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Short communication

Prediction of isotope patterns for partially deuterated analytes in HPLC/MS

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Abstract

A probability model is given which accurately predicts the electrospray mass patterns of substance P arising from full or incomplete deuterium exchange in solution. For accurate determination of the number of exchangeable protons the solvent should have a deuterium purity of at least 98%. At 25 °C several minutes are required before the degree of exchange reaches the equilibrium value.

Keywords: HPLC/MS; Deuterium exchange; Prediction of isotope patterns

1. Introduction

The use of deuterium exchange to provide chemical information is well established in HPLC/MS. Hydrogen atoms bound to heteroatoms such as oxygen and nitrogen are in general more reactive than those bound to carbon and can exchange much more readily with other exchangeable hydrogens in the surrounding environment. By comparing the mass spectra of the analyte with and without deuterium exchange the presence of functional groups such as amides, amines, alcohols, and acids can be established in both molecular and fragment ions. This approach is an important tool in drug development, for example, in the elucidation of chemical structures of impurities and metabolites.

Given the right conditions deuterium exchange can occur in either the gas phase (for example, by using ND_3 in place of N_2 as nebulisation gas [1]) or in solution [2–11]. With HPLC/MS the solution phase deuterium exchange can be achieved either by using deuterated solvents in the mobile phase [3,4,6,7,9,11] or by post-column addition [10].

However whilst deuterium exchange is established in HPLC/MS, there are relatively few reports of work covering the required purity of the deuterated mobile phase and the consequences of partial deuteration ([2] covers FAB with a partially deuterated matrix, and [3] thermo spray). For compounds with many exchangeable protons incomplete exchange results in a complex pattern of isotope peaks which can make interpretation difficult. The problem is more serious for compounds of moderate mass, such as small peptides, where the numbers of carbon and other atoms means that the pattern due to the naturally occurring isotopes is already complex.

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2. Probability model

Probability models can be used to predict H/D mass distribution patterns based upon the number of exchangeable protons in the analyte and the deuterium purity of the surrounding environment [2]. In this work the expected overall mass patterns were predicted by combining the patterns expected from H/D exchange, with those arising from the naturally occurring isotopes in the non-deuterated parent.

For an analyte with n exchangeable hydrogens the relative intensity of the peak with a mass shift of p (where $p < n$) can be described by Eq. (1).

$$I_p = d^p(1-d)^{(n-p)}\frac{n!}{(n-p)!p!} \quad (1)$$

where d is the deuterium purity of the solvent system (between 0 and 1).

The relative intensity depends upon the chance of p of the n sites being deuterated and the number of ways in which p can be chosen from n. Multiplication of the deuteration probability distribution with those arising from the naturally occurring isotopes (e.g. ^{13}C and ^{12}C) gives the overall expected mass distribution. The predicted relative intensities of the individual isotope peaks can then be compared with those measured from the mass spectra.

The importance of a high degree of deuterium purity increases with the number of exchangeable sites n. This importance is illustrated by examining the relative intensities of the mass peak arising from all n sites being deuterated, with that due to only $n-1$ being deuterated. Using Eq. (1) the relative intensities of the two peaks can be described by Eq. (2).

$$\frac{I_n}{I_{n-1}} = \frac{d^n}{(1-d)n} \quad (2)$$

Fig. 1 shows the values obtained from Eq. (2) for analytes with different numbers of exchangeable protons in solvents with deuterium purities in the range 85–100%. From Fig. 1 it is seen that for small values of n a moderate value of solvent deuterium purity is sufficient to ensure that the mass peak due to n is much more intense than that due to $n-1$. As the number of exchangeable sites increases so must the deuterium

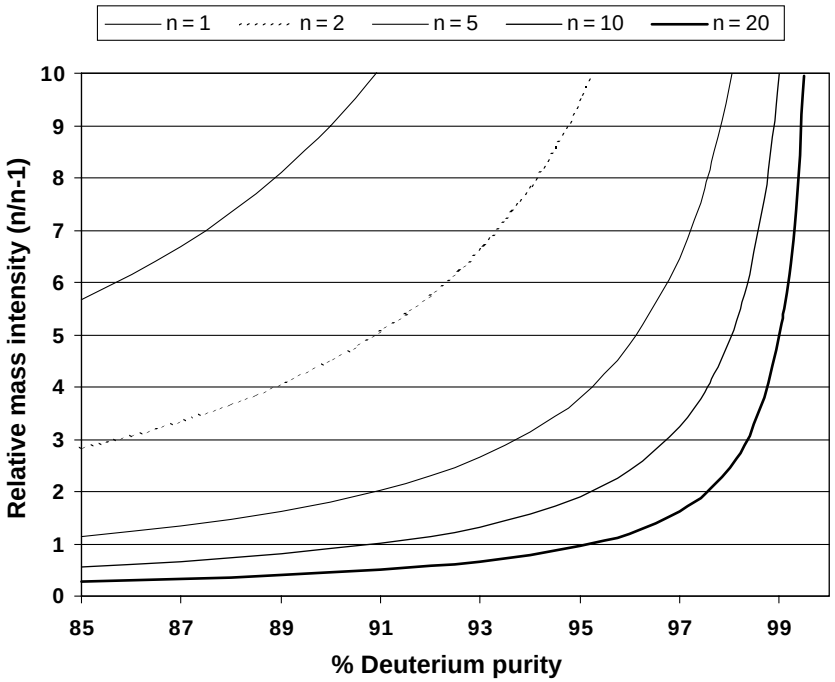

Fig. 1. Relative intensities of the mass peaks arising from complete and partial ($n-1$) deuteration for analytes with different numbers of exchangeable protons.

purity of the solvent. With $n = 20$ a deuterium purity of more than 95% is required for the fully deuterated mass peak to be more intense than that due to only 19 positions being deuterated.

3. Experimental

Mass spectra were recorded using a Micromass ZQ (Manchester, UK), in the range *m/z* 1340–1380 with a cone voltage of 100 V. Samples were infused using a syringe pump at 50 μl/min. Deuterium oxide (99.9%) and TFA were from Fluorochem (Glossop, Derbyshire, UK), and substance P was from Sigma (Gillingham, UK). Two stock solutions were prepared by mixing 0.5 mg of substance P, 8 μl of TFA, and 8000 μl of either H_2O or D_2O. The stock solutions were then allowed to stand at room temperature overnight before analysis. For the kinetic study (25 °C) an 80% D_2O solution was prepared by mixing the two stock solutions and then analysing it at times ranging from 230 to 64,580 s. For the other experiments appropriate proportions of the two stock solutions were mixed and allowed to stand for 24 h at room temperature before analysis.

4. Results and discussion

Based upon the structure of the peptide substance P (see Fig. 2), the most intense MH^+ ion would be expected to give an *m/z* value of 1347.7, and full deuteration to give a mass shift of +23 ($M'D^+$). In the first experiment the kinetics of deuterium exchange were investigated by mixing a solution of substance P dissolved in H_2O/0.1% TFA with one of substance P dissolved in D_2O/0.1% TFA, to give a resultant solution of 80% D_2O/20% H_2O/0.1%TFA. Mass spectra of substance P in the 80% D_2O solution were then recorded at times ranging from 230 to 64,580 s after mixing. Fig. 3 shows the intensities of the ions with *m/z* values ranging from 1362 to 1373 (i.e. mass shifts between +14 and +25 from 1348), as a function of time. Fig. 3 shows that the intensities of the different ions alter slightly at the early time points but show no significant changes after 790 s. These data indicate that a finite period of time is required to reach equilibrium in the deuterium exchange process if the mass analysis is to give accurate results. Fig. 3 also shows that the mass ion representing full deuteration (+23) is only the eighth most intense.

Fig. 4 shows the predicted mass shift patterns obtained from substance P dissolved in 99.8, 90, and 80% D_2O, respectively. In Fig. 4a deuteration is almost complete with an $M'D^+$ ion shifted by +23 being the most intense ion. Higher masses are due to full deuteration and the presence of the naturally abundant ^{13}C, ^{15}N, ^{34}S, and ^{18}O isotopes. Fig. 4b and c show that as the deuterium purity of the solvent drops so does the relative intensity of the fully deuterated peak. With 90% D_2O mass +23 is the third most intense peak and with 80% D_2O only the eighth most intense peak.

Fig. 5 compares the mass shifts predicted by the probability model, and the measured mass spectrum for substance P dissolved in 98% D_2O/2% H_2O/0.1% TFA. Fig. 5 shows an excellent agreement between the mass pattern expected from the probability pattern and that obtained by MS. The most intense mass peak has an *m/z* value of 1371, which is +23 from the

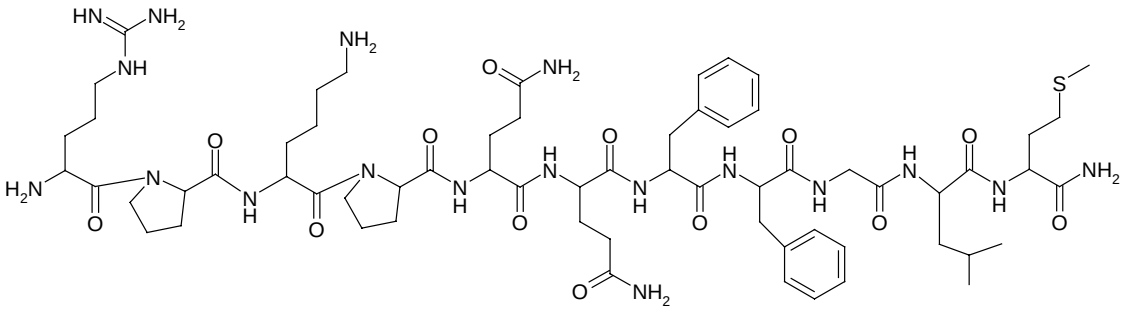

Fig. 2. The structure of the peptide substance P.

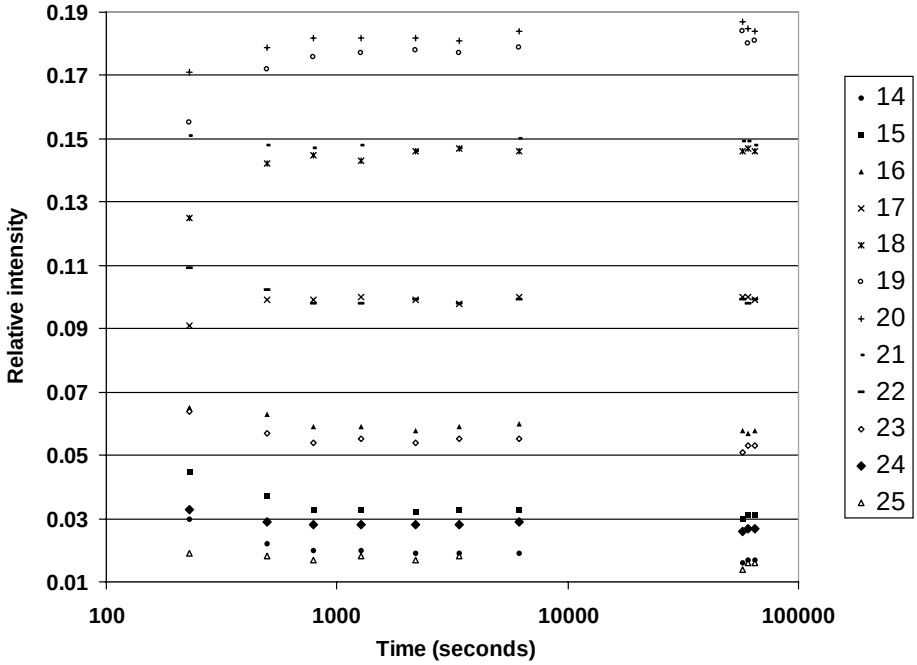

Fig. 3. Measured mass intensities for substance P dissolved in 80% D_2O as a function of time.

un-deuterated parent and in agreement with the probability model. The relative proportions of the other major mass ions at *m/z* 1370, 1372, and 1373 (+22, +24, and +25 from the parent), are also correctly predicted by the probability model.

In order to check the probability model further the predicted and experimental mass distributions were measured in solutions ranging from 80% D_2O to 99.8% D_2O. Fig. 6 shows good agreement between the predicted relative intensities (solid lines),

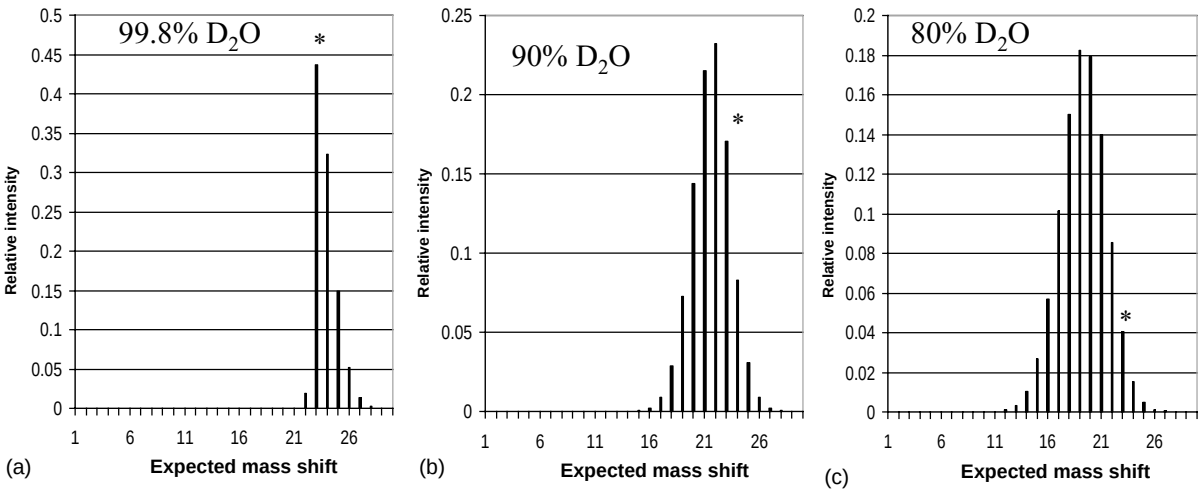

Fig. 4. Predicted mass shifts for substance P dissolved in 99.8, 90, and 80% D_2O.

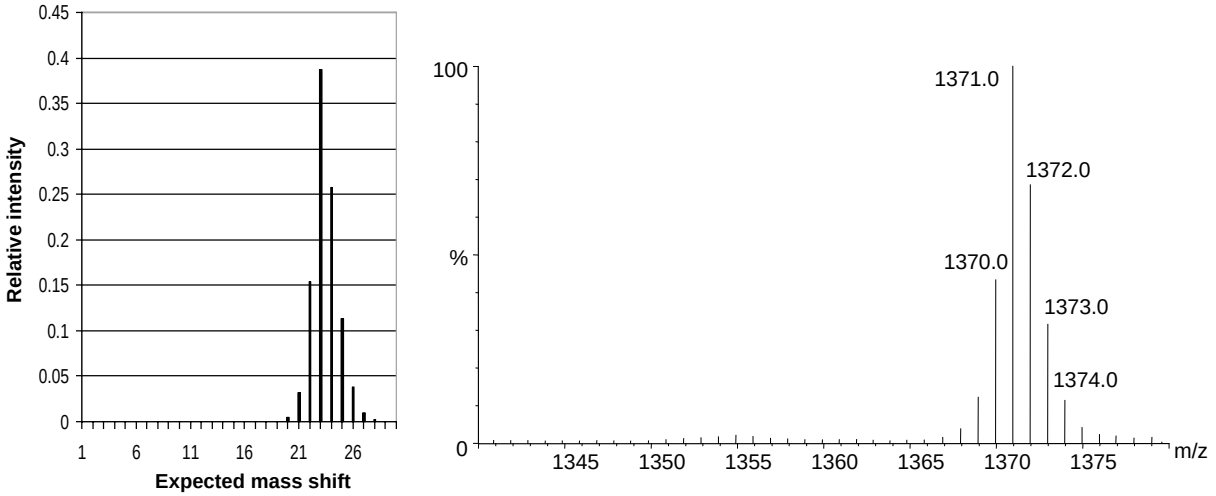

Fig. 5. Predicted mass shifts and measured masses for substance P dissolved in 98% D_2O.

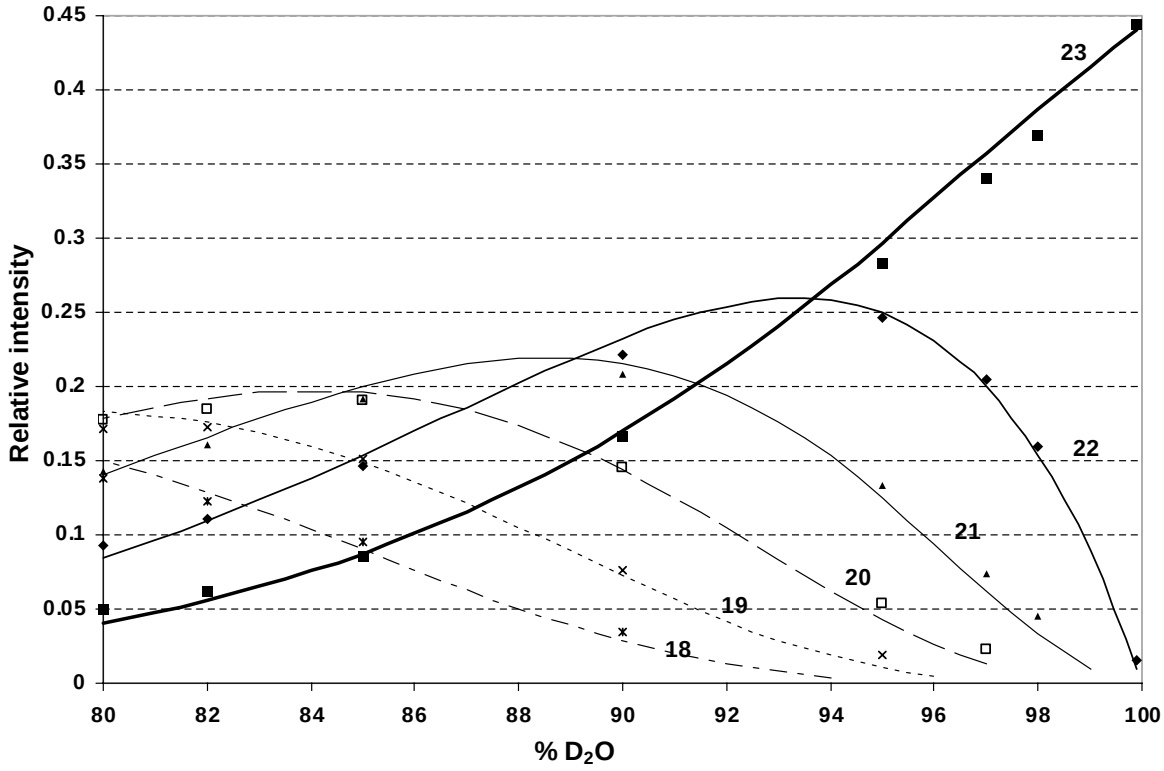

Fig. 6. Predicted and measured mass patterns for substance P as a function of the deuterium purity of the solvent.

and the measured values (individual data points) for ions shifted in mass from 18 to 23. Fig. 6 shows that the relative intensities of the mass peaks arising from partial deuteration increase rapidly as the deuterium purity of the solvent drops below 99.8%. For example, at a deuterium purity of 95% the peak due to full deuteration is only slightly more intense than the partially deuterated $n - 1$ peak. Because of the complexity in the mass pattern arising from both the partial deuteration and the naturally occurring isotopes, the deuterium purity must be significantly higher than 95%, and ideally of 98% for n to be determined reliably.

5. Conclusions

For the peptide substance P the unambiguous determination of the number of exchangeable protons requires the use of a solvent with a high deuterium purity, ideally 98% or more, and an equilibration period of several minutes. These observations support the use of fully deuterated mobile phases rather than post-column addition as a reliable way of determining the number of exchangeable protons by LC/MS. For fast LC methods, or analytes and mobile phase conditions which give slower exchange kinetics, the sample may also need to be incubated with deuterated solvents prior to analysis.

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