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CONTINUOUS-FLOW NUCLEAR MAGNETIC RESONANCE*

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SUMMARY

The effect of the volume of the detector flow cell upon peak broadening in high-performance liquid chromatography with nuclear magnetic resonance detection has been monitored using a modified fluorescence detector. The results of solvent-resonance suppression using 1331 hard pulse sequences are described. The possibility of obtaining ^{13}C distortionless enhancement by polarization transfer spectra in the flow system is demonstrated.

INTRODUCTION

Continuous-flow nuclear magnetic resonance (NMR) spectroscopy is beginning to become an accepted analytical tool[1]. It allows the monitoring of kinetics and metabolic events by ^{1}H, ^{19}F or ^{13}C NMR spectroscopy[1–6] and can be used as a special detector in direct on-line coupling of high-performance liquid chromatography (HPLC) and NMR spectroscopy[4–8].

The optimum volume of the continuous-flow cell for HPLC–NMR is still a matter of discussion. On the one hand, HPLC peak broadening should remain as small as possible, on the other hand the limited sensitivity of the NMR method necessitates a relative large volume for detection. Up to now the effect of peak broadening caused by a NMR flow cell was measured by connecting a UV detector after the NMR flow probehead[4,7]. With this arrangement, the dead volume of the effluent also contributed to peak broadening. The true cell-volume effect may be obtained by directly observing a test separation within the NMR flow cell. In this case UV methods cannot be used because of the optical cut-off of the Pyrex glass used in the construction of the flow cell. For this reason, we resorted to fluorescence detection using a modified commercially available fluorescence detector.

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An additional and significant problem in HPLC–^{1}H NMR is that of solvent suppression. Up to now most applications of direct HPLC–NMR have been with adsorption chromatography[1,3–5,8]. In routine HPLC however, about 80% of separations are performed in the reversed-phase mode. When water–acetonitrile mixtures are employed as the mobile phase the resonances of both solvent components have to be suppressed. This was achieved by the use of the gated homodecoupling technique[7]. In a typical HPLC analysis, a total presaturation time of 2 sec was needed. Therefore in these cases the stop-flow technique is obligatory.

Recently, a new effective 1331 hard pulse solvent-suppression technique was described by Hore[9]. The application of this technique to reduce the delay time between acquisition scans and improve suppression factors during continuous flow ^{1}H NMR spectroscopy has been investigated.

It has been demonstrated that broadband-decoupled ^{13}C NMR spectra of acceptable quality can be obtained in a flowing system[2,6]. Nowadays, in ^{13}C NMR spectroscopy, elegant signal-multiplicity assignment techniques using polarization transfer between ^{1}H and ^{13}C nuclei (distortionless enhancement by polarization transfer, DEPT) are employed[10,11]. Thus, we also investigated the possibility of polarization transfer in continuous-flow systems, and of obtaining DEPT spectra.

EXPERIMENTAL

Constant and reproducible flow-rates in all experiments were obtained by use of the pump of a Bruker LC 31 system.

Fluorescence measurement

In order to obtain information about HPLC peak broadening, a Biotronik fluorescence detector was modified to allow different types of flow cell to be adjusted in the beam (Fig. 1). A halogen lamp provided the exciting radiation at a wavelength of 360 nm. Photodiode detection was performed at 470 nm. Dansyl-amino acids used for the test separation (Fig. 2) were prepared as described earlier[12].

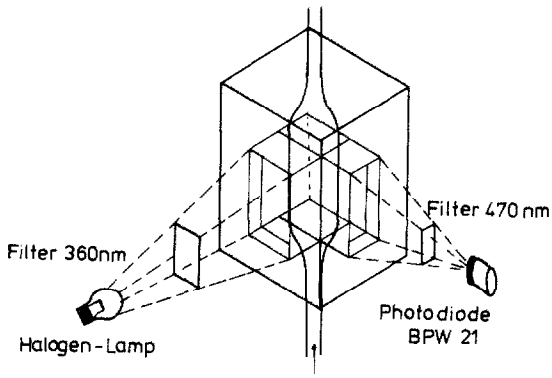

Fig. 1. Modified fluorescence detection arrangement for the determination of peak broadening in different flow cells.

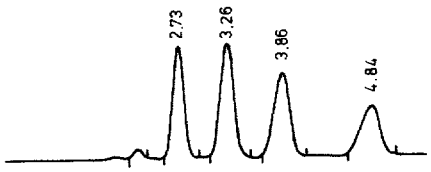

Fig. 2. Separation of dansyl-amino acids: aspartic acid (retention time, t_R = 2.73 min), glycine (t_R = 3.26 min), arginine (t_R = 3.86 min) and alanine (t_R = 4.84 min). Mobile phase: methanol–water (60:40), pH 2.0, flow-rate 1 ml/min. Fluorescence detection: excitation wavelength 360 nm, emission wavelength 470 nm (Biotronik BT 6630).

NMR measurements

^{1}H NMR spectra were recorded on a Bruker AM 300 NMR spectrometer (7.0 T) controlled by the computer system Aspect 3000 including an array processor. A disk system (Control Data Corporation) with a transfer time of less than 2 sec per 8 K interferogram was used for data storage. ^{13}C NMR spectra were recorded on a Bruker WM 400 NMR spectrometer (9.4 T) controlled by the computer system Aspect 2000. Data were stored on a 80-Mbyte disk system (Control Data Corporation). For continuous-flow spectra, NMR flow cells[6,7] were used. The internal diameter of the glass tube in the measuring range was 4 mm, thus resulting in a total measuring volume of 188.5 μl and a residence time of 11.3 sec at a flow-rate of 1 ml/min. In the case of the ^{13}C NMR flow probehead, an additional decoupling coil was mounted axially to the measuring coil.

Solvent suppression was carried out using the 1331 hard pulse method of Hore[9]. The timing of the sequence is: P_1, D_2, P_3, D_2, P_3, D_2, P_1, Acquisition, D_1, where P_n = excitation pulse and D_n = delay time. The $\pi/2$ pulse is divided into four hard pulses in which $P_3 = 3\ P_1$. By adjusting the pulse carrier frequency to the that of the resonance of water, further nulls in the net magnetization (for instance at the acetonitrile resonance) occur at intervals of $1/D_2$. The original pulse of 5.8 μsec was attenuated to 32.0 μsec with the help of a 20-dB attenuator. According to the 1331 sequence, this value was divided into pulse lengths of 4 and 12 μsec, respectively. For data acquisition at a flow-rate of 1 ml/min, a delay, D_1 between scans of 0.1 sec was used. The delay, D_2, between pulses was set according to the frequency difference between the resonances of water and of acetonitrile. In all experiments the total sum of transients was 16.

DEPT spectra were recorded by appropriate change of $\pi/2$ pulse of 42 μsec with a delay time between scans of 2 sec. Thirty-two scans were accumulated in all cases.

RESULTS AND DISCUSSION

Peak broadening in on-line HPLC–NMR

For maximum sensitivity of the NMR measurement, a cell volume equivalent to the chromatographic peak volume is required. On the other hand, chromatographic peak broadening becomes significant when the volume of the detector cell approaches the chromatographic peak volume. The influence of the detector volume upon peak broadening was observed for two analytical columns (4.6 mm I.D., length 125 and 250 mm, Figs. 3 and 4) and a semipreparative column (250 × 8 mm I.D.,

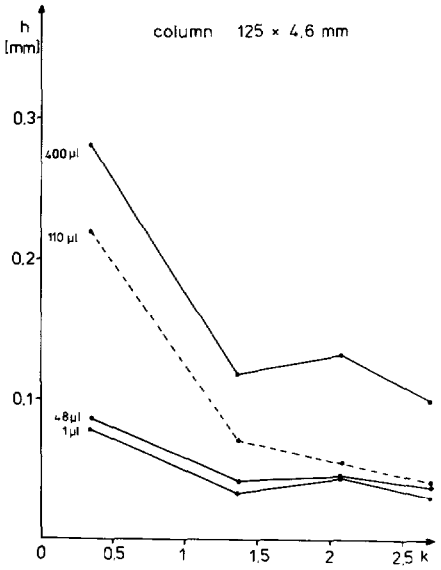

Fig. 3. Effect on plate height (h) of different flow cell volumes using an analytical column (125 × 4.6 mm).

Fig. 5) using the experimental arrangement shown in Figs. 1 and 2. In all cases LiChrosorb RP-18, 7 μm (Merck, Darmstadt, F.R.G.) was used as column packing material. For peaks with capacity factors (k') above 2, detector volumes up to about 200 μl can be tolerated without significant loss in resolution in all cases investigated. Shorter analytical columns and peaks with small k' values require smaller cell volumes. In these cases stop-flow measurement is advantageous.

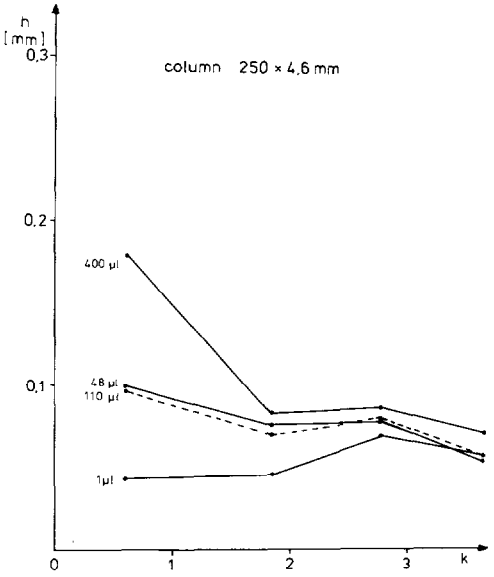

Fig. 4. Effect on plate height of different flow cell volumes using a long analytical column (250 × 4.6 mm).

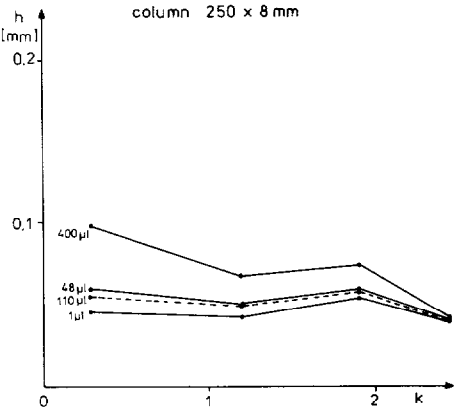

Fig. 5. Effect on plate height of different flow cell volumes using a semipreparative column (250 × 8 mm)

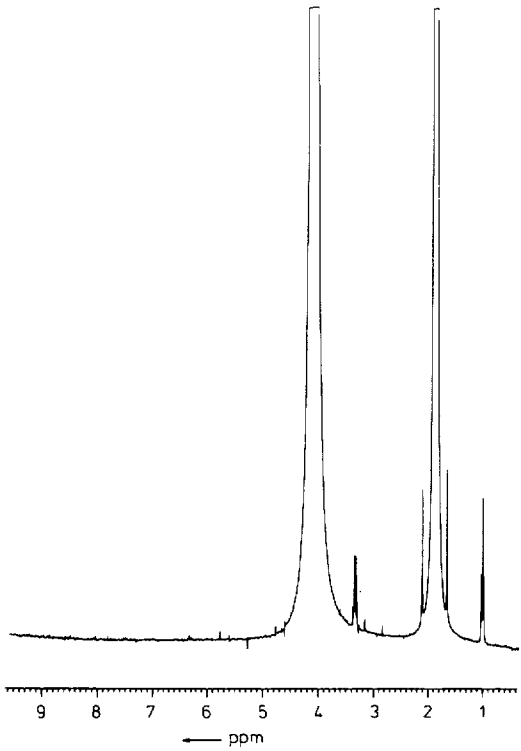

Fig. 6. Continuous-flow ^1H NMR spectrum (300 MHz) of a 1% solution of diethyl ether in acetonitrile–water (50:50). Flow-rate: 1 ml/min. Pulse repetition time: 2.05 sec. Number of data points: 16K. Number of scans: 16. Spectral width: 4000 Hz.

Solvent suppression

The continuous flow ^1H NMR spectrum of a 1% solution of diethyl ether in acetonitrile–water (50:50) is shown in Fig. 6. Using the 1331 hard pulse sequence the solvent signals are effectively reduced (Fig. 7a). By treating the raw data with weighting functions used in two-dimensional experiments, the spectrum of Fig. 7b results. Thus suppression ratios of approximately 200:1 are obtained. In principle, a further decrease in the signal hump and refinement of the hard pulse sequence is possible and can lead to even higher suppression ratios.

Continuous-flow DEPT spectra

The multiplicity of ^{13}C NMR signals may easily be recognized by using the DEPT sequence introduced by Doddrell and co-workers[10,11]. This pulse sequence consists of two spin-echo experiments ($\pi/2$, τ, π) for ^1H and for ^{13}C nuclei, together with a delay time t_1. Polarization transfer is caused by a variable pulse, θ, after the correlated motion of ^1H and ^{13}C nuclei has occurred. The delay time, t_1, is related to the median ^1H–^{13}C coupling constant, J, by $t_1 = 1/2\,J$. Fig. 8 shows the spectra obtained with the ^{13}C NMR flow cell (volume 188 μl). In the decoupled spectrum of cholestryl acetate (BB) all resonances appear. In this continuous-flow spectrum even the signals of an impurity, ethylbenzene, are visible. In the DEPT CH_x spectrum ($\theta = \pi/4$) all quaternary carbon atoms are suppressed. In the CH spectrum ($\theta = \pi/2$), suppression of the CH_2– and CH_3– signals is imperfect because of pulse-angle irregularities at the end of the detection coil. The $\theta = 3\,\pi/4$ continuous-flow DEPT spectrum clearly distinguishes between primary, secondary and tertiary carbon atoms. Using this technique the signal multiplicity in flowing liquids can rapidly be obtained. This is of great interest for continuous process control.

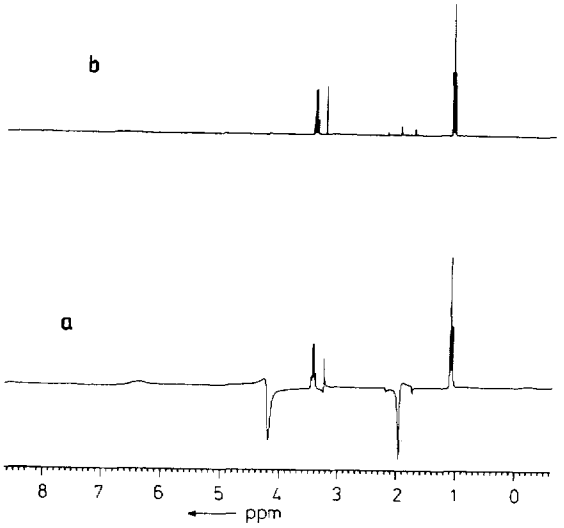

Fig. 7. Continuous-flow ^1H NMR spectrum as in Fig. 6 but using 1331 hard pulse solvent suppression. Pulse repetition time: 2.15 sec. Other details as in Fig. 6. a, Spectrum without further data manipulation; b, spectrum after multiplification of the free induction decay with a sine-square filter function.

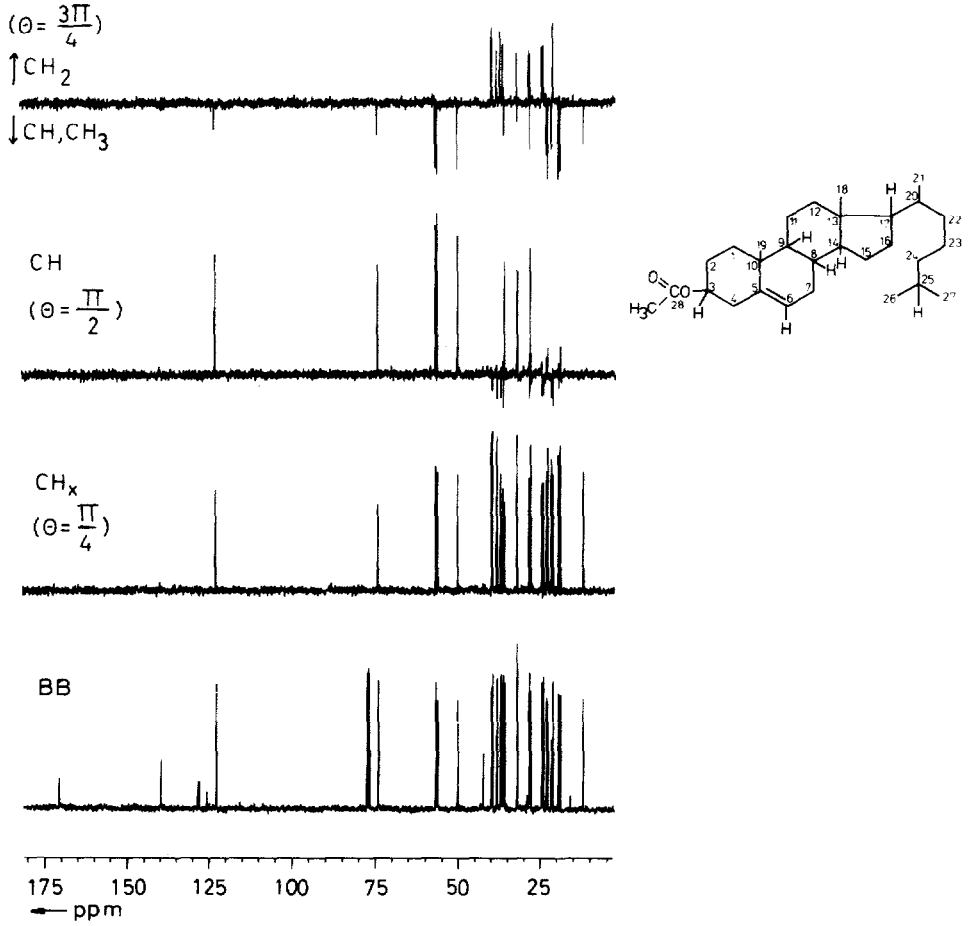

Fig. 8. Continuous-flow ^{13}C NMR spectra (100.6 MHz) of cholesteryl acetate under various conditions. Flow-rate: 1 ml/min. Number of data points: 32 K. Number of scans: 32. Spectral width: 23 800 Hz. Line broadening: 1.5 Hz. BB-spectrum: pulse repetition time, 1.7 sec; composite pulse decoupling. DEPT spectra: pulse repetition time, 2.7 sec; variable pulse angle θ (π = 42 μsec).

CONCLUSION

Continuous-flow NMR spectroscopy is approaching the status of static-rotation NMR. The NMR peak broadening in the flow system is compensated by an increased sensitivity obtainable at low flow-rates[7,8] with adequate detector volumes. The peak broadening effect of these detector volumes in HPLC separation may be neglected. Newly developed one-dimensional pulse sequences are also possible in continuous-flow NMR spectroscopy, allowing many applications.

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