HPLC–SPE–NMR in pharmaceutical development: capabilities and applications†

Martin Sandvoss,1* Ben Bardsley,2 Tony L. Beck,2 Emma Lee-Smith,2 Stephanie E. North,1 Peter J. Moore,2 Andrew J. Edwards2 and Richard J. Smith2

1 DMPK Structural ID Group, GlaxoSmithKline R&D, Park Road, Ware SG12 0DP, UK
2 Analytical Sciences, Chemical Development, GlaxoSmithKline R&D, Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, UK

Received 29 September 2004; Revised 24 March 2005; Accepted 12 April 2005

High-performance liquid chromatography–solid phase extraction–NMR spectroscopy (HPLC–SPE–NMR) has recently become commercially available and has been evaluated with regard to its applicability in a pharmaceutical environment. The addition of an automated SPE unit to an HPLC–NMR system for peak trapping results in an improved NMR signal-to-noise ratio (S/N) and also has other practical advantages. The trapping efficiency is shown to depend on compound polarity and is highest for compounds eluting late on reversed-phase HPLC systems. Multiple peak trapping further increases the S/N, again with the best results for less polar compounds. For polar compounds, multiple peak trapping resulted in no S/N gain as the amount of material retained on the SPE cartridge was equivalent to that from a single injection. When compared with conventional HPLC–NMR, a S/N gain of up to five-fold could be achieved for some compounds in a single trapping step. A major advantage of the technique is the independence of the chromatographic step from the NMR step, resulting in greater versatility than conventional HPLC–NMR in the HPLC solvents and NMR solvents that can be used. Practical applications from both drug metabolite and drug impurity identification are presented. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: HPLC–SPE–NMR; hyphenated techniques; drug development; solid phase extraction; metabolite identification

INTRODUCTION

The hyphenation of NMR with high-performance liquid chromatography (HPLC) was pioneered in the 1980s, found wide application from the mid-1990s and is now a well-established analytical technique, especially in pharmaceutical and natural products research.1,2 However, partly owing to the intrinsic insensitivity of NMR and partly owing to practical limitations, the absolute sensitivity of the method has remained below that of other structural elucidation techniques (e.g. HPLC–MS). In recent years, some of this inherent insensitivity has been overcome by improvements in spectrometer hardware and solvent suppression pulse sequences which allow small molecules in the low to sub-microgram range to be analysed. Further improvements in spectrometer hardware such as cryogenic probe technology and miniaturized probes are now becoming widely available and these are leading to greater steps in sensitivity enhancement.

Recently, a different approach to performing HPLC–NMR experiments has become available in which the separation step of HPLC–NMR has been modified so as to increase the absolute sensitivity of the technique. In this method, analytes eluting from the HPLC column are trapped on solid phase extraction (SPE) cartridges prior to subsequent elution into the NMR flow probe for analysis using an NMR-compatible solvent: this is HPLC–SPE–NMR. First published as a concept by Griffiths and Horton in 19983 and later applied using SPE cartridges in a custom set-up,4,5 this technique has recently become commercially available6,7 and its application to natural product analysis has been reported elsewhere.8

The traditional use of SPE in analytical chemistry is as a sample purification and enrichment tool prior to HPLC analysis.9 However, in HPLC–SPE–NMR it is used as a means of trapping and concentrating analytes after HPLC separation and through the use of strong solvents the analyte can be eluted from the cartridge in a sharp, highly concentrated elution band, which results in an improved signal-to-noise ratio (S/N) compared with conventional HPLC–NMR.

The use of NMR probe dimensions matched to the elution volume (flow cell volume of 30 µl) makes optimal use of the available sample as the sample volume for the NMR analysis is now independent of the original chromatographic peak volume. This has always been a problem with conventional HPLC–NMR where loss of sensitivity is often seen because of inefficient collection of broad peaks. In general, the column dimensions can now be freely chosen so that, for example, classical 4.6 mm i.d. columns can be combined with highly mass-effective small-scale probes.

†Presented as part of a special issue on Hyphenated NMR Techniques.
*Correspondence to: Martin Sandvoss, DMPK Structural ID Group, GlaxoSmithKline R&D, Park Road, Ware SG12 0DP, UK. E-mail: martin.x.sandvoss@gsk.com

Copyright © 2005 John Wiley & Sons, Ltd.
The use of fully deuterated solvents for NMR ensures maximum NMR compatibility, often rendering the need for solvent suppression unnecessary and leading to significantly cleaner baselines in the NMR spectra obtained. The solvent can be chosen to prevent NMR signal overlap and is independent of the chromatographic solvent requirements. Hence, by choosing fully organic, non-protic solvents, it is still possible to observe exchangeable protons in the NMR spectrum. Finally, owing to the small dimensions of the NMR probe, only small and therefore cost-effective amounts of deuterated solvent of the order of 240–300 µl are required.

On the chromatographic side, the choice of HPLC mobile phases is independent of the NMR solvent considerations. This not only allows the use of inexpensive protonated solvents or NMR-incompatible buffer systems for chromatography but also allows the original chromatographic method to be used without any redevelopment. In HPLC–NMR it has typically been found that the switch from water to deuterium oxide as an HPLC eluent generally results in slightly different elution characteristics. Since the separation is performed using protonated solvents (water), the hyphenation of a mass spectrometer to the system is straightforward as no shift in mass due to deuterium exchange is observed.

A further benefit of HPLC–SPE–NMR over conventional HPLC–NMR is that the S/N can be further increased by trapping of peaks from consecutive injections on to a single SPE cartridge without overloading the separation column or without the need to use a semi-preparative-scale system. In addition, this trapping of peaks can be performed off-line from the spectrometer, allowing it to be used for other purposes (e.g. conventional tube NMR).

On the downside, there is a possible complication to NMR inherent in the HPLC–SPE–NMR approach. NMR is a non-selective detection method used for the identification of unknown analytes. Because of its non-discriminatory detection of protons, it is often used for screening mixtures of unknowns when other analytical techniques such as UV/VIS or MS methods do not yield a sufficiently uniform response. Even in conventional HPLC–NMR it can be assumed that any proton-containing compound that elutes from the column will be detected by the NMR. However, the interfacing of an SPE step to the system adds a degree of chemical discrimination to the system. It can no longer be assumed as a given fact that every analyte of a mixture of unknowns will be detected. An analyte with unknown characteristics might not be retained on the trapping cartridge or might irreversibly bind to the stationary phase material. This dilemma cannot be solved in principle, although with further understanding we may be able to select particular solid phases for the retention of particular types of molecule (e.g. polar, non-polar, specific functional groups). It is the aim of this paper to report our practical experiences and describe the applicability and benefits of the HPLC–SPE–NMR approach from a drug development perspective.

**EXPERIMENTAL**

**HPLC–SPE–NMR**

The HPLC–SPE–NMR systems used consisted of an Agilent (Waldbonn, Germany) 1100 HPLC system and a Bruker/Spark Holland Prospekt II SPE unit under the control of Bruker Hystar 3.0 software (Bruker, Rheinstetten, Germany). The NMR spectrometers used were a Bruker DRX600 (for metabolite ID work) and an AV700 (for impurities), equipped with 30 µl dual inverse $^1$H/$^13$C and 30 µl triple resonance inverse $^1$H/$^{13}$C/$^{15}$N z-gradient flow probes, respectively.

**SPE method**

Unless noted otherwise, the following standard SPE method was applied: after HPLC separation on reversed-phase columns using acetonitrile–water gradients, the peaks of interest were trapped on Spark Holland Hypersphere Resin GP cartridges (2 × 10 mm) by adding water to the eluent flow at three times the flow-rate. The loaded cartridges were then dried using a stream of nitrogen for 30 min and the trapped analytes were subsequently eluted from the cartridge with 240 µl of acetonitrile-$d_3$ (Sigma Aldrich, Milwaukee, WI, USA) dispensed by a syringe pump of the Spark Prospekt II unit.

**HPLC conditions**

The following HPLC column and eluents were used for analysis of the test compounds and drug impurities: 150 × 4.6 mm Phenomenex Luna C18(2), 5 µm particle size, flow-rate 1 ml min$^{-1}$, 0.05% (v/v) aqueous trifluoroacetic acid–acetonitrile (ACN) gradient (0 min, 0% ACN; 25 min, 95% ACN). For metabolite identification work the conditions were as follows. GSK-A: 250 × 4.6 mm ACT ACE5 C18 column, 5 µm particle size, flow-rate 1 ml min$^{-1}$, 50 mM ammonium acetate (pH 5)–acetonitrile gradient (0–5 min, 5% ACN; 10 min, 25% ACN; 45 min, 50% ACN; 50 min, 95% ACN). GSK-B: 250 × 4.6 mm Waters Symmetry Shield RP8 column, 5 µm particle size, flow-rate 0.8 ml min$^{-1}$, 50 mM ammonium acetate–acetonitrile gradient (0–6.25 min, 4% ACN; 12.5 min, 10% ACN; 58.75 min, 40% ACN; 66.25 min, 95% ACN). GSK-C: 250 × 4.6 mm Hypersil BDS column, 5 µm particle size, flow-rate 1 ml min$^{-1}$, 50 mM ammonium acetate (pH 4.5)–acetonitrile gradient (0–8 min, 15% ACN; 28 min; 22% ACN; 50 min, 55% ACN; 55 min, 95% ACN). GSK-D, E and F: 150 × 4.6 mm Zorbax SB-C8 column, 3.5 µm particle size, flow-rate 0.8 ml min$^{-1}$, 25 mM ammonium acetate (pH 8.6)–acetonitrile gradient (0 min, 1% ACN; 37.5 min, 30% ACN; 40 min, 90% ACN).

**Test compounds**

A test mixture comprising equimolar quantities (3.75 mM) of commercially available phthalic acid, benzoic acid, propiophenone, valerophenone and octanophenone dissolved in methanol was used for measuring system performance. A 10 µl volume of this mixture was injected on to the column.

**NMR**

Quantitative $^1$H NMR experiments employed a relaxation delay of 10 s, a pulse width of 30°, 128 scans, 32 K data points.
processed into 32 K points and all were recorded using a fixed receiver gain to ensure comparability of spectra. Spectra were processed with exponential line broadening of 0.3 Hz. Typical conditions for metabolite identification NMR were NOESY presaturation solvent suppression, a relaxation delay of 1 s, 64 K data points processed into 32 K points and an exponential line broadening of 1 Hz.

Quantification of compound recoveries

The quantification of absolute amounts in the flow cell was achieved by using the fixed concentration, residual acetonitrile-$d_2$ signal in the $^1$H NMR spectra. For a given charge of acetonitrile-$d_3$ (typically $>99.8$ at.% D) the acetonitrile-$d_2$ signal was quantified against a known reference standard and then itself used to quantify the concentration of analytes in the active volume (30 µl) of the NMR flow cell.

RESULTS AND DISCUSSION

System capabilities

In order to achieve trapping results with a maximal S/N, the SPE system should ideally meet the following demands:

- Quantitative trapping of peaks on solid phase cartridges without elution of any material from the cartridge while in contact with mobile phase.
- Elution of the analyte from the SPE cartridge in a sharp, complete front without any peak broadening.
- Applicability of the solid phases to a wide range of different classes of chemical compounds (drug-related metabolites, impurities and degradants) and an elution solvent which dissolves a wide range of classes of compounds.

The following describes to what degree HPLC–SPE–NMR meets the above demands for such an ideal system.

Pharmaceutical compounds, for example actual drug substances, intermediates, impurities, degradants and metabolites, span a wide range of chemical classes. One of the key points to making the most efficient use of HPLC–SPE–NMR therefore is knowing how these different classes of compound will behave, in terms of retention and elution from the solid phase of the cartridges that are used. A test mixture composed of a group of compounds spanning a range of polarities and retention and elution characteristics was investigated so as to try to understand how the behaviours of particular pharmaceutical compounds can be predicted for future HPLC–SPE–NMR work.

The test sample that was used was composed of an equimolar mixture of five commercially available compounds (Fig. 1). This test mixture gives rise to a reversed-phase chromatogram (acetonitrile gradient) where the most polar compound elutes at $\approx$32% acetonitrile and the least polar component elutes at $\approx$92% acetonitrile. Our work with these compounds used the HySphere resin-GP general-purpose polydivinylbenzene-based resin, which in preliminary experiments yielded significantly better results than RP-18 cartridges from the same manufacturer (Spark Holland). The effectiveness of the trapping and elution process was measured by transfer of the peaks eluting from the HPLC column into the NMR probe and measurement of the amount of signal generated for each compound under the same NMR conditions. Theoretically, all of the compounds should generate the same level of signal in the NMR spectrum if they are all trapped on the SPE cartridges quantitatively and eluted quantitatively into the active volume of the NMR probe. Integral values, rather than S/N values, were used to compare results for the selected compounds to negate any effects from variations in peak widths, either intrinsic or from shimming differences. To ensure comparability of the integral results for the different compounds, the $^1$H NMR spectra were recorded under identical conditions.

The results (Fig. 2) show that there is a significant variation in the integral value (normalized to one proton)

![Figure 1. HPLC trace for an HPLC–SPE–NMR test mixture (phthalic acid, benzoic acid, propiophenone, valerophenone and octanophenone). The diagonal line indicates the percentage of acetonitrile in the mobile phase.](image-url)
obtained, and hence on the amount of material transferred into the NMR flow cell, for each of the compounds in the test mixture. In particular, it can be seen that the best results were obtained for the relatively non-polar propiophenone and valerophenone whereas the results for the more polar benzoic acid and phthalic acid indicate that the trapping and elution process for these compounds is not as efficient as for the less polar compounds. The integral value obtained for phthalic acid is a factor of 3.5 lower than that for valerophenone. The likely reason for this difference is that the polar compounds are not retained on the cartridges as well as the non-polar compounds. However, at the other end of the scale, octanophenone, the most non-polar compound, is expected to have a high affinity for the solid phase. It is possible that the reduced level of signal observed for octanophenone compared with propiophenone and valerophenone is due to incomplete elution from the cartridge resulting from this high affinity.

The multiple trapping of compounds on cartridges involves repeat injections of the same solution on to the HPLC column and successive trapping of a particular fraction on the same cartridge. Multiple trapping has the potential to increase the amount of material available for NMR and significant improvements in experiment time for achieving a given signal strength may be possible, particularly for peaks from low-level impurities. To evaluate the effect of multiple trapping on the NMR response, the trapping process was repeated either two or five times. For each experiment, the compounds in the test mixture were trapped on SPE cartridges. From the results of these experiments (Fig. 2), it appears that for polar compounds, such as phthalic acid, there is no significant benefit from multiple trapping, whereas for the less polar compounds there are significant increases in signal obtained for the two-times and five-times trapping experiments.

The increases in integral that are obtained through multiple trapping can be quantified by comparing the ratio of the signal achieved in the multiple trapping experiment with that achieved in the single trapping experiment (Fig. 3). The least polar compound, octanophenone, demonstrates that multiple trapping can be as effective as the theory suggests, although given that octanophenone does not appear to elute fully from the cartridge (Fig. 2) this multiple increase is relative only to that original result. However, as the polarity of the compounds increases, the efficiency of the multiple trapping decreases. This is most evident for the experiment with five injections of phthalic acid, and these results support the idea that phthalic acid has poor affinity for the stationary phase of the SPE cartridge. The indication is that although multiple trapping may be effective for non-polar compounds, the benefit of each subsequent injection decreases as the polarity of the analyte increases up to the point where the results for multiple trapping are no better than for single trapping.

To evaluate the recovery of the SPE–NMR experiments, expressed as the amount of material in the active volume of the NMR flow cell relative to the amount injected on to the column, the sample amounts in the flow cell were measured using the residual acetonitrile-$d_2$ signal from the solvent as an internal standard. The results are presented in Fig. 4 as the percentage of the amount of material observed in the NMR flow cell compared with the amount originally injected on to the HPLC system for each compound versus the compound polarity.

The maximum recovery into the flow cell active volume observed in the single trapping experiments was ~40%, for valerophenone and propiophenone. As the number of trappings increases, the overall recovery is reduced in all cases except for octanophenone. The recovery into the active volume of ~40%, as the best value, may not appear at first sight to be particularly high. However, it should be noted that this value represents the percentage of the injected amount of material in the active volume of the flow cell. The full flow cell volume is double the active volume and it can be assumed

---

Figure 2. Plot of relative integral (arbitrary scale) in the $^1$H NMR spectrum versus percentage of acetonitrile at which the compound elutes, for the spectra obtained for each of the compounds in the test mixture resulting from a single trapping experiment, a double trapping experiment and a five-times trapping experiment. The five compounds in order of elution are phthalic acid, benzoic acid, propiophenone, valerophenone and octanophenone.

Figure 3. Plot showing factor increase in signal obtained in multiple trapping experiments when compared with the result obtained from the single trapping experiment for each of the compounds in the test mixture. By definition, the single trapping experiment gives a result of 1.0 in all cases.
that owing to diffusion the concentration of material in the active volume will be the same throughout the flow cell. Therefore, the actual amounts of material recovered in the flow cell from the initial injections will be double the values shown in Fig. 4 (alternatively stated, 50% can be considered as the maximum possible recovery). Bearing this in mind, the recovery for the less polar compounds actually appears very successful with up to 80% of the injected material transferred to the flow cell. In these cases, it appears that the retention step has been highly efficient in trapping the compound on the cartridge and also that the elution step has also been successful in transferring the trapped material into the NMR probe in a concentrated plug of solvent. For the more polar compounds, it appears that the retention step has not been as successful as the measured recoveries into the active volume are much lower, e.g. below 10% for phthalic acid. For the recovery from the five-times trapping experiment only ~2% of the total injected amount of phthalic acid is recovered into the active volume. It may, of course, be possible to recover greater total amounts of material than are described above from the cartridges since the results depend on a number of factors not investigated here. However, for practical purposes under the conditions described, these values indicate the relative success of the transfer process for the different polarity compounds.

The above findings are put into perspective when the S/N levels achievable by HPLC–SPE–NMR are directly compared with those obtainable with conventional HPLC–NMR. A sample of dog bile containing the compound GSK-A and its metabolite M1 was analysed by both HPLC–SPE–NMR and conventional HPLC–NMR (120 µl flow cell, 700 MHz, standard HPLC–NMR system). Figure 5 shows that the S/N is improved 4.4-fold (5.5-fold if compensating for the different field strengths) when using the HPLC–SPE–NMR system with a 30 µl flow probe. Even though the two systems may not be directly comparable because of their different flow cell dimensions, this figure illustrates the sensitivity gain which can be achieved by HPLC–SPE–NMR. Eluting from an RP-18 column at >60% acetonitrile, GSK-A is a compound which is in the polarity optimum for the resin GP/RP-18 method (cf. Fig. 3) and the efficiency of the triple trapping is indeed three times greater than for the single trapping experiment (compare Fig. 4). For metabolite M1 (spectra not shown), which elutes at 40% acetonitrile, the sensitivity improvement over conventional HPLC–NMR was 2.8-fold (3.5-fold when compensating for field strengths).

An important aspect of the sensitivity gains of HPLC–SPE–NMR is that in many cases in conventional HPLC–
NMR, a chromatographic peak is not ideally trapped in the NMR flow cell, owing either to peak broadness or miscalibration of the transfer times. In the case of GSK-A, the peak width was in the region of 30 s, which would lead to a peak volume much larger than the flow cell volume. With peak trapping, these problems are eliminated since broader peaks are trapped and therefore focused on the SPE cartridge. In addition, the selection of a peak is much less critical than in HPLC–NMR since the exact detection of the middle of a chromatographic peak is not necessary for trapping and the subsequent peak transfer. Also, sample recovery is very straightforward in SPE–NMR as the content of the NMR probe can simply be blown out into a vial by applying a nitrogen gas flow.

One further characteristic of HPLC–SPE–NMR to be considered is that although a constituent of a sample mixture might be soluble in the initial solvent for HPLC injection and during the chromatographic run, it may not be soluble in the organic solvent used for cartridge elution. Therefore, when dealing with unknown substances such as metabolites or impurities, potential solvent incompatibilities need to be considered. Figure 6 shows a practical example of this, with a glutathione conjugate of radiolabelled [14C]GSK-B, which was well separated by chromatography but did not give an SPE–NMR spectrum despite its relatively strong 14C radio signal. The first assumption was that the compound was not retained on the SPE cartridge. This assumption was disproved as radioactivity was shown to remain on the cartridge, indicating that the metabolite was retained but had not eluted in pure acetonitrile-d3. The addition of 33% deuterium oxide to the elution solvent was required to elute the glutathione conjugate from the cartridge. Owing to the lack of standards, effects such as this are difficult to predict (the transformation of rather lipophilic compounds into water-soluble metabolites and conjugates is a fundamental principle in drug metabolism), but it should be noted that this is the only such example of organic solvent incompatibility that we have encountered so far.

**Practical applications**

**Metabolite identification**

In contrast to chemical transformations and degradations, when dealing with metabolic biotransformations not only are slight modifications of physico-chemical properties of a molecule such as polarity and solubility by phase I reactions (e.g. oxidation, hydroxylation) to be expected, but also more dramatic changes caused by conjugation reactions (such as sulfation or glucuronidation). It is therefore difficult to predict the properties of the unknowns in order to optimize the SPE trapping conditions. In this section we give examples of the applicability of HPLC–SPE–NMR to drug metabolite identification using a generic method.

Figure 7 demonstrates a typical metabolite application of HPLC–SPE–NMR: the analysis of fractions from a preparative separation where components are not sufficiently resolved and hence need further purification. The 1H spectrum of a fraction from a semi-preparative HPLC separation of rat bile dosed with GSK-C, recorded with a conventional 5 mm tube TXI probe at 700 MHz, is given in Fig. 7(a). The spectrum shows one major and two minor metabolites of GSK-C, which required further purification in order to be identified. Traditionally, HPLC–NMR is used for such

---

**Figure 6.** Solvent compatibility of an unknown radiolabelled drug metabolite: a glutathione conjugate of GSK-B (peak 1) from a rat bile fraction was not soluble in acetonitrile-d3 (spectrum b). An acetonitrile-d3/D2O mixture (2:1) was used for elution instead (spectrum c).
problems where further purification is needed but a full semi-preparative work-up of the fraction would not be justified. In such cases, the use of HPLC–NMR generally results in a decrease in S/N for a variety of reasons such as losses in sample handling, broad peak shape (causing non-optimal sample recovery) or restrictions in receiver gain when using partly protonated solvents. With HPLC–SPE–NMR the S/N of the minor metabolite (O-methoxy-O-glucuronide) of GSK-C [Fig. 7(b)] is better than that for the off-line spectrum (even at 700 MHz) after full purification and removal of both the main metabolite and the endogenous components from the sample.

In biotransformation studies, the metabolites are usually more polar than the parent compound, which poses a potential problem because HPLC methods are usually optimized by using the parent molecule as a reference standard. Since metabolite standards are typically not available at this stage of a drug metabolism study, it is necessary to rely on standard compounds with polarities and properties comparable to those of the expected metabolites, but this cannot guarantee retention of metabolites in all possible cases. However, SPE trapping has generally worked without the need for a specialized method, even with polar metabolites. For example, a sample of rat bile containing
Figure 8. Example of a fraction of rat bile from a GSK-D, GSK-E and GSK-F study. It can be seen that even very early eluting peaks, which elute at less than 8% acetonitrile (a) can be retained allowing NMR spectrum acquisition.

GSK-D, GSK-E and GSK-F was analysed by reversed-phase chromatography using a gradient of 0–30% acetonitrile over 37.5 min (Fig. 8). All components were sufficiently retained on the cartridges and NMR spectra could be recorded for them with the first peak eluting in 8% acetonitrile. For the successful trapping of a peak the chemistry of both molecule and cartridge material needs to be taken into consideration and this example proves that if only polar–apolar interactions are present, trapping of a relatively polar compound is still possible with the standard resin GP/RP method, although this may require an increased water make-up flow for trapping.

Apart from the sensitivity gain, another important argument for using SPE in the HPLC–NMR system is centred on the practical considerations of metabolism studies. Metabolites need to be quantified using HPLC with radiodetection and their structures adequately elucidated using MS and NMR. The chromatographic systems developed to resolve the metabolites employ MS-compatible solvents, which are not necessarily ideal for NMR work. With HPLC–SPE–NMR trapping, however, the incompatibility of solvents for both techniques becomes irrelevant as it is now possible to use the original chromatographic system with the SPE system without any redevelopment of the initial method. As a consequence, the unequivocal link between the quantification and the identification of metabolites is simplified and becomes more efficient.

Chemical process development

The application of HPLC–SPE–NMR in a process development environment is primarily for the identification of unknown impurities and degradants. In order to perform this kind of work, samples derived from a variety of sources are used: on the one hand samples relatively rich in impurities such as mother liquors and reaction mixtures and on the other hand samples with relatively low levels of impurities such as purified samples of drug substances and samples from forced degradation studies. The reasons for needing to identify these impurities are also various. For reaction mixtures and mother liquors the identification of unknowns is typically required to support chemistry scale-up development or synthetic route development. For drugs which are about to be launched on to the market, there is a regulatory requirement to identify all the observed impurities in the drug substance that are present at a level of 0.1% (w/w) or above.

Mother liquors from work-up procedures and stressed reaction mixtures are potentially two of the most productive means of obtaining samples of process-related impurities for structure elucidation as they are often enriched in impurities, compared with the final, isolated product. In addition, large quantities of these samples may often be available. For such high-concentration samples the main advantage of HPLC–SPE–NMR is the ready availability of the high separation power of analytical-scale HPLC for fractionating
the samples, as in the case of closely eluting isomers which are difficult to separate in preparative HPLC. The ability to record routinely one- and two-dimensional spectra (e.g., HSQC, HMBC) from such multiple trapings is one of the great advantages of HPLC–SPE–NMR over conventional HPLC–NMR and for samples in process development this technique is therefore a powerful tool for rapid, unequivocal structure elucidation from mixtures.

In cases where the components of interest in the mixture are present in small quantities it may still be possible to obtain enough data to allow full structure elucidation, although it may also be desirable in such cases to enrich the mixture to increase the proportions of those components. This is demonstrated in the following example, where two impurities of interest were present in a sample, both at levels below 0.1% (w/w). An enriched sample of these impurities was obtained by mass-directed preparative HPLC, producing a sample containing ~8% (w/w) of each of the impurities. This was analysed by HPLC–SPE–NMR, trapping the peaks of interest four times and recording the ^1^H NMR spectra after elution into the NMR flow cell.

Portions of the ^1^H NMR spectra for the two components are shown in Fig. 9. These also demonstrate one of the important advantages of HPLC–SPE–NMR over conventional HPLC–NMR, namely that exchangeable proton signals may be readily observed using this technique. In the first spectrum, an indole NH signal is observed in the spectrum, confirming that the indole nitrogen present in the parent molecule is not substituted, whereas in the second spectrum no indole NH signal is observed, suggesting that the impurity is an N-substituted indole compound.

**CONCLUSIONS**

HPLC–SPE–NMR represents an interesting and important development in hyphenated NMR technology. The sensitivity gain which can be obtained for compounds of medium and low polarity comes at little cost apart from the price for the additional equipment and the consumables, which is compensated for by the savings of deuterated solvent. More importantly, practical considerations such as the improved compatibility with most chromatographic methods, the use of H_2O as an HPLC solvent and the decoupling of the chromatography from the acquisition of NMR data should ensure that HPLC–SPE–NMR will play an important role in the pharmaceutical NMR laboratory.

**REFERENCES**