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On-line liquid chromatography coupled with high field NMR and mass spectrometry (LC-NMR-MS): a new technique for drug metabolite structure elucidation

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Abstract

High performance liquid chromatography has been coupled simultaneously to high field NMR and MS detectors, giving UV, NMR and mass spectra for each component in a mixture, after on-line separation. This powerful new tool for the structure elucidation of components in mixtures without isolation has been successfully applied to the analysis of the metabolites of paracetamol in human urine. © 1997 Elsevier Science B.V.

Keywords: Chromatography; Mass spectrometry; NMR; Human urine; Paracetamol

1. Introduction

NMR Spectroscopy is a powerful method for the analysis of endogenous and exogenous components in biological fluids [1]. The main advantages of NMR spectroscopy for the analysis of biofluids are that; (i) NMR is a non-selective detector, monitoring the levels of all low molecular weight components in free solution above the detection threshold, (ii) the technique is fast and requires only low sample volumes, (iii) the information content of NMR spectra is very high, with, for instance, hundreds of human urine components being detected in ^{1}H NMR spectra of human urine, and most importantly, (iv) NMR spectra are inherently rich in structural information on each one of the components that are detected.

For the structure elucidation of novel components in matrices as complex as human urine, it is extremely advantageous to couple a separation technology to the NMR, as has been achieved by LC-NMR [2,3]. Improvements in NMR sensitivity and solvent suppression efficiency have made LC-NMR a viable and important tool for the identification of components in complex mixtures [2,3]. The recent introduction of suppression methods which can be tailored to the width of the solvent peak allows the observation of signals close to the solvent, on-flow [4,5]. However, to solve the molecular structure of a novel substance by NMR spectroscopy alone is often impossible. LC-MS is a well established technology for determining the molecular weights of components in

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mixtures with excellent sensitivity but, as the ionisation process used for most LC-MS applications is relatively 'soft', little structural information is obtained [6]. Coupled, on-line LC-NMR-MS is the logical combination of these two powerful and complementary analytical techniques. We have recently applied this technique to simple chemical mixtures [7] and have extended its application to the analysis of biological fluids. This technology can be implemented using commercially available equipment, paying particular attention to operation of the chromatograph and mass spectrometer within the stray field of the NMR magnet. The ionisation characteristics of biomolecules differ sufficiently that the mass spectrometer used in the system should be equipped to perform the full range of ionisation modes typically used in metabolism studies. Using LC-NMR-MS we can obtain UV, NMR and mass spectral data from components in mixtures as they elute from an HPLC in a single chromatographic run. This avoids the potential problem of mis-interpreting data from chromatographic components which change their elution order with small variations in HPLC conditions. The LC-NMR-MS technique gives far more structure elucidation power than available previously to solve the structures of components in complex mixtures rapidly and efficiently. In this paper we demonstrate the effectiveness of this new technology by its application to the analysis of the structures of the metabolites of the analgesic paracetamol (1) in human urine [3].

2. Experimental

2.1. Sample

Human urine was obtained from a healthy male volunteer 1.75–4.75 h after taking 1 g of paracetamol (1). A portion (10 ml) of the sample was freeze-dried and reconstituted in 1.5 ml deuterium oxide before chromatographic separation.

2.2. Apparatus

The equipment used for these experiments consisted of a Varian UNITY plus 500 NMR spectrometer (Varian NMR Instruments, 3120 Hansen Way, Palo Alto, CA, 94304-1030, USA) fitted with a prototype Varian ^{1}H LC-NMR probehead, a Finnigan Navigator mass spectrometer (Finnigan Masslab, Crewe Road, Wythenshawe, Manchester, UK (formerly Fisons Instruments)) fitted with an electrospray interface switchable for positive or negative ion detection, and an HP 1050 HPLC system (Hewlett Packard, Cain Rd., Bracknell, Berkshire, UK). The three instruments were linked to ensure that NMR and MS data acquisition started in synchronisation with the injection from the autosampler on the HPLC system. The flow from the HPLC was split 50:1 (NMR:MS) post-column using an Acurate splitter (Presearch, 13 Business Centre West, Avenue One, Letchworth Garden City, UK). All connections were made using 0.125 mm i.d. PEEK tubing and zero dead volume couplings (Upchurch Scientific, Oak Harbor, Washington, USA). The respective lengths of PEEK tubing post splitter were adjusted so that each component from the LC run arrived at the mass spectrometer 30 s before arriving in the NMR flow cell. All the retention times quoted refer to the mass spectrometry data.

2.3. HP 1050

Of the solution 25 μl was injected using an autosampler. The mobile phase was composed of CH_3CN (HPLC grade, Fisons, Loughborough, solvent A) and D_2O containing 0.1% v/v trifluoroacetic acid-d (Aldrich, Milwaukee, Wisconsin, USA, solvent B). Gradient elution was employed starting at 1% A increasing linearly to 35% A, over 50 min, flowing at 0.5 ml min^{-1}. The HPLC column was a 5 μm Spherisorb ODS-II 4.6 mm × 25 cm (Phase Sep, Deeside Ind. Park, Queensferry, Clwyd, UK). Peaks were detected by UV at 210 nm.

2.4. Varian UNITY plus 500

The LC-NMR probehead contained a flow cell with an active volume of 60 μl and was fitted with an actively shielded z-axis pulsed field gradient coil capable of generating gradients of 32 G cm^{-1}.

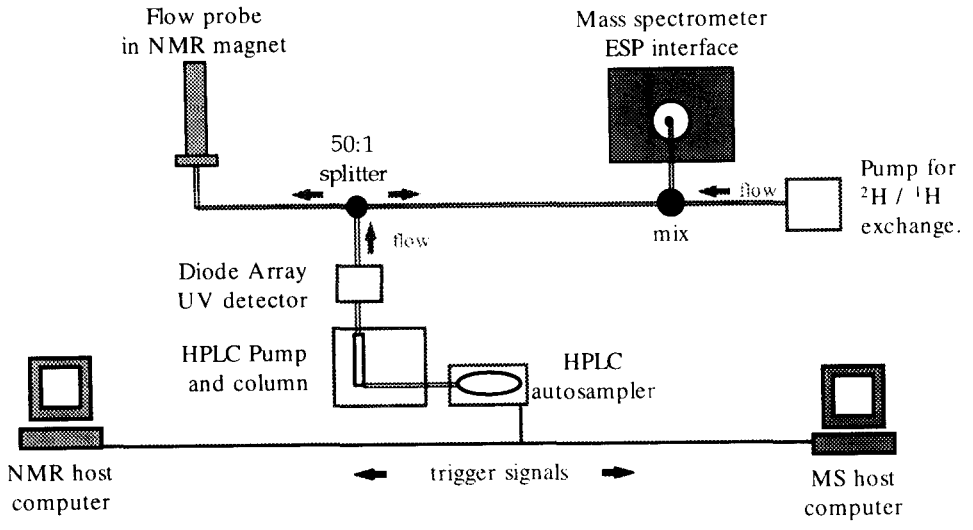


Fig. 1. Schematic diagram of the LC-NMR-MS system.

The ^2H resonance of the D_2O was used for a field-frequency lock and the spectrum was centred on the acetonitrile methyl resonance. Solvent suppression was obtained using a modified T_1- and B_1-optimised wet pulse train [5]. 500 MHz ^1H NMR spectra were recorded in blocks of 16 transients with a total recycle time of 1 s per transient. The NMR data were recorded and processed using Vnmr version 5.1 (Varian NMR Instruments, 3120 Hansen Way, Palo Alto, CA, 94304-1030, USA).

2.5. Finnigan navigator

A mass range of 100–500 Da was scanned in 1 s using nebulised electrospray. The needle voltage was 4.0 kV and the nebulising gas was nitrogen at 80 psi pressure. Data was obtained in both positive and negative ion modes, switching between scans. The data were acquired using Fisons Mass Lynx version 2.2 (Fisons Instruments, Tudor Road, Wythenshawe, Manchester, UK). The make up flow for the deuterium 'back exchange' was provided by an LDC Constametric 3500 LC pump (LDC Instruments, Riviara Beach, Florida, USA) at a flow rate of 200 μl min^{-1}. The solvent system used was 1% aqueous acetic acid (Fisons, Loughborough, UK): methanol (Fisons, Loughborough, UK) 50:50 v/v.

3. Results

Fig. 1 shows a schematic representation of the equipment used to record the LC-NMR-MS data. We chose to connect the spectrometers in parallel after the UV detector. The splitter ensured that a high proportion (98%) of the eluent was directed to the NMR spectrometer. With this arrangement it was possible to collect the eluent from the NMR probe for further study, if required. The relative sensitivities of electrospray mass spectrometry and NMR are ideal for parallel connection. Alternative configurations including connecting the mass spectrometer serially to the outlet of the NMR probe could cause problems by (i) generating excessive back-pressure causing damage to the NMR probe and (ii) potentially degrading the effective chromatographic separation at the mass spectrometer by band broadening due to the relatively large volume of the NMR flow-cell. The start of acquisition of the NMR and MS data was triggered simultaneously from the HPLC autosampler when the sample was injected onto the column. The 'on-flow' NMR chromatogram is shown in Fig. 2. The change in the relative positions of the acetonitrile and HOD resonances with time reflects the gradient of solvent composition during the LC run. Although

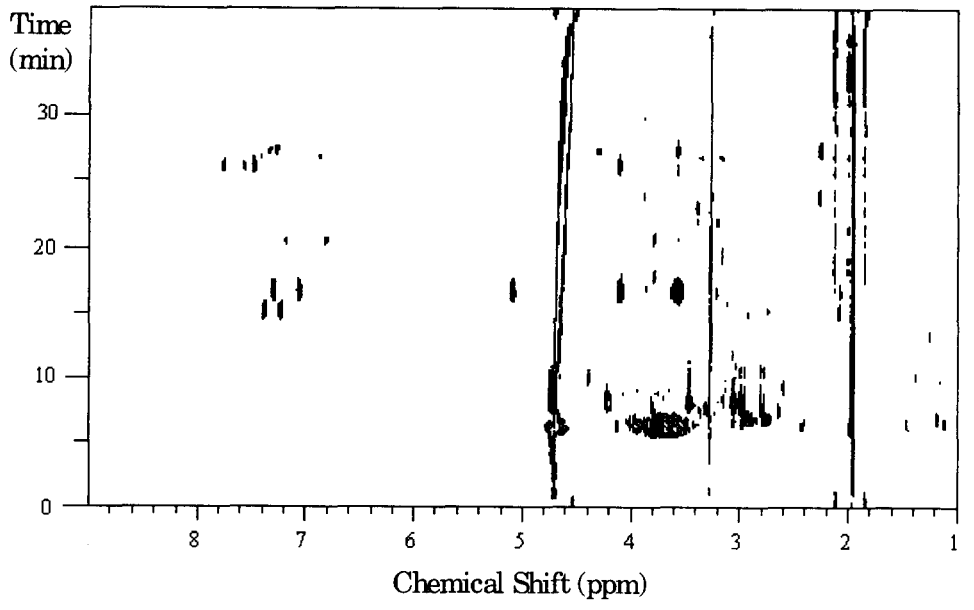


Fig. 2. The 'on-flow' NMR chromatogram: x-axis, chemical shift (ppm), y-axis, time (mins). The NMR data lags behind the mass spectrometry data by 30 s.

the spectrometer was locked onto the D_2O resonance, the centre of the spectrum was automatically maintained at the chemical shift of the acetonitrile by periodic re-referencing. This ensured that the chemical shifts of eluted compounds were insensitive to solvent composition. The frequency difference between the two solvent resonances was also checked periodically and used to update the doubly frequency selective pulses used for the solvent suppression [5]. Phase consistency at the two excitation frequencies was obtained using phase modulation of the selective pulses [8]. This ensured that baseline disturbances were minimised. A resonance is observed at 3.3 ppm in each ^1H NMR spectrum. This is due to the presence of a trace level of methanol in the acetonitrile LC solvent. The corresponding UV trace and total ion mass chromatogram (TIC) in positive ion mode are shown in Fig. 3a and b, respectively.

Using this LC-NMR-MS technique we have been able to detect 'on-flow' paracetamol and some of its metabolites, as well as a number of endogenous metabolites from human urine (see Scheme 1). Endogenous metabolites, including sugars, citrate and lactate can clearly be seen eluting in the first few minutes of the LC run. Fig. 4a shows the positive ion mass spectrum of the component at a retention time of 16.1 min. The mass spectrum shows a pseudomolecular ion (MD +) at m/z 334 consistent with paracetamol glucuronide (2). A second experiment was carried out, adding methanol:acetic acid to the mass spectrometer flow post splitter. This enabled all the deuterons to be exchanged for hydrogens. The mass spectra produced during this experiment showed MH^+ ions for all the components and, by comparison of the two experiments, the number of exchangeable hydrogens on each component could easily be determined from the mass differences. The MH^+ ion for the glucuronide appeared at m/z 328 and by mass difference from the first experiment it can be seen that there were five exchangeable hydrogens present on the molecule (Fig. 4b). The ^1H NMR spectrum (Fig. 4c) clearly shows the para-disubstituted benzene ring hydrogens at δ_H 7.3 and 7.05, and the aliphatic hydrogens from the sugar ring at δ_H ~3.6, 4.1 and 5.1. The magnitude of the H1′–H2′ coupling constant (ca. 7 Hz) confirms the β stere-

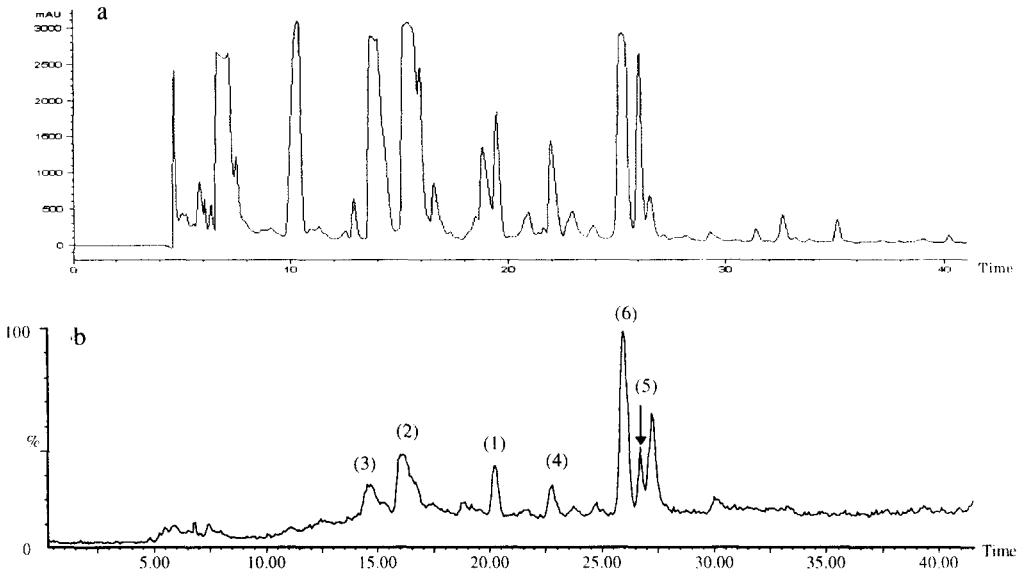


Fig. 3. (a) The UV trace (at 210 nm). (b) The mass spectrometry Total Ion Chromatogram (TIC).

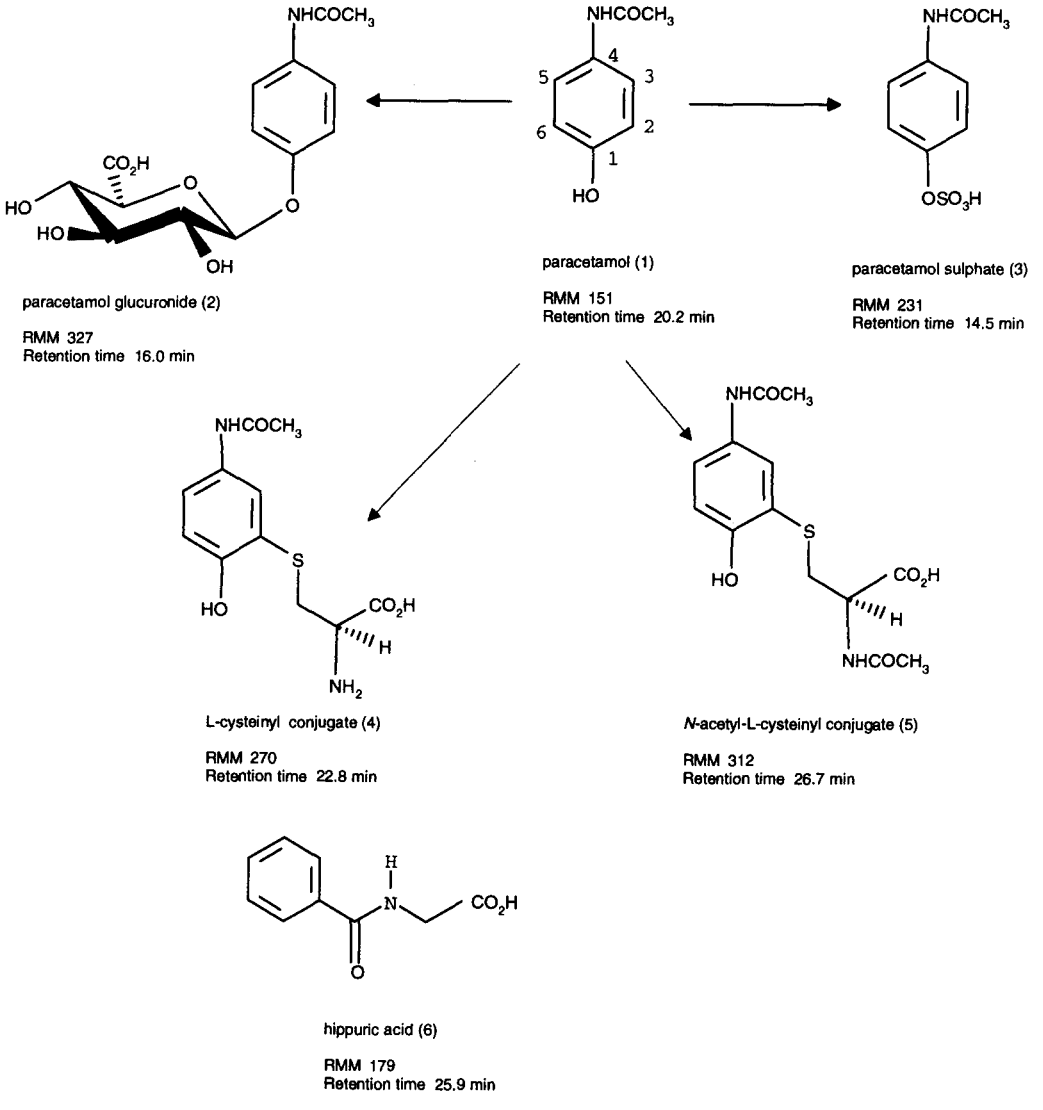
ochemistry at the anomeric centre. The acetyl methyl resonance of the paracetamol glucuronide can be clearly seen inside the ^{13}C satellites of the acetonitrile, albeit with reduced intensity, highlighting the selective nature of the ^{1}H NMR solvent suppression.

Fig. 5 shows the mass spectra and ^{1}H NMR for the peak, eluting at a retention time of 14.5 min. The mass spectrum for the fully deuterated form shows a pseudomolecular ion (MD^+) at m/z 235 (Fig. 5a), and for the protonated form (MH^+) at m/z 232 (Fig. 6b). This is consistent with the addition of an $-SO_3$ moiety to paracetamol,but does not reveal where it is attached. The NMR data (Fig. 5c) show characteristics resonances for the para-disubstituted benzene ring and the acetyl methyl group. The chemical shifts of the aromatic hydrogens show that the additional $-SO_3$ moiety must be attached to the ring oxygen, and not to the nitrogen or oxygen of the amide group. This information, which unambiguously identifies the metabolite as paracetamol sulphate (3), could not have been obtained from either technique alone.

Hippuric acid (6) gives one of the most intense UV peaks in the LC run and is identifiable using the combination of mass spectrometry and ^{1}H NMR at 25.9 mins (Fig. 6a–c). The MD^+ is observed at m/z 183 and the MH^+ at m/z 180, and ^{1}H NMR shows the presence of the mono-substituted benzene ring and the methylene adjacent to the acid moiety. Other components that were observed in this work are listed in Scheme 1.

4. Discussion

The experiments described here serve to illustrate the power of LC-NMR-MS in providing rapid information on the structure of drug metabolites. Obtaining UV, MS, and NMR data at the same time provides unambiguous structural information which is not available from any other combined technique. The ability to identify the number of exchangeable hydrogens by mass spectrometry provides the NMR spectroscopist with essential information to aid the analysis of the NMR data. The relatively high cost of this combined technique can be offset against the efficiency of this approach when compared to the laborious extraction of metabolites from body fluids followed by MS or NMR analysis, or the synthesis and use of radiolabelled drug.



Scheme 1.

The LC connections to both the NMR and MS were kept to a minimum and no degradation in chromatographic resolution was observed. The operation of the Navigator mass spectrometer within the influence of the NMR magnetic field did not have any significant adverse affect on the sensitivity or resolution of either instrument. This contrasts with our experience of using a Fisons Trio-1000 mass spectrometer, previously reported [7]. We believe this improvement is due to the use of RF lenses in this instrument, in contrast to the conventional electrostatic lenses used in the Trio-1000. All the work described here was acquired 'on-flow'; the use of stop-flow acquisition would improve the detection level of the NMR spectrometer and we are investigating ways in which the mass spectrometer can be used to trigger stop-flow experiments.

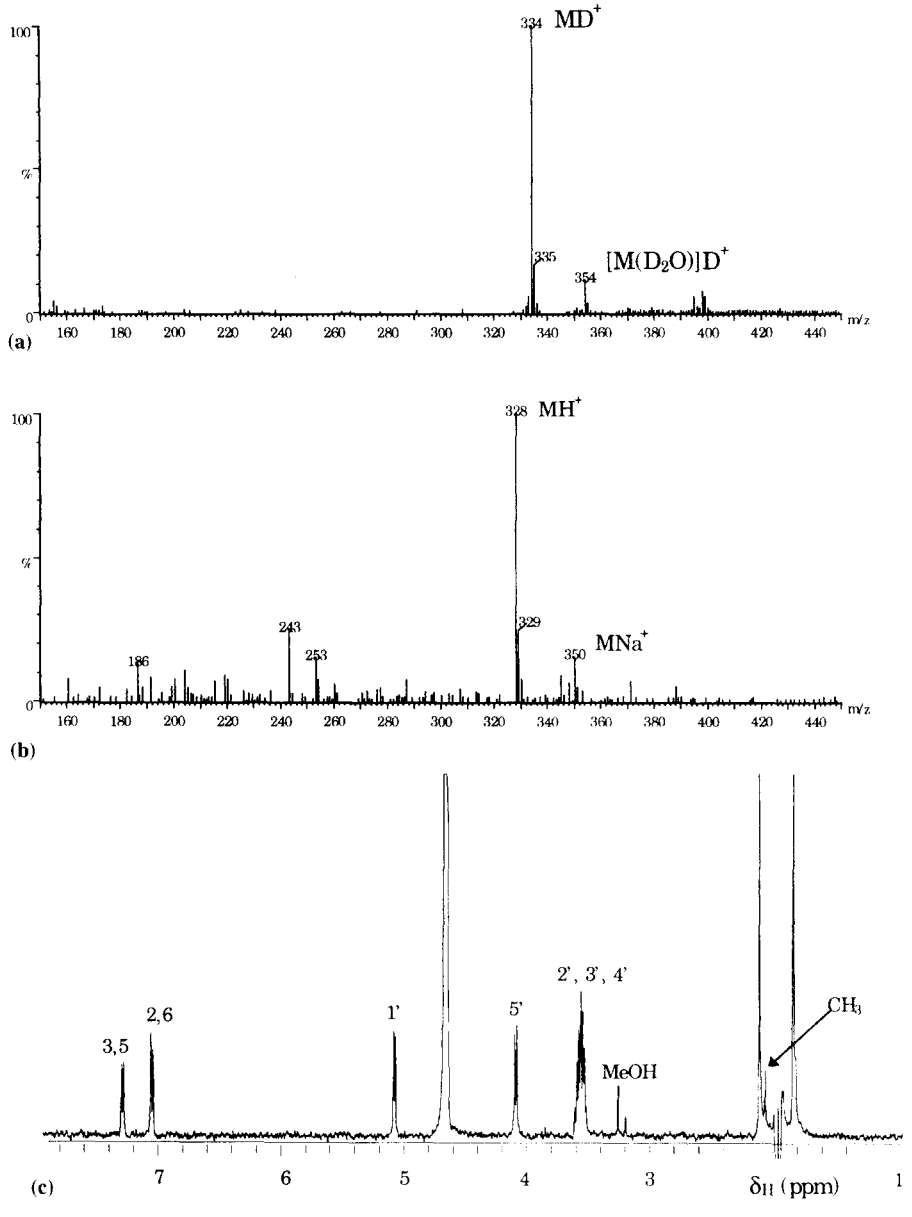


Fig. 4. 'On flow' spectra of paracetamol glucuronide (2): (a) deuterated mass spectrum, (b) mass spectrum after D/H exchange, (c) ^{1}H NMR spectrum (note: the two large singlets at ca. $\delta_H 2.0$ are the ^{13}C satellites of the CH_3CN solvent and the large signal at ca. $\delta_H 4.7$ is due to residual HOD).

We have demonstrated the viability and usefulness of LC-NMR-MS as a powerful tool for obtaining structural information on metabolites from complex biological matrices in a single experiment. This approach to structure elucidation represents a significant advance over existing technology and we predict that it will be increasingly used in the future.

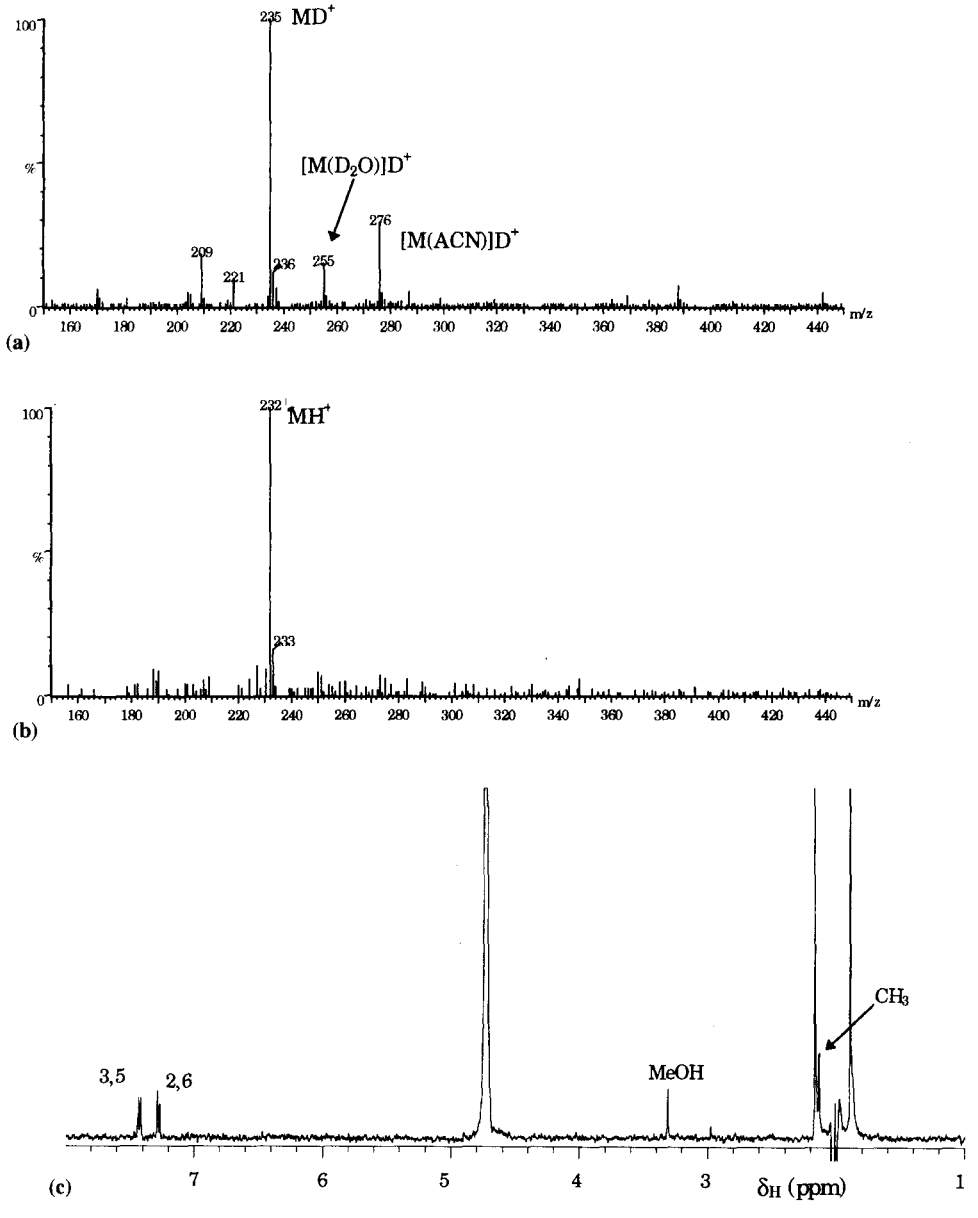


Fig. 5. 'On flow' spectra of paracetamol sulphate (3): (a) deuterated mass spectrum, (b) mass spectrum after D/H exchange, (c) ^{1}H NMR spectrum.

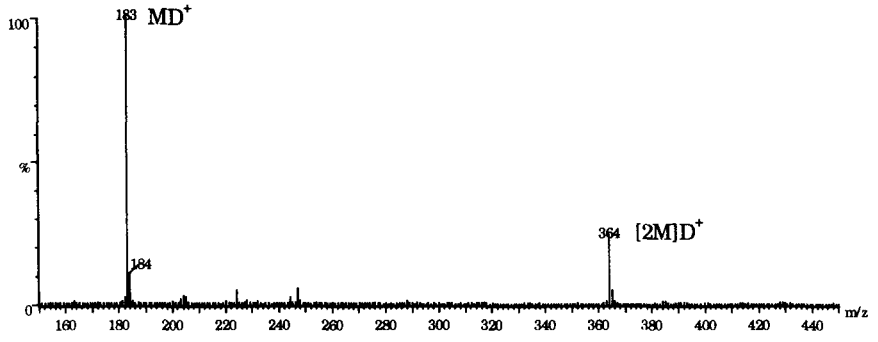


Figure 6a

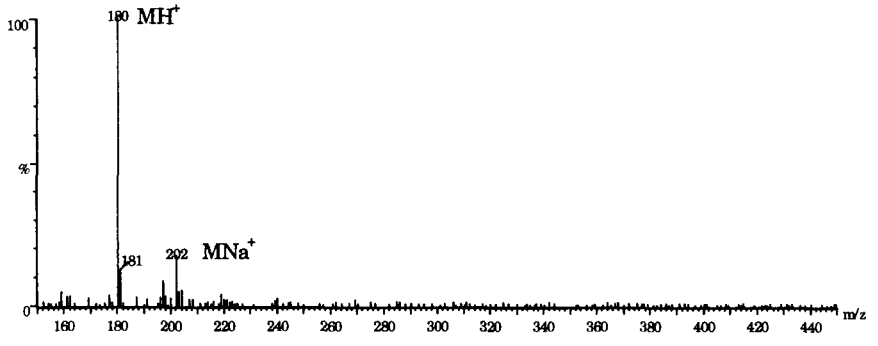


Figure 6b

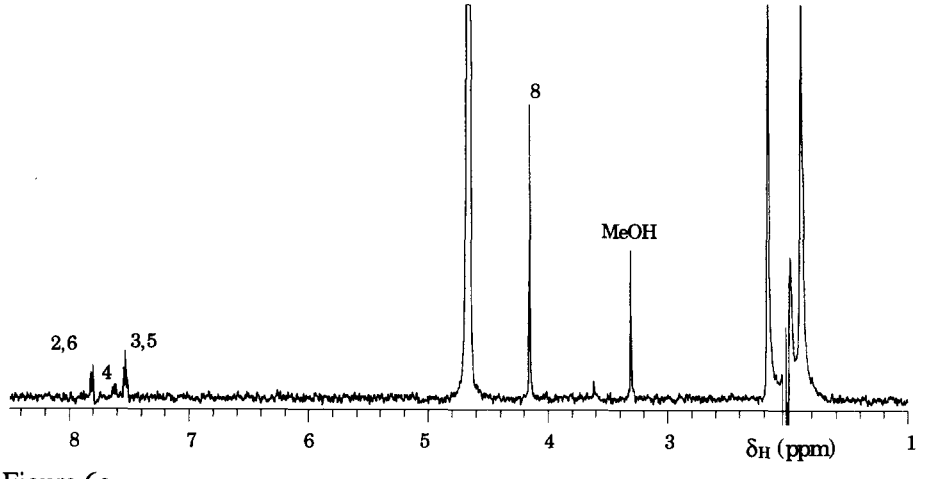


Figure 6c

Fig. 6. 'On flow' spectra of hippuric acid (6): (a) deuterated mass spectrum, (b) mass spectrum after D/H exchange, (c) ^{1}H NMR spectrum.

Acknowledgements

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