen served both as sheath and auxiliary gas, argon as collision gas. Positive ions were detected by scanning from 200 to 500 u with a total scan duration of 1.0 s for a single spectrum. For loop injections (5 μl) with nondeuterated solvents, an isocratic mixture of 0.01% (v/v) TFA in $^{1}H_2O$–MeCN (1:1, v/v) was delivered by an Applied Biosystems 140b pump. For analysis under deuterated conditions, $^{1}H_2O$ was replaced by $^{2}H_2O$. The flow-rate was set to 0.2 ml min^{-1}.

3. Results and discussion

As an initial step we undertook a hydrogen/deuterium exchange ESI-MS experiment to determine the protonation degree and the number of exchanged protons of our reference samples under acidic conditions (pH 3). For this purpose, standard samples of dioncophylline A (**1a**), *N*-methyldioncophylline A (**1b**), korupensamine A (**2**), ajmalicine hydrochloride (**3**·HCl), and (−)-nicotine (**4**) (Fig. 1) were prepared by dissolving the respective alkaloid in $^{2}H_2O$–MeCN and in $^{1}H_2O$–MeCN.

Their $[M_H+{}^{1}H]^{+}$ and $[M_D+{}^{2}H]^{+}$ pseudo-

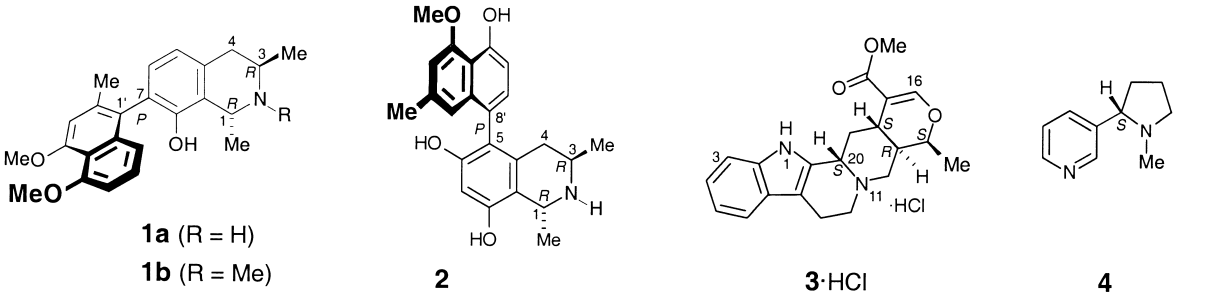


Fig. 1. Structures of dioncophylline A (**1a**), *N*-methyldioncophylline A (**1b**), korupensamine A (**2**), ajmalicine hydrochloride (**3**·HCl), and (−)-nicotine (**4**).

molecular ion masses (with M_H and M_D representing the neutral molecular species protonated or deuterated solely by chemical exchange) were determined after loop injection with ^1H_2O–MeCN or ^2H_2O–MeCN as solvents (Table 1).

The ESI-MS data clearly show that all reference compounds are detected as singly protonated (and thus singly ionized) species, and that in ^2H_2O all exchangeable proton sites are fully deuterated. This gives rise to mass shifts of up to +5 u. This result stimulated us to search for a possibility to detect such attached protons by ^{1}H-NMR, too.

In a first NMR experiment, we investigated the naphthylisoquinoline alkaloid dioncophylline A (**1a**) (Fig. 1). Under normal HPLC–NMR conditions (^2H_2O–MeCN), the region of 6.5–9 ppm of its ^{1}H-NMR spectrum only exhibits the signals of six aromatic protons (Fig. 2A). If, however, a twig extract from *T. peltatum*, a main source of **1a** [18], was examined by HPLC–NMR with ^1H_2O (^1H_2O–^2H_2O–MeCN, 56.7:5:38.3, acidified with 0.01% TFA) and MeCN as eluents, two broad signals in the region of 8.7–8.8 ppm became visible (Fig. 2B).

Table 1
Determination of *m*/*z* of protonated and deuterated pseudomolecular ions of compounds **1**–**4**

Compound	*m*/*z*		Exchangeable protons[a]
	$[M_H+{}^1H]^+$	$[M_D+{}^2H]^+$	
1a	378	381	3
1b	392	394	2
2	380	385	5
3	353	355	2
4	163	164	1

[a] Inclusive ionizing proton or deuteron.

The presence of these two signals, which integrated as two protons with equal intensity, suggested that they originate from two diastereotopic hydrogen atoms attached to a quaternary nitrogen. The formation of such a protonated tetrahydroisoquinoline species **1a**·H^+ is also in accordance with the chemical shifts of the signals between 8 and 9 ppm [21].

To confirm the origin of these – never observed in LC–NMR – signals, a TOCSY experiment was performed on a reference sample of a similar tetrahydroisoquinoline alkaloid, namely korupensamine A (**2**) (Fig. 1), in ^1H_2O–MeCN solution (for a high-resolution proton spectrum of **1a** in $CDCl_3$ see Ref. [22]).

Under acidic conditions, again two signals with

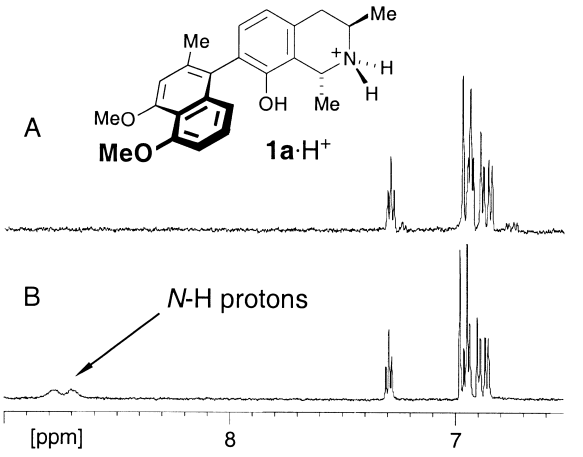
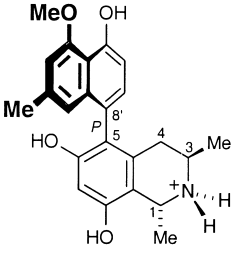


Fig. 2. (A) ^{1}H-NMR spectrum of a reference sample of **1a**, 200 μg ml^{-1} in ^2H_2O–MeCN (1:1, v/v), directly injected in flow cell, eight scans acquired; (B) stop-flow HPLC–NMR spectrum of **1a**·H^+ from a twig extract of *T. peltatum*, stop at 23.7 min (^1H_2O–^2H_2O–MeCN, 56.7:5:38.3, v/v), 32 scans acquired.

similar intensities and chemical shifts were observed. In a TOCSY experiment, both protons showed correlation peaks to all other protons of the saturated part of the tetrahydroisoquinoline ring (Fig. 3). This fact identified them as ammonium protons resulting from the protonated tetrahydroisoquinoline species **2**·H^+. Furthermore, in combination with ^{1}H-NMR data, it was now possible to assign the complete $CH(CH_3)$-NH_2-$CH(CH_3)$-CH_2 connectivity from TOCSY data. This is an important result, because under deuterated conditions only the – isolated – fragments $CH(CH_3)$ and $CH(CH_3)$-CH_2 are deducible from TOCSY data. If the nitrogen atom is deuterated, it prevents a correlation between H-1 and H-3. By contrast, the doubly protonated quaternary nitrogen species allows a Hartmann–Hahn transfer [23] between the two fragments.

This new feature in HPLC–NMR is not limited to correlation spectroscopy based on scalar coupling, which is demonstrated for ajmalicine hydrochloride (**3**·HCl) (Fig. 4).

This time, in contrast to the examples seen above, only one exchangeable proton around 10.3 ppm is observable, probably H-1. However, as this proton is not spin coupled to any others, its assignment cannot be accessed by TOCSY experiments. Therefore a NOESY experiment, based on dipolar coherence transfer, was carried out. Fig. 4 displays two peaks from the resulting detailed view of the obtained 2D



2·H^+

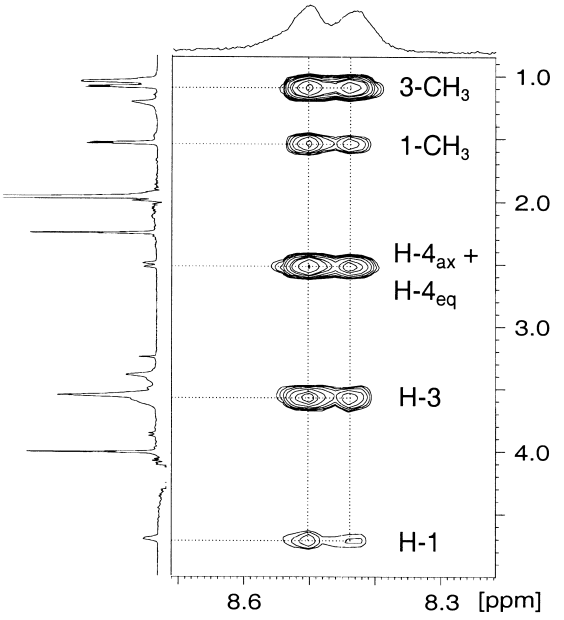
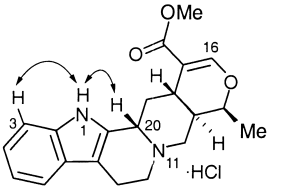


Fig. 3. 2D-TOCSY spectrum of **2**·H^+ (0.5 mg ml^{-1} in ^2H_2O–^1H_2O–MeCN, 5:45:50, v/v, acidified with TFA, directly injected in flow cell); acquisition parameters: 16 scans, 256 increments in F1, total acquisition time 2.5 h.



3·HCl

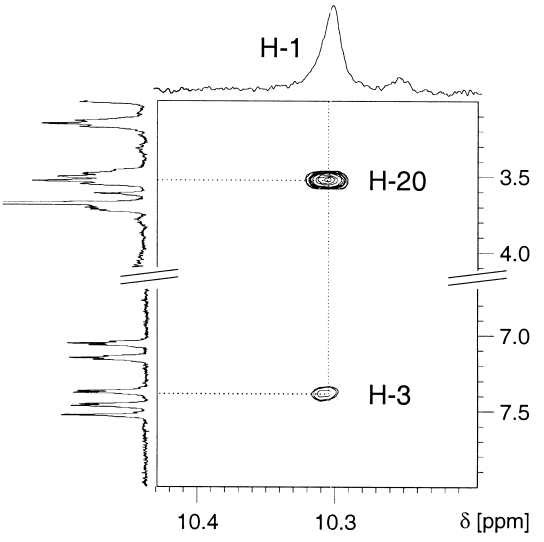


Fig. 4. 2D-NOESY spectrum of ajmalicine hydrochloride (**3**·HCl) (0.5 mg ml^{-1} in ^2H_2O–^1H_2O–MeCN, 5:45:50, v/v, directly injected in flow cell; acquisition parameters: 48 scans, 256 increments in F1, total acquisition time 9.5 h); NOE correlations between N-H and neighboring protons are indicated by arrows.

spectrum, which arise from NOE interactions between H-1 and its neighboring protons H-3 and H-20. This shows that it is not only possible to identify exchangeable amino protons by comparing "^1H_2O" and "^2H_2O" HPLC–NMR spectra and HPLC–MS data, but it likewise demonstrates that this concept also permits a distinction between different potentially proton bearing nitrogen atoms under HPLC–NMR conditions, and their positions in the molecule can be deduced, e.g., by TOCSY or NOESY measurements. Interestingly, although N-11 should show basicity, no peak arising from an attached hydrogen could be observed.

This feature was also observed in the case of (−)-nicotine (**4**) (Fig. 1), which has two tertiary nitrogen atoms. No signals from amine protons could be observed under the same acidic conditions as used above, despite the fact that in LC–MS **4** shows a mass shift of +1 u when using ^2H_2O as the cosolvent (see Table 1). That this has to do with the character of **1b** as a tertiary amine, is confirmed by the fact that the *N*-methyl analogue (**1b**) [19] of dioncophylline A (**1a**) (Fig. 1) does not give an N-H peak either.

The $^1H/^2H$-exchange rate of nitrogen bound protons depends strongly on the pH value of the solution [24,25]. Englander and co-workers describe a p*D* value ($[D^+]=10^{-pD}$) of about 3 for the lowest proton/deuterium-exchange rate of poly-D,L-alanine [24] and a pH of about 5 for proton/tritium exchange in myoglobin studies [25]. These values are generally valid for many proteins although the specific exchange rate depends on the electron withdrawing character of the neighboring groups [24].

However, since amino protons of alkaloids show a somewhat different character as compared to the N-H protons in proteins, where these are covered by the macrostructure, our measurements worked well at pH 3. Under these conditions, the observed secondary amino protons show obviously slow exchange relative to the NMR time scale.

We note that in no case hydroxyl protons, either from aliphatic or from aromatic hydroxyl protons, could be detected. The reason may be either an accidental overlap and suppression of these protons with the residual $^1HO^2H$ peak, or a fast proton exchange. Under similar conditions, protons from aliphatic hydroxy groups were detected if sophisticated solvent suppression schemes were used [14]. These protons showed no intramolecular interactions, but gave rise to solvent–solute NOE correlations. A possible solution to this problem could be the careful cooling of the NMR probe to decrease the rate of proton exchange, or the addition of expensive (since deuterated) and viscous solvents like glycerol [26].

To conclude, we have demonstrated that protic solvents can be utilized in HPLC–NMR to detect and characterize exchangeable protons, e.g., from secondary amino groups. Their position within the carbon skeleton can be assigned unambiguously by 2D-NMR experiments like NOESY or TOCSY. In combination with HPLC–MS, HPLC–NMR exchange experiments can provide a valuable contribution to the elucidation of natural products in hyphenated analysis.

Acknowledgements

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