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Application of on-line capillary high-performance liquid chromatography–nuclear magnetic resonance spectrometry coupling for the analysis of vitamin A derivatives

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Abstract

The direct on-line coupling between capillary high-performance liquid chromatography (capillary HPLC) and proton high-field nuclear magnetic resonance (NMR) spectrometry has been used to derive structural information about constituents of a mixture of vitamin A derivatives. ^{1}H NMR spectra were recorded in the stopped-flow and continuous-flow mode within a 180 μm I.D. capillary column mounted in a micro probe on a 600 MHz NMR spectrometer. The resolution of the ^{1}H NMR spectra obtained in capillary HPLC–NMR coupling experiments is sufficient to determine coupling constants in the order of 1.5 Hz. The detection limit is in the lower nanogram range. A stopped-flow 2D-TOCSY experiment of a 1% solution of vitamin A acetate acquired within 4 h reveals that the acquisition of 2D NMR spectra is possible in the nanoliter detection scale without any loss of structural information.

Keywords: Detection, LC; Nuclear magnetic resonance spectrometry; Vitamins; Ethyl phthalate; LC–NMR

1. Introduction

The application of directly coupled HPLC–NMR spectrometry for the investigation of complex mixtures of organic compounds in polymer, pharmaceutical and biomedical research is starting to become a routine analytical technique [1–23]. This established hyphenated technique employs conventional analytical HPLC columns (250×4.0 mm) and NMR flow-cells with detection volumes between 40–180 μl. The fact that deuterated solvents, which are conventionally applied for NMR spectrometry, are extremely expensive forbids their use in LC–NMR. Instead HPLC grade protonated solvents with consequent solvent signal suppression are used. The need for the reduction of solvent consumption as well as higher separation efficiencies promote the increasing interest in miniaturized chromatographic separation techniques such as capillary HPLC and capillary electrophoresis. Therefore the direct on-line coupling of these separation techniques with NMR spectrometry to yield the same spectrometric resolution and improved sensitivity with respect to already established HPLC–NMR techniques is a current challenge [24–29]. The on-line use of capillary chromatographic separation–NMR coupling offers several advantages compared to the conventional hyphenated technique. Fully deuterated solvents can be used because the required flow-rates are low. Therefore suppression of strong solvent signals is no longer necessary and the whole proton chemical shift range can be used for structural elucidation purposes. The use of capillaries with internal diameters be-

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tween 75–900 μm results in NMR detection volumes between 5 nl to 1 μl.

The first use of nanoliter NMR detection was described by Wu et al. [24,25]. They used a 5 nl detection volume by wrapping a solenoidal microcoil around a horizontal fused-silica capillary with a 75 μm internal diameter. Whereas a detection limit in the nanogram range could be reached with the 5 nl flow cell, the NMR resolution values reported in the first experiments were between 7 to 11 Hz. By continuously improving the applied solenoidal flow cell design, the authors finally could reach NMR signal line widths of 0.6 Hz with a 300 MHz NMR spectrometer [27].

An alternative flow cell design has been used for the first capillary HPLC–NMR experiments [28,29]. Here the capillary is mounted vertically in a modified microprobe using a double saddle Helmholtz coil for NMR detection. This design enables an easy exchange of the capillary for different chromatographic application fields. Because the shim systems used for homogenization in cryomagnets are optimized in the vertical z-direction parallel to the magnetic field lines, the vertical "insert-design" of a capillary in the NMR probe resulted in continuous-flow NMR spectra with a resolution in the order of 1.5 Hz. The principal disadvantage of the "insert design" is the lower filling factor (ratio of coil volume to sample volume) and thus the lower signal/noise ratio compared to the solenoidal design [29]. This drawback can be partially compensated by decreasing the internal diameter of the Helmholtz detection coil. Whereas in the first experiments a microprobe with a 3 mm radio frequency (r.f.) coil was used, we report here upon the use of a capillary microprobe with a 2 mm r.f. coil. It is of great practical importance to check the applicability of coupled micro HPLC–NMR spectrometry for real chromatographic separation problems such as detection and identification of vitamin A acetate and its dimers.

2. Experimental

2.1. Samples

Diethyl phthalate was obtained from Aldrich (Steinheim, Germany) and vitamin A acetate and a mixture of vitamin A acetate reaction products were obtained from BASF (Ludwigshafen, Germany).

2.2. Capillary HPLC

Fused-silica capillaries of 180 μm I.D.×350 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). The packing of the capillary columns with a 3 μm C_{18} stationary phase was performed by Grom (Herrenberg, Germany).

2.2.1. Chromatographic conditions

Capillary HPLC analysis of a mixture of vitamin A derivatives (1 μl sample) was carried out using a 180 μm I.D.×350 μm O.D. fused-silica column packed with 3 μm GromSil ODS-2 (Grom). An isocratic elution (in order to recycle the solvent) in deuterioacetonitrile was performed to elute the mixture of vitamin A derivatives. The HPLC system consisted of a Bischoff pump, a Valco injection device, a stainless-steel T-piece and a resistance capillary. Solvent splitting was accomplished by a stainless-steel T-piece and a resistance capillary of 50 μm I.D.×15 cm, yielding a split ratio of approximately 1:100. The splitted solvent was recycled. The pump and the T-piece were connected by fused-silica capillaries of 250 μm I.D. Injection was performed by filling the 1 μl loop of the Valco injection device and connecting it to the pump.

2.3. Capillary HPLC–NMR coupling

NMR spectra were recorded on a Bruker AMX 600 spectrometer equipped with a ^1H 2.0 mm capillary microprobe. The experimental set-up for a capillary HPLC–NMR coupling is outlined in Fig. 1. HPLC pump, T-piece and resistance capillary were located at a distance of about 3 m from the 14 T cryomagnet (Fig. 1).

2.3.1. Static measurements

Static measurements were performed by injecting a 0.1% solution of diethyl phthalate in deuterioacetone (99.8%, Merck, Darmstadt, Germany) directly into the capillary cell. 16 K data points with a spectral width of 5208 Hz, resulting in an acquisition time of 1.57 s were recorded. The relaxation delay was set at 2 s, 128 transients were

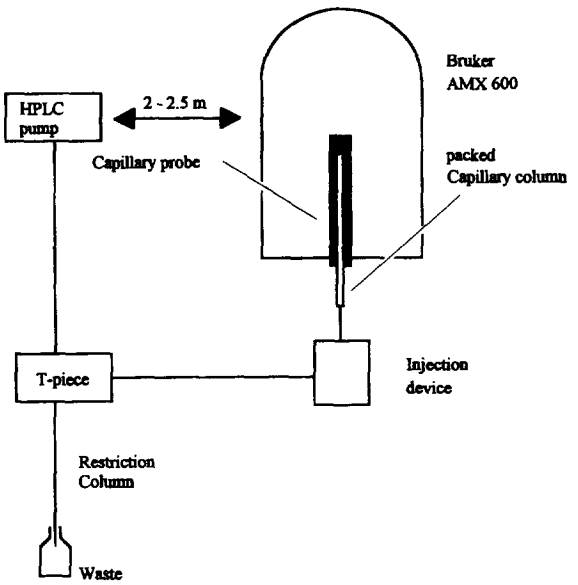

Fig. 1. Experimental arrangement for capillary HPLC–NMR coupling.

coadded with a total acquisition time of 7 min 43 s. The data were processed without applying a window function.

2.3.2. Stopped-flow measurements

Stopped-flow spectra were recorded when the peak maximum of the chromatographic peak had entered the detection volume by coadding 128 transients. This event could only be checked by continuously monitoring the chromatographic separation by continuous-flow ^{1}H NMR spectrometry.

1D NMR spectra

1D NMR spectra were recorded with 16 K data points and a spectral width of 5435 Hz, resulting in an acquisition time of 1.5 s. A relaxation delay of 1 s was used, and 128 transients were usually coadded with a total acquisition time of 320 s. A multiplication with an exponential window function with an 0.5 Hz line-broadening factor was applied before the Fourier transformation step.

2D NMR spectrum

A phase sensitive, two-dimensional total correlation spectrum (2D-TOCSY) was carried out on a sample of 1% of vitamin A acetate. Experimental parameters were 400 t_1 increments with 24 transients with 4 K complex data points acquired in simultaneous mode with a spectral width in both dimensions of 5345 Hz. With an acquisition time of 0.38 s and an applied mixing time of the MLEV spin lock of 65 ms a total acquisition time of 4 h occurred. The data were apodized with a shifted squared sine bell window function in both dimensions.

2.3.3. Continuous flow measurements

A packed capillary column (150 mm×0.180 mm) was located directly before the NMR detection window. The polyimide coating was removed over the length of the NMR detection window. The separation was performed at a flow-rate of 0.5 μl/min in deuterioacetonitrile (99%, Deutero Herresbach, Germany). A 1-μl volume of a 1% solution of a mixture of vitamin A derivatives was injected.

For the on-line measurements, 24 transients with 4 K complex data points and a spectral width of 5345 Hz were recorded per retention time increment. A relaxation delay of 1 s and an acquisition time of 0.38 s per transient were used. The pulse angle was set to 45°. During the separation 64 FIDs with an acquisition time of 33.1 s per FID were recorded. Data were treated as a 2D NMR matrix (t_1=retention time) and processed with UXNMR software. A phase sensitive Fourier transformation was performed in the t_2 direction only. The pseudo 2D matrix was apodized with a shifted sine bell function (shift 2.0) in f_2 only.

3. Results and discussion

3.1. NMR characteristics of the capillary microprobe

Fig. 2 shows the ^{1}H NMR spectrum of a 0.1% solution of ethyl phthalate in deuterioacetone recorded in the static mode in the 180 μm capillary. The sample is injected directly to the detection cell in the static mode in order to simulate stopped-flow experiments for an estimation of the detection range of the NMR probe. The coaddition of 128 transients performed within a total acquisition time of 7 min is appropriate and resulted in an acceptable *S/N* ratio for all signals of ethyl phthalate. Here, about 200 ng

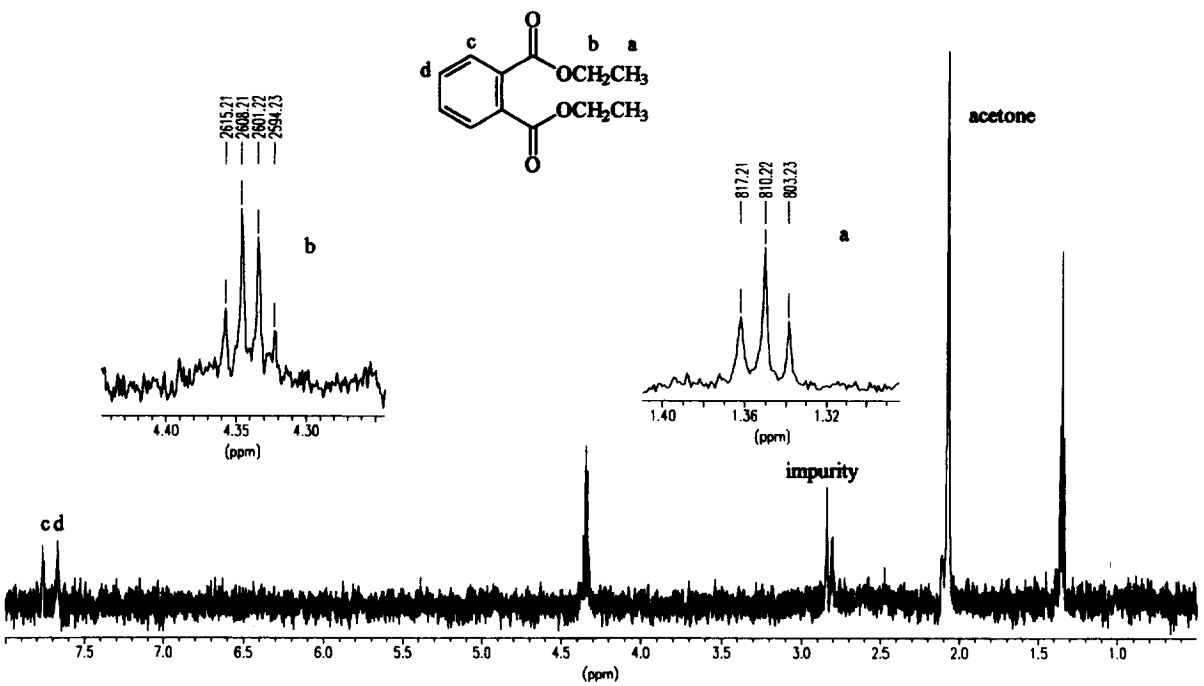

Fig. 2. Stopped-flow ^{1}H spectrum (600 MHz) of a 0.1% solution of ethyl phthalate in deuterioacetone.

(900 pmol) of ethyl phthalate are present in the 200 nl detection volume.

The stopped-flow ^{1}H NMR spectrum of a 1% solution of all-*trans*-vitamin A acetate (Fig. 3) demonstrates the NMR resolution obtained in the nanoliter detection volume. In stopped-flow experiments at the chromatographic peak maximum the separation is stopped to acquire NMR spectra. After the data acquisition the separation will be continued and other peaks of interest can be examined. A loss of chromatographic separation efficiency is more than compensated by gaining structural information of the NMR detection. The apparent resolution allows the determination of all coupling constants present in the ^{1}H NMR spectrum of all-*trans*-vitamin A acetate. The observed line width is in the order of 1.2 Hz.

3.2. On-line separation

Fig. 4 shows the on-line NMR detected separation of a mixture of vitamin A derivatives. Here, about 1 μl of a 5% solution of a vitamin A mixture was injected. In the contour plot of the separation, the ^{1}H chemical shifts are plotted against the retention times. During the separation NMR spectra were recorded continuously so that in contrast to stopped-flow experiments only limited time for NMR data acquisition is available. Nevertheless the on-line contour plot shows that even with this in terms of NMR spectrometry small amounts of sample spectra could be recorded in a 200 nl volume in a limited time. The signals of the first eluting compound at t_r=7.2 min can clearly be assigned to all-*trans*-vitamin A acetate. Two additional sets of proton patterns are seen in the contour plot at retention times of 12 and 13 min. Differences in the aliphatic region at δ=1.85 ppm indicate the existence of at least two compounds. A detailed analysis of 1D and 2D NMR spectra of reaction products of vitamin A acetate is in progress.

3.3. Stopped-flow measurement

To provide further information upon the applicability of capillary HPLC–NMR a ^{1}H–^{1}H stopped-flow 2D-TOCSY spectrum of all-*trans*-vitamin A acetate has been recorded (Fig. 5). The H–H

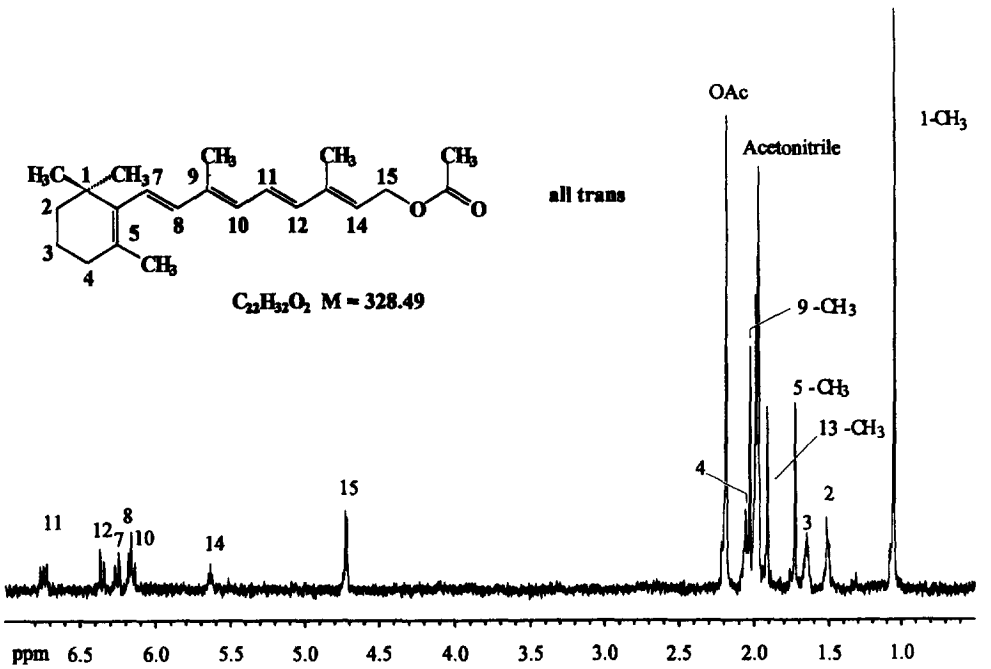

Fig. 3. Stopped-flow ^{1}H NMR spectrum (600 MHz) of a 1.0% solution of vitamin A acetate in deuterioacetonitrile.

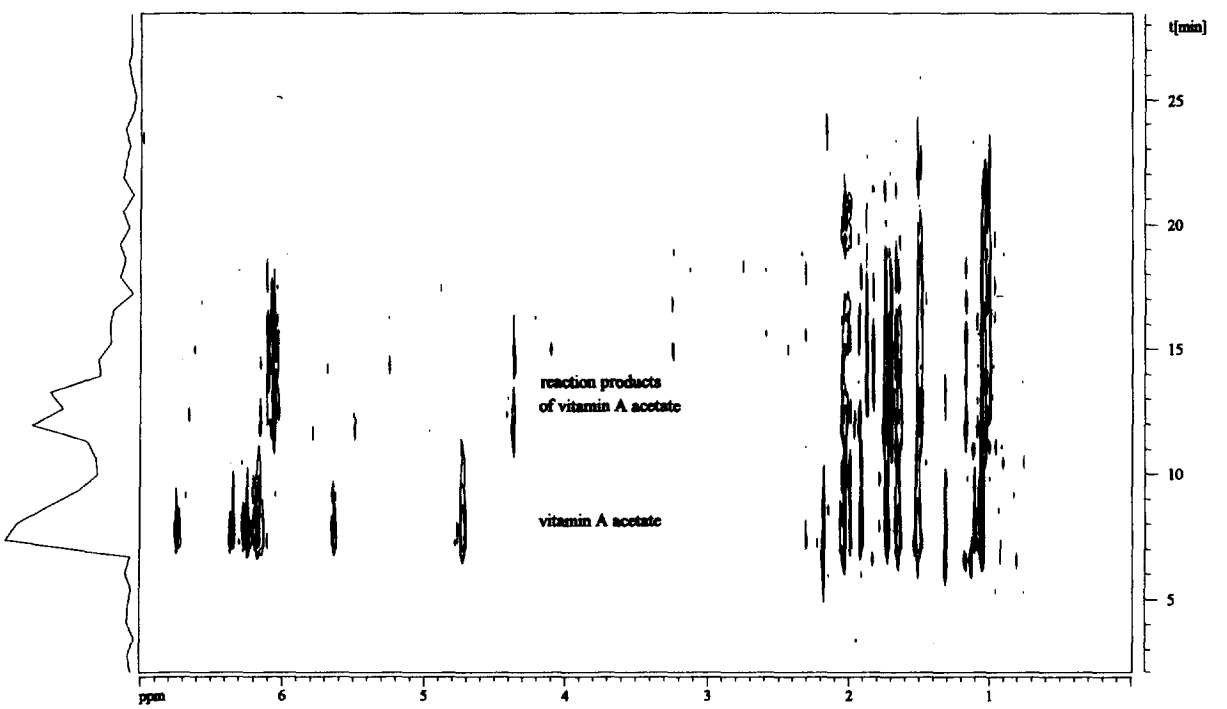

Fig. 4. ^{1}H NMR chromatogram (contour plot, 600 MHz) of a capillary HPLC separation of vitamin A derivatives: vitamin A acetate and vitamin A dimerization products.

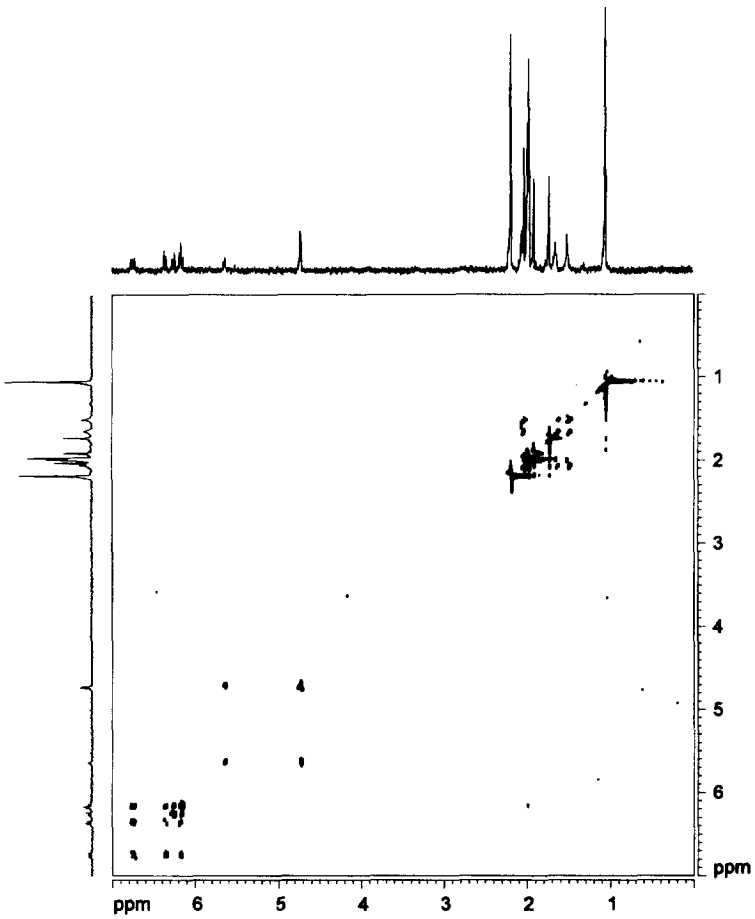

Fig. 5. Contour plot (600 MHz) of a TOCSY correlation of a 1% solution of all-*trans*-vitamin A acetate.

connectivity pattern between the neighbouring protons H-10, H-11 and H-12 is clearly seen in the lower left corner of the contour plot in the chemical shift range between 5.9 and 7.0 ppm. The TOCSY spectrum recorded in the 180 μl capillary contains the same stereochemical information content as a conventionally recorded spectrum in a 5 mm tube. This example indicates that a straightforward 2D application of organic compounds below the μg-range without any loss of spectral information is possible in the nanoliter scale.

4. Conclusion

The obtained data clearly show that on-line capillary HPLC–NMR coupling can be used for characterization of instable compounds as for example vitamin A derivatives, which decompose or isomerize under air and light. Continuous-flow detection allows the use of ^{1}H NMR chemical shift values as second dimension, whereas with stopped-flow detection 2D assignment techniques can be used. With an improved design for a small volume, high-sensitivity capillary probe, high-resolution NMR spectra could be recorded. Because deuterated solvents can be used in capillary separations solvent signal suppression is no longer a problem. A 2D NMR spectrum could be obtained in the nanoliter scale, which allows the assignment of all spin connectivities present. This experiment demonstrates the current progress in capillary HPLC–NMR coupling. It took six years between the first reported high-resolution NMR spectrum [1] and the first reported

2D NMR spectrum [5,7] in HPLC–NMR coupling. The time interval between the first ^{1}H NMR spectra in the nanoliter scale [24,25,28,29] and the first reported 2D NMR spectra is only a few months. However, the hyphenation of capillary separation methods with NMR spectrometry and electrospray mass spectrometry or FT mass spectrometry (capillary HPLC–NMR–MS) is still the next challenge.

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