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High-performance Liquid Chromatography/NMR Spectrometry/Mass Spectrometry: Further Advances in Hyphenated Technology

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The earlier use of combined liquid chromatography/NMR spectrometry/mass spectrometry (LC/NMR/MS) involved the use of a particle beam interface. This paper describes further developments of this hyphenated technology, in particular the incorporation of an electrospray interface into the LC/NMR/MS system. This improved LC/NMR/MS system was designed for the support of a combinatorial library program. The power of this technique is demonstrated in the successful structural elucidation of each compound in a mixture of commercially available peptides.

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INTRODUCTION

The ability to perform spectroscopic analysis of complex mixtures without prior separation into individual components is often needed by chemists and biochemists. The use of liquid chromatography/mass spectrometry (LC/MS) for these analyses can now be considered a standard analytical tool. A number of LC/MS interfaces are available to cope with a variety of compound types and a wide range of LC solvent systems.[1,2] Most LC/MS interfaces involve the use of soft ionization techniques. Consequently, the mass spectral data usually provide good molecular mass information but limited structural information, as little fragmentation occurs.

NMR spectroscopic data provide structural and stereochemical information. However, mixtures normally have to be separated prior to analysis in order to acquire NMR spectra on the individual components. Recent advances in NMR probe design have permitted the use of ‘on-line’ LC/NMR to study complex mixtures. This, combined with the progress made with solvent suppression techniques, has allowed the use of protonated solvent systems.[3–6] Hence the technique is applicable to a wide range of analytical problems[7,8] and is now used routinely in some laboratories.

A logical next step is to combine LC/MS with LC/NMR to provide both structural and molecular mass information, enabling molecular structures to be determined with greater ease and certainty. The first experiments in our laboratories[9] demonstrated the feasibility of this technique. The practical issues about linking NMR and MS to an HPLC system and operating the mass spectrometer in close proximity to the NMR’s magnetic field are reported here.

The use of combinatorial chemistry to produce libraries of compounds is becoming of increasing importance in the drug discovery process.[10] Screen hits need to be analysed to identify the structure of the individual active component. Large numbers of samples containing only small quantities of complex mixtures require analysis and so the ability to obtain the maximum amount of information from a single analytical run is very important. In the work reported here we investigated the potential of the LC/NMR/MS technique for the analysis of combinatorial chemistry mixtures by using a mixture of commercially available peptides.

EXPERIMENTAL

The equipment used for these experiments consisted of a Varian Unity*plus* 500 MHz NMR spectrometer (Varian NMR Instruments, Palo Alto, CA, USA) fitted with a prototype Varian LC/NMR probe head, a Trio 1000 mass spectrometer (Fisons Instruments, Manchester, UK) with a 2000 mass range analyser and an HP1050 HPLC system (Hewlett–Packard, Bracknell, UK) with a 100 vial autosampler and multi-wavelength UV detector. The flow from the HPLC system was split using an IC-20 splitter (Presearch, Letchworth, UK). All connections were made using 0.25 mm i.d. PEEK tubing and Upchurch zero dead volume couplings (Anachem, Luton, UK). The three systems were linked so that a simple contact start from the HPLC injector initiated both the NMR and MS acquisitions. A schematic diagram of the instrument configuration is shown in Fig. 1.

In order to investigate the effect of the magnetic field on the mass spectrometer performance, the system was

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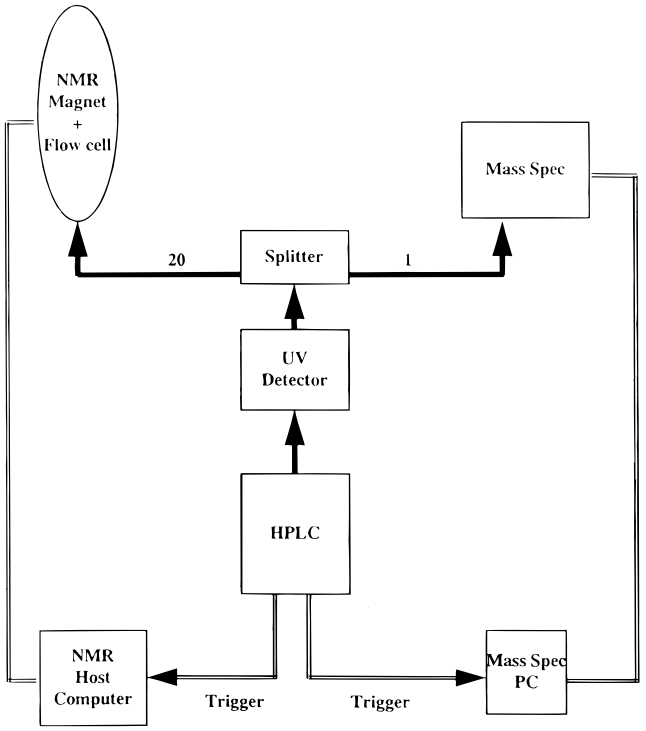

Figure 1. Schematic diagram showing the configuration of the instruments.

set up on a laboratory trolley outside the NMR's magnetic field. The electron impact (EI) source was installed in the mass spectrometer without an LC interface, and the system was tuned and calibrated using the internal reference, heptacosa (perfluorotributylamine). The magnetic field was measured using a Gaussmeter 100 (Redcliffe Magtronics, Bristol, UK). The mass spectrometer was moved into the magnetic field, retuned and calibrated. The effect of proximity and orientation to the magnet on the ion beam was investigated by adjusting the position of the trolley. The optimum position for the mass spectrometer was found to be approximately 2 m from the magnet centre with the quadrupole perpendicular to the field gradient. At this position the magnetic field was 5 G.

Tuning the mass spectrometer within the NMR's magnetic field presented some difficulties. Although the source tuning characteristics appeared to be unchanged, the quadrupole performance was different. The resolution was much greater than normal,[9] requiring a reduction of the low mas resolution parameter and an increase in the ion energy. The resultant mass spectrometer performance was ~100-fold less sensitive than normal. With a comparable decrease in the level of noise, however, this proved not to be a problem and the sensitivity was adequate to obtain good MS data.

The HPLC system was set up next to the mass spectrometer and the eluent from the UV detector was split 20:1 (NMR:MS). Samples were injected using the autosampler and a contact closure was used to trigger both the NMR and MS acquisitions.

The sample was prepared from a dry mixture of nine peptides (Sigma Chemical, Poole, Dorset, UK) dissolved in the mobile phase under the initial gradient conditions and at a concentration of 1 mg ml^{-1} for each component. The peptides are listed in Table 1. The HPLC column used was an Inertsil ODS 2, 5 cm × 4.6 mm i.d. (Hichrom, Reading, UK). The mobile phase consisted of CH_3CN and 0.1% trifluoroacetic acid (TFA) in D_2O (99.9% D) (Aldrich, Milwaukee, WI, USA). A linear gradient of 5–50% CH_3CN over 50 min at a flow rate of 0.5 ml min^{-1} and an injection volume of 100 µl were used. The CH_3CN and TFA were both of HPLC grade (Fisons, Loughborough, UK). The UV absorbance was monitored at 220 nm.

The LC/NMR probe head contained a flow cell with an active volume of 60 µl and was fitted with an actively shielded pulsed-field gradient coil. The ^2H resonance of the D_2O was used for the field frequency lock and the spectrum was centred on the acetonitrile methyl resonance. The probe was tuned and the 90° pulse calibrated at the initial HPLC solvent gradient conditions. The tuning was also checked at the final gradient conditions and found not to be significantly different. The ^1H NMR spectra were recorded in blocks of 16 transients with a total recycle time of 1 s per transient. The CH_3 and HOD resonances from the solvents were suppressed using a train of four selective WET[11] pulses each followed by a B_0 gradient pulse. The suppression sequence was followed with a composite 90° read pulse. The data were acquired using Vnmr software version 5.1 (Varian NMR Instruments).

The electrospray interface was used in the positive ionization mode. A mass range of 50–750 Da was scanned in 2 s. A source temperature of 250 °C, a needle temperature of 120 °C and a needle voltage of 3000 kV were used. Nitrogen (high purity) (Air Products,

Table 1. Molecular masses of peptides and their deuterated analogues and the expected deuterated molecular ion

Peptide	^1H M_r (Da)	No. of exchangeables	^2H M_r (Da)	$^2H^+$ molecular ion expected (Da)
Gly-Gly-His	269	6	275	277
Pro-Phe-Gly-Lys	447	7	454	456
Trp-Gly	261	5	266	268
Thr-Val-Leu	331	6	337	339
Val-Ala-Ala-Phe	406	6	412	414
Phe-Gly-Gly-Phe	426	6	432	434
Lys-Phe-Ile-Gly-Leu-Met-NH_2	706	11	717	719
N-tBOC-Glu-Glu-Leu-methyl ester	503	5	508	510
p-Glu-Gly-Arg-Phe-amide	488	10	498	500

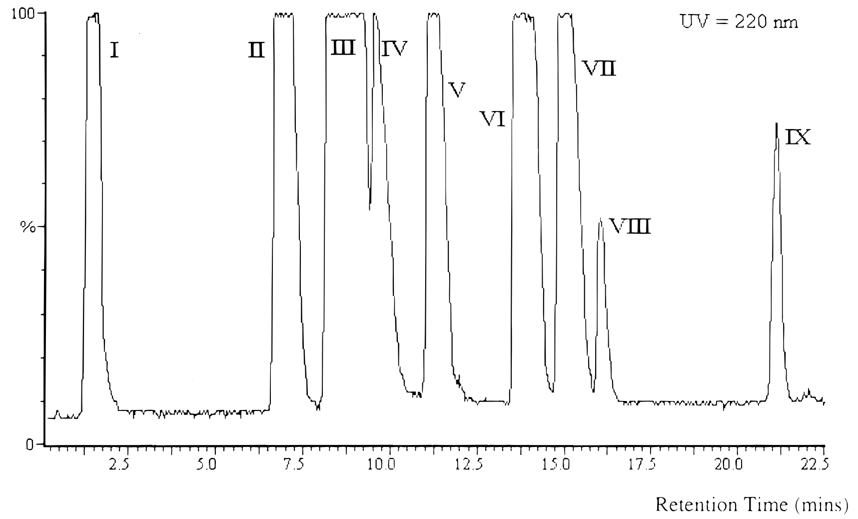

Figure 2. UV chromatogram recorded at 220 nm showing nine peptide peaks.

Walton-on-Thames, UK) was used for both the co-axial flow and the bath gas. The data were acquired using LabBase software version 2.14 (Fisons Instruments).

RESULTS AND DISCUSSION

The UV chromatogram recorded at 220 nm (Fig. 2) shows nine peaks. Two of the peptides co-elute as peak III and another of the peptides gives rise to two peaks (VII and VIII) as both the amide and the acid forms are seen. We were able to identify all nine peptides by both NMR and MS.

Owing to the use of D_2O in the mobile phase (in order to acquire the NMR data), 'normal' MH^+ ions were not expected to be observed in the mass spectra. All of the NH and OH hydrogens in the peptides exchanged with deuterium, and the ions observed in the mass spectrometer were deuterated rather than protonated molecular ions. Table 1 shows the number of exchangeable hydrogens and the expected m/z for the deuterated molecular ion of each of the peptides.

The TIC and BPI traces were too weak for the individual components to be detected. Mass spectra were examined at retention times estimated from the UV chromatogram. The delay between the UV detector and both the mass spectrometer and the NMR flow cell was 0.5 min (Fig. 1). Unambiguous mass spectra for nine of the components in the mixture were observed, allowing the relative molecular mass (M_r) for the deuterium-exchanged molecule to be determined. The non-deuterated M_r can be obtained from Table 1. The M_r information allowed assignments of the chromatographic peaks to be made; these are given in Table 2.

The ^1H NMR data (Fig. 3) shows the chemical shift along the abscissa and time on the ordinate. The separate components can be clearly seen in this pseudo-two-dimensional NMR plot. Spectra of the individual

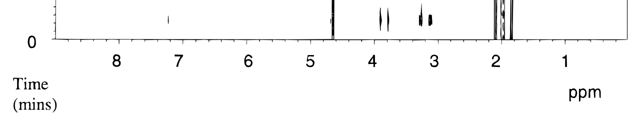

Figure 3. Pseudo-two-dimensional ^1H NMR plot showing chemical shift along the abscissa and retention time along the ordinate.

Table 2. Assignment of the nine chromatographic peaks

Peak No.	Observed $[M + D]^+$	Expected $[M + D]^+$	Peptide
I	277	277	Gly-Gly-His
II	456	456	Pro-Phe-Gly-Lys
III	268	268	Trp-Gly
	500	500	p-Glu-Gly-Arg-Phe-amide
IV	339	339	Thr-Val-Leu
V	414	414	Val-Ala-Ala-Phe
VI	434	434	Phe-Gly-Gly-Phe
VII	719	719	Lys-Phe-Ile-Gly-Leu-Met-NH_2
VIII	719	719	Lys-Phe-Ile-Gly-Leu-Met-OH
IX	510	510	N-tBOC-Glu-Glu-Leu-methyl ester

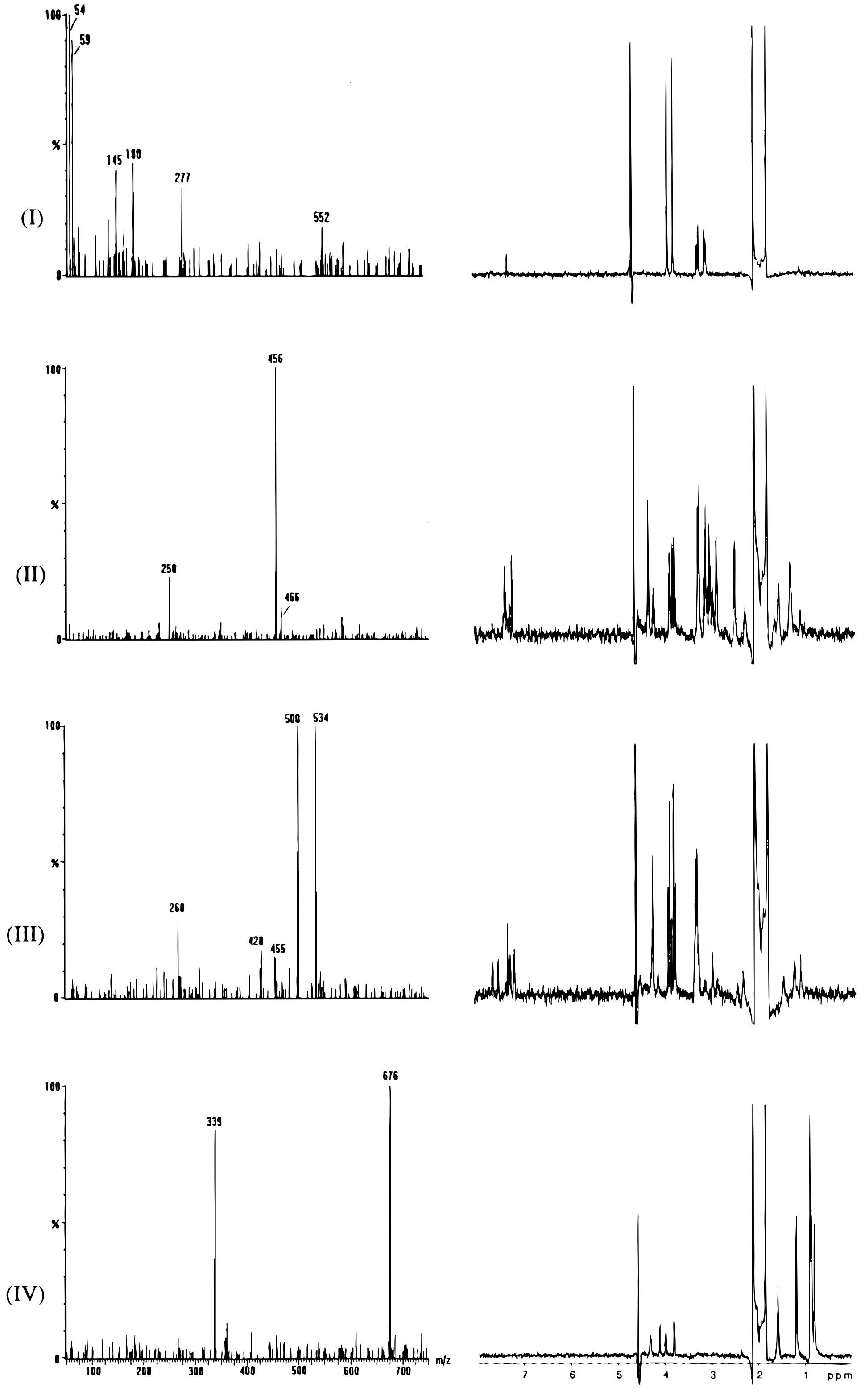

Figure 4. Electrospray mass spectra and 500 MHz ^{1}H NMR spectra of peaks I–IV.

peptides are displayed by taking slices along the ordinate. The relative positions of the methyl resonance of the acetonitrile and the HOD signals in the NMR spectrum changed through the run as the organic content of the HPLC eluent increased. This gives rise to the HOD signal shifting upfield from ~4.65 to ~4.40 ppm as the run proceeds. The Scout Scan[11] method was used to measure the chemical shift of the HOD and methyl acetonitrile signals. This allowed the transmitter frequency to be set on the acetonitrile resonance, thereby maintaining it at the centre of the spectrum and ensuring that the chemical shifts of the different components were kept constant. The solvent suppression was also modified using the scout results.

The mass spectra and NMR spectra for each peak identified in the mixture are shown together in Figs 4 and 5. None of the exchangeable protons in the peptides are observed in the NMR spectra owing to the presence of D_2O in the mobile phase. The signal at ~ 2 ppm is due to the residual acetonitrile solvent signal and the unsuppressed ^{13}C satellites.

Peak I

The mass spectrum shows ions at 277 and 552 Da corresponding to MD^+ and M_2D^+ of the peptide Gly-Gly-His, respectively. In the NMR spectrum the α-hydrogens from the Gly residue are seen as a doublet

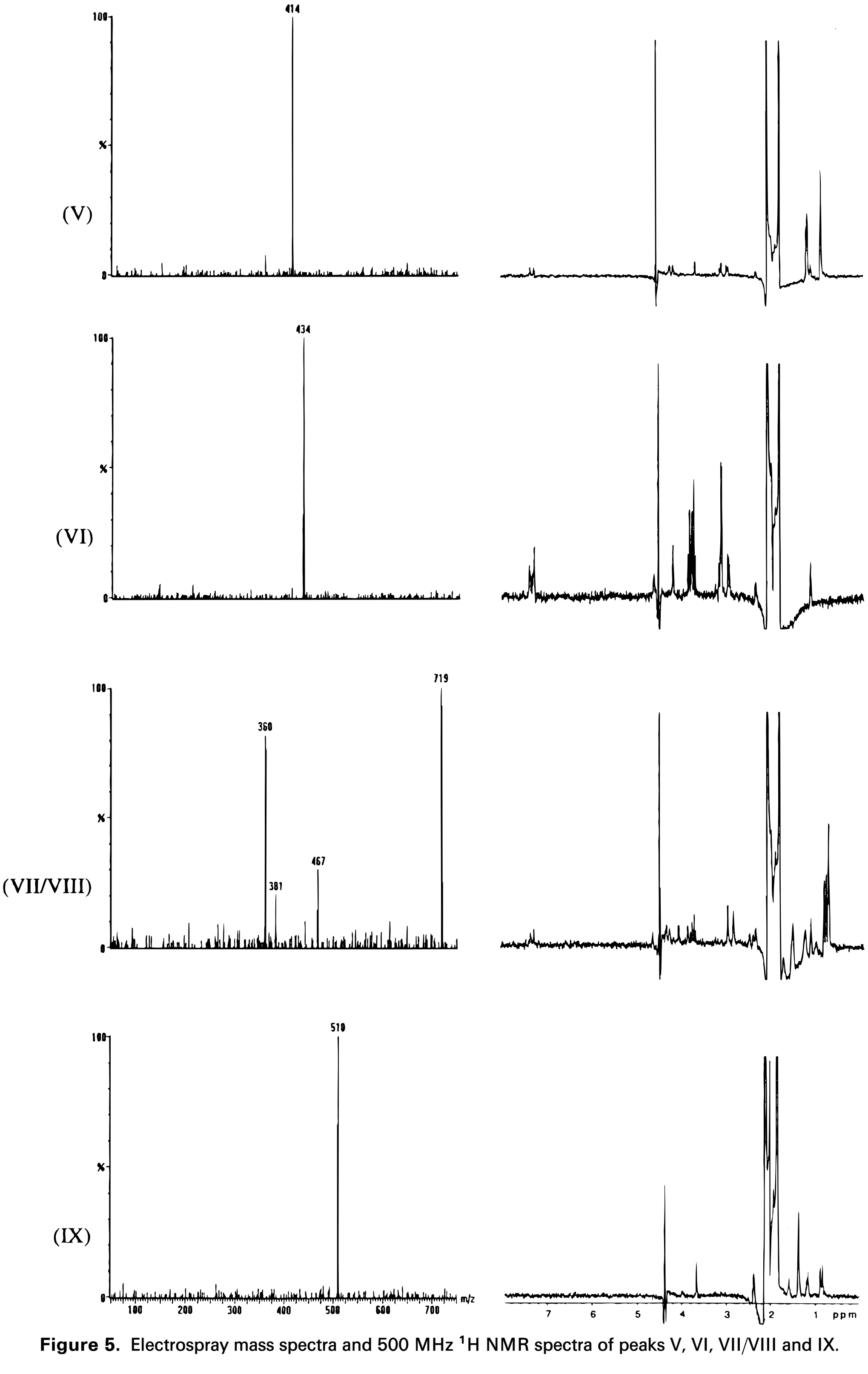

Figure 5. Electrospray mass spectra and 500 MHz ^1H NMR spectra of peaks V, VI, VII/VIII and IX.

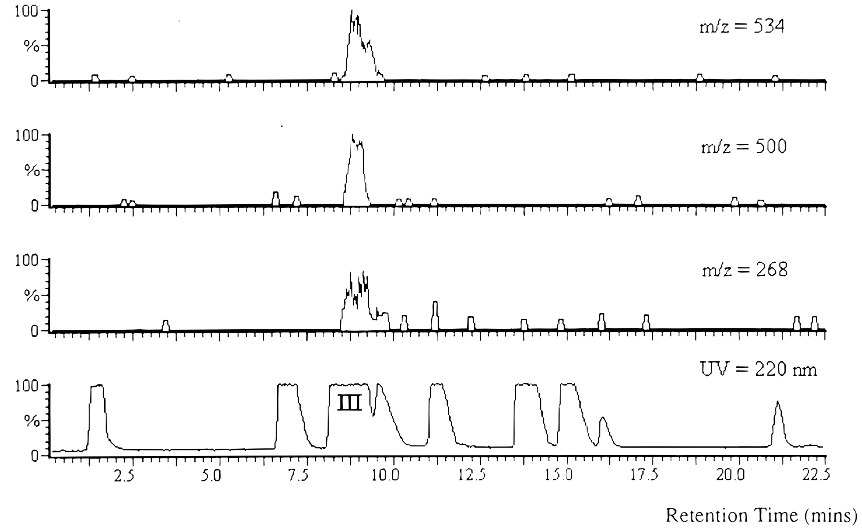

Figure 6. Single-ion chromatograms for *m/z* 534 (M_2D^+ for Trp-Gly), *m/z* 268 (MD^+ for Trp-Gly) and *m/z* 500 (MD^+ for p-Glu-Gly-Arg-Phe-amide) showing these peptides co-eluting as peak III in the UV trace.

at 3.8 ppm. The His α-hydrogen is obscured by the residual water signal and only one of the histidine ring protons is seen at 7.2 ppm. The β-hydrogens however, can be seen at 3.1 and 3.3 ppm.

Peaks II, IV, V, VI and IX

These peaks are easily identified from their NMR and mass spectra. The mass spectra all show clear MD^+ ions, and in the case of peak IV an M_2D^+ ion is also observed. The relative molecular masses of each of the components are thus readily determined. The NMR spectra show the expected signals for the different peptide residues. In most cases the aromatic hydrogens and the α-hydrogens are clearly seen along with the methyl and methylene signals. The Phe α-hydrogens, however, are obscured by the residual water signal.

Peak III

The mass spectrum shows the MD^+ and M_2D^+ ions (268 and 534 Da) for the peptide Trp–Gly. Also observed is the MD^+ ion at 500 Da for the peptide p-Glu-Gly-Arg-Phe-amide. Examination of the single-ion mass chromatograms for *m/z* 534, 500 and 268 (Fig. 6) suggests that these two peptides co-elute. The aromatic protons from the Trp and Phe residues appear between 7.1 and 7.6 ppm in the NMR spectrum. The Trp and Phe α-protons are obscured by the residual water signal. The Glu, Gly and Arg α-hydrogens are seen between 3.7 and 4.4 ppm. The Trp methylene signal appears at 3.3 ppm and the Phe methylene can be seen at 3.0 ppm.

Peaks VII and VIII

Peaks VII and VIII in the UV trace are thought to arise from the same component. Previous LC/MS work using a fully protonated solvent system, and analysing the single peptide component Lys-Phe-Ile-Gly-Leu-Met-NH_2, showed two closely eluting peaks that had very similar mass spectra. The spectrum of the first peak showed ions at 707 and 559 Da corresponding to MH^+

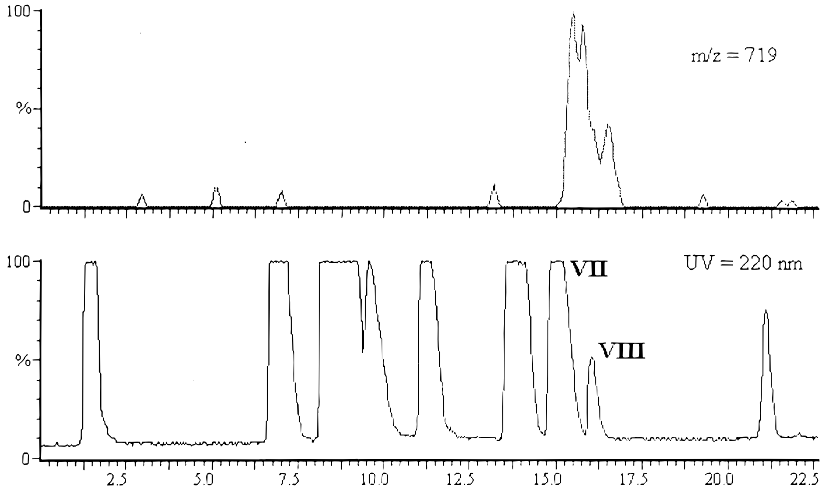

Figure 7. Single-ion chromatogram for *m/z* 719 showing that peaks VII (Lys-Phe-Ile-Gly-Leu-Met- NH_2)and VIII (Lys-Phe-Ile-Gly-Leu-Met-OH) both possess MD^+ ions of 719 Da.

for Lys-Phe-Ile-Gly-Leu-Met-NH_2 and the loss of the Met-NH_2 residue, respectively. The spectrum of the second peak, however, showed ions at 708 and 559 Da, this time corresponding to the MH^+ for Lys-Phe-Ile-Gly-Leu-Met-OH and the fragment ion for the loss of Met-OH. In the deuterated system, however, with all of the exchangeable protons deuterated, the M_r of the amide and the acid forms are the same (717 Da). The single-ion chromatogram for this MD^+ ion at *m/z* 719 (Fig. 7) shows two peaks corresponding to peaks VII and VIII in the UV trace. The mass spectra of the two peaks are identical, the base peak being the MD^+ ion at 719 Da. The ions at 361 and 382 Da are both doubly charged species corresponding to $[M + 2D]^{2+}$ and $[M + 2Na]^{2+}$, respectively. The ion observed at 467 Da is currently unassigned.

In the two-dimensional NMR plot, only one of these peaks is seen. It is not possible to tell whether this peak is the acid or the amide from the NMR data as their spectra would be similar. In the UV chromatogram (Fig. 2), however, the peak corresponding to the amide is much more intense than that of the acid. This suggests that it is likely that the peak seen by NMR is that of the amide. The NMR spectrum shows the aromatic Phe protons at 7.1–7.4 ppm. The α-hydrogens for the Lys, Ile, Leu and Gly residues can be seen between 3.6 and 4.4 ppm. Both the Met and Phe α-hydrogens are obscured by the residual water signal. The Phe β-hydrogen signals can be seen between 2.8 and 3.0 ppm. Methyl signals from Ile and Leu residues appear between 0.7 and 0.9 ppm. The methionine methyl resonance is obscured by the residual acetonitrile solvent signal.

The NMR and MS systems were set up in parallel rather than in series. Parallel detection provides two sets of already synchronized data, making interpretation much easier. The parallel configuration avoids concerns that the choice of LC/MS interface might produce too much back-pressure for the NMR flow cell.

CONCLUSIONS

This work has demonstrated the power of on-line LC/NMR/MS as an analytical technique. The ability to obtain UV, NMR and MS data for individual components in a complex mixture within a single LC run is advantageous, particularly when sample quantity is limited. Also, the reduction in total analysis time and increase in sample throughput provide considerable cost savings.

A number of problems associated with the operation of a quadrupole mass spectrometer close to a high-field magnet have been investigated. One of the major problems, a 100-fold loss of sensitivity, may be improved by shielding the mass spectrometer from the magnet. This might be achieved if the ion optics and analyser housing were manufactured from μ-metal.

The technique has been evaluated on a mixture of known peptides using electrospray ionization and the effect of deuterium exchange on the MS results have been discussed. Further experiments are required to investigate methods of reversing the deuterium–hydrogen exchange process after splitting the flow to the mass spectrometer. This would improve the ability to identify components in a mixture of unknowns.

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