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Short Communication

Use of liquid chromatography–nuclear magnetic resonance spectroscopy for the identification of impurities in drug substances

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Abstract

There are many complex analytical problems in the pharmaceutical industry which require the use of more than one instrumental technique for their solution. Hyphenated techniques such as GC–MS, LC–MS and LC–NMR have been developed to help solve such problems in a time-efficient manner. The on-line combination of HPLC and NMR offers the potential for rapid collection of detailed structural information from samples in a way no other hyphenated technique can. LC–NMR, however, is an inherently insensitive technique but this paper shows that worthwhile data can be obtained on a medium-field (400 MHz) instrument.

1. Introduction

Direct coupling of liquid chromatography (LC) to ^{1}H NMR using stopped-flow methods was reported in 1978 [1] and flowing LC–NMR was reported the year after [2]. Since then, great improvements have been made in spectrometer design and field strength, in flow-cell design and in pulse sequences for solvent suppression [3,4] which have enabled wider exploitation of the technique [5]. LC–NMR combines a powerful separation technique with a detector that offers a wealth of structural and stereochemical information. It would thus be expected to have wide application in the pharmaceutical industry in, for example, investigating impurities in drug compounds at both the research and development stage [6]. Unpublished work from this laboratory has shown that the technique is particularly powerful for the analysis of compounds which cannot be isolated. LC–NMR is an inherently insensitive technique and consequently some recent publications have exploited the increased sensitivity of high- and ultra-high-field NMR spectrometers [5,7,8]. For the identification of impurities in drug substances, however, this paper shows that spectra on impurities at around the 3% level can be obtained in a respectable time frame using a medium-field spectrometer.

2. Experimental

2.1. HPLC

All HPLC was performed using a Unicam

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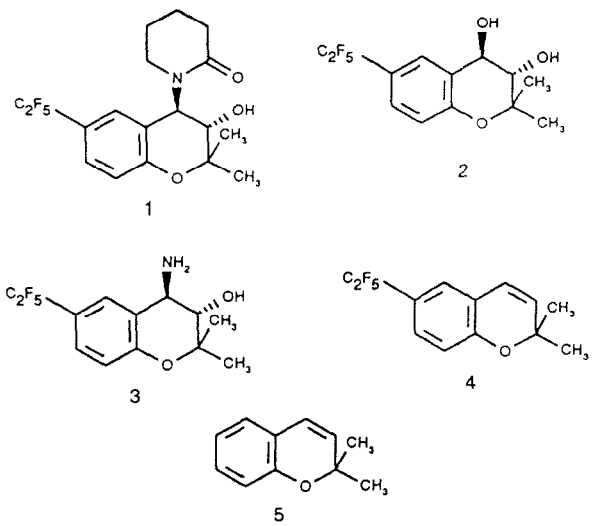

Crystal 200 pump and 250 photodiode array detector. The spiked sample of **1** was dissolved at a concentration of approximately 10 mg/ml in eluent and injections of 100 or 200 μl made using a Rheodyne 7125 manual injector, equipped with a 200-μl loop. The outlet of the detector was connected to the inlet of the LC–NMR probe by 3 m of polyether ether ketone (PEEK) tubing (0.010 in. internal diameter; 1 in. = 2.54 cm). The transfer time from the UV detector to the flow cell of the probe was approximately 30 s at 1 ml/min. Analysis of **4** was performed by injecting on to the column 100 or 200 μl from an approximately 10 mg/ml solution.

2.2. NMR

All spectra were recorded on a Bruker AMX 400 NMR spectrometer fitted with an inverse-geometry LC–NMR probe with a 240-μl flow cell. Flowing data were accumulated using a pseudo-two-dimensional version of the "noesyprtp" pulse programme. A number of 8192 data points was used in f2, with each increment in "f1" corresponding to 16 scans and taking approximately 27 s. The acetonitrile peak was suppressed by presaturation; the residual water peak was also suppressed if its height was significantly greater than that of the acetonitrile ^{13}C satellites. Spectra were referenced to the position of the water peak, taken to be 4.41 ppm at 40% MeCN and 4.16 ppm at 60% MeCN. Stopped-flow spectra were also accumulated into 8192 data points and zero-filled once before transformation. Presaturation of acetonitrile and water resonances and referencing was performed as for the flowing experiments.

3. Results and discussion

To investigate to what extent sensitivity is a limiting factor in LC–NMR, a sample of a research drug substance **1** was spiked with the related compounds **2** and **3**, at levels of 9 and 4%

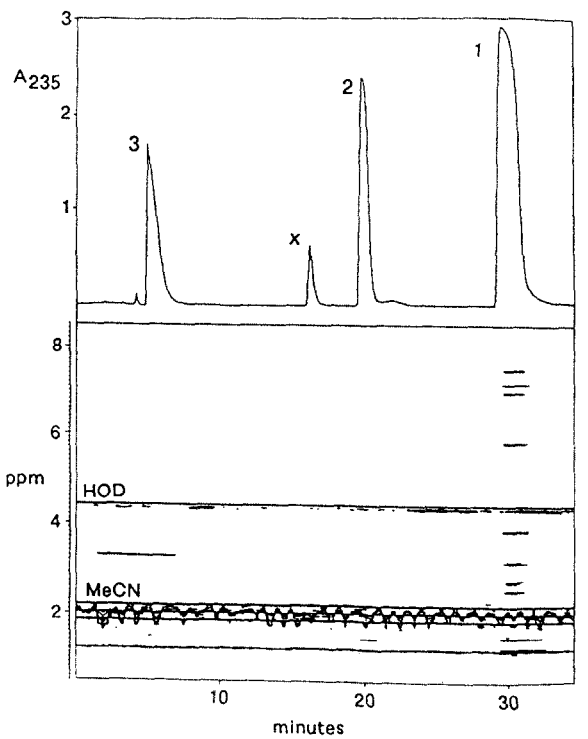

Fig. 1. Typical UV (absorption at 235nm) and ^1H NMR data for sample of **1**, spiked with **2** and **3** at levels of 9 and 4% (w/w), respectively. Approximately 100 μl of sample were injected on to a Spherisorb S5 ODS2 column, 250 × 4.6 mm, and eluted with acetonitrile–0.05 *M* potassium dihydrogen-orthophosphate in ^2H_2O (40:60), adjusted to pH 3.5 with orthophosphoric acid, at a flow-rate of 1.0 ml/min. The signals from the solvent are indicated by HOD (water) and MeCN (acetonitrile). The peak labels correspond to the structures in the text. The peak labelled x is a 1% impurity not investigated in this study. D = Deuterium.

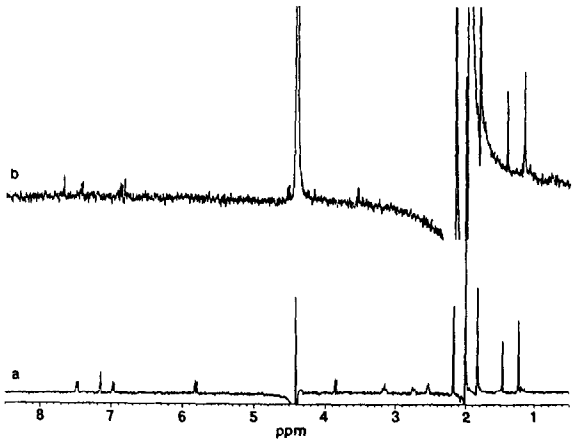

Fig. 2. Rows abstracted from the data matrix in Fig. 1, corresponding to **1** (trace a) and **2** (trace b). The former is a single row (16 scans), the latter is a summation of four rows.

(w/w), respectively and then examined by LC–NMR, using a 400 MHz spectrometer. Typical UV and ^{1}H NMR data for such an experiment are shown in Fig. 1. The two-dimensional plot of chemical shift versus retention time represents the spectrum observed at approximately 27-s time intervals. Two species are observed in the ^{1}H plot and they correspond to the main component, **1**, and the 9% component, **2**. For the latter, only the methyl resonances at approximately 1.54 and 1.79 ppm are clearly visible in the two-dimensional plot, indicating that the limit of detection for this compound under the flowing conditions used here is approximately 50 μg injected on column. Rows from the data matrix were abstracted for compounds **1** and **2** and the corresponding spectra are shown in Fig. 2. Considerable improvements in spectral quality are obtainable by performing stopped-flow LC–NMR. Stopped-flow ^{1}H spectra for all three components are shown in Fig. 3. The LC–NMR data on **3** clearly show that useful structural information can be obtained on relatively minor components of mixtures. At present on our equipment it appears that sample amounts in the range 25–50 μg injected on column are needed to obtain good quality ^{1}H data in reasonable accumulation times (30 min). Minor components of mixtures (say 1%) would therefore be investi-

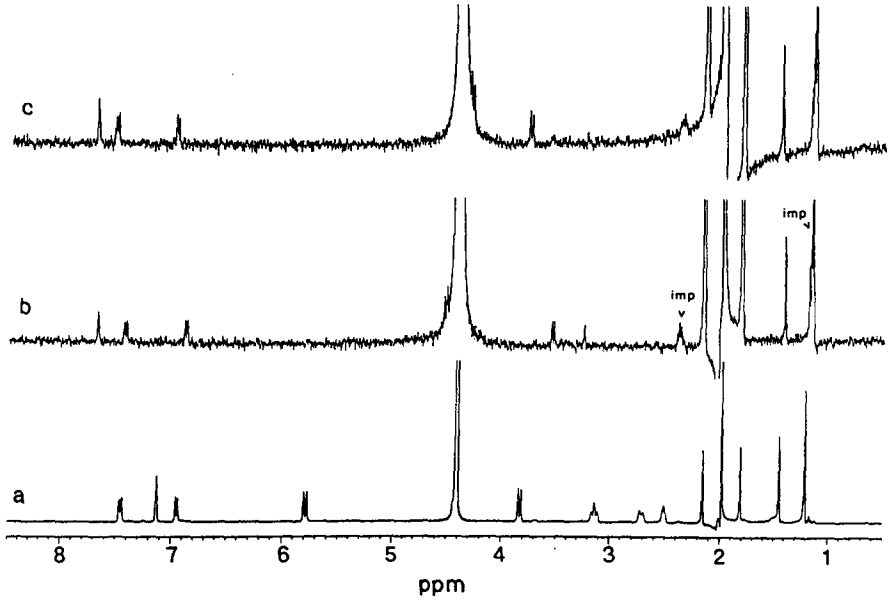

Fig. 3. Stopped-flow ^{1}H spectra for **1** (trace a), **2** (trace b) and **3** (trace c). Chromatographic conditions as in Fig. 1, except that a separate injection of 200 μl of the mixture was used to obtain the data on **3**. A number of 512 scans was acquired for **1**, 1024 for **2** and 1255 for **3**. The multiplets indicated by "imp" at 1.17 and 2.39 ppm are due to an impurity/impurities in the acetonitrile. Note the impurity resonance at 1.17 ppm obscures one of the methyl resonances of **2** (trace b) and **3** (trace c).

gated by injecting more of the mixture on to the column and/or by allowing longer accumulation times.

Having demonstrated the use of LC–NMR in a model system, a "real" sample was examined. This sample was presented as consisting mainly of compound **4** but containing an unknown, early-eluting impurity at a level estimated to be 3%. The chromatogram is shown in Fig. 4, together with the ^{1}H spectra obtained for the main peak, **4**, and the unknown. The ^{1}H spectrum of the latter is very similar to that of **4** with a singlet for the two methyl groups (1.35 ppm) and two doublets for the olefinic protons (5.65 and 6.35 ppm) indicating that these groups are common in both the unknown impurity and **4**. The fact that both methyl groups appear as a singlet resonance in the ^{1}H NMR spectra of **4** and **5** implies that an olefinic bond is present in both molecules. If this bond were saturated, individual resonances would be observed for the methyl groups as in structures **3**, **2** and **1** (Fig. 3). Compound **4** has three aromatic resonances (6.86, 7.33 and 7.37 ppm) with chemical shifts and coupling patterns as expected for a trisubstituted ring. The four aromatic resonances of the unknown impurity (7.08, 7.00, 6.83 and 6.69 ppm) are typical of a 1,2 disubstituted aromatic ring and indicate that the unknown impurity has the structure **5**. J values for **4** and **5** are not quoted because of the low digital resolution of the data. On-flow data will necessarily have low digital resolution in order to obtain sufficient increments in "f1". Stopped-flow data could have been acquired with sufficient digital resolution to measure J coupling constants, but in this work, lower digital resolution was used to maximize the S/N ratio of the spectrum. It is obviously useful to obtain UV spectra on each peak at the same time as the ^{1}H NMR spectra, using a photodiode array detector in-line (as was the case in this work). In this case the UV spectrum confirmed that **5** was structurally distinct from other known possible impurities. LC–MS and LC–NMR would normally be combined to identify unknown impurities, but here using

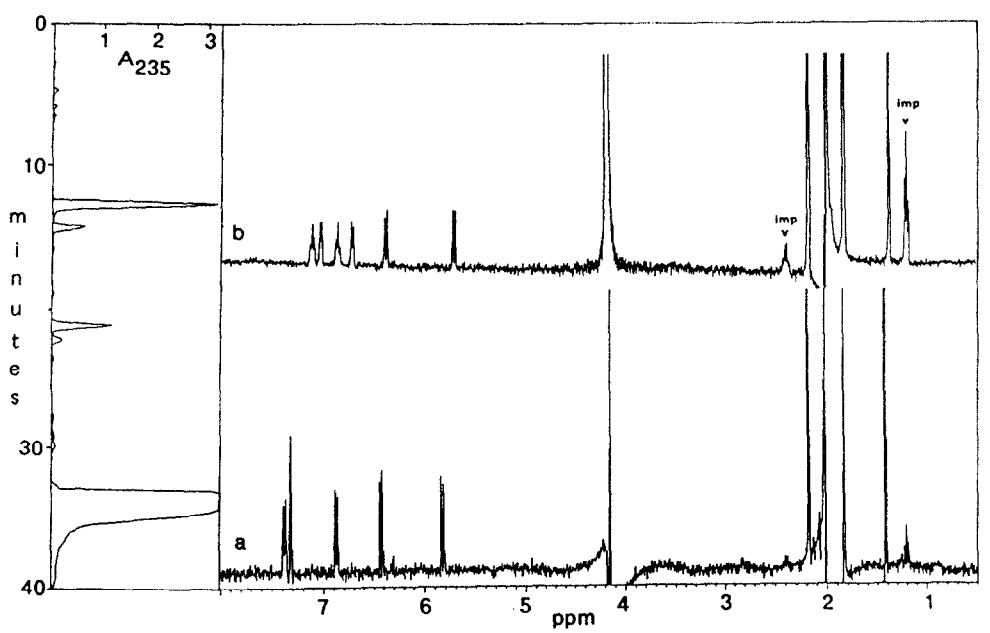

Fig. 4. Chromatogram for sample consisting mainly of **4**, with ^{1}H spectra of **4** (flowing, trace a) and the impurity **5** (stopped-flow, 1024 scans, trace b). Conditions as in Fig. 1 except that acetonitrile–buffer ratio was 60:40. Approximately 100 μl sample injected on to the column for the flowing data and 200 μl for the stopped-flow data. The multiplets indicated by "imp" at 1.20 and 2.40 ppm are due to an impurity/impurities in the acetonitrile.

both frit fast atom bombardment and thermospray, LC–MS failed to identify **5**. It is also advantageous that typical reversed-phase eluents pose no problem of compatibility with LC–NMR. For example, LC–NMR has been performed in this laboratory using ammonium formate buffers and tetrahydrofuran–aqueous trifluoroacetic acid mixtures as eluents in addition to the phosphate buffers used in this study.

4. Conclusions

LC–NMR can provide very useful structural information on relatively minor components of mixtures, even using a 400 MHz spectrometer. Relatively high loadings may be needed to obtain data on low-level impurities (depending on flow cell size and spectrometer field strength) but this is not usually a problem with the robust separation methods used to examine pharmaceuticals.

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