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Directly coupled HPLC–NMR and HPLC–NMR–MS in pharmaceutical research and development

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Abstract

The methodology for the direct coupling of HPLC with NMR spectroscopy and the simultaneous double coupling of HPLC with NMR and mass spectrometry (MS) is described. Indications of the necessary technical developments to achieve this are given, and the applications of these new techniques to studies of pharmaceutical relevance are reviewed. These include studies of combinatorial chemistry libraries, synthetic chemical impurities, characterisation of drug mixtures, identification of natural products of possible pharmaceutical interest and identification of xenobiotic metabolites in human, animal and in vitro systems. In addition, HPLC–NMR has been used to investigate xenobiotic metabolite reactivity. Finally, the potential future directions of the techniques are discussed.

Keywords: Combinatorial chemistry; Pharmaceuticals; Natural products; Impurities

1. Introduction

High-performance liquid chromatography (HPLC) is one of the principal methods for the analysis of chemical mixtures but conventional detectors used to monitor the separation, based on refractive index, UV, fluorescence and electrochemical properties, provide only a limited amount of information on molecular structure. In addition, in studies of drug metabolism, it has generally been necessary to incorporate radioisotopes (such as ^{3}H and ^{14}C) into the xenobiotic substance under investigation to ensure subsequent detection of compound-related material. Thus, for this particular application, HPLC with in-line radioactivity monitoring has provided a rapid and efficient means of metabolite detection. However, all of these detectors often do not provide sufficient information to allow molecular structural determination. Real advances in on-line minor component structure determination have only resulted from the relatively recent advent of the reliable hyphenation of HPLC and mass spectrometry (MS). This new technology is now widely exploited and there has been a huge growth of applications of HPLC–MS in the pharmaceutical industry, especially in the identification and quantification of drugs and metabolites in biofluids and extracts of excreta. These advances notwithstanding, mass spectrometry

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by itself does not always provide unambiguous structural identification, and NMR spectroscopic data is often needed. However, conventionally NMR spectroscopic analysis has until recently required time-consuming isolation and purification steps, and sometimes this can conflict with the efficient HPLC–MS approach. It should be noted that even HPLC–NMR can be considerably more time-consuming than HPLC–MS.

High-resolution NMR spectroscopy is demonstrably one of the most important methods of structure elucidation with an extensive range of biochemical and chemical applications. The direct linking of HPLC with NMR spectroscopy has been a remarkable success story [1,2] and has transformed the technique from a research tool to the stage where routine analytical applications are possible. As the number of HPLC–NMR systems have increased in user laboratories, there has been a logical extension of the hyphenation of HPLC–NMR and HPLC–MS into a single combined system for structure elucidation. Here, we describe the operation of HPLC–NMR and describe advances and applications in HPLC–NMR–MS. The operational difficulties of this double hyphenation are discussed with practical solutions.

2. Technical developments in HPLC–NMR and HPLC–NMR–MS

2.1. Introduction

Direct on-line coupling of an NMR spectrometer as a detector for chromatographic separation, analogous to the use of MS for such applications, has required the development of technical features such as flow-probe hardware, efficient NMR solvent suppression pulse sequences and new software. The technical problems that needed resolution for successful practical commercial exploitation are summarised below.

2.2. The requirement for high dynamic range in NMR spectroscopy

It is necessary to detect signals from low concentrations of analytes in the presence of large ^{1}H NMR signals from the HPLC solvents. The solution to this problem has been the development of new techniques for suppressing the solvent NMR resonance. These are able to cope with mixed solvents, such as methanol–water, acetonitrile–water and even more complex solvent combinations, including the problems which are associated with eluent proportions changing during a gradient run [3,4].

A related factor which delayed the implementation of practical HPLC–NMR spectroscopy was the earlier need to use deuterated solvents for chromatography to overcome the dynamic range problem and these are prohibitively expensive except for microbore separations. Whereas solvents such as CCl_4 could be used for ^{1}H NMR in normal-phase applications, they are hardly ideal and are environmentally unacceptable. Furthermore, this approach would necessitate the use of a probe with a separate external sample compartment containing a deuterated liquid to provide a signal for stabilising the magnetic field. This type of probe has been developed specifically for use with supercritical fluid chromatography linked to NMR spectroscopy (SFC–NMR) where CO_2 is used as the eluent [5]. The solution to this difficulty for reversed-phase HPLC–NMR has come about because modern NMR spectrometers can perform solvent NMR resonance suppression very efficiently, thus negating the need for deuterated solvents. In practice D_2O is still often used to prepare eluents rather than H_2O simply because this makes multiple solvent suppression easier (D_2O currently costs about \$200/l). Also, acetonitrile-d_3 is being used increasingly in pharmaceutical laboratories because the cost of this solvent is negligible in relation to the other operating costs of such a laboratory and it allows a substantial gain in quality of the results.

The commonly used solvents for reversed-phase HPLC separations are methanol and acetonitrile. Acetonitrile and methanol both give rise to a singlet resonance in the ^{1}H NMR spectrum which can be suppressed easily. However, the ^{13}C satellite peaks, caused by the one-bond ^{1}H–^{13}C spin couplings from the 1.1% of molecules with the naturally abundant ^{13}C isotope at the methyl carbon remain following suppression of the main peak and this can still cause problems. This is because these satellite peaks are often much larger than the signals for the analytes, and thus must also be suppressed. One approach is to set the suppression irradiation frequency over the

central peak and the two satellite peaks in a cyclical fashion. Alternatively, if an inverse geometry probe is used which includes a ^{13}C coil, then broadband ^{13}C decoupling is possible, collapsing the satellite peaks under the central peak, enabling conventional single frequency suppression. An automatic method for carrying out solvent suppression has also been developed. Thus, in the case of gradient elution using acetonitrile as the organic modifier, the NMR resonance frequency of the acetonitrile will change during the run as the solvent composition changes. An NMR software routine searches for the ^{13}C satellite peaks in the ^{1}H NMR spectrum of the solvent mixture, interpolating to find the main signal and then setting the suppression frequency accordingly. This has the advantages of being non-perturbing to the NMR observation of the analyte signals, being implemented in real-time during the separation and being independent of NMR solvent shifts induced by chromatographic solvent gradients.

2.3. Avoidance of compromised chromatographic resolution

Because of the necessary layout of an NMR laboratory, HPLC–NMR requires the use of long column-to-detector transfer times and the use of relatively high volume flow cells (by chromatographic standards) and this was originally considered to be a potential cause of chromatographic peak broadening. For HPLC–NMR probes, a compromise has to be made between the needs of chromatography and NMR for the detection volume of the flow cell. Ideally this should be as low as possible for good chromatography and as high as possible for NMR detection. The low volume for chromatography can only be compensated for by increasing the filling factor which can be achieved by fixing the RF coil directly on to the NMR flow cell wall. The whole issue of flow-rates and flow cell sizes for coupled chromatography–NMR spectroscopy has been addressed by Albert [2]. This of course makes it impossible to spin the sample, as is performed in conventional probes, to improve magnetic field inhomogeneities. Practically, however, this turns out not to be a problem as field homogeneity with this small volume is good. Further, modern computer-controlled methods for optimising field homogeneity reduce the requirement for spinning. A major factor in determining the sensitivity or peak heights is the observed lineshape as, if the peaks have wide bases, poor signal-to-noise ratio results because a significant part of the signal intensity is found in this part of the peak. Thus good magnetic field homogeneity, giving narrow NMR lines, is also a prerequisite for a good signal-to-noise ratio. A detailed analysis of the flow and NMR requirements for optimum operation of HPLC–NMR has been given recently [6,7].

2.4. The need for high NMR sensitivity

The low sensitivity of NMR in relation to the quantities of metabolites often separated using HPLC with UV detection must be recognised. However, as will be seen later, impressive sensitivities can be achieved using HPLC–NMR in the stop-flow mode. The detection limits of HPLC–NMR are continually being revised downwards [8] as new technical advances are made. Recently, these have included the use of high magnetic field strengths (operating at 750 and 800 MHz for ^{1}H NMR spectroscopy), the incorporation of digital filtering and oversampling into NMR data acquisition and the introduction of microbore HPLC methods. By combining the use of digital electronics with microbore HPLC [9,10], it appears that detection limits for structural characterisation are in the region of 5 ng even at lower ^{1}H NMR observation frequencies of 500 and 600 MHz.

In principle, it is possible to effect NMR detection for any of the magnetically active nuclei, but those of most importance in pharmaceutical studies are ^{1}H, ^{2}H, ^{19}F, ^{13}C, ^{15}N and ^{31}P. In addition because of the generally low levels of minor components, the most sensitive nuclei, ^{1}H, ^{19}F and ^{31}P have been used most extensively. The use of ^{13}C NMR in HPLC–NMR can be facilitated through indirect detection of ^{13}C resonances via the much more sensitive ^{1}H NMR signals of attached protons. A major benefit of using ^{19}F NMR spectroscopy for detection of fluorine-containing molecules is that the background, unlike that for ^{1}H NMR spectroscopy, is usually negligible.

2.5. Additional considerations for double coupling of NMR and MS to HPLC

HPLC–MS has been employed for many years but only since the advent of electrospray ionisation has it become a truly robust and routine method for the

analysis of mixtures. Now that NMR spectroscopy has been coupled to HPLC, it has, for the first time, become possible to acquire both NMR and MS data simultaneously from a single chromatographic analysis.

Solvent selection for HPLC–NMR–MS has to be a compromise between the ideal requirements of each instrument. Thus for HPLC–NMR the use of inorganic buffers, e.g., sodium phosphate, for pH modification is preferred because no additional signals are introduced into the NMR spectrum but this type of buffer system is currently incompatible with most HPLC–MS systems using an electrospray interface. An alternative acidic modifier is trifluoroacetic acid (TFA), which has no protons to cause interferences in the NMR spectrum. Initial experiments, using paracetamol metabolites or propranolol as model analytes [11], showed that 0.1% TFA could be used with HPLC–MS for a limited range of analytes present at high concentration (>1 μg on column) in positive ion mode. However, with acidic analytes such as ibuprofen and its metabolites, ion suppression was complete and even at high sample concentrations MS data could not be obtained. Formic acid was found to provide a suitable compromise between the needs of MS on the one hand and NMR on the other. The interference of the single proton of formic acid, which has a sharp, readily suppressable NMR singlet near δ8.5, gives minimal interference in the resulting NMR spectra and enables MS data to be acquired for acidic analytes.

There are two ways to configure the NMR and mass spectrometers, either in parallel or in series. As NMR is a relatively insensitive technique, large volumes and high concentrations of analytes are used wherever possible to compensate and to reduce analysis time. As a result, generally 4.6-mm HPLC columns are used to avoid problems of overloading. This means flow-rates of the order of 0.5–1.0 ml/min must be employed to meet the requirements of the NMR without compromising the chromatography. Such high flow-rates can easily be accommodated by modern mass spectrometers. However, as electrospray-MS is concentration dependent, as opposed to mass sensitive, operating the NMR spectrometer and the MS in parallel, and thus splitting the flow such that a minor fraction goes to the MS, has little effect on sensitivity but greatly enhances the source lifetime allowing the mass spectrometer to be operated at optimum sensitivity for longer. If the flow is split prior to the NMR with the length of the capillary to the MS adjusted so that the analyte peak has just passed completely through the MS as it fills the NMR flow cell, the MS can be used to supplement the UV data to direct NMR experiments. Further, splitting in this manner enables the use of stop-flow NMR with minimum degradation of the integrity of the chromatography [11].

Running in series, i.e., with the sample-destructive MS after the non-destructive NMR, allows for the completion of all NMR experiments whether on-flow or stop-flow before MS analysis begins, but introduces the possibility of peak dispersion before MS analysis for any peaks trapped between the NMR and MS when the flow is stopped. Series operation causes the NMR flow cell and its connections to be operated at higher pressures than they were designed for, with the consequent possibility that leaks are more likely. Series operation also fails to take advantage of the mass spectrometrist's ability to flag up peaks of interest quickly. Nevertheless, some applications have used serial coupling of NMR and MS to HPLC because of the ease of disassembling the components for separate mass spectrometry studies, and have obtained useful results.

Given the strength of the magnetic field surrounding an NMR magnet, there is obviously the potential for interference with the operation of the mass spectrometer. There is also the potential for the presence of the MS to interfere with the operation of the NMR spectrometer. Some experiments have been carried out to investigate this in the situation where the mass spectrometer was sited at the 10 Gauss line with its axis radial to the NMR. Varying the angle of the mass spectrometer to the NMR had no effect on either the data from the mass spectrometer or the NMR. The HPLC, NMR and MS were situated approximately at the corners of an equilateral triangle but this was largely dictated by the size of the laboratory and the position of the supplies to the mass spectrometer. Nevertheless, the mass scale needed to be re-calibrated and there may also have been a drop in low mass sensitivity (below $m/z=$ 150) but this was not quantified. No effect on the operation of the NMR with the MS located in this position was observed [11]. In addition, the advent of

shielded superconducting NMR magnets has alleviated the potential problems considerably.

3. Operational methods in HPLC–NMR and HPLC–NMR–MS

There are currently five main options which can be employed for HPLC–NMR using either isocratic or gradient elution. These are continuous-flow, stop-flow, 'time-sliced' stop-flow, peak collection into capillary loops for post-chromatographic analysis and automatic peak detection with UV-detected triggered NMR acquisition.

The simplest of these is continuous-flow detection, but this is usually only practical when using ^{1}H or ^{19}F NMR for detection unless isotopically enriched compounds are available. However, there are examples of HPLC–NMR studies using ^{2}H and ^{31}P NMR detection in the drug metabolism field. Where continuous-flow NMR detection is used for gradient elution, the NMR resonance positions of the solvent peaks shift with the changing solvent composition. For effective solvent suppression, these solvent resonance frequencies must be determined as the chromatographic run proceeds.

If the retention times of the analytes are known, or there is an efficient method for their detection on-line, such as UV, MS or radioactivity, stop-flow HPLC–NMR becomes a viable option. In the stop-flow technique, all the usual techniques available for high-resolution NMR can be used. In particular, these include valuable techniques for structure determination such as two-dimensional NMR experiments which provide correlation between NMR resonances based on mutual spin–spin coupling such as COSY or TOCSY. In practice, it is possible to acquire NMR data on a number of peaks in a chromatogram using a series of stops during elution without on-column diffusion causing an unacceptable loss of chromatographic resolution.

There are two further special categories of stop-flow experiment. Firstly fractions eluting from the column can be stored in capillary loops for later off-line NMR study ('peak picking'). Secondly, the flow can be halted at short intervals during the passage of the eluting peak through the NMR flow cell ('time-slicing') in a manner analogous to the use of a diode-array UV detector to obtain spectra from various portions of the peak. This allows chromatographic peak purity to be estimated. Time-slicing is most useful where the separation is poor, or where the compounds under study have weak/no UV chromophores making it difficult to determine the retention times.

Fully automated analysis is also an option wherein the samples are placed in an autosampler and predefined HPLC–NMR experiments are performed. The software allows automatic detection of UV peaks in the chromatogram based on predetermined time-windows or peak intensities. The successful detection of each UV peak triggers the system to stop the flow at an appropriate time to isolate the peak in the NMR flow probe. Then data relating to the peak (intensity, retention time) are transferred to the NMR host computer and used to define the parameters for the automatically acquired NMR spectrum. This automatic NMR operation includes field homogeneity optimisation, setting and optimisation of all NMR acquisition parameters and the predefinition of the resultant signal-to-noise ratio required in the spectrum. The measurement of two-dimensional NMR spectra can also be performed. With currently available commercial software, the automated run can be halted at any time with reversion to manual control if desired.

It is not usually necessary to make any compromises in a desired chromatographic procedure to accommodate the various types of HPLC–NMR experiment. In addition, HPLC–NMR analyses are generally robust with respect to changes in chromatographic conditions. The powerful structural elucidation capabilities of NMR spectroscopy often ensure that complete chromatographic separation is not necessary for full characterisation of the peak.

In the case of HPLC–NMR–MS experiments there are some additional considerations. So far, the principal MS ionisation method has been electrospray in either positive or negative ion mode (using either single quadrupole or ion-trap mass spectrometers) and this puts further constraints on the chromatographic solvent systems as outlined earlier. When using HPLC–NMR, the chromatography is often developed off-line from the NMR using non-deuterated solvents. It is not always simply a matter of replacing non-deuterated solvents with deuterated

solvents to reproduce the chromatography for HPLC–NMR or HPLC–NMR–MS as this can give rise to changes in retention times. For this reason it is standard practice to run an initial chromatographic run with a small injection volume (e.g., 10 μl) and then scale-up (e.g., 50 μl) for stop-flow NMR, when optimum conditions have been established. We have found that during the initial run it is often possible to acquire a great deal of valuable MS data which can then be used to guide the selection of peaks for study by NMR spectroscopy. Ideally this also allows the second run to be acquired while mixing the eluent just prior to the mass spectrometer with a non-deuterated solvent to back-exchange the deuterium atoms in exchangeable situations (e.g., NH and OH groups) for hydrogens. In this way, if these initial data cannot be readily understood, the number of exchangeable hydrogens in any compound can be counted as it elutes [12].

Finally, the double hyphenation of NMR and MS to HPLC [12–15] brings some additional benefits of MS. These include the often superior sensitivity of MS, although this is not a universal factor and the ability of MS to be used to search for particular diagnostic groups or fragments such as an increase in m/z of 16 for Phase I hydroxylated drug metabolites or an increase of 196 for a glucuronide.

4. Applications in combinatorial chemistry

Characterisation of the structure and conformation of small biologically active molecules is part of the standard approach to lead generation in drug design studies. In particular, it is now possible to automatically synthesise many thousands of small molecules and then rapidly measure their effects in a given pharmacological test system. The power of such techniques comes from the immense number of compounds which can be generated and screened for activity. Two studies have evaluated HPLC–NMR in the field, one based on a mixture of 27 closely related tripeptides [16] and the other on two separate mixtures of four aromatic compounds and three pentapeptides [17].

The approach is illustrated with the tripeptide application comprising a synthetic mixture of the 27 combinations of the tripeptide formed from alanine (A), methionine (M) and tyrosine (Y), as the C-terminal amide, $H_2N{\cdot}CH(R_1){\cdot}CONH{\cdot}CH(R_2){\cdot}CONH.CH(R_3){\cdot}CONH_2$ where R_1, R_2 and R_3 take all combinations of A, M and Y [16].

HPLC analysis was carried out using a 250×4.6-mm I.D. Spherisorb ODS-2 column at 35°C with elution using isocratic D_2O–phosphate buffer at pH 2.5 for 5 min followed by a linear gradient of acetonitrile to 50% after 50 min with UV monitoring of the eluent peaks at 225 nm. The NMR measurements were carried out with solvent suppression at a ^1H NMR frequency of 600 MHz. The assignment of the resonances were based on those of standard compounds such as A–A–A–OH and Y–Y–Y–OH using standard 1D and 2D methods such as ^1H–^{13}C and ^1H–^{15}N HMQC spectra.

The ^1H NMR on-flow detected separation is shown in Fig. 1. This is a contour plot with ^1H NMR chemical shift on the horizontal axis and retention time on the vertical axis. This figure includes all of the resonances including those of the residual peaks from suppressed HDO (δ4.8) and acetonitrile (δ2.0). The chemical shifts of the individual amino acids fall into characteristic ranges. Thus, all alanyl methyl signals appear between δ1.6 and δ1.35. The methionyl β-CH_2 and γ-CH_2 signals appear in a narrow range between δ2.6–2.7 and the methionyl *S*-methyl signal gives rise to a singlet near δ2.1, but this is obscured by the suppressed acetonitrile resonance. The tyrosyl β-CH_2 signals appear around δ3.0–3.2 and the aromatic signals give the resonances around δ6.9 and δ7.3 for the protons *ortho* and *meta* to the hydroxyl substituent, respectively. All of the α-CH resonances for all of the peptides appear between δ4.0 and δ4.6. On expansion (not shown), those due to alanyl residues appear as quartets and those arising from methionyl or tyrosyl residues appear as triplets.

Based on chemical shifts and peak multiplicities, the on-flow HPLC–NMR characterisation of the majority of the components in the mixture of 27 tripeptides was achieved and it demonstrated that this approach is likely to be an effective method for compound mixtures. The elution positions of all of the alanyl-containing peptides were determined with the exception of A–M–M–NH_2 which may have co-eluted with another peptide or may have been synthesised in a much smaller quantity. The only

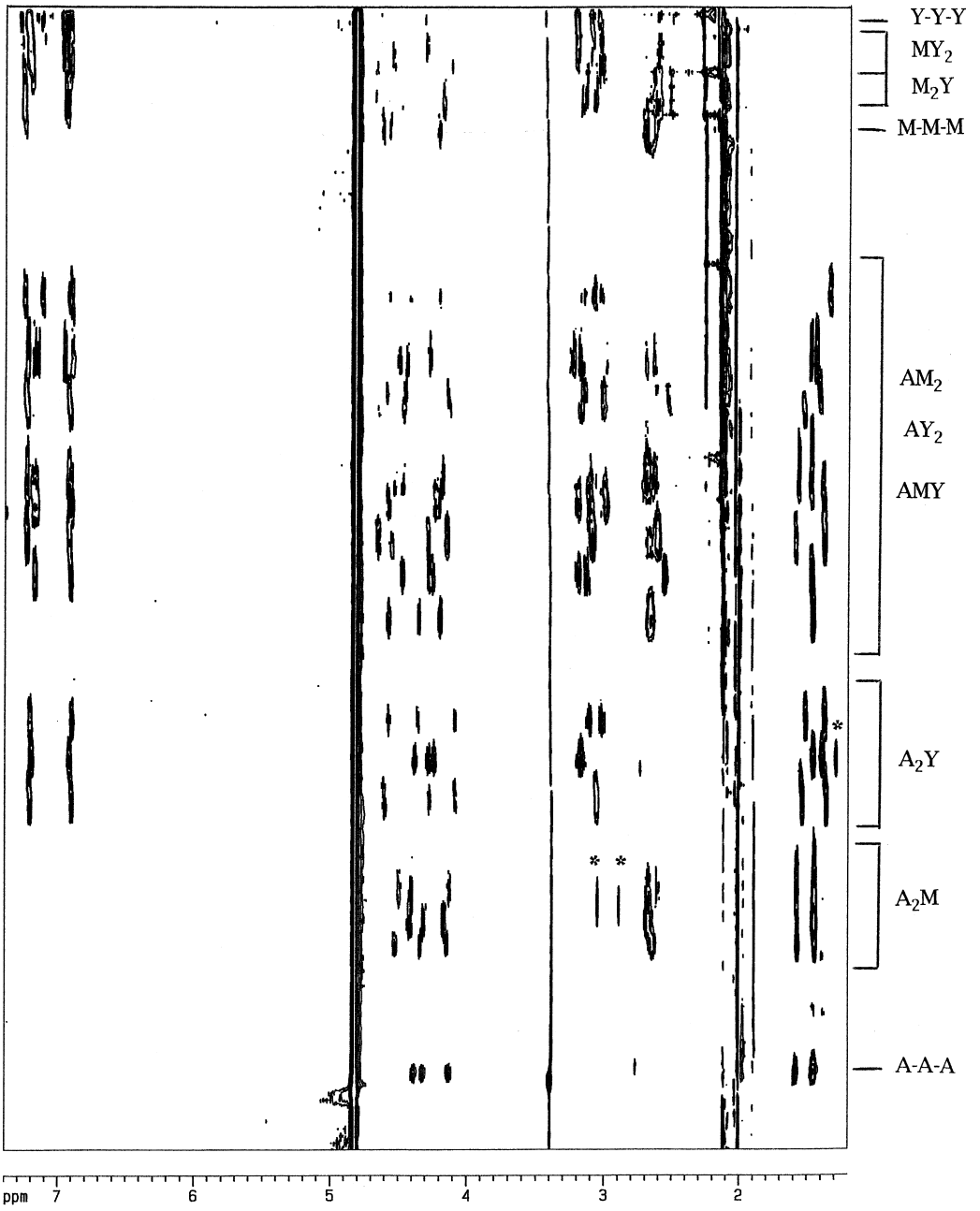

Fig. 1. On flow 600 MHz ^{1}H NMR spectral detection of the HPLC separation of the peptide mixture. The horizontal axis corresponds to the ^{1}H NMR spectrum and the vertical axis represents time, the total acquisition period being 50 min. The asterisks denote non-peptide impurity peaks. The labels at the right hand side denote the classes of tripeptide, e.g., A_2M refers to the three compounds, A–A–M–NH_2, A–M–A–NH_2 and M–A–A–NH_2.

other tripeptides for which assignments have not been obtained are the MY_2–NH_2 isomers and two of the three M_2Y–NH_2 isomers. These elute towards the end of the gradient run and may not be as well resolved under these HPLC conditions. Additionally with changes in the relative chemical shifts of the solvent signals, the intensities of the non-N-terminal α-CH protons and the methionyl β-methylene signals from these peptides may have been reduced by the effects of the solvent suppression irradiation of the water and acetonitrile resonances, respectively. With further optimisation of the elution conditions, it is possible that all 27 analytes could have been resolved and characterised.

5. Application to drug impurities

The manufacture and quality control of a drug are controlled by a variety of national regulatory authorities. As well as drug efficacy, there is also a strong emphasis on the purity of final drug substances and it is necessary to obtain full characterisation and identification of any impurities at the level of ≥0.1% of the UV peak area using HPLC analysis [18]. In order to characterise such impurities, currently it is necessary to isolate individual components by preparative HPLC. This work is often time consuming and expensive, and yet may not give conclusive identification. Furthermore it is possible for the impurities to be degraded during sample extraction and purification. There is, therefore, a considerable need to develop and validate new methods for determining product purity.

Following the lengthy development of the technique and with the availability of commercial equipment, one of the first published real applications of HPLC–NMR was concerned with the identification of an impurity in a synthetic drug precursor [19] and a number of examples are now in the literature including characterisation of impurities in a bulk batch of fluticasone propionate [20] an anti-inflammatory drug used for the treatment of the underlying inflammatory component of asthma, a GART inhibitor AG2034 [21] and the degradation products of a HIV protease inhibitor [22]. The approach can be exemplified by our study on fluticasone propionate [20] with the structure shown in Fig. 2(2.1).

A chromatography method was developed using a 250×4.6-mm column packed with 5 μm ODS-2. Gradient elution was performed using D_2O (containing 0.05% trifluoroacetic acid) and acetonitrile (ACN), starting at ACN–D_2O (45:55, v/v) increasing linearly to 60:40 (v/v) by 25 min and finally increasing to 75:25 (v/v) by 50 min. The HPLC–NMR spectra were acquired at 600 MHz in the stop-flow mode and by time-slicing. In time-slice mode, the HPLC elution was halted at 15-s intervals and ^{1}H NMR spectra were acquired. Separate HPLC–MS data were also acquired on a single quadrupole mass spectrometer equipped with a standard thermospray source operated in positive ion mode. The chromatographic details were the same as used for HPLC–NMR spectroscopy, except for substitution of H_2O for D_2O in the mobile phase. The common fragment ion of 435, with the structure shown in Fig. 2(2.2), was used to identify fluticasone related material.

The UV-detected chromatogram containing the peak of fluticasone propionate and the four impurity peaks of interest (see Fig. 2(2.3–2.6) for structures) gave retention times for the impurities of 20, 26, 30 and 36 min, respectively. Identification of the different peaks in the HPLC chromatogram was achieved by one-dimensional ^{1}H HPLC–NMR spectroscopy in the stop-flow mode and by HPLC–MS using positive ion thermospray ionisation. All the proposed structures for the four impurity peaks were consistent with the known chemical synthesis of fluticasone. The purity of the HPLC UV peak of fluticasone propionate itself was investigated by HPLC–NMR using the time-slice mode of operation. The elution was halted every 15 s over the peak and a ^{1}H NMR spectrum acquired. Near the leading edge of the UV peak where the signal-to-noise ratio is poor, through the main part of the peak where the signal-to-noise ratio increases and finally to the trailing edge of the peak where the signal-to-noise ratio is again lower, the spectra showed no evidence for components other than fluticasone propionate.

Directly coupled HPLC–NMR and HPLC–NMR–MS can greatly enhance the ability to characterise impurities in a pharmaceutical product. As such, this approach is potentially significant as a general tool for purity analyses and would be expected to be important in speeding up production chemistry pro-

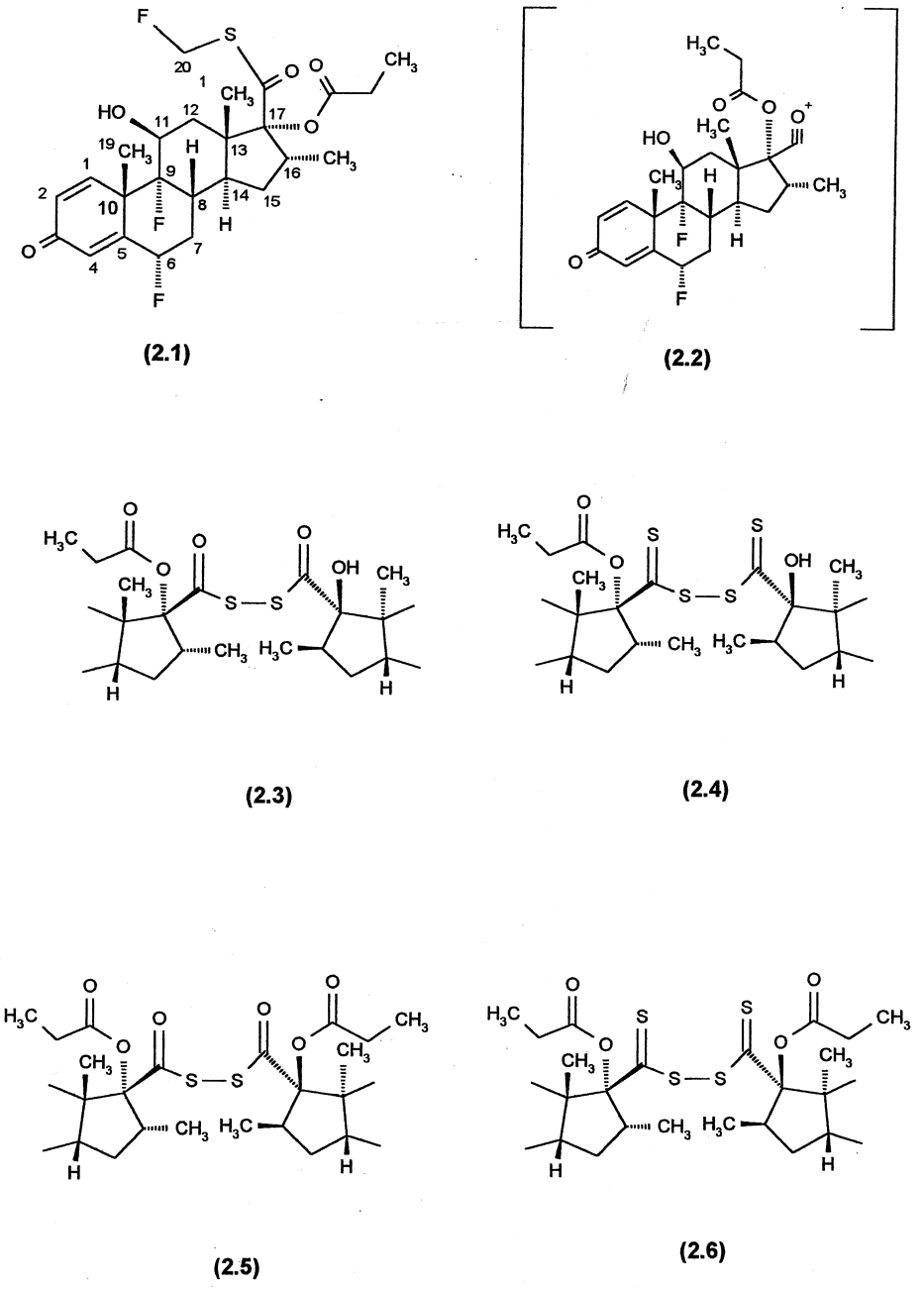

Fig. 2. Structure of fluticasone propionate (2.1), the diagnostic ion used in HPLC–MS (2.2) and partial structures for four impurities (2.3–2.6).

cesses and for regulatory affairs. In this study, it was shown that HPLC–NMR spectroscopy could detect and characterise impurities below the 0.1% peak level [20], the relevant limit for submissions to regulatory authorities.

However, NMR spectra on the peaks which were

at a level of 0.2% or less of the parent drug required considerable and time-consuming data acquisition to achieve acceptable signal-to-noise ratios. Although this appears to involve a high cost in NMR analysis time it would be justified in cases such as that found with fluticasone propionate where there are few alternative analytical approaches. It may, therefore, be beneficial to concentrate the impurities, if stable, before the HPLC–NMR by the application of solid-phase extraction chromatography, column switching or concentration/enrichment of the impurities by preparative HPLC. Such techniques can be feasible because of the availability of large amounts of sample from drug production batches.

This work focussed on the evaluation of HPLC–NMR spectroscopy for characterising impurities in raw drug substances. However, the technique could to be applicable to the investigation of formulated drug substances where impurities often appear as a result of the drug substance reacting with the formulation compound or on samples from degradation studies. These types of adduct can sometimes be unstable during sample extraction and purification. This area is of considerable practical importance in the pharmaceutical industry and is worthy of further study.

6. Chiral HPLC–NMR and HPLC-CD for pharmaceutical mixtures

Many pharmaceutical products are chiral molecules either as single isomers or more commonly as racemic mixtures. In addition, many formulated products are mixtures of active compounds together with a number of additives such as excipients. For chiral molecules, the pressure to develop single isomer forms as therapeutics in preference to racemic mixtures arises from the fact that one enantiomer is usually more biologically active than the other and also that enantiomers can have very different toxicity profiles.

We have combined chiral HPLC on-line with NMR to demonstrate the application of chiral HPLC–NMR spectroscopy to the separation and characterization of different isomers present in a drug substance using, as an example, atracurium besylate, a neuromuscular blocking agent used widely in surgery [23]. Atracurium besylate, (2,2′-(3,11-dioxo-4,10-dioxatridecamethylene)-bis-(2-methyl-1,2,3,4-tetrahydropapaverinium benzenesulfonate), is prepared from racemic 1,2,3,4-tetrahydropapaverine, and has four chiral centres. However, because of the symmetry of the molecule, atracurium has 10 distinct species with the structure given in Fig. 3, where the configuration at C1 can be *R* or *S*. Since the final stage of synthesis is quaternization at N2, the isomers have been distinguished, simply for convenience, by the configuration of the substituents at the C1–N2 bond, such that when a tetrahydroisoquinoline residue has the benzyl group at C1 and, arbitrarily, the 3,11-dioxo-4,10-dioxatridecamethylene chain is in a *cis* configuration, this is called a '*cis*' residue. Thus Fig. 3 shows atracurium in the *R-cis*/*R-cis* isomer form.

In achiral media, different NMR spectra are expected for each of the four types of enantiomeric pairs and for the two *meso* compounds. In general because of the synthetic approach, the ratio of *cis* to *trans* residues is about 3 which, assuming that

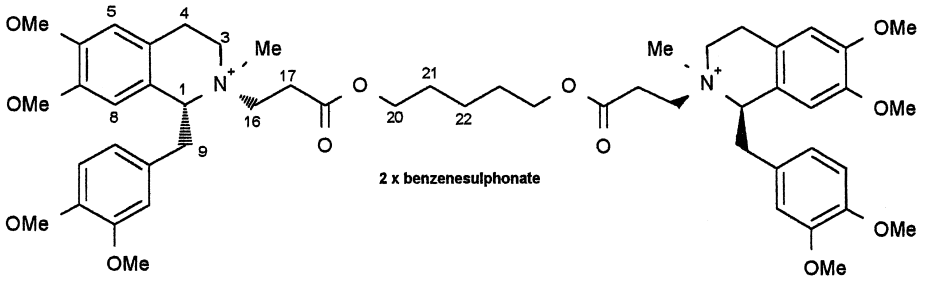

Fig. 3. The structure of atracurium besylate. Only the *R-cis*/*R-cis* isomer is shown. The material is formulated as a mixture of *cis* and *trans* isomers (see text for definition) with *R* and *S* tetrahydroisoquinoline residues.

quaternization at one tetrahydroisoquinoline residue does not affect quaternization at the other, leads to proportions of *cis–cis*, *cis–trans* and *trans–trans* isomers in the ratio of 11:6:1. The preference for *cis* residues has been proved using NOE NMR measurements and by X-ray crystallography on related substances. The ^{1}H NMR chemical shifts, principally of the H8 proton, can be affected not only by whether the C1–N2 configuration in the residue is *cis* or *trans* but also by the configuration of the remote tetrahydroisoquinoline unit.

In summary, therefore, after on-line chiral HPLC separation, NMR spectroscopy has been used to characterize compounds in terms of the *cis* and *trans* isomers and to identify the racemic pairs on the basis of their identical NMR spectra. In addition, HPLC-CD was used to identify the absolute configuration of the enantiomers based on the known CD spectrum of *R*-laudanosine hydrochloride, a closely related molecule. The application to pharmaceutical analysis of HPLC separation with on-line CD detection has been the subject of one publication [24].

Chiral HPLC was performed isocratically at a flow-rate of 3 ml/min using two 250×4.6 mm, 5 μm Chiracel OD-H columns connected to a Chiracel OD guard column of 50×4.6 mm I.D. and an eluent comprising 0.5 *M* $NaClO_4$ buffer (pH 2.0)–ACN (60:40, v/v).

A full assignment of the 750-MHz ^{1}H NMR spectrum of the atracurium besylate mixture sample was achieved using standard one- and two-dimensional NMR methods. From the ^{1}H NMR spectrum of the mixture it was possible to resolve and assign different chemical shifts for the *cis–cis*, *cis–trans*, *trans–cis*, and *trans–trans* isomers for various protons in the molecule. The HPLC–NMR spectroscopic data were also acquired at 750 MHz in the stop-flow mode. For HPLC-CD spectroscopy, the separation was performed as for the HPLC–NMR experiments using a J-600 CD spectrometer with a specially constructed HPLC cell (5 mm path length, 2 mm aperture, 16 μl volume), including quartz doublet focussing and defocussing optics, connected to the HPLC column via a PEEK capillary. Spectroscopic detection was performed in stop-flow mode at 236 nm (at which the atracurium isomers have a CD peak). The instrument was continually flushed with dry nitrogen to ensure optimal spectroscopic performance.

Fig. 4a shows the resulting UV chromatogram used for HPLC–NMR giving good resolution of nine out of the 10 isomers. These eluted with retention times of between 60 and 100 min. This chromatogram also shows some early eluting substances in the atracurium besylate mixture which were seen at retention times between 20 and 35 min. These peaks

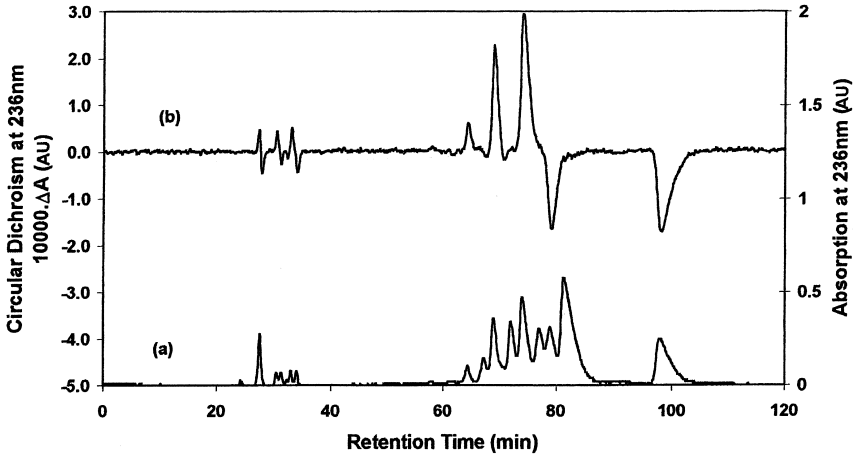

Fig. 4. (a) HPLC separation of the atracurium mixture, using UV detection at 280 nm. (b) HPLC separation of the atracurium mixture, using CD detection at 236 nm, and with the same chromatographic conditions as for UV detection. The left hand vertical scale refers to the CD detection and the right hand vertical scale refers to the UV detection. The peaks with retention times between 60 and 100 min arise from atracurium isomers and correspond in order to NMR spectra A to K shown in Fig. 5.

are minor impurities or degradation products related to atracurium.

Expansions of key reporter resonances in the 750-MHz ^{1}H NMR spectra obtained in stop-flow mode are given in Fig. 5. It is clear from the chiral HPLC chromatogram that although some resolution of nine out of the 10 peaks has been achieved, most of the peaks show considerable overlap. It might therefore have been expected that NMR spectra produced from the individual chiral HPLC peaks would not show single isomers. However, it has been found that these NMR spectra were in general remarkably pure. Although the HPLC peaks were in some cases as much as 8 min wide, the NMR flow probe used in this work only had a 65-μl volume. Hence, only a small slice from the centre of each HPLC peak was collected in the NMR flow cell for spectral data acquisition, giving maximum probability of a spectrum uncontaminated by other components.

Peaks A and B were identified as the *trans–trans* isomers by NMR. This is because not only are they the smallest peaks in the chromatogram, but also give a high field shift for a single H8 proton at ~δ5.6 which is consistent with the assignments of the *trans* isomers from the whole mixture. It was not possible to obtain an NMR spectrum for peak D. Peaks F, J and K are identified as the *cis* isomers. These peaks are the largest in the HPLC chromatogram and give a single lower field ^{1}H chemical shift for the H8 proton at ~δ5.74 which is also consistent with the assignments of the *cis* isomers from the whole mixture. This leaves peaks C, E, G and H which have been identified as the *cis–trans* isomers because they each give two H8 resonances in their spectra for the different *cis* and *trans* parts of the isomer. Other key resonances in the ^{1}H NMR spectrum including H5, H1, H20, H21 and H22 also show differences which are consistent with the assignments made on the whole mixture to confirm the stereochemistry. The identification of the enantiomeric pair from the three *cis–cis* isomers was possible from the NMR spectra in that the spectra from components F and K were essentially identical and that from component J showed distinct chemical shift differences (e.g., as indicated by resonances for H20 in particular, see Fig. 5). From this it can be deduced that Peaks F and K were the *R*/*cis–R*/*cis*, *S*/*cis–S*/*cis* enantiomeric pair and that peak J was

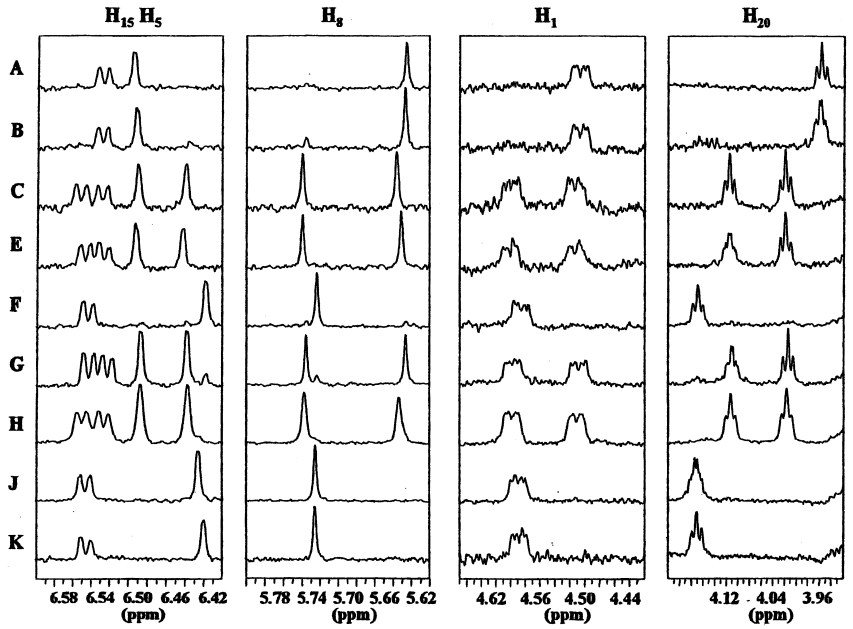

Fig. 5. Expansions of 750 MHz ^{1}H NMR spectra for key peaks in the chiral HPLC–NMR spectra for the different isomers of atracurium besylate.

the *R*/*cis*–*S*/*cis*–*meso* compound. Similarly it could be shown that peaks C and H formed a *cis*–*trans* enantiomeric pair as did peaks E and G as indicated by resonances for H5 and H20 in particular. Finally, although good NMR spectra of only two out of the three *trans*–*trans* isomers were obtained (peaks A and B), these were clearly different and thus one is from the *meso* compound and the other is one of the enantiomeric pair isomers.

The results from the HPLC-CD spectroscopy are given in Fig. 4b. This shows a HPLC-CD trace on the same time scale as the UV chromatogram. The result was a series of CD peaks, some of which were positive, others negative and some which gave virtually no CD spectrum. An additional CD spectrum was also collected on a reference compound *R*-laudanosine hydrochloride which gave a negative CD spectrum. From this it was therefore possible to confirm that for the previously identified *cis*–*cis* isomers, Peak F had *S*/*S* configuration, Peak K was *R*/*R* and peak J was the *R*/*S*–*meso* compound. Similar arguments could be applied to the peaks from the *trans*–*trans* isomers in the chromatogram in that Peak A was *S*/*S*, Peak D was *R*/*R* and Peak B was the *R*/*S*–*meso* compound. Finally for the *cis*–*trans* isomers, again in agreement with the NMR results, Peak C was *S*/*S*, Peak E was *R*/*S*, Peak G was *R*/*S* and Peak H was *R*/*R*.

The HPLC–NMR spectroscopy was useful for identifying the isomeric configuration at the C1–N2 bond, for identifying the enantiomeric pairs of compounds and for distinguishing them from the *meso* forms. The HPLC-CD experiments were complementary in that, whilst unable to distinguish the C1–N2 isomers (*cis* or *trans*), it was possible to determine the absolute stereochemistry at C1 at each tetrahydroisoquinoline residue as either *R*/*R*, *S*/*S* or *R*/*S* based on the sign of the CD response at a chosen wavelength. A consistent finding was that the *S* isomers eluted before the *R* isomers and the *trans* forms eluted before the *cis* forms. By these means a full characterization of all of the 10 isomers of atracurium was achieved.

7. Application to natural products

Natural products have been and remain a rich source of leads for the pharmaceutical industry and many marketed drugs are either natural products or are modifications of such substances. Hence considerable effort is spent in isolating and characterising chemicals from natural sources which can be tested in a variety of biological screens. Often it is necessary to carry out laborious extraction and purification steps and the advent of directly coupled HPLC–NMR has been explored as an alternative technique for natural product identification.

The use of HPLC–NMR, and other hyphenated techniques such as HPLC–MS–MS, for identification of natural products from plant sources has been reviewed by Wolfender [25,26]. The same group have used the technique extensively to characterise plant products including polyphenols and bitter components from Gentianaceae species [27] including the assignment of stereochemistry at a double bond in a new secoiridoid glycoside, seemannoside [28]. Also it was possible to identify antifungal materials from the African plant *Swertia calycina* [27,28], compounds from the Leguminosae family [29], prenylated flavanones from dichloromethane extracts of *Monotes engleri* [30], naphthoquinones from *Cordia linnaei* [31], pyrrolizidine alkaloids from *Senecio* species [32] and antioxidant compounds from the leaves of *Orophea enneandra* [33]. Brinkmann et al. have also identified new naphthylisoquinoline alkaloids from a root extract of *Ancistrocladus likoko* using directly coupled HPLC–NMR spectroscopy [34].

Other studies on natural products using, amongst other techniques, HPLC–NMR include the characteristion of vitamin derivatives [35], saponins from *Bacopa monniera* Wettst [36], antibacterial sesquiterpene lactones from an extract from *Vernonia fastigiata* [37], components from *Hypericum perforatum* L. [38] and ecdysteroids from *Silene otites* [39]. In this latter system, directly coupled HPLC–NMR–MS was also used to identify additional compounds [40].

Microbial production of secondary metabolites is also an important source of novel therapeutic agents. However, the physiological and biochemical factors that determine the onset of production of a specific secondary metabolite in a particular species are incompletely understood. Generally, a range of analytical techniques, often elaborate, time-consuming and involving extensive sample pre-treatment, have to be developed in order to monitor the details of the

metabolic changes and substrate consumption that accompany secondary metabolite production. To provide rapid multi-parametric information about the microbial fermentation process, ^{1}H HPLC–NMR has been applied to characterize microbial metabolites directly in the broth supernatants from a wild-type strain of *S. citricolor* [41]. This species produces aristeromycin, the carbocyclic analogue of adenosine, a secondary metabolite with antibiotic properties. The 600-MHz ^{1}H NMR spectrum of the broth was particularly complex in the chemical shift region between δ4.2 and δ3.4, since it contains major sugar signals obscuring minor metabolites. Hence an HPLC method with on-line ^{1}H NMR detection was employed to physically separate components which gave signals in this region.

8. Application to drug metabolism

8.1. Introduction

By far, the largest body of work to date using HPLC–NMR and HPLC–NMR–MS is in the field of drug metabolism where the methodology has been used extensively for the identification of metabolites in studies from clinical trials involving human subjects, the investigation of model drugs in animals in vivo and also through the use of in vitro systems such as liver microsome incubations. The results in the literature are summarised briefly below and one example, the identification of the metabolites of 2-bromo-4-trifluoromethylaniline found in rat urine [42], is given in somewhat more detail since it encapsulates many of the different aspects which comprise HPLC–NMR–MS and thus serves to illustrate the various modes of operation of the use of the technology.

8.2. A summary of human metabolism studies

Antipyrine has been extensively employed as a probe to investigate the induction of drug metabolism. In man, the main metabolites are the glucuronic acid conjugates of norantipyrine, 4-hydroxyantipyrine and 3-hydroxymethylantipyrine. Norantipyrine can tautomerise to the 5-enol and it is this *O*-glucuronide which is formed rather than an *N*-glucuronide. Stop-flow HPLC–NMR, on a 500-MHz NMR spectrometer, was used to analyse urine samples obtained from a human volunteer following the oral administration of antipyrine [43]. This experiment enabled the unambiguous determination of the structures of the major antipyrine metabolites, rapidly and without any pretreatment (other than preconcentration by freeze-drying) of the sample. By this means, it was possible to identify the ether glucuronide of 4-hydroxyantipyrine, norantipyrine-glucuronide and 4-hydroxyantipyrine itself, the latter either excreted as such or produced by degradation of the glucuronide. In addition, a minor component showed signals for both olefinic hydrogen and glucuronide proton resonances and was probably 3-hydroxymethylantipyrine glucuronide.

Ibuprofen is a widely used non-steroidal anti-inflammatory drug which is subject to extensive metabolism, via both Phase I (hydroxylation and oxidation) and Phase II (glucuronidation) pathways. The principal Phase I metabolites are hydroxy and carboxy oxidation products. In man, the metabolites of ibuprofen are rapidly excreted in the urine following administration of normal therapeutic doses. HPLC–NMR studies, employing gradient elution, performed on freeze-dried urines obtained from a healthy male volunteer, have been carried out [44]. From a continuous-flow HPLC–NMR run it could be seen that three ^{1}H NMR signals around δ5.6 were assignable to anomeric protons from glucuronide conjugates in the samples. Confirmation of this identification was obtained via stop-flow measurements. This allowed identification of the glucuronide of the side chain hydroxylated metabolite of ibuprofen, the glucuronide of ibuprofen itself and the glucuronide of the diacid metabolite. Also identified were unconjugated hydroxy-ibuprofen, and the side chain oxidized diacid metabolite. The two-dimensional TOCSY spectrum (with double solvent suppression) in stop-flow mode was useful in confirming the proposed structures in that the cross peaks due to the glucuronide spin system, and those for the methyl–methine spin system, were readily visible. More recently, a comprehensive study employing HPLC–NMR–MS has been used to confirm the identities of these metabolites [45].

A similar application of HPLC–NMR in drug

metabolism was the study of the metabolic fate of racemic flurbiprofen ((±)-2-(2-fluoro-4-biphenylyl)propionic acid) [46] which, in the same way as seen for ibuprofen is converted to a number of Phase I and II metabolites. The principal metabolite is 4′-hydroxy-flurbiprofen and 3′,4′-dihydroxy-flurbiprofen is a minor metabolite, but all metabolites are excreted mainly as the glucuronide conjugates.

The presence of fluorine in a drug molecule enables selective detection of drug metabolites for compounds such as flurbiprofen. Apart from fluoride ion, usually present at low concentration in biofluids and giving a single broad resonance, there are virtually no endogenous fluorine-containing compounds and hence ^{19}F NMR spectroscopy provides a rapid diagnostic method for assessing the number and level of metabolites of fluorine-containing drugs in body fluids. The 600-MHz ^{1}H NMR spectrum for a urine sample from a human volunteer, following ingestion of 200 mg of flurbiprofen was complex, showing a multiplicity of signals from endogenous metabolites, as well as those due to flurbiprofen-related compounds. This complexity precluded any detailed structural or quantitative analysis demonstrating the limitations of ^{1}H NMR in this case. The corresponding ^{19}F NMR spectrum (with ^{1}H decoupling) indicated the presence of four major fluorine containing species. The chemical shifts of the major fluorinated metabolites were all suggestive of modifications to the drug distant to the fluorine-containing phenyl ring, whilst a total of some 24 separate fluorinated components were detected in this spectrum altogether.

The pseudo-two-dimensional contour plot for the continuous-flow HPLC–NMR experiment, with ^{19}F detection showed four ^{19}F resonances, present as two pairs at retention times of 30.5 and 36.6 min. Of these, the peaks eluting at 30.5 min corresponded in chemical shift to the two largest resonances seen in the ^{1}F NMR spectrum of the whole urine, whilst those eluting at 36.6 min corresponded to the remaining major components. The pairing of the chromatographic peaks was due to the glucuronides being present as diastereoisomers as a result of the conjugation of β-D-glucuronic acid with the *R* and *S* isomers of flurbiprofen or its metabolites. Interestingly the intensities of the signals for the two diastereoisomers in each pair of resonances were not equal indicating either differential excretion or metabolism of the two isomers of the racemic drug or inversion of the chiral centre in vivo.

The experiment was repeated using stop-flow ^{1}H NMR detection at 600 MHz at the appropriate retention times for the ^{19}F NMR-detected metabolites. The resulting ^{1}H NMR spectra were consistent with the β-D-glucuronic acid conjugate of the hydroxylated metabolite eluting at 30.5 min and flurbiprofen glucuronide at 36.6 min. Using the ‘time slicing’ technique, the ^{1}H NMR spectrum was obtained for this peak after elution for a further 30 s. This spectrum was only one of the diastereoisomers of flurbiprofen-β-D-glucuronide, revealing the inhomogeneity of the chromatographic peak. Further studies with ^{1}H NMR in stop-flow mode enabled a further, minor, flurbiprofen metabolite to be identified as the free 4′-hydroxyflurbiprofen.

Paracetamol, or acetaminophen, is one of the most widely studied of all xenobiotics and its metabolic fate is well documented. A number of ^{1}H NMR studies of paracetamol metabolism in man have been conducted, and the major metabolites, namely, the phenolic ether glucuronide, the sulfate and the product with *N*-acetylcysteinyl substituted at position C3, together with paracetamol itself were all detected and quantified in urine [47,48]. More recently HPLC–NMR and HPLC–NMR–MS has been used to characterise these metabolites in biofluids including urine [49].

Blood plasma is, physico-chemically, a more complex biofluid than urine, with high concentrations of proteins and lipoproteins with multiphasic elements. The low-molecular weight substances present may also bind to the plasma proteins resulting in complications for analysis by ^{1}H NMR methods. However, the determination of drug metabolites in human blood plasma by HPLC–NMR has been demonstrated in a study of plasma from dialysis patients suffering from chronic renal failure [49]. The 750-MHz ^{1}H NMR spectra of plasma samples from these subjects indicated the presence of a paracetamol-like metabolite which subsequently, using ^{1}H HPLC–NMR at 600 MHz, was identified as paracetamol glucuronide. This is a somewhat unusual example as, in most cases, drug metabolites are rapidly eliminated from the plasma. However, for patients with renal failure the reduced ability of the

subject to eliminate these compounds results in a build up in the plasma.

Tolfenamic acid is a non-steroidal anti-inflammatory agent which undergoes oxidation in vivo with the metabolites being conjugated with β-D-glucuronic acid. This metabolism has been investigated using 800-MHz ^{1}H HPLC–NMR spectroscopy of human urine following oral administration of the drug to a volunteer [50]. The stop-flow approach was used and a number of glucuronide conjugates were identified. These included those of the parent compound and of compounds with both methyl group and ring hydroxylation.

GW524W91 is a compound which was intended for use as an anti-HIV infection agent. The human metabolite profile has been determined via a combination of methods including ^{19}F NMR of human urine [51]. Urine from a clinical trial has also recently been analysed by HPLC–^{1}H NMR spectroscopy at 500 MHz [52]. In this work the stop-flow technique was used to examine each UV-absorbing peak in the chromatogram. ^{1}H NMR spectroscopy was used to detect the presence of the characteristic doublet resonance (at a chemical shift near δ8) due to the nucleoside base proton. This approach enabled the identification of unchanged GW524W91 as the most abundant of the compound-related material in the sample. In addition, the de-aminated compound, and one of the diastereomeric sulfoxides were also observed. In the case of the glucuronide, identification was possible from the characteristic anomeric proton chemical shift appearing at δ4.51 and spectral assignment was confirmed via the use of a ^{1}H–^{1}H 2-D TOCSY experiment, obtained by overnight data acquisition in stop-flow mode. For GW524W91 itself, it was also possible, due to the large amounts of material present in the sample, to obtain a ^{1}H–^{13}C HMQC spectrum from an overnight experiment.

8.3. Animal metabolism studies of pharmaceuticals and model compounds

In the rat, paracetamol and its metabolites are excreted via the bile as well as in urine. HPLC–NMR has been used to analyse bile from cannulated animals dosed orally with paracetamol using reversed-phase chromatography with gradient elution. The major paracetamol metabolite present in the bile was the phenolic glucuronide with smaller quantities of the sulfate also present. Using HPLC–NMR, characteristic spectra for both of these compounds were obtained without difficulty, using the stop-flow technique [49]. Employing the same HPLC conditions used for the analysis of bile, ^{1}H HPLC–NMR spectra of the urine from the paracetamol-dosed rats were obtained, confirming the presence of the sulfate and glucuronide conjugates and paracetamol. The *N*-acetylcysteinyl conjugate was also detected and a ^{1}H NMR spectrum obtained by stop-flow analysis. This spectrum required about 50 min to collect and still gave a poor signal-to-noise ratio as expected for such a minor metabolite.

In this type of application it is often necessary to compromise on the data acquisition regime, both in terms of achieved signal-to-noise ratio and in the spectral digital resolution. This may be the result of the need to preserve sample viability, or because the time scale of events being monitored does not permit long acquisition times. In such instances, advantages, in terms of information content, may be more readily obtained if data are subject to further processing after acquisition and so the maximum entropy technique [53] was applied to the NMR spectrum from the *N*-acetylcysteinyl metabolite of paracetamol and the optimum lineshape (Lorentzian) and linewidth were both obtained by maximising the probability value of the derived solution. The result clearly showed the loss of symmetry of the phenyl ring as a result of the formation of the *N*-acetylcysteinyl adduct with substitution *meta* to the *N*-acetyl of paracetamol confirmed by the ^{1}H chemical shifts of the remaining aromatic protons. The non-equivalent methylene and the methine resonances of the cysteine moiety are also present in the spectrum, with chemical shifts consistent with *S*-substitution. Furthermore, signals could be observed for the *N*-acetyl protons, albeit somewhat attenuated by the acetonitrile solvent signal irradiation.

Phenacetin was once in widespread use as an analgesic but, after being implicated as a cause of kidney toxicity, it was withdrawn from the market. Recently, the rat metabolism of phenacetin has been re-investigated using HPLC–NMR and HPLC–NMR–MS [54]. This approach showed that the compound is metabolised principally to paracetamol with subsequent conjugation producing paracetamol

glucuronide and paracetamol sulfate. *N*-hydroxy-paracetamol was also tentatively identified.

There are a number of other HPLC–NMR and HPLC–NMR–MS studies which include the characterisation of xenobiotic metabolites. The metabolites of the potential antipsychotic agent iloperidone have been elucidated. Identification of metabolites in biological fluids from rats, dogs and humans was achieved using HPLC–MS–MS and, for bile in particular, HPLC–NMR was used to identify a number of structures [55]. Metabolites of the multi-drug resistance inhibitor LY335979 have been characterised using HPLC–NMR from rat bile and from human liver microsome incubations. An *N*-oxide metabolite was produced from oxidation of a quinoline nitrogen and, in addition, three glucuronide metabolites were identified, formed by conjugation after oxidation in the quinoline ring [56]. Finally, the implementation of HPLC–NMR–MS, connected in series, has been demonstrated and used to identify metabolites of the non-nucleoside HIV reverse transcriptase inhibitor, GW420867. Again the major substance proved to be glucuronide conjugate of a ring-hydroxylated derivative of the compound [57].

Relatively few drugs contain phosphorus, probably the most important class being those related to cyclophosphamide which are used in chemotherapy. However, the presence of phosphorus in such a molecule does provide the opportunity for using ^{31}P NMR as a specific method for detection of metabolites. The toxicity of the anticancer drug ifosfamide has been studied using extracts from the urine of patients on ifosfamide therapy [58] and this study was combined with investigations of ifosfamide metabolism in the rat. In this case, using urine samples which had been freeze-dried and reconstituted at a 12-fold concentration, it was possible to locate the drug-related substances using ^{31}P-detected HPLC–NMR in the continuous flow mode and subsequently to use stop-flow methods to characterise the metabolites using ^{1}H NMR. Although ^{31}P NMR spectroscopy is much less sensitive than ^{1}H or ^{19}F NMR spectroscopy, it proved useful for the identification of the chromatographic retention times of phosphorus-containing species in this study, particularly since the metabolites of ifosfamide have poor UV absorption characteristics. Under the HPLC conditions used, the parent drug and three metabolites were identified, ifosforamide mustard, 4-hydroxyifosfamide and 2-de(chloroethyl)ifosfamide.

The metabolic fate and urinary excretion of 2-bromo-4-trifluoromethylaniline has been studied in the rat using ^{19}F NMR spectroscopy and directly coupled HPLC–NMR–MS [42]. The ^{19}F NMR spectrum of whole rat urine collected 0–8 h after i.p. dosing with 50 mg kg^{-1} of the compound is shown in Fig. 6 indicating the number and relative levels of fluorinated molecules in the urine. It was clear that there was very little of the parent compound in the urine from addition of authentic material. After a solid-phase extraction step, HPLC–NMR and HPLC–NMR–MS experiments were carried out to identify the three most abundant species seen in Fig. 6. Thus, Fig. 7 shows the continuous-flow ^{19}F NMR-detected chromatogram as a contour plot and this gives the retention times of the major fluorine-containing species. Subsequent HPLC–NMR–MS experiments with ^{19}F and ^{1}H NMR detection and negative ion electrospray MS at these retention times demonstrated that the major metabolite (labelled A in Fig. 6) was 2-amino-3-bromo-5-trifluoromethylphenylsulfate accounting for 23% of the dose being excreted in the 0–8 h urine. The spectra corresponding to this metabolite are shown in Fig. 8, with Fig. 8a being the ^{19}F NMR spectrum, Fig. 8b showing the ^{1}H NMR spectrum with the expected *meta*-coupled aromatic protons and Fig. 8c being the negative ion electrospray mass spectrum of the fully deuterated molecule. A similar approach was used to identify Peak B in Fig. 6 as 2-bromo-4-trifluoromethylphenylhydroxylamine-*N*-glucuronide (7% of the dose) and Peak C in Fig. 6 as 2-amino-3-bromo-5-trifluoromethylphenylglucuronide (1.4% of the dose). In addition, MS could be used to detect and identify a number of minor metabolites below the NMR detection limit [42]. Similar studies have also been carried out on 2-chloro-4-trifluoromethylaniline [59] and 3-methyl-4-trifluoromethylaniline and its acetanilide [60].

Some preliminary work has been carried out to determine the possibility of using ^{2}H NMR spectroscopic detection in HPLC–NMR. To this end, the metabolism of dimethylformamide-d_7, DMF-d_7, in the rat was investigated using ^{2}H NMR spectroscopy [61]. ^{2}H NMR detection of the deuterated metabolites from DMF-d_7 in rat urine was attempted in con-

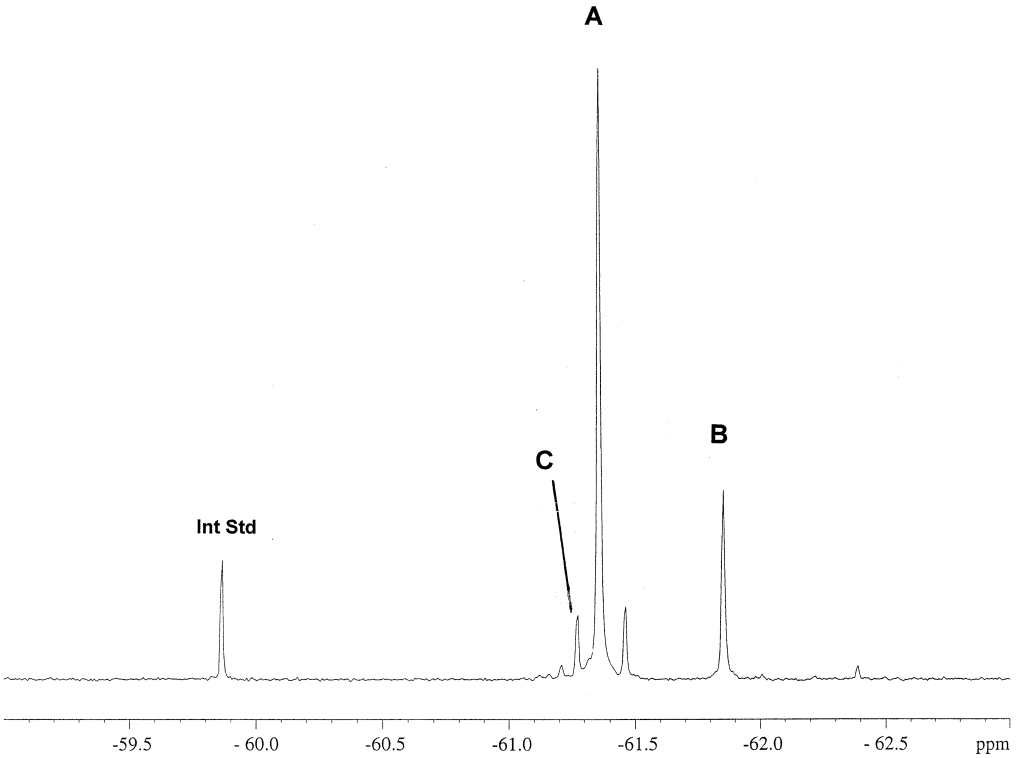

Fig. 6. 376-MHz ^{19}F NMR spectrum of rat urine after intra-peritoneal administration of 2-bromo-4-trifluoromethylaniline. The internal standard used (Int Std) was 2-trifluoromethylbenzoic acid. A, B and C denote the metabolites identified using HPLC–NMR–MS.

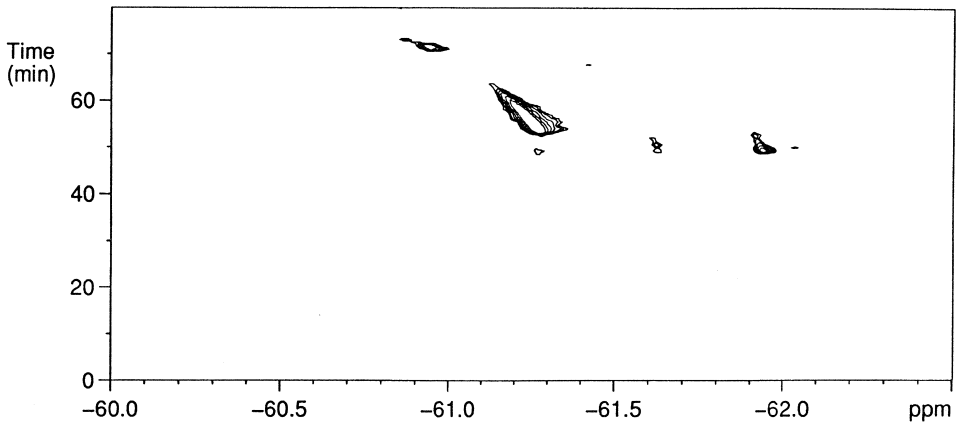

Fig. 7. Continuous flow 470-MHz ^{19}F NMR-detected HPLC chromatogram from rat urine following administration of 2-bromo-4-trifluoromethylaniline. The ^{19}F NMR chemical shift is along the horizontal axis, the HPLC retention time is on the vertical axis and the eluting peak intensity is shown in a contour plot.

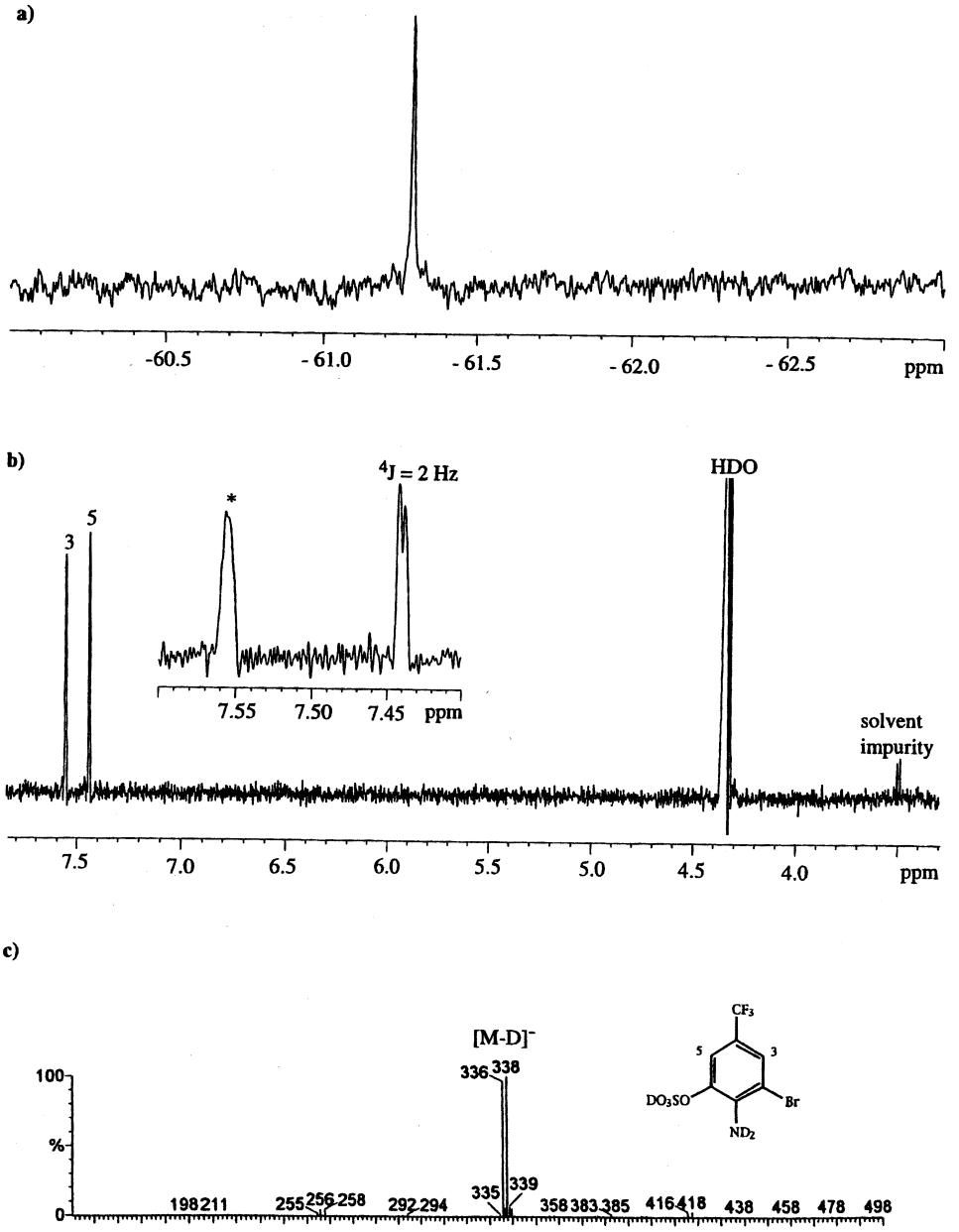

Fig. 8. NMR and MS data for the major metabolite of 2-bromo-4-trifluoromethylaniline, namely 2-amino-3-bromo-5-trifluoromethylphenyl sulfate, in rat urine obtained by stop-flow HPLC–NMR–MS. (a) 470-MHz ^{19}F NMR spectrum, (b) 500-MHz ^{1}H NMR spectrum showing resolution-enhanced expansion of the region of the aromatic protons (* denotes unresolved meta spin coupling) and (c) negative ion electrospray MS of the molecule with all exchangeable hydrogens replaced by deuterium.

tinuous-flow HPLC–NMR [62]. These experiments were carried out at a ^{2}H observation frequency of 92.1 MHz (which corresponds to ^{1}H at 600 MHz). Based on chemical shifts and the known metabolism of DMF-d_7, the parent compound was the major drug-related material present. The next two most abundant species had very similar retention times, with each containing one type of methyl group. One

of these arises from dimethylamine-d_6 whilst the other was one rotational form of *N*-hydroxymethyl-*N*-methylformamide-d_6. In addition, one peak was seen which was detected in the 0–8 h urine only and which was not then assigned. This compound also shows a formyl deuteron resonance and possibly arises from a demethylated product such as *N*-methylformamide-d_4. It is clear that ^{2}H NMR spectroscopy is not likely to be of major use in drug metabolism, but because the ^{2}H nucleus has a rapid NMR relaxation time, data can be acquired rapidly and for small molecules the linewidths are reasonably sharp. This means that for equal numbers of nuclei (i.e., the same level of isotopic substitution), it has approximately the same sensitivity as ^{13}C NMR spectroscopy although the chemical shift range and hence spectral dispersion is much higher for the latter.

8.4. Application to in vitro metabolism studies

In vitro techniques, such as the use of tissue slices and cell suspensions, are being used increasingly to obtain insights into the metabolism of new compounds in both animals and man. GW1370U87, 1-ethyl-phenoxathiin-10,10-dioxide, is intended for use as a monoamine oxidase-A inhibitor and its metabolism has been studied in human liver microsomes [63]. The supernatant from the cells was collected, concentrated and examined using 600-MHz ^{1}H HPLC–NMR in stop-flow mode. The UV-detected chromatogram was uncomplicated with most, but not all, of the UV peaks being due to GW1370U87 or its metabolites. Chromatography was stopped at the top of each peak and a ^{1}H NMR spectrum obtained. In all, six GW1370U87-related HPLC peaks were characterised.

Directly coupled 750-MHz HPLC–^{1}H NMR spectroscopy has been applied to the characterisation of low level metabolites of 3-amino-2-(2-fluorophenoxy)pyridine in rat microsomes [64]. In stop-flow HPLC–NMR mode, the direct injection of microsomal extracts enabled the separation and characterisation of minor metabolites. Unequivocal identification of the metabolites was achieved without the use of radiolabel or synthetic standards. In addition, the metabolism of the multi-drug resistance inhibitor LY335979 in human liver microsome incubations has been studied [56].

8.5. Application to drug metabolite reactivity

Many drugs containing carboxylate groups form β-1-*O*-acyl glucuronides as major metabolites. Such ester glucuronides are potentially reactive due to the susceptibility of the acyl group to nucleophilic reactions and they can undergo hydrolysis, acyl migration and covalent adduct formation. The acyl migration reactions result in positional isomers and anomers as shown below and these may be reactive towards serum proteins with toxicological consequences. The acyl group migrates successively to the 2-, 3- and 4-hydroxyl groups of the glucuronic acid moiety, thereby allowing the formation of both α- and β- anomers of the positional isomers (see Fig. 9).

Synthetic fluorobenzoic acid and trifluoromethylbenzoic acid glucuronide conjugates were chosen as model compounds of carboxylate group-containing drugs and an HPLC method has been developed for the simultaneous determination of the 1-, 2-, 3- and 4-positional isomers of the acyl glucuronides, and their α- and β-anomers for 2-, 3- and 4-fluorobenzoic acids together with the aglycones formed via hydrolysis. A typical result is shown in Fig. 10 which depicts the continuous flow 750-MHz ^{1}H HPLC–NMR characterisation of the glucuronides from an equilibrium mixture of transacylated glucuronides of 4-fluorobenzoic acid, measured in the continuous flow mode [65]. The ^{1}H NMR frequency is on the horizontal axis and the chromatographic retention time is on the vertical axis. Each of the glucuronide isomers is eluted separately and can be identified from its NMR spectrum. It has been noted that in general the elution order of transacylated glucuronides is β-4-*O*-acyl-, α-4-*O*-acyl-, α-3-*O*-acyl-, β-3-*O*-acyl-, β-2-*O*-acyl- and α-2-*O*-acyl- irrespective of the nature of the carboxylic acid-containing moiety.

This directly coupled HPLC–NMR method has been used to investigate the acyl migration kinetics of individual isomers of 2-, 3- and 4-fluoro-, and 2- and 3-trifluoromethylbenzoyl-D-glucopyranuronic acid separated from an equilibrium mixture of the β-1-*O*-acyl isomer, the α- and β-2-*O*-acyl isomers,

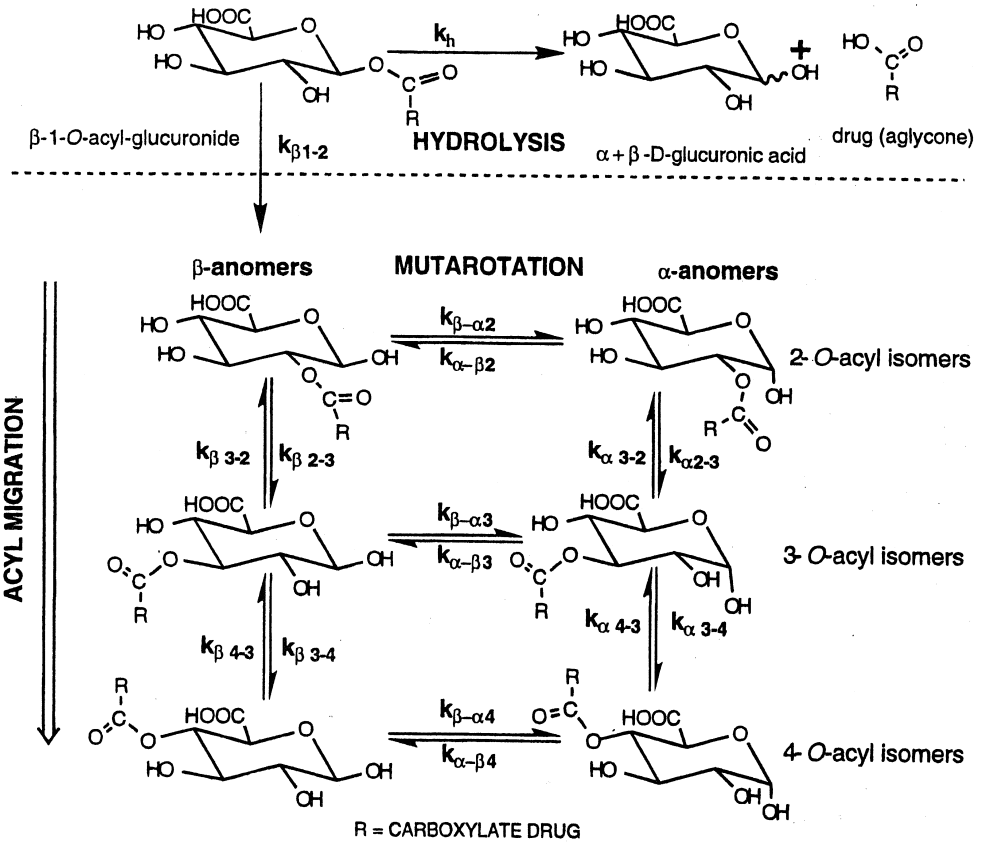

Fig. 9. The reaction scheme depicting acyl migration in glucuronide conjugates.

the α- and β-3-*O*-acyl isomers and the α- and β-4-*O*-acyl isomers at pH 7.4 and 25°C [65–68]. Both continuous-flow HPLC–NMR at 750 MHz and stop-flow methods have been used. For detailed kinetic studies, each isomer was separated using reversed-phase HPLC and then led into an NMR flow probe in a 600-MHz NMR spectrometer. The flow was stopped and sequential ^{1}H NMR spectra collected, thus allowing the direct observation of the appearance of the glucuronide positional isomers of that particular glucuronide isomer which had been isolated. This is illustrated in Fig. 11, which shows the build-up of other products following the introduction of the β-4-*O*-acyl-glucuronide of 2-fluorobenzoic acid into the NMR probe after HPLC separation. The rate constants for the decomposition of the various isomers were determined and the acyl migration reactions were simulated using a mathematical model of the kinetics of the glucuronide rearrangement (incorporating nine first-order rate constants determining acyl migration reactions and six first-order rate constants describing the mutarotation of the 2-, 3- and 4-positional isomers).

Finally, the acyl migration of the glucuronide metabolite of the model drug 6,11-dihydro-11-oxo-dibenz[*b,e*]oxepin-2-acetic acid has also been investigated in pH 7.4 buffer and urine using directly coupled 600- and 750-MHz stop-flow HPLC–^{1}H NMR spectroscopy [69,70].

It is clear that directly coupled HPLC–NMR spectroscopy offers a unique analytical approach to obtain structural information of inter-converting compounds in a complex mixture of isomers. This method will be of value in the elucidation of the reactivity of drug glucuronides in terms of acyl migration and enable an investigation of the potential for protein binding. Furthermore, this HPLC–NMR approach to the study of glucuronide acyl migration

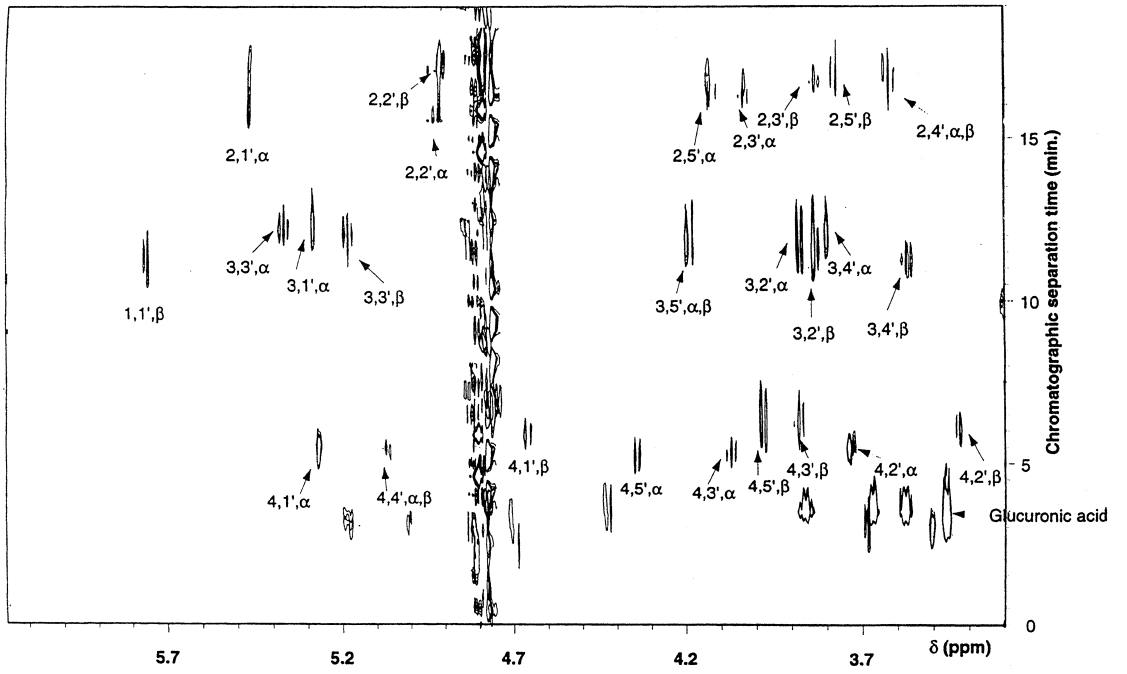

Fig. 10. Continuous flow 750-MHz ^{1}H HPLC–NMR spectrum of an equilibrium mixture of transacylated glucuronides of 4-fluorobenzoic acid. The nomenclature 2,1′-α denotes the 1′ proton in the α-anomer of the 2-*O*-acyl glucuronide.

reactions allows unique kinetic information to be obtained relating to glucuronide reactivity, and this approach will be useful in future structure–activity studies on the toxicity of drug ester glucuronides.

It has recently been shown using NMR spectroscopy in conjunction with isotope-labelling studies that there is a significant degree of deactylation–reacetylation (futile deactylation) of paracetamol metabolites in vivo in the rat [71]. If this also occurs in humans, then it may help to explain the observed incidence of nephrotoxicity of paracetamol, in that the process would result in levels of the potent nephrotoxin 4-aminophenol in vivo. Confirmation of the levels of futile deactylation in individual metabolites of isotopically labelled paracetamol in man has been achieved using directly coupled HPLC–NMR spectroscopy at 600 MHz. In this study a solid-phase extract of a 0–4-h urine after dosing with paracetamol-d_3 was separated using HPLC with a methanol–water gradient elution. Methanol was used instead of the more usual acetonitrile to avoid the large methyl NMR resonance of the latter which would obscure any observation of transacetylated products. Good ^{1}H NMR spectra were obtained from the sulfate and glucuronide conjugates of paracetamol-d_3 and quantitation of the level of transacetylated products for both of these metabolites was obtained by integration of the observed acetyl peak relative to the peaks from the aromatic protons. In man, it was shown that paracetamol glucuronide underwent transacetylation to an extent of 1%, and for the sulfate conjugate the level was 2% [72]. This compares with results for the sulfate in the rat of about 10% [71].

Further work has been carried out on the now withdrawn drug, phenacetin or 4-ethoxyparacetamol. Using the same HPLC–NMR approach, the level of futile deacetylation in phenacetin in the rat was much higher than for paracetamol. This has been quantified in the major metabolites, paracetamol sulfate and paracetamol glucuronide, as 30 and 36%, respectively, using HPLC–NMR spectroscopy. The level of futile deacetylation for paracetamol and a further, tentatively assigned, metabolite, *N*-hydroxy-paracetamol sulfate, was quantified at 32% [54].

The metabolism of paracetamol proceeds through

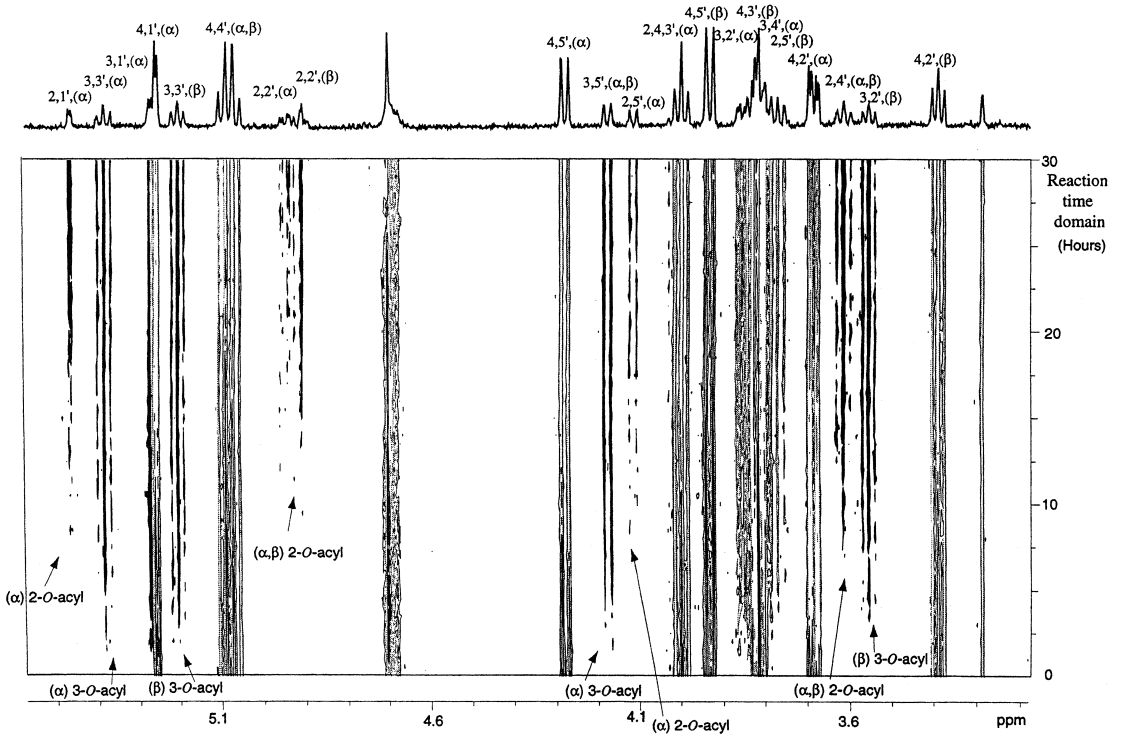

Fig. 11. Stop-flow 600-MHz ^{1}H NMR spectrum obtained after the β-4-*O*-acyl-glucuronide of 2-fluorobenzoic acid had been isolated in the NMR flow cell. This shows the successive formation of 3-*O*-acyl- and 2-*O*-acyl isomers with time.

a reactive intermediate known as NAPQI which can react with glutathione at the 2, 3, or *ipso* carbons. This reaction has been investigated using directly coupled HPLC–NMR at 500 MHz [73]. The two reactants, (NAPQI and glutathione, GSH) were mixed and the product mixture separated using directly coupled HPLC–NMR. In fact all three isomers were shown to be produced with the *ipso* derivative being most abundant and the 2′-isomer the least. By holding the *ipso*-NAPQI–GSH adduct in the flow probe of the NMR spectrometer for 1 h the rate at which it decomposed to the other isomers and other species could be monitored.

9. Future developments

It is possible to identify areas where rapid advances are to be expected that will further increase the utility of HPLC–NMR. One such is automation where the ability for increased automation in coupled separation–NMR techniques will be a major factor in enabling the more widespread application of the technology. Indeed, it is not difficult to envisage a time when HPLC–NMR combined with in-line HPLC–MS will, automatically, provide the complete qualitative and quantitative metabolic fate of xenobiotics in a single chromatographic run.

New technical developments are also occurring which, in the forseeable future, will provide greatly increased NMR sensitivity. These developments include the use of higher magnetic field strengths and hence observation frequencies. In addition the development of NMR probes and preamplifiers cooled with cryogenic liquids will provide lower detection limits and higher sensitivities to a degree surpassing any arising from increases in magnetic field [74]. Horizontal detector coils (i.e., perpendicular to the magnetic field) have been demonstrated and this approach can be expected to be applied to HPLC–NMR and SFC–NMR. Micro-bore methods are also under development for HPLC–NMR and initial tests

[9,10] have been made. This approach with its consequent low solvent usage allows fully deuterated NMR solvents to be employed at reasonable cost and alleviates the solvent suppression problems referred to earlier. It has been found that detection of low nanogram quantities of material can be achieved in 3–4 min under stop-flow conditions and two-dimensional NMR spectra are therefore possible also [75,76]. It is expected that new eluent systems for HPLC which are advantageous for NMR and MS detection will be developed and some preliminary results have been given on the use of superheated D_2O in this respect [77,78].

Capillary electrophoresis (CE) is a relatively new technique but has been shown to be a very powerful addition to the armoury of separative methods [79,80]. This differs from HPLC in that the separation results from the influence of an applied voltage. These voltages may generate both electroosmotic and electrophoretic flow of buffers and ionic species within the capillary. The technique is very simple experimentally, with all that is required being a length of fused-silica capillary with an optical window to enable detection, a detector (UV, fluorescence or mass spectrometry), a high voltage source, two electrode assemblies and buffer solutions in suitable reservoirs. The technique has been shown to provide very high separation efficiencies but the small injection volume (a few nl) means that high sensitivity can only be achieved if concentrations of the analyte in the sample are high. The use of NMR spectroscopy for detection in CE has been demonstrated [81–83]. This has an active volume of ~5 nl and limits of detection using ^1H NMR in the ng range for acquisition periods of the order of 1 min. The limit of detection in concentration terms is about that of HPLC–NMR but in mass terms represents about two orders of magnitude less. The use of CE–NMR, and the related technique CEC–NMR at an observation frequency of 600 MHz, has been applied to the detection and characterisation of paracetamol metabolites found in human urine [84,85].

Finally, further hyphenation of other spectroscopic techniques for analyte identification such as infra-red spectroscopy has been demonstrated [86]. In addition, it is expected that publications on the use of other types of mass spectrometric detection in HPLC–NMR–MS, such as time-of-flight (TOF) and ion-cyclotron resonance MS, which allow accurate masses and hence empirical molecular formulae to be determined, will be forthcoming. A recent interesting development is the coupling of HPLC to inductively coupled plasma MS (ICPMS) which can be used as a specific atom detector. This has been applied to the detection of the brominated metabolites found in rat urine after dosing with 4-bromoanaline and the resulting chromatogram can be dubbed a 'bromatogram' [87]. It is possible to foresee many applications of this technology.

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