Deuteriated Solvents in High Performance Liquid Chromatography–Fast Atom Bombardment Mass Spectrometry

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Complete exchange of protons with deuterons in conventional FAB-MS can be achieved by introducing samples into the mass spectrometer *via* microbore-HPLC using a deuteriated mobile phase; structural information of analytes in a mixture can be obtained by using this system.

Although several biologically active molecules, in particular peptides and proteins, are often characterised by complex structures, a common feature is the number of labile hydrogen atoms normally bonded to hetero atoms. This number usually increases with an increase in molecular size. Moreover, such molecules are often isolated in small amounts and samples may contain substantial impurity levels of other closely related compounds. Separation of the substance of interest by high performance liquid chromatography (HPLC) directly followed by mass spectrometric analysis has been found to be a useful technique, especially for the characterisation of low levels of simple molecules.[1,2] In this paper we have extended this technique by showing that HPLC resolution of a mixture of peptides using deuteriated solvents and analysis by continuous flow fast atom bombardment–mass spectrometry (CF/FAB-MS) offers greater potential for the rapid acquisition of valuable structural information, related to the number of exchangeable (mobile) protons.

FAB-MS has been reported in proton/deuteron exchange experiments on thermally labile biomolecules.[3–5] Examination of a sample dissolved in a deuteriated solvent, such as D_2O or DCl and in a matrix of [$^2H_3$]glycerol gives rise to a multi-peak envelope of a molecular ions of both protonated and deuteriated molecules due to proton/deuteron exchange.[6] The intensities of ions in the envelope depends on the degree of deuterio exchange on dissolving the sample and on the amount of H/D back-exchange from reaction with atmospheric water. The amount of back-exchange can be kept to a minimum by using fresh deuteriated solvents and by replacing air in the collision cell with argon. Repeated treatment with D_2O on the FAB probe tip has also been shown recently[7] to give high levels of deuterium substitution. We have found that the use of a microbore HPLC reverse-phase system equilibrated with deuteriated mobile phase[8] gives complete deuterium exchange. This system besides being 'closed' has other advantages: any H_2O present in the applied sample is considerably diluted by the mobile phase and is eluted at much faster rate than the material analysed. Figs. 1(*a*) to (*c*) show mass spectra for PRO-PHE-GLY-LYS **1**, run under different conditions. This tetrapeptide contains seven exchangeable protons. Application of **1** into the mass spectrometer *via* an HPLC system using non-deuteriated solvents as the mobile phase[8] gives the expected molecular ion $(M + H)^+$ of 448 [Fig. 1(*a*)]. Replacement of this mobile phase with deuteriated components gives full exchange of all 'mobile' protons and an observed molecular ion $(M + D)^+$ of 456 [Fig. 1(*c*)]. In contrast to the latter situation, application of **1** in deuteriated solvents (D_2O/[$^2H_3$]glycerol) to the tip of the MS probe leads to partial exchange as shown in Fig. 1(*b*).

The signal to noise ratio using the microbore-HPLC system is lower than using the static probe because of the reduced amounts of analytes reaching the probe tip. This leads to poor peak detection using the VG 11-73 data system used in this study. Hence, some distortion of isotope ratios away from the expected values was observed. This effect is further compounded by the reduced ionisation when using deuteriated

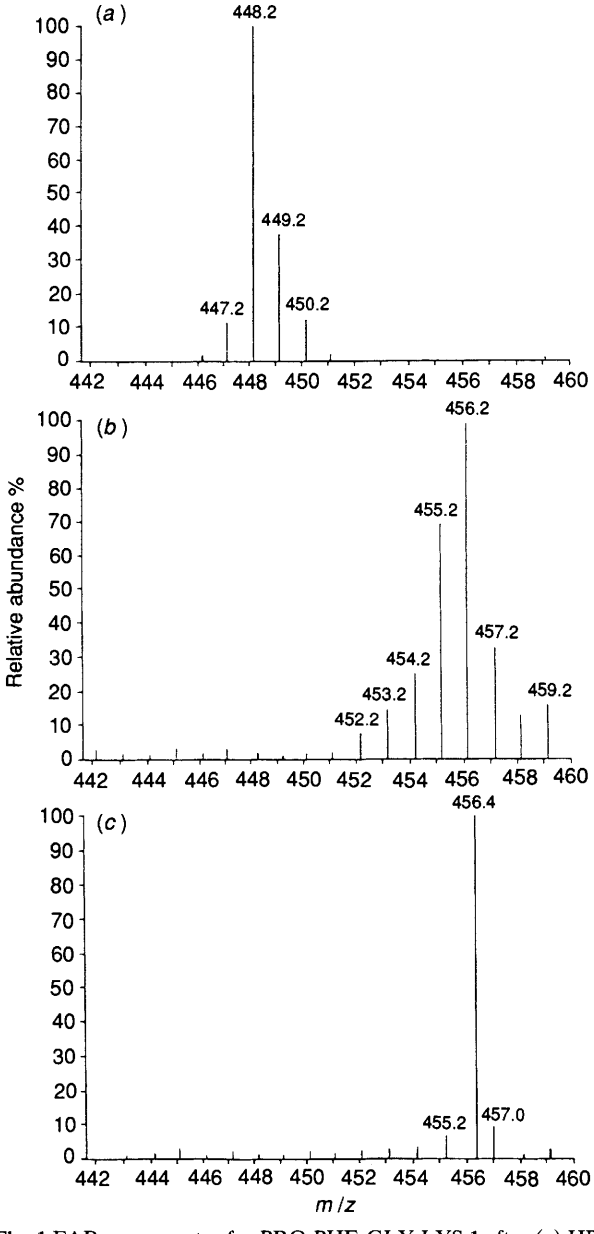

Fig. 1 FAB mass spectra for PRO-PHE-GLY-LYS **1** after (*a*) HPLC using an aqueous mobile phase, (*b*) direct application to the probe in deuteriated solvents and (*c*) HPLC using a deuteriated mobile phase

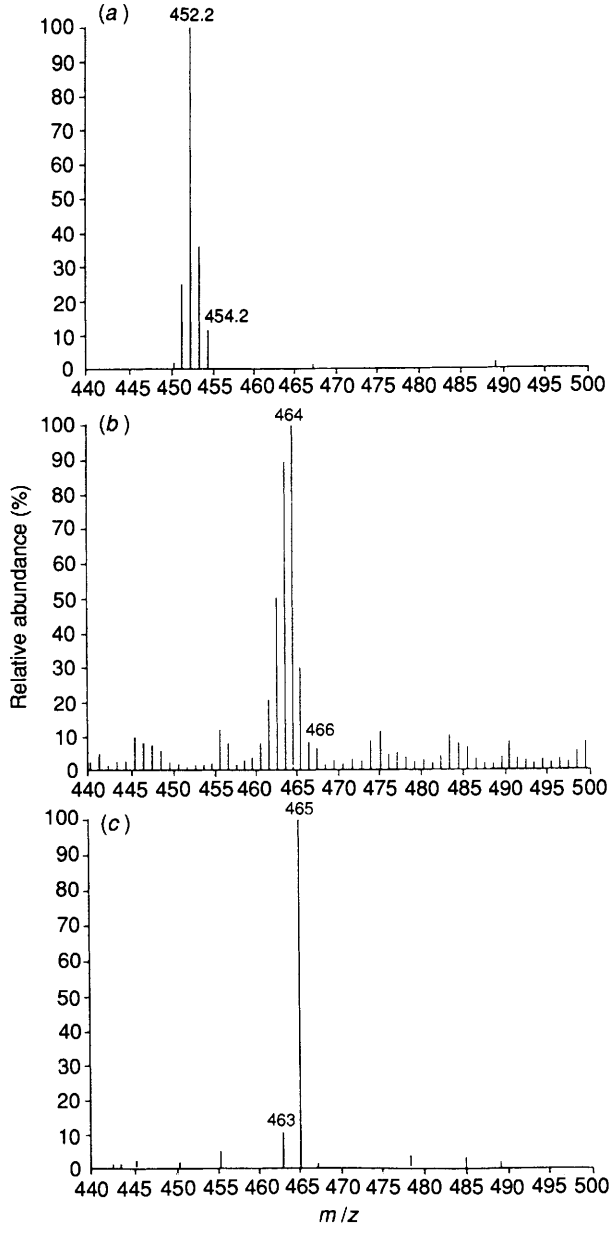

Fig. 2 FAB mass spectra for GLY-GLY-TYR-ARG **2**. The conditions for spectra (*a*), (*b*) and (*c*) are the same as in Fig. 1.

solvents, presumably because of the increased strength of deuterium bonding in the solution. However, background subtraction of spectra produced data where the protonated/deuteriated molecules were clearly observed but the isotope ratios expected for the natural ^{13}C abundance could still not be accurately measured. The major advantage of using the continuous flow probe is the substantial reduction in the chemical noise obtained, compared with using the static probe; the noise in the latter system is due to the high concentration of glycerol present.

Figs. 2(*a*) to (*c*) show the positive ion FAB mass spectra for another test compound, GLY-GLY-TYR-ARG **2**. This molecule contains twelve exchangeable protons, clearly seen on comparison of Figs. 2(*a*) and (*b*). It is interesting that direct application of **2** on the MS probe in deuteriated solvents gives the most abundant molecular ion of 464, that is one less than that observed for full exchange as shown in Fig. 2(*b*). This may be due to intramolecular hydrogen bonding between the hydrogen in the hydroxy group of tyrosine and the arginine moiety. In the LC separation this strong hydrogen bond will be broken when the molecule is distributed between the stationary and mobile phases.

The combination of mass spectrometry with HPLC has also the obvious advantage of separating the components of a mixture before spectrometric analysis. Tables 1 and 2 show data obtained for the HPLC-MS analysis of a mixture of fourteen peptides which include **1** and **2**. Microbore-HPLC was carried out using either conventional (aqueous) conditions or a mixture of deuteriated solvents as the mobile phase.[8] Comparison of the two tables shows that results are complementary. Although retention times are generally shorter using the deuteriated mobile phase it appears that *N*-terminal tyrosine, as shown for compounds **3**, **7**, **10** and **13**, has a different effect. The *N*-terminal tyrosyl peptides apparently have a relatively longer retention time in deuteriated rather than aqueous mobile phase. This has led to differences in elution order, leading in some cases to complete resolution in one phase and not in the other. Although molecules **3** and **4**

Table 1 HPLC–MS data using non-deuteriated solvents in the mobile phase[a]

Peptide (compound number)	Retention time/min	Molecular mass	Observed $(M + H)^+$
TYR-GLY **3**	9.12	238	239
GLY-TYR **4**	11.75	238	239
GLY-GLY-TYR-ARG **2**	13.57	451	452
TRP-GLY-GLY **5**	27.58	318	319
TRP-GLU **6**	35.00	333	Not observed
PRO-PHE-GLY-LYS **1**	42.90	447	448
TYR-LEU **7**	48.85	294	295
VAL-TYR-VAL **8**	55.27	379	380
PHE-LEU **9**	62.70	278	279
TYR-PRO-PHE **10**	71.60	425	426
PHE-GLY-PHE-GLY **1**	71.60	426	427
PHE-GLY-GLY-PHE **12**	74.25	426	427
TYR-GLY-GLY-PHE-LEU **13**	85.77	555	556
ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE **14**	96.63	1044	Not observed

[a] The HPLC conditions used were as follows: column, RP-3007 micron 250 × 1.0 mm C8; aqueous mobile phase, reservoir A—H_2O, 0.07% trifluoroacetic acid (TFA), 1% glycerol; reservoir B—70% MeCN, 30% H_2O, 0.08% TFA, 1% glycerol; flow rate, 50 μl min^{-1}; gradient time (min)/%A/%B, 0/99/1, 35/96/4, 65/79/21, 90/74/26, 110/65/35, 130/50/50. Deuteriated mobile phase, reservoir A—D_2O, 0.07% TFA, 1% [$^2H_3$]glycerol; reservoir B—70% MeCN, 30% D_2O, 0.08% TFA, 1% [$^2H_3$]glycerol; gradient, time (min)/%A/%B, 0/100/0, 15/98/2, 50/86/4, 65/79/21, 90/74/26, 110/65/35, 135/50/50.

have the same retention time in deuteriated solvents they are still distinguishable on the basis of their mass spectra applying deconvolution techniques.

In conclusion, we have shown that the use of microbore-HPLC as an interface for FAB-MS can provide valuable and complementary information on the primary structure of peptides when experiments are carried out with either normal (aqueous) or deuteriated mobile phases. Using deuteriated solvents complete exchange of the 'mobile' (exchangeable) hydrogens is obtained. Complete exchange cannot be obtained when samples are applied in D_2O to a static probe.

Table 2 HPLC–MS data using deuteriated solvents in the mobile phase[a]

Peptide (compound number)	Retention time/min	Observed $(M + D)^+$	Number of exchangeable protons
TYR-GLY **3**	7.15	245	5
GLY-TYR **4**	7.15	245	5
GLY-GLY-TYR-ARG **2**	12.75	465	12
TRP-GLY-GLY **5**	26.10	326	6
TRP-GLU **6**	29.90	341	6
PRO-PHE-GLY-LYS **1**	47.83	456	7
TYR-LEU **7**	62.15	301	5
VAL-TYR-VAL **8**	70.22	387	6
PHE-LEU **9**	74.50	284	4
PHE-GLY-PHE-GLY **11**	81.75	434	6
PHE-GLY-GLY-PHE **12**	83.88	434	6
TYR-PRO-PHE **10**	85.37	432	5
TYR-GLY-GLY-PHE-LEU **13**	92.77	565	8
ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE **14**	97.05	1064	18

[a] See footnote *a* Table 1.

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