Liquid Chromatography–Nuclear Magnetic Resonance–Mass Spectrometry

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Introduction

As part of the development process of any new chemical entity as a drug substance, evidence is required that its metabolism in humans is similar to its metabolism in the animal species selected for toxicological evaluation. In order to obtain this information a number of studies are carried out using both radiolabelled and unlabelled material. Xenobiotics are generally metabolized to generate more polar compounds that are then more readily eliminated from the body. The metabolism of drugs commonly occurs in two main phases: phase I, functionalization type reactions such as oxidation, reduction and hydrolysis and phase II where metabolism reactions are generally conjugative reactions such as glucuronidation, sulfation, methylation, and acetylation whereby an endogenous group is attached to the molecule.

Historically drug metabolite identification has usually been based on the comparison of UV detected high-performance liquid chromatography (HPLC) retention times of isolated 'unknown' metabolites with authentic standards. This method of detecting drug metabolites, and subsequently characterizing, is not only a time-consuming process, and hence expensive, but yields no or very limited structural information.

The application and use of mass spectrometry in drug metabolism studies has contributed significantly to the improved structural characterization of novel drug metabolites. More recently, tandem mass spectrometry (MS/MS) has been increasingly used in both the characterization and quantitation of metabolites in microsomal incubates, urine, plasma and faecal extracts derived from both *in vitro* and *in vivo* sources.

Even though mass spectrometry is an exquisitely sensitive and specific detector capable of providing drug metabolite information from complex biological matrices, it does not always provide unequivocal structural identification, and in these instances NMR spectroscopy is often needed to provide conclusive site specific structural characterization. Increasingly the complementary information provided by MS and NMR is used in conjunction with HPLC separation techniques and indeed reports exist where all three techniques have been linked to generate on-line LC/NMR/MS.

A Systematic Approach to Drug Metabolite Identification

This section describes an approach to drug metabolite identification which utilizes semi-preparative HPLC, LC/MS^n, NMR and HPLC/NMR. The potential of coupling NMR and tandem mass spectrometry (MS^n) in the form of an ion trap mass spectrometer is also highlighted. The advantage of using an ion trap mass spectrometer over a quadrupole instrument is primarily the ability to generate MS^n spectra by repetitively isolating and fragmenting stored ions, and thereby producing second, third and subsequent generation product ions. This enables advantage to be taken of both structural and molecular weight information generated by the MS to complement the NMR data. The power of the use of these techniques in combination with each other is illustrated, using the example of novel non-nucleoside reverse transcriptase inhibitor, GW420867 (see **Figure 1**) which serves to demonstrate the approach currently taken to drug metabolite characterization in our laboratory.

Sample Fractionation

Urine samples collected following oral administration of GW420867 to animal species and man were pooled to provide 100 mL of sample in each case. The pooled urine samples were separately freeze-dried and then reconstituted in distilled water to give a final volume equal to one tenth of the initial volume. The resulting concentrated urine samples were centrifuged and the supernatants removed and stored at 0–8°C prior to isolation by preparative HPLC. Injections of the concentrated urine samples (10 mL) were made onto a preparative HPLC column, and analytes separated with reversed-phase gradient elution. The column eluent was collected into 96 well microtitre plates at 15 s fraction intervals. The microtitre plates were transferred to a 96 well dry down station

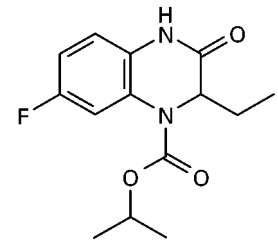

Figure 1 Structure of GW420867.

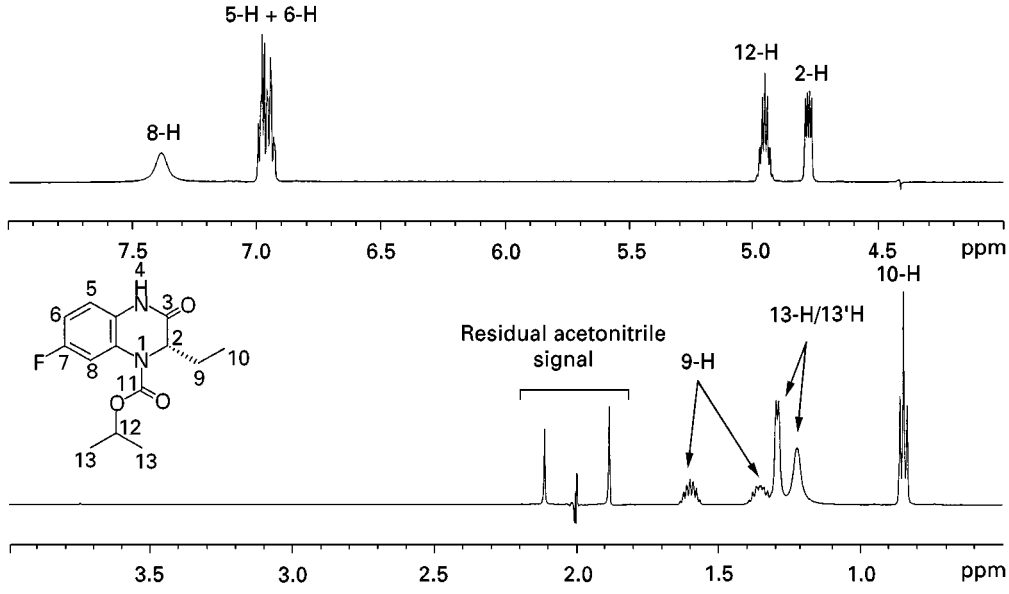

Figure 2 ^{1}H-NMR spectrum of a 1 mg mL^{-1} solution of authentic GW420867X in 50 : 50 acetonitrile – deuterium oxide, obtained on a Bruker DRX-600 spectrometer over 256 scans with dual solvent suppression.

and evaporated to dryness under a constant flow of nitrogen at 25°C. The dried fractions were re-dissolved in 50 : 50 acetonitrile – deuterium oxide. The microtitre plates were sonicated for 10 min in an ultrasonic bath to aid the resolvation process and the resulting dissolved fractions transferred individually to 5 mm NMR tubes for analysis by ^{1}H and ^{19}F NMR spectroscopy for the presence of drug-related material.

Fraction Screening by ^{1}H and ^{19}F NMR

In this particular example the ^{19}F NMR spectra may be used to ascertain the distribution of drug-related material in the processed fractions as the drug molecule contained a fluorine atom and there are little or no endogenous fluorinated background signals in urine. However comparison of the ^{1}H NMR spectra obtained for the isolated fractions with that acquired for the authentic parent compound may also be used for this purpose. The ^{1}H NMR spectrum of the parent drug is shown in **Figure 2**. The aromatic protons at 6.78 and 6.94 ppm and the aliphatic protons at 0.85 and 1.29 ppm were used to identify possible drug metabolites and also to indicate structural changes where appropriate. By contrast the ^{19}F NMR spectra only provides limited structural information on changes in the immediate vicinity of the fluorine atom.

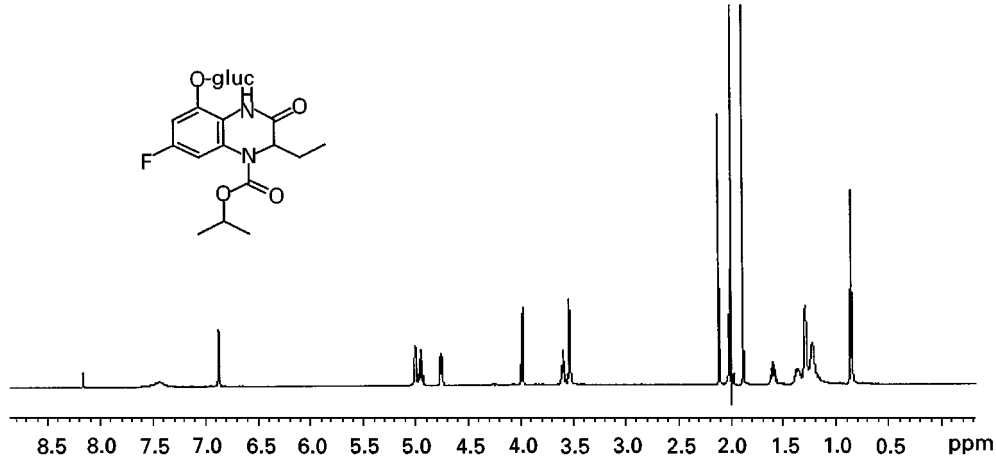

Figure 3 ^{1}H-NMR spectrum of the H-5 hydroxyl glucuronide metabolite of GW420867X in–acetonitrile – deuterium oxide (1 : 1) obtained on a Bruker DRX-600 spectrometer over 64 scans with dual solvent suppression, following isolation from rabbit urine.

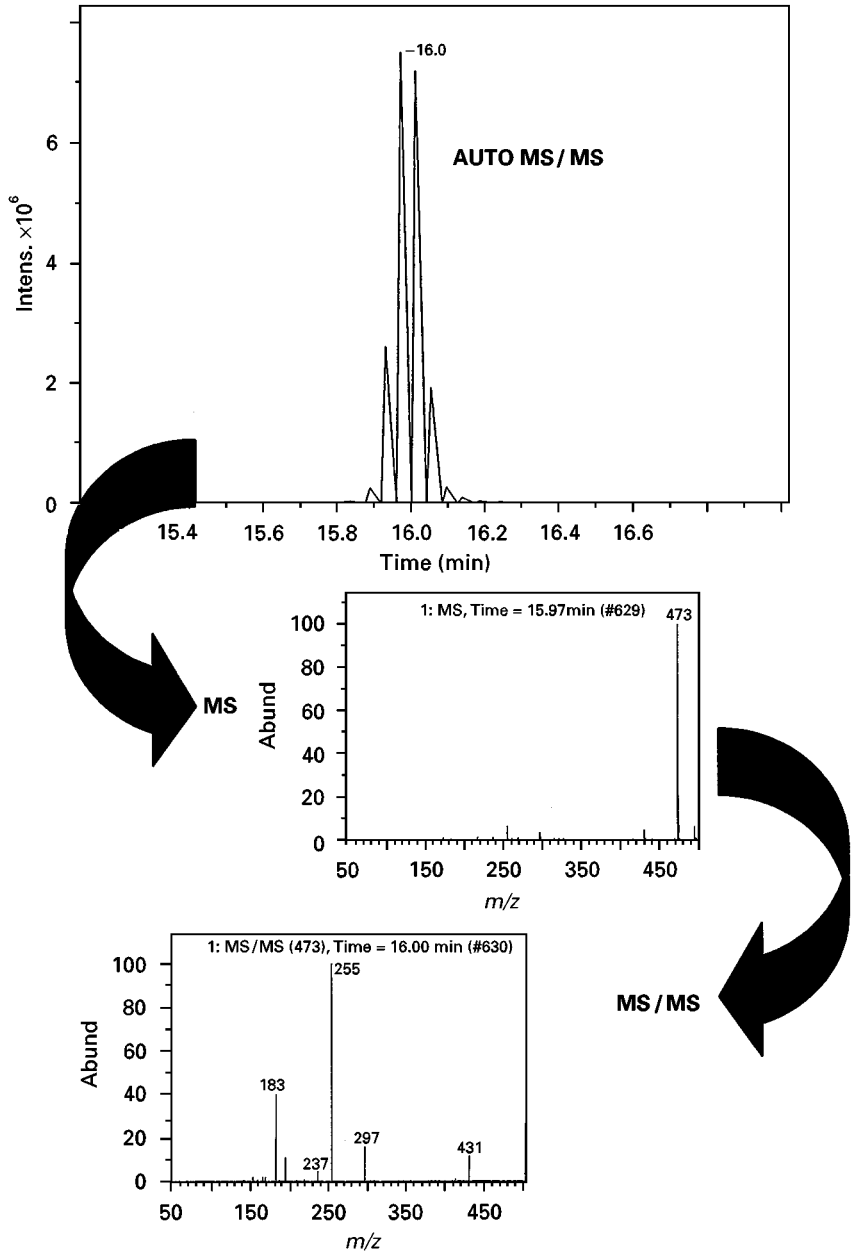

Figure 4 LC/MS/MS analysis of the H-5 hydroxyl glucuronide metabolite of GW420867X following injection of a rabbit urine fraction on to a 250 × 4.6 mm Zorbax RX-C8 5 μm column. Analytes were eluted with a 0–100% aqueous 0.1% formic acid/acetonitrile gradient over 30 min at 1 mL min^{-1}. The column eluent was monitored using the Esquire ion trap operating in positive ion electrospray 'auto' MS/MS, with a resonance fragmentation amplitude of 1.2 V.

A ^{1}H NMR spectrum from an isolated rabbit urine fraction acquired using only 64 scans, is given in **Figure 3** and illustrates that metabolites can potentially be isolated with high degree of purity. Inspection of the ^{1}H NMR spectrum readily enabled the metabolite to be identified as a glucuronic acid conjugate of the 5-hydroxylated species (as shown in Figure 3); the anomeric H1 (5.1 ppm) and H2–H5 (3.4–3.6 ppm) glucuronyl protons being clearly visible.

Analysis of Metabolite Containing Fraction by Mass Spectrometry

Fractions highlighted using ^{19}F and ^{1}H NMR spectroscopy as containing drug-related material were further investigated using electrospray LC/MS/MS operating in data dependent MS/MS mode. Small injections enabled discernible MH^+ or $[M - H]^-$ ions for all metabolites to be obtained to provide complemen-

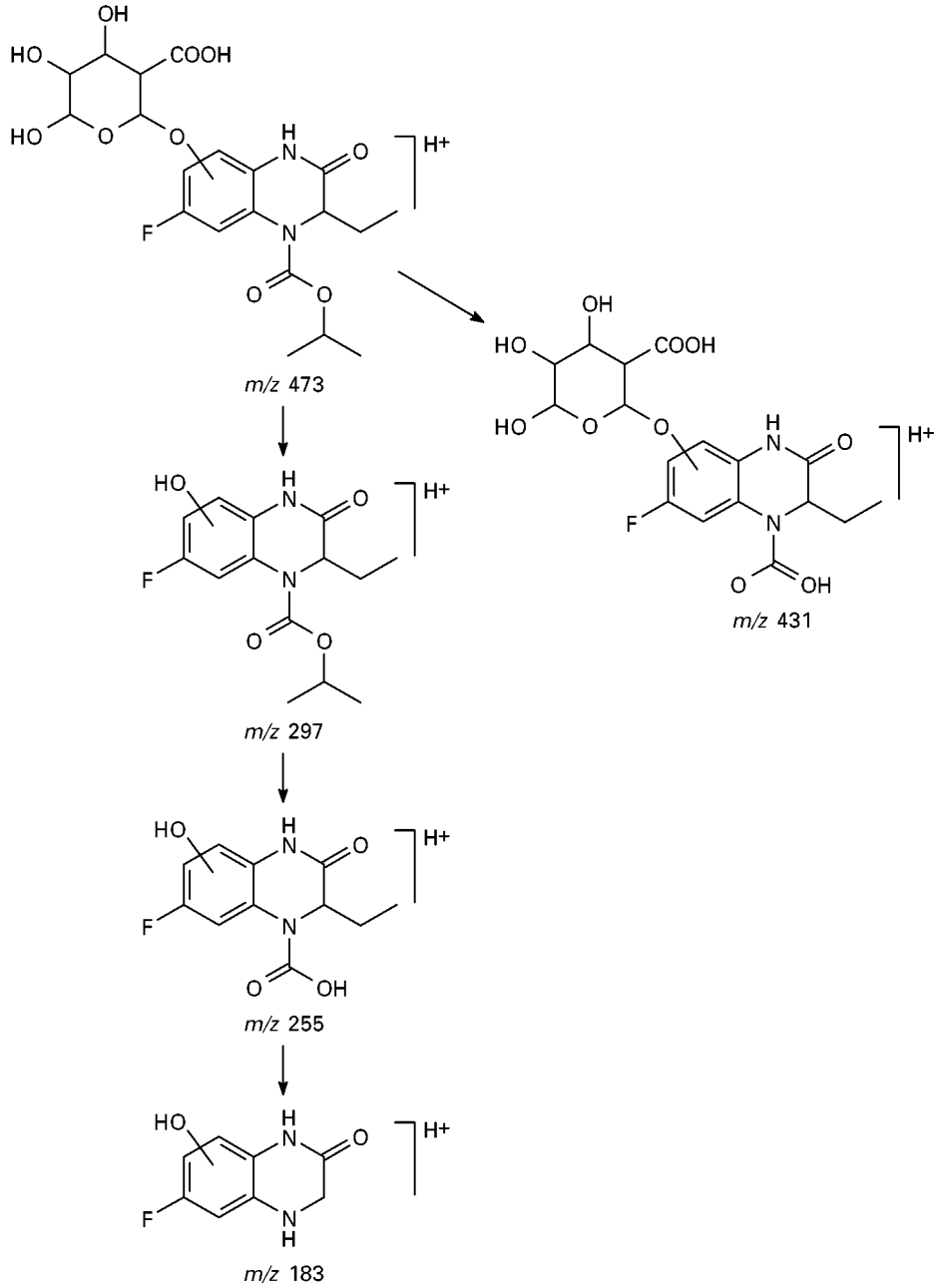

Figure 5 Rationalization of the product ions resulting from MS/MS of the isolated H-5 hydroxyl glucuronide of GW420867.

tary molecular weight and structural information to supplement. LC/MS/MS chromatograms and spectra corresponding to the NMR spectrum in Figure 3 are given in **Figure 4**. This metabolite could be rationalized as a hydroxyl glucuronide (see **Figure 5**), and therefore complemented the NMR data already acquired. In common with many glucuronide conjugates, the loss of the glucuronyl moiety in the MS/MS spectrum (-176 amu) is clearly observed. MS/MS data interpretation of all spectra was assisted by comparison with the MS/MS spectrum of an authentic standard.

The example cited above is an extremely common and representative example where the precise position of aromatic protons are established unequivocally through the use of ^{1}H NMR spectroscopy. The MS data provided added confidence by providing both molecular weight and some structural information. Thus by combining the NMR and MS data full structural elucidation is more effectively and rapidly achieved. Thus for GW4208657 using NMR and MS as described above, a total of 17 metabolites were unequivocally identified following extraction from various matrices.

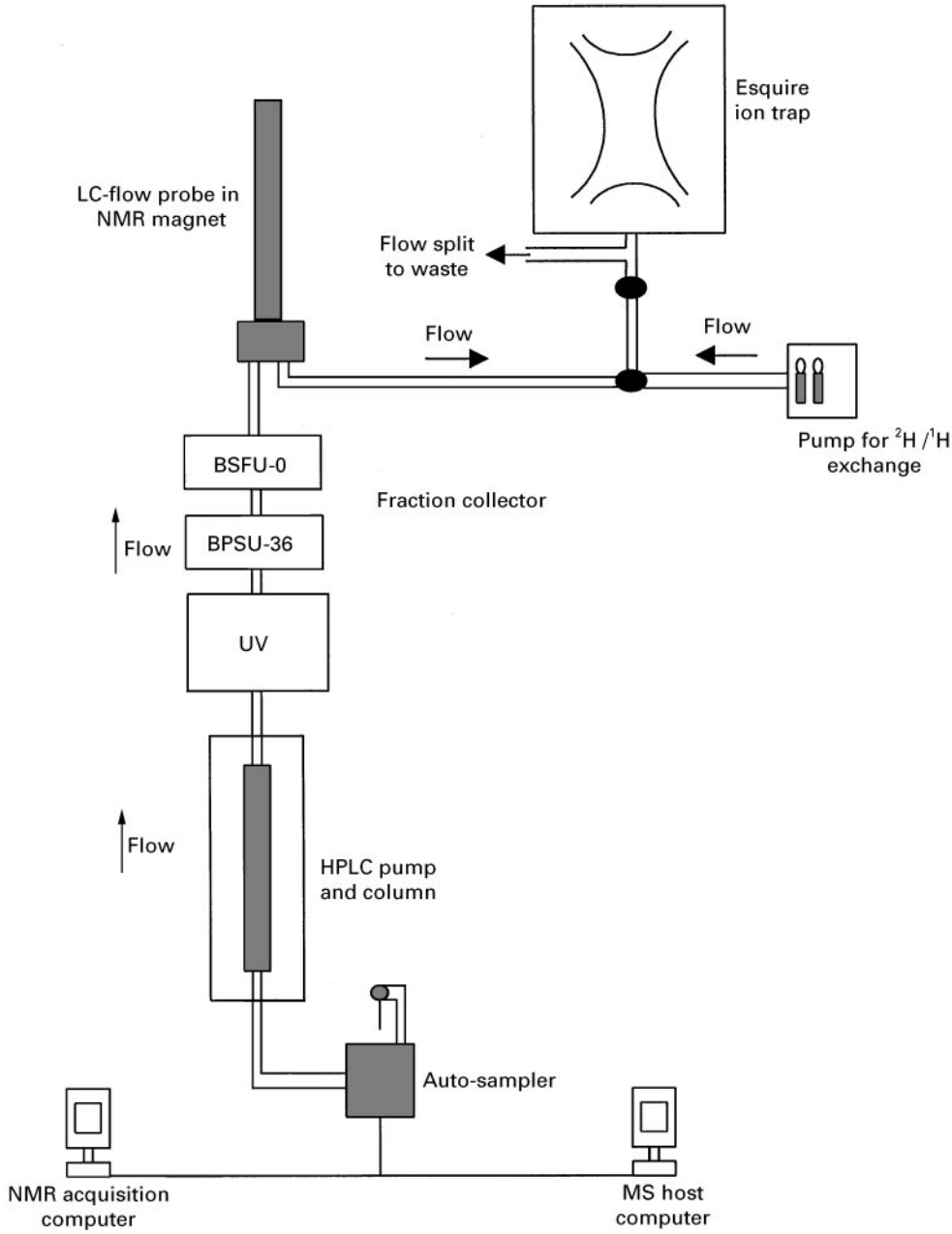

Figure 6 The LC/NMR/MS^{n} system was configured as depicted by connecting the Bruker DRX-600 NMR spectrometer and the Bruker–Esquire mass spectrometer in series. In this configuration the mass spectrometer was operated just outside the 5 Gauss line of the NMR magnet.

Further Analysis Using Directly Coupled LC/NMR/MS^{n}

In most cases metabolites isolated directly from the preparative HPLC fractions alone gave satisfactory ^{1}H NMR and MS spectra and could therefore be readily characterized without further investigation. However some fractions which had either a low concentration of metabolite or which were impure mixtures of metabolites required further separation and or longer data acquisition using LC/NMR/MS^{n}.

LC/NMR probes are generally more sensitive than standard NMR probes as the sample is concentrated into a smaller volume by the chromatographic process and the superior filling factors (this is the proximity of the NMR RF coils to the sample) obtained in LC/NMR probes.

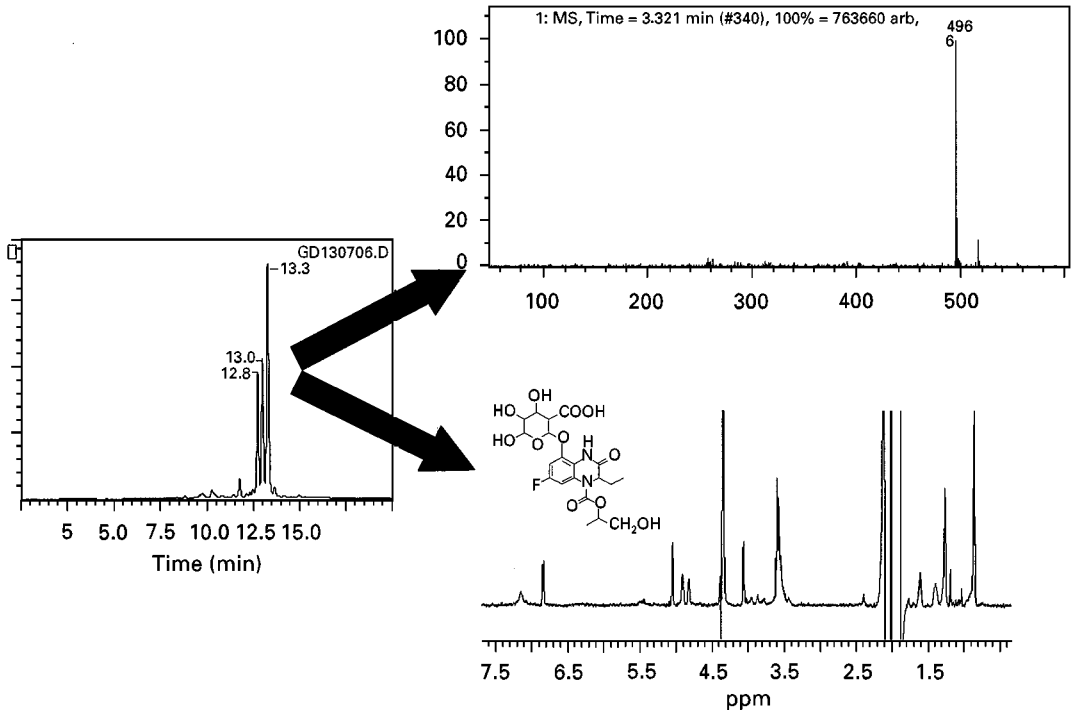

Figure 7 Chromatogram, mass spectrum and ^{1}H NMR spectrum acquired following the injection of a human urine fraction onto the LC/NMR/MS^n system illustrated in Figure 6. The peak at 13.3 min was trapped in the NMR flow probe, and a ^{1}H NMR spectrum acquired over 256 scans. The peak was then pumped into the ion trap through the electrospray source and the subsequent mass spectrum acquired in positive ion mode using deuterated solvents. Chromatography was performed on a 250 × 3.2 mm Phenomenex Magellen C18 column, eluted with a 0–80% aqueous 0.1% formic acid/acetonitrile gradient, over 35 min.

Additionally the combination of LC/NMR/MS allows an impure fraction to become further purified through the use of an analytical chromatographic system step following the initial semipreparative isolation. An example of the use of LC/NMR/MS for the identification of a novel human urine metabolite of GW420867 is given below.

For the application of LC/NMR/MS the instruments are configured in series as shown in **Figure 6**. This arrangement allows easy operation of peak storage followed by transfer to the NMR spectrometer and stop flow analysis, and it avoids difficulties with synchronization of the NMR and MS data capture; NMR data acquisition occurs first.

Following acquisition of the ^{1}H-NMR spectrum, the metabolite was transferred from the NMR probe to the ion trap mass spectrometer, where MS and MS^n spectra were obtained. The system was operated in one of two ways; in one approach, the flow from the NMR probe was split directly into the electrospray interface. In this mode, the presence of deuterium oxide in the mobile phase produced MD^+ ions in which all labile hydrogens had been exchanged with deuterium. In the second mode, an aqueous make up flow was employed to effect deuterium 'back exchange', resulting in undeuterated MH^+ ions.

An UV chromatogram acquired from an on-line LC/NMR/MS^n analytical run from a human urine fraction is shown in **Figure 7** and indicates the presence of three main peaks. The ^{1}H NMR spectrum and the resulting MS spectrum acquired for peak III, eluting at 13.3 min is also shown in Figure 7. Inspection of the ^{1}H NMR spectrum indicates that aromatic substitution has occurred in the position meta to the fluorine-bearing carbon, resulting in the loss of an aromatic proton. The remaining 2 aromatic protons at 6.84 ppm (doublet of doublets, J = 10.6 Hz and 1.47 Hz) and 7.15 ppm (broad due to restricted rotation at the amide bond) are consistent with substitution in the 5-hydroxy position.

The presence of glucuronic acid anomeric H1 proton at 5.04 ppm suggests the presence of a glucuronide. Calculation of the signal integrals in the aliphatic region at 1.26 ppm indicated the loss of one of the isopropyl methyl groups. Hydroxylation at the isopropyl group would generate a CH_2OH group whose protons will be evident in the NMR spectrum in the region of 3.5 ppm. Although these signals could not be clearly observed, as the signals from the H2–H4 of

the glucuronic acid were obscuring this region, the sum total of integral values of all the protons in this region indicated the presence of five protons. This was in support of the hypothesis of hydroxylation at the isopropyl methyl.

The MS spectrum shown in Figure 7 was acquired by directly coupling the LC/NMR probe outlet to the MS. As NMR spectra are acquired in a mixture of D2O/ACN the resulting ion at *m/z* 496 from this directly coupled arrangement is in a deuterated form (MD^+). In order to simplify matters an MS spectrum of the same peak was acquired, using the 'back exchange' configuration, which allows all the deuterium atoms to be exchanged with hydrogens. This is given in **Figure 8**. In this spectrum the MH^+ ion can be observed at *m/z* 489, which indicates that there are six exchangeable hydrogens on the original metabolite. Subsequent MS^4 experiments on *m/z* 489 were conducted as the peak eluted into the ion trap and these MS/MS spectra are also given in Figure 8. MS^n spectra enhance the interpretation of the original MS/MS spectrum and can be used to assist the elucidation of the structure and fragmentation pathway, as shown in the case of this novel human urinary metabolite. In this example, comparison of the product ions at *m/z* 183 and 255 to their equivalents formed from parent drug (*m/z* 167 and 239 respectively, data not shown), indicates aromatic hydroxylation the position of which was confirmed by the ^1H NMR spectrum. Loss of the isopropyl group to generate *m/z* 255 from *m/z* 313 suggested that the site of secondary hydroxylation was the isopropyl group, and this is supported by the NMR data. The use of an ion trap mass spectrometer providing molecular weight and structural fragmentation information, from MS and MS^n experiments respectively, when combined with NMR provides an extremely powerful structure elucidation tool.

Conclusions

The importance of combining data from different analytical techniques has been demonstrated. NMR and MS^n have been coupled with preparative and

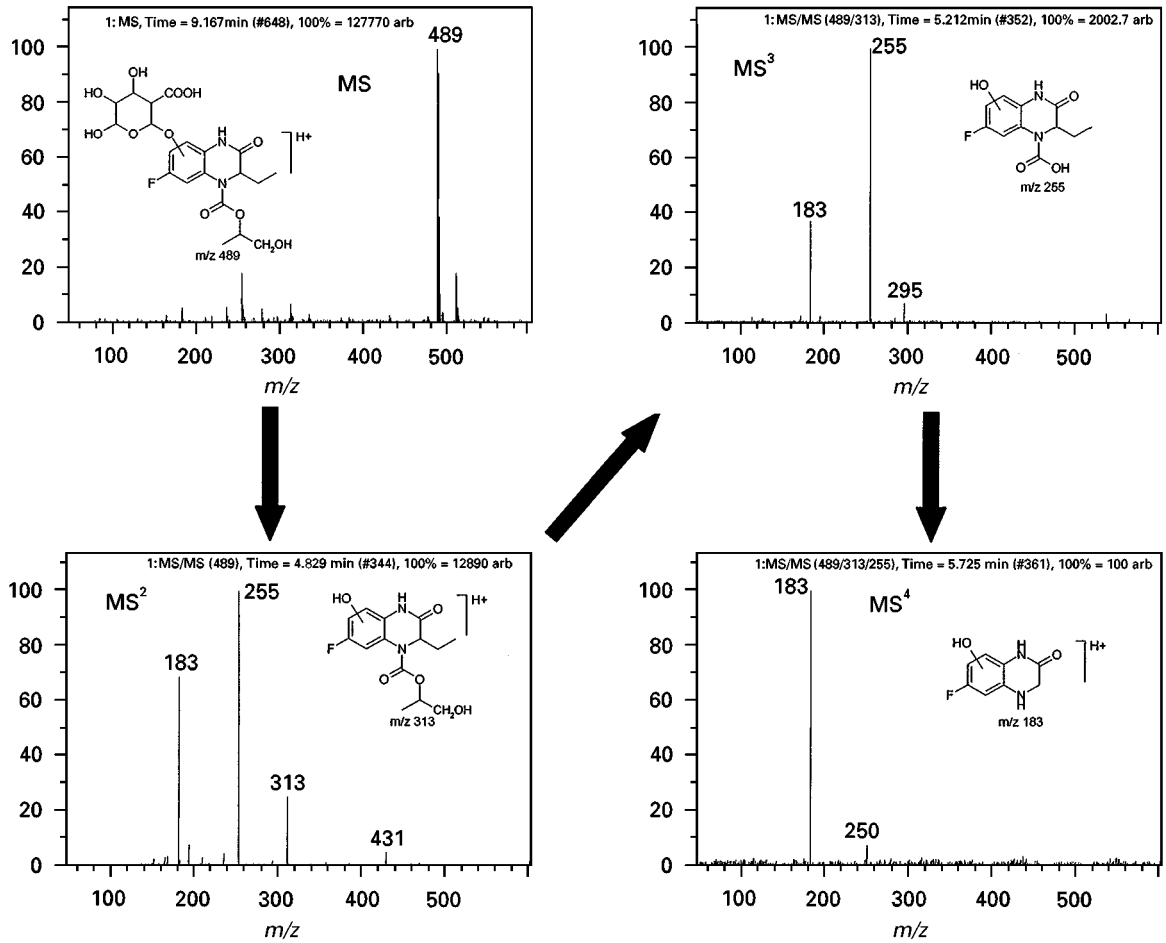

Figure 8 MS and MS^n spectra acquired following the injection of human urine fraction onto the LC/NMR/MS^n system operating in 'back-exchange' mode. Fragmentation was induced using a resonant excitation amplitude of 1.2 V, following mass isolation.

analytical HPLC to allow rapid and effective identification of metabolites in urine, following the oral administration of GW420867. The combination of these techniques enables structural and molecular weight information to be interpreted with greater efficiency and accuracy.

Future Prospects

The advantages of capillary HPLC and nanospray MS, both 'miniaturized' versions of their predecessors, HPLC and electrospray, are well documented and there is also a move towards miniaturization in most other modern analytical techniques. The enhanced sensitivity obtained by capillary LC using a capillary UV flow cell may be extended to NMR, with the development of capillary NMR flow cells. The higher efficiency separations and reduced band broadening that result from capillary LC has the effect of producing a more concentrated sample eluting from the column and therefore a higher sample concentration into the NMR flow cell. This potentially leads to a better signal to noise ratio at lower analyte concentrations in the acquisition of NMR data. A further advantage of capillary LC is the reduced solvent consumption associated with this technique, which can lead to particularly significant cost-savings when dealing with the expensive deuterated solvents used in NMR spectroscopy. Residual protonated solvent suppression in the NMR spectrum then becomes an easier task.

Capillary LC and capillary electrochromatography (CEC) coupled to mass spectrometry are already in widespread use within the pharmaceutical industry and therefore the connection of capillary LC or CEC to both NMR and MS would be a natural progression.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry; Large-Scale Liquid Chromatography; Nuclear Magnetic Resonance Detectors.

Further Reading

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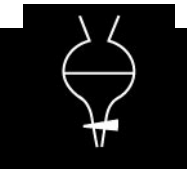
DRUGS OF ABUSE: SOLID-PHASE EXTRACTION



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Introduction

In toxicological analysis, two basic approaches can be distinguished: first, a directed search, geared to a limited number of substances, such as in workplace testing or the analysis of alcohol or special drugs in traffic offences; and second, an undirected search, also called systematic toxicological analysis (STA). STA can be defined as an undirected search for potentially harmful substances whose presence are uncertain and whose identities are unknown. STA is required if little or no information is available in so-called general unknown cases. However, if one toxicant is known, the analyst is required to establish whether other compounds of toxicological relevance are present.

The drug screening process can generally be divided into two stages, sample preparation and analysis of the drugs. Some forms of sample work-up – isolation and concentration – are required for most analytical techniques, such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) coupled to various detector systems. The samples available for analysis are complex biological matrices, in which toxicologically relevant substances are present in trace amounts compared to the endogenous compounds present. Therefore, work-up procedures should retain all relevant substances, at the same time removing all irrelevant substances and interferences.

Liquid–liquid extraction (LLE), often combined with sample pretreatment procedures such as conjugate hydrolysis, digestion or protein removal, was the standard method in the past. Although LLE proved to be suitable in a great number of cases, there are many disadvantages of this technique, e.g. matrix interferences, emulsion formation or the use of large