Direct on-line hyphenation of capillary liquid chromatography to nuclear magnetic resonance spectroscopy: Practical aspects and application to drug metabolite identification

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Abstract

In combining the high peak concentrations of capillary liquid chromatography (CapLC) with the high mass sensitivity of micro scale nuclear magnetic resonance (NMR) the hyphenation of CapLC to micro NMR offers a substantial gain in overall sensitivity. This paper deals with our experiences gained using a commercial CapLC-NMR system which has very recently become available. The limits of detection (SNR > 3) for a test compound of a molecular weight of M 318 were found to be ∼100 ng (0.35 nmol) within an hour acquisition time and ∼25 ng over night (85 pmol). Practical aspects such as the feasibility of stopped-flow experiments and sample handling issues are discussed in detail and first possible drug metabolite applications to hepatocyte incubations and direct analysis of plasma samples are presented.
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1. Introduction

Over the last decade liquid chromatography (LC)-nuclear magnetic resonance (NMR), the hyphenation of the most powerful separation method for non-volatile molecules with the most information-rich spectroscopic method for structural elucidation, has become an established analytical tool in particular for drug metabolism studies and natural products analysis [1].

Even though significant progress has been made through the development of spectrometers of higher field strengths and improvements to signal processing, pulse sequences and probe design—the sensitivity of LC-NMR is still low in comparison to other analytical methods.

However two major trends promise to deliver significant improvements to the sensitivity of NMR detection: cryogenic NMR probe technology and miniaturisation of the probe design.

While conventional cryogenic NMR probes are just becoming commercially available and cryogenic flow probes are still in the prototype stage yet, miniaturisation, on the other hand, is currently a major trend in analytical chemistry in general, the advantages of which are particularly apparent in LC-NMR hyphenation.

In capillary liquid chromatography (CapLC)-NMR the concentrations of the eluted peaks are higher than in conventional HPLC (for a given amount of sample the concentration at the peak maximum is typically inversely proportional to the square of the inner diameter of the separation column).

In addition, miniaturised NMR probes show a higher mass sensitivity than conventional ones. Apart from the reduced amount of noise that is generated from a sample that contains less solvent, this improvement is due to the geometry of small solenoid coils—mainly because of the higher inductance per unit volume of microcoils [2].

Furthermore, due to its low solvent consumption, miniaturised chromatography allows the use of fully deuterated eluents, which renders solvent suppression unnecessary, and thus results in better spectral quality.

While the first reports on capillary LC-NMR were published in 1995/1996 [3,4], several publications have appeared since which either dealt with further improvements to the probe design or first applications to standard mixtures

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of natural products [5–9]. Applications on drug metabolites have been reported for CE and CEC-NMR [10,11]. However, apart from a recent report from the spectrometer manufacturer [12] all of these studies were performed using custom-made prototype NMR probes. In this paper, we report on our experiences with a capillary LC probe (MRM/Protasis, Savoy, IL) which has become commercially available only very recently. This probe is based on a design using a solenoidal microcoil, which was developed by Sweedler and others at University of Illinois [13–16] who, along with Albert and others [17] at University of Tuebingen have led the development of micro NMR probes. This paper deals with first practical experiences and applications of a CapLC-NMR probe in an industrial working lab.

2. Experimental

2.1. CapLC system

NMR spectroscopy was performed on a Bruker DRX 600 spectrometer (Bruker Karlsruhe, Germany) equipped with a 1.5 μl capillary NMR probe head (MRM Corp., Savoy, IL, USA) coupled to a Waters CapLC® capillary chromatography system (Waters, Milford, MA, USA) equipped with a UniFlows Degasys Ultimate DU4001 vacuum degasser.

The autosampler of the Waters CapLC® was replaced by a Rheodyne (Rohnert Park, CA, USA) 7725i injection valve with a 10 μl custom-made injection loop.

A VICI/Valco four-port valve was added to the flow path in order to shut off the flow for stopped-flow experiments (valve positions both up and down stream of the column were tried). The fluidic connections were made using fused silica capillary with an i.d. of 50 μm and a o.d. of 360 μm (PolymicroTechnologies, USA). The standard UV cell of the Waters chromatography system was placed externally by using two 3 m long 400 μm i.d. light fibre connectors (Anglia Instruments, Soham, UK).

The separation column used was a 0.3 mm × 100 mm i.d., 3 μm, Targa C-18 from Higgins Analytical, Mountain View, CA, USA.

The eluents used were (a) 0.1% formic acid-d3 (Sigma–Aldrich) in D2O (99.9 at.%, Fluorochem, Old Glossop, UK) and (b) Acetonitrile-d3 (99.8 at.%, Sigma–Aldrich, Milwaukee, WI, USA). The eluent gradient used was 1–90% B in 30 min unless otherwise stated.

Flow rates were determined by connecting a 10 μl micro syringe to the outlet of the chromatography system and measuring the amount of time taken to fill a 5 μl volume.

^{1}H NMR spectra were recorded with or without presaturation of the residual HOD signal using the following parameters: time domain size 16 k data points, sweep width 9.2 ppm, acqisition time 1.36 s, relaxation delay 1 s; processed into 32 k data points using an exponential multiplication of 1 Hz. The 90° pulse width was 11.5 μs at 22 dB attenuation.

The 2D COSY spectra were recorded using the pulse sequence cosypr and the following settings: time domain size 2 k data points, 512 increments, 8 scans per increment, sweep width 9.2 ppm, acquisition time 0.17 s, relaxation delay 1 s; processed into 2 k × 512 data points using unshifted sinusoidal windows in both dimensions.

2.1.1. Off-line NMR

Off-line NMR spectra for comparison purposes were recorded using a Bruker DRX 600 and a DRX 700 spectrometer, each equipped with a 5 mm TXI probe head. Samples were dissolved in 600 μl of a D2O/acetonitrile (50:50) mixture and spectra were recorded using the dual presaturation sequence lc1d12 according to our standard laboratory procedure. NMR parameters were chosen to correspond to those of the CapLC-NMR experiments.

2.1.2. Sample preparation

Sample preparation for CapLC-NMR analysis was generally done by a two-step drying process (in a 1.5 ml Eppendorf vial, subsequently in a 200 μl HPLC vial) under a stream of nitrogen. If necessary, samples were centrifuged beforehand. Prior to injection samples were dissolved in approximately 5 μl eluent.

Protein from plasma samples was precipitated by adding four times the sample volume of acetonitrile and subsequent centrifugation at 5000 rpm for 60 min.

3. Results and discussion

In order to gain experience with the CapLC-NMR system, diclofenac a common non-steroidal anti-inflammatory drug with two aromatic rings and a molecular weight of 318 was chosen as a test compound.

All preliminary tests were aimed at assessing the suitability of the system for drug metabolite identification applications including the feasibility of gradient elution and stopped-flow experiments. The handling of mass limited samples, the detection limits obtainable and the spectral quality in the presence of matrix were also of significant interest.

3.1. Practical aspects

Since even microcoil NMR is insensitive in comparison to other detection methods there is still a need to perform stopped-flow experiments in order to allow sufficient acquisition time so that lower detection limits may be achieved through signal averaging. So far, in most publications on CapLC-NMR stopped-flow experiments were triggered by NMR detection, and UV triggered stopped-flow experiments have only been reported very recently [6].

Although it has been demonstrated that a long transfer capillary between the UV cell and the NMR probe of 3–5 m does not negatively affect the chromatographic peak integrity

[18], a long transfer capillary proved to cause problems in gradient elution mode in our hands.

It was found that in isocratic mode (50:50 D2O/ACN-d3) the peak transfer time from UV cell to NMR probe was 2:45 min, whereas in gradient mode (1–90% ACN-d3 in 30 min) this reduced to less than 2:25 min. Since the stop-flow transfer time determined for isocratic mode did not significantly differ from the transfer time in on-flow mode it can be assumed that this variation is mainly due to a variation in solvent flow rates.

Measurement of the actual flow rate revealed up to a 10% variation in flow rate over a 30 min 1–90% ACN gradient. In periods of falling backpressure (due to changes in solvent viscosity) an increased flow rate was observed and similarly the flow rate decreased when backpressure rose.

These findings suggest variations in flow caused by changes in the compressibility and viscosity of the eluent. Since the variations are inherent to the instrument, the best option is to reduce the transfer time so as to minimise the effects of the flow rate variation. This was achieved by relocating the original Waters-UV cell to the base of the NMR magnet and connecting it via a 3 m long light fibre connectors. This reduced the measured transfer time to around 45 s. which reduced the effects of the flow rate variation to an acceptable level.

This arrangement maintained approximately 50% of the original UV sensitivity whereas evaluation of a custom-made capillary scale Bruker/J&M prototype micro flow cell showed less sensitivity. In addition, the stopped-flow shut-off valve is best placed downstream of the column to prevent compressed solvent from relaxing off the column.

3.1.1. Probe dimensions

In order to gain optimal NMR response in LC-NMR experiments the peak volume should not exceed the active volume of the NMR probe, i.e. the probe dimensions should be tailored to the dimensions of the chromatographic system. In our hands, the eluted peak volumes (calculated as peak width at half height × flow rate) of a 100 ng injection of diclofenac were 0.85 μl and thus even below the active volume of the NMR probe. Due to peak concentration in gradient elution (1–90% B/30 min) there was no difference whether the sample injection volume was 0.1 μl or 6.4 μl. Very polar compounds however might show some broadening if injected in large injection volumes, depending on their retention properties and the solvent, they are dissolved in. In such cases a smaller injection volume should be preferred. The dimensions of the capillary LC-NMR probe are thus well adjusted to the dimensions of the capillary chromatographic system.

3.2. Limits of detection for the CapLC-NMR system (LODs)

The limits of detection for the capillary LC-NMR (defined as the absolute amount of sample, that gives a S/N of 3 or above for the weakest signal) were again determined using diclofenac as a test compound. Fig. 1 shows a dilution series of 536, 134 and 67 ng diclofenac injected on column. The
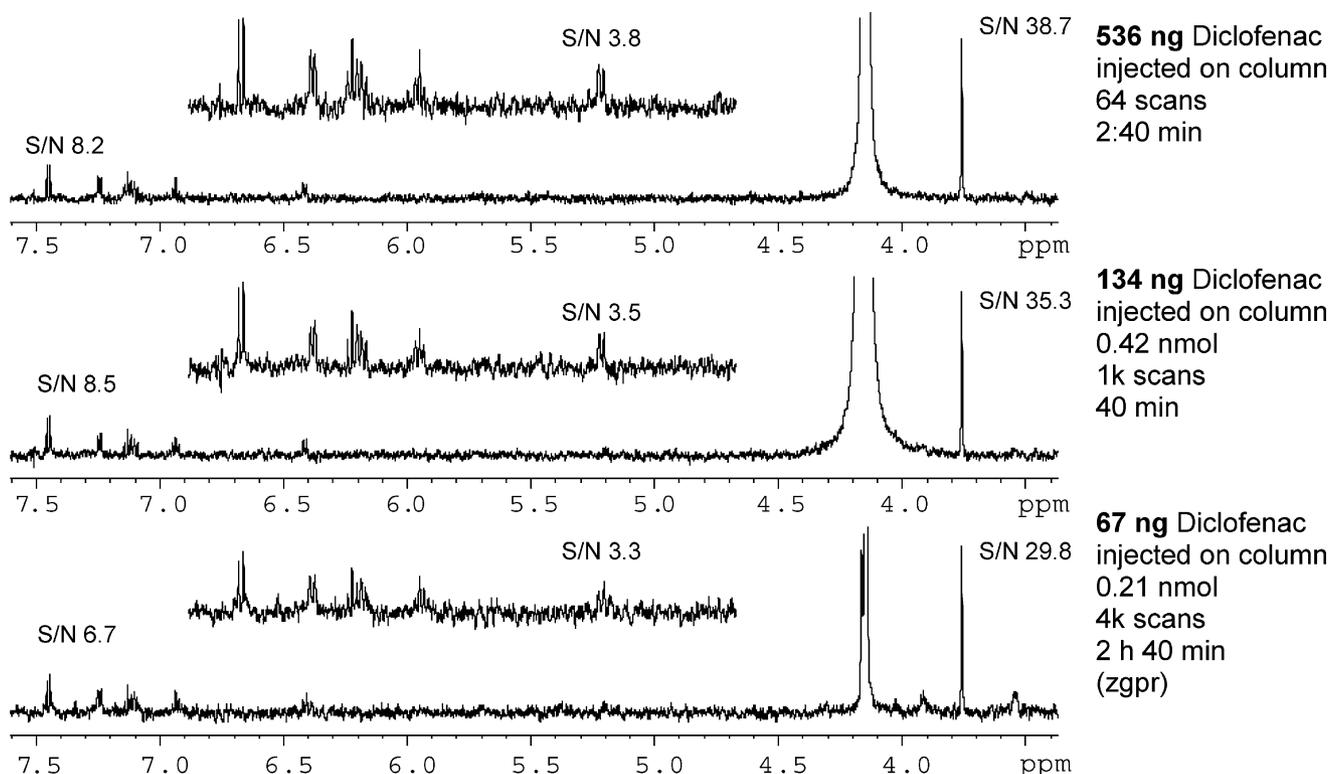
Fig. 1. Limits of detection—stopped-flow on-column injections of diclofenac (MW 318).
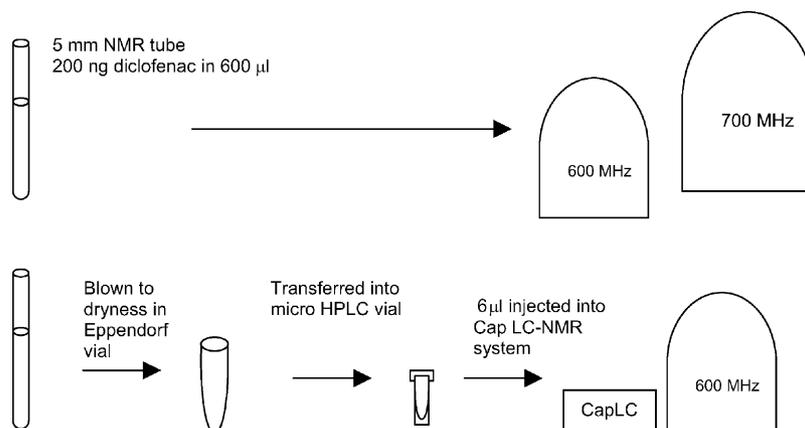
Fig. 2. Diclofenac (200 ng) comparison of 5 mm tube NMR with CapLC-NMR—experimental scheme.

limit of detection for diclofenac was found to be 0.35 nmol within 1 h. Note that in theory the S/N should be the same for all three injections ($S/N \approx N_{scans}^{1/2}$). The decrease in S/N with increasing experiment time is probably due to diffusion of the sample from the active volume (1.5 μl) to the total volume (5 μl) of the NMR flow cell. The detection limit was found to be 25 ng diclofenac (85 pmol) for overnight data acquisition.

3.3. Sample handling compared to conventional tube NMR

In mass limited applications such as drug metabolite identification or natural products elucidation it is a prerequisite for optimal results to gather all available sample in the active volume of the NMR flow cell. In order to do so small amounts of sample need to be handled and the aim is to inject the whole sample on to the system without too much loss due to sample handling. In our hands, it has been of advantage to use a conventional Rheodyne HPLC injector with a 10 μl sample loop rather than a (micro injection) autosampler. In this way the sample (typically dissolved in 5 μl eluent) can be injected directly using the microliter syringe that is used for sample handling and make-up. Whereas with an autosampler, even working in microliter pick-up mode a considerable amount of a 5 μl sample will always be lost on the walls of the HPLC vials.

In order to demonstrate the overall advantage of the capillary scale NMR detection with respect to conventional tube NMR, a 200 ng diclofenac sample was recored in a 5 mm tube at 600 and 700 MHz. The sample was then concentrated and injected on to the CapLC-NMR system (Fig. 2). This experiment takes into account the amount of sample that is lost due to handling very low sample volumes. Even taking these losses into account, CapLC-NMR is still 7× more sensitive than conventional off-line NMR (Fig. 3).

3.4. Hepatocyte incubations

Hepatocyte incubations are gaining more and more importance in the early stages of metabolite investigations. Due
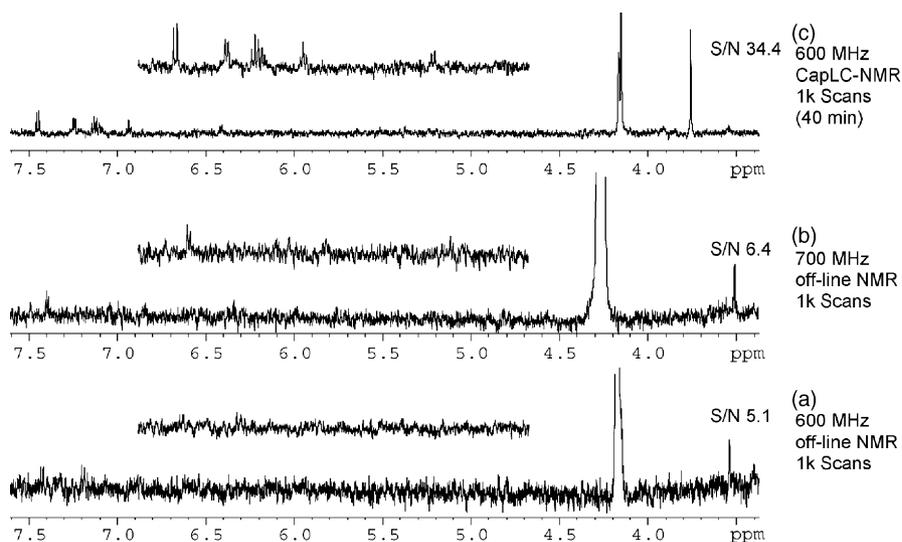
Fig. 3. Diclofenac (200 ng) comparison of 5 mm tube NMR with CapLC-NMR—experiment results (see Fig. 2).
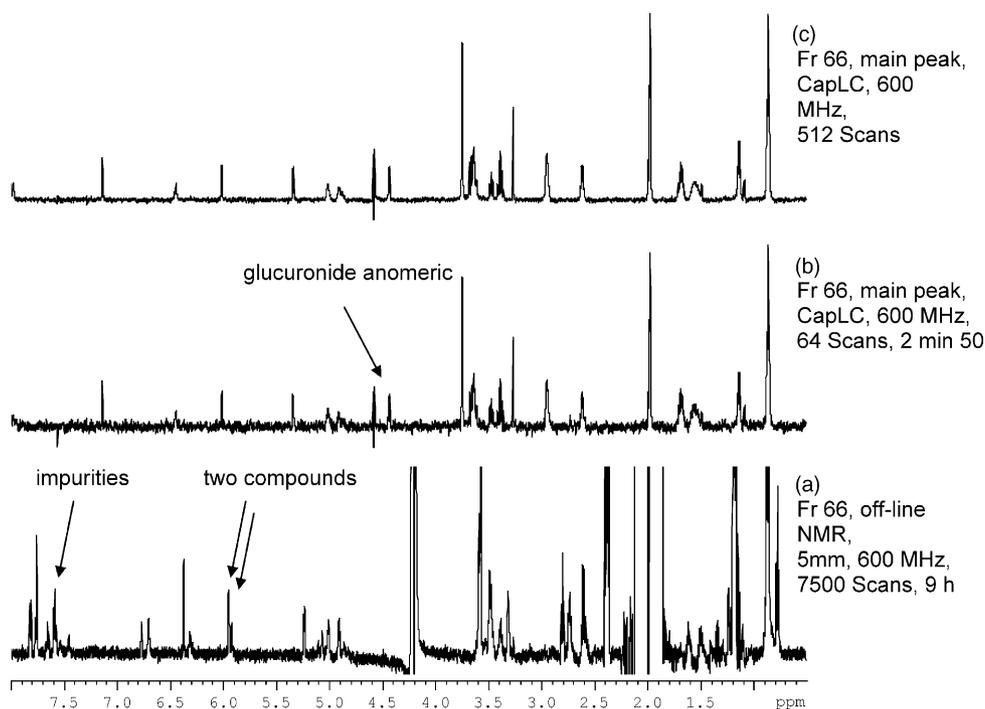
Fig. 4. GW433737 rat hepatocyte incubation (50 μm), preparative fraction no. 66—comparison of 5 mm tube NMR (inverse configuration) to CapLC-NMR at 600 MHz.

to small incubation sizes though, the absolute amount of metabolite is limited and such samples suggest themselves for CapLC-NMR analysis. Fig. 4a shows a spectrum of a fraction that was obtained from a preparative separation of a rat hepatocyte incubation of GW433737 (50 μM). This type of sample usually contains only small amounts of metabolites and thus requires long acquisition times using our standard NMR procedures. The two upper spectra (Fig. 4b and c) show the results of the CapLC-NMR experiments: a signif-

icant increase in sensitivity (2:50 min acquisition time compared to 9 h before), the absence of endogenous impurities as well as the separation from a further co-eluting metabolite.

3.5. High dose plasma studies

Direct NMR analysis of circulating metabolites in blood plasma represents another mass limited drug metabolite application. Due to limited NMR sensitivity, this type of
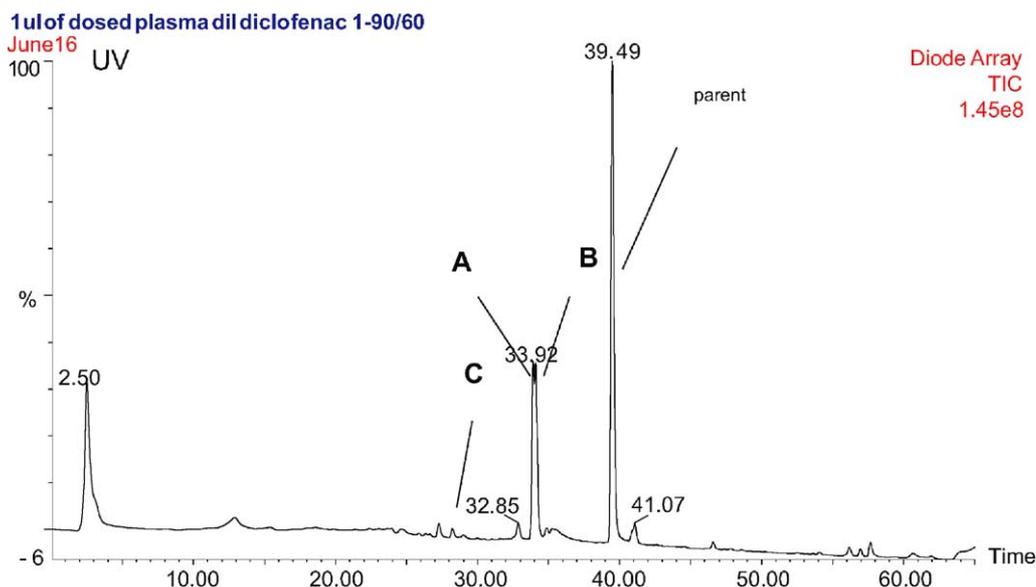
Fig. 5. UV chromatogram of rat plasma after 50 mg/kg i.v. dose of diclofenac (pooled time points 10–60 min after dose), equivalent of 200 μl plasma injected onto column, injection volume ca. 5 μl.
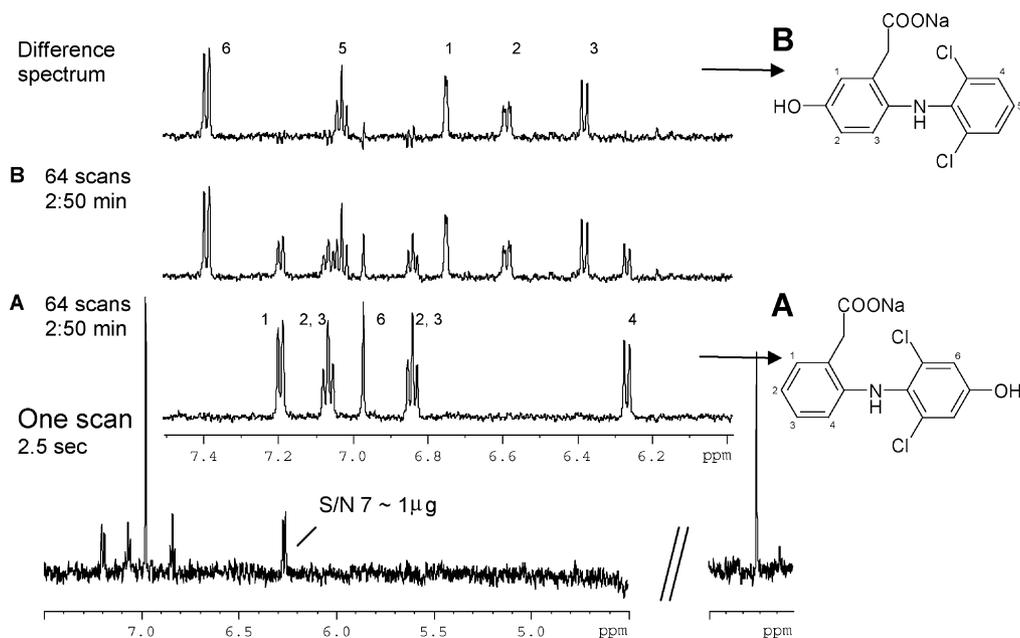
Fig. 6. CapLC-NMR spectra of the two main metabolites of diclofenac in rat plasma (50 mg/kg i.v. dose, pooled time points 10–60 min after dose), equivalent of 200 μl plasma injected onto column, injection volume ca. 5 μl. Estimated amount of substance in probe: ∼1 μg (based on S/N).

sample has not been accessible to conventional scale LC-NMR until now. Figs. 5–8 show the results obtained from a high dose Diclofenac study (50 mg/kg, i.v.) which was commissioned in order to gain first experiences on the suitability of CapLC-NMR for this kind of samples. The sample preparation consisted of a simple acetonitrile precipitation and after centrifugation and concentration the equivalent of 200 μl plasma was injected on to the CapLC-NMR system. As shown in Fig. 6, the two main

metabolites in the sample could be readily detected with only one scan each and 64 scans yielded spectra of off-line NMR-like quality. The use of fully deuterated solvents that is possible in capillary scale contributes to the high spectral quality (straightforward phase correction and excellent baseline).

Fig. 7 shows a 2D COSY spectrum (8 scans, 512 increments) acquired from the mixture of the two main metabolites in stopped-flow mode within 90 min.
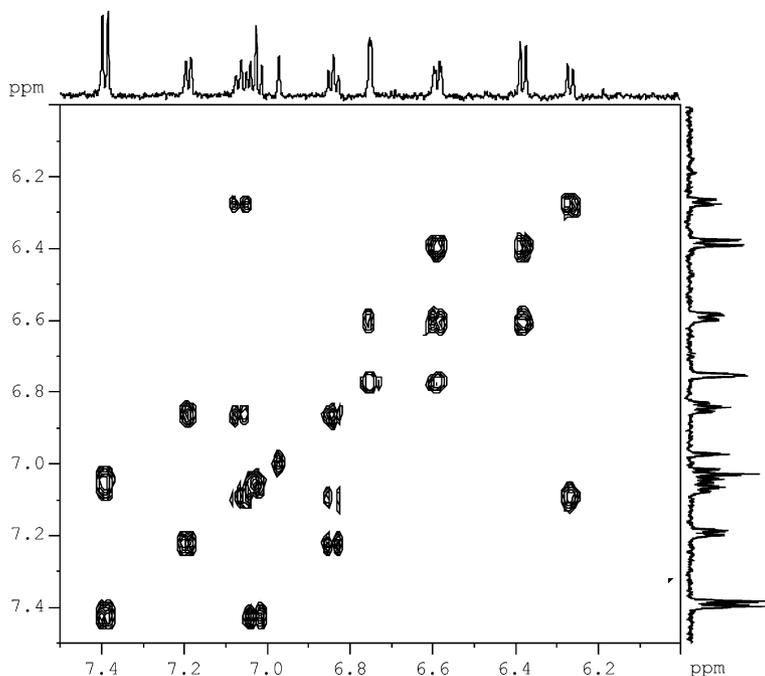
Fig. 7. CapLC-COSY spectrum of the coeluting two main metabolites of diclofenac in rat plasma.
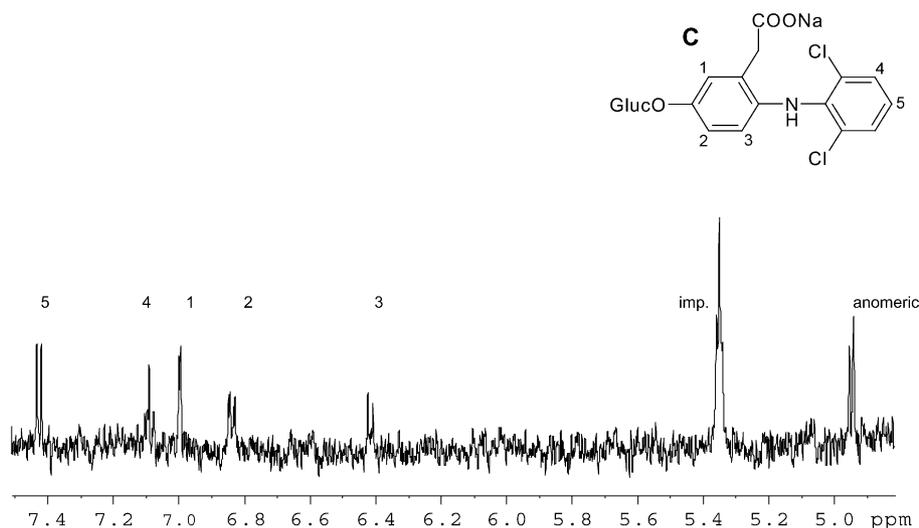
Fig. 8. NMR spectrum of a minor glucuronide metabolite (peak C in Fig. 5) of diclofenac in rat plasma (sample had been recovered and re-injected), 2924 scans, 1 h and 55 min acquisition time, equivalent of 200 μl plasma injected onto column, assignments based on chemical shifts.

The suitability of CapLC-NMR to characterise minor compounds from a plasma sample is illustrated in Fig. 8, a spectrum of the minor glucuronide metabolite of diclofenac which was acquired within 2 h, even though the sample had been recovered from the earlier stopped-flow experiments and had been re-injected. These results suggest that it should be possible to record high quality spectra of the main metabolites from 10 mg/kg dose studies.

4. Conclusions

CapLC-NMR shows excellent sensitivity which is about an order of magnitude higher than conventional NMR set-ups. It thus opens fields of research for direct LC-NMR analysis that have not been accessible so far.

In our hands, the samples best suited for this technique are mass limited ones with medium or low matrix complexity such as plasma samples or pre-fractionated samples, for which it is used routinely in our laboratory. This is mainly due to the difficulty of detecting the peaks of interest for stopped-flow analysis by pure UV detection if the sample is too complex, since the more selective detection methods of choice—radio detection or parallel MS detection—are not available for capillary scale.

The handling of small sample volumes requires special attention but can be reliably managed. Some samples however, might precipitate if dried down to high concentrations in small volumes. In this respect conventional scale LC-NMR might be more widely applicable. Apart from the gain in sensitivity, the main advantage of CapLC-NMR over LC-NMR is the use of fully deuterated solvents which results in higher spectral quality without the need to set-up suppression sequences and without the loss of important parts of the NMR spectrum.

At the present time the reliable trapping of the peaks for stopped-flow acquisitions due to flow rate variations represents the main obstacle to CapLC-NMR-becoming a widely used routine technique. External UV cells and suitably placed switching valves circumvent these problems and the manufacturers are currently developing solvent delivery units that address these problems.

Acknowledgements

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