Journal of Chromatography, 647 (1993) 31–38
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2788

Deuterium oxide as a reagent for the modification of mass spectra in electrospray microcolumn liquid chromatography–mass spectrometry

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ABSTRACT

Microcolumn liquid chromatography was used in combination with a quadrupole mass spectrometer equipped with an electrospray ionization interface. Deuterium oxide was used as a reagent to induce peak shifts in mass spectra. The peak shift obtained gives the number of heteroatoms carrying hydrogen atoms that are present in a molecule. Examples relating to amino alcohols, peptides and sugars are given. Two different modes of operation were investigated; postcolumn addition of the reagent to the eluent from the column and the use of deuterium oxide as a mobile phase component. The latter technique makes use of a dynamic exchange reaction on-column. Exchanged hydrogen atoms are carried away from the migrating zone (as H^2HO or H_2O) containing the target compound, leading to a high yield of the deuterated compound. On-column exchange was found to be more useful, especially if the compounds contained several exchangeable hydrogen atoms. No significant changes were observed with regard to chromatographic selectivity or efficiency.

INTRODUCTION

Recent developments in instrumentation for mass spectrometry, particularly the introduction of the electrospray interface (ESI), have had an enormous impact in the field of bioanalytical separations. Several review papers have already been published, dealing with both fundamentals and applications [1–4]. In parallel, liquid chromatography (LC) using packed fused silica columns has been developed into a powerful separation technique offering high separation efficiency and the capability to deal with small amounts of sample [5,6]. Another attractive feature of microcolumn LC is the low volumetric flow-rate of the mobile phase, of the order of 1 μl/min. This makes it possible to use fairly expensive or for other reasons uncommonly used mobile phases. High-resolution LC can now be combined with a powerful detector without compromises to either LC or the mass spectrometer, at least with respect to liquid flow-rates. Consequently, several papers have been published [7–9] describing the combination of slurry-packed capillary columns and a mass spectrometer using the ESI. High separation efficiency and high sensitivity were demonstrated.

Many of the new types of interface for LC–MS, especially the ESI, produce in the positive-ion mode only the protonated molecular ion with no or very few structurally significant fragment ions. Additional information can be obtained by using tandem mass spectrometry (MS–MS). Unfortunately, the resulting daughter ion spectrum is often difficult to interpret without a comparison with some structurally related compounds. An additional disadvantage with this technique is that more of the sample is usually needed. Other methods that have been used to induce fragmentation include thermally induced

0021-9673/93/$06.00

dissociation [10] and the technique of accelerating the ions in the intermediate pressure region between the nozzle and the skimmer [8].

Derivatization of the target molecule has been used successfully in GC–MS for many years but also to some extent in LC–MS [11,12]. In an LC–MS system, it is sometimes beneficial to modify the properties of the mobile phase. Halogenated mobile phase additives improved the sensitivity in the negative ion mode [13,14] and recently sodium acetate was used in a thermospray source to help detect oligosaccharides [15]. A postcolumn modification of the mobile phase is commonly used in ESI. The sheath liquid introduction [8,16] of an organic solvent is aimed at changing the bulk properties of the mobile phase such as reducing the surface tension, changing the pH [17] or adjusting the total flow-rate into the interface as in capillary zone electrophoresis (CZE) [3,17].

Deuterium oxide has been used as a reagent for determining the number of exchangeable hydrogen atoms in organic molecules using various analytical techniques including NMR [18] and mass spectrometry [19]. More recently, exchange reactions were investigated using thermospray [20,21], electrospray [22] and fast atom bombardment (FAB) [23].

The aim of this work was to evaluate the use of deuterium oxide as a mobile phase constituent in an *on-column* exchange reaction and to compare the results obtained with those of continuous postcolumn addition.

EXPERIMENTAL

Chemicals

LC-grade acetonitrile was obtained from Rathburn (Walkerburn, UK) and used as received. Water was purified using a Milli-Q system (Millipore). Deuterium oxide (99.8% isotopic purity) was purchased from Dr. Glaser (Basle, Switzerland) and angiotensin I and II (human, synthetic) from Sigma (St. Louis, MO, USA). Other compounds studied were obtained in-house.

Sheath liquid and postcolumn addition of deuterium oxide

Deuterium oxide as a mixture [acetonitrile–deuterium oxide (50:50)] was delivered at various flow-rates by a low-pressure syringe pump (Harvard Apparatus, South Natic, MA, USA) and mixed with the sample stream using either of the two configurations, A and B, as shown in Fig. 1. The sample [1×10^{-5} *M* in acetonitrile–water (50:50)] was infused continuously through a short column (200 mm × 250 μm I.D., creating a slight back-pressure) using a Carlo Erba (Milan, Italy) Phoenix 20 CU pump and a Model 7010 500-μl loop injector (Rheodyne, Berkeley, CA, USA) at a flow-rate of 1 μl/min. Ion intensities were determined in the profile mode and averaged for 1 min.

The correction for the heavy isotope effect was made by measuring the natural isotopic ratio for the $[M+1]^+$ (C-12) and $[M+2]^+$ (C-13) ions before using deuterium oxide. In cases where two hydrogen atoms were exchanged also the $[M+3]^+$ (S-34) ion had to be measured. The area ratios determined will be constant in experiments with deuterium oxide also. In the presence of deuterium oxide, using the area of the remaining (original) M + 1 ion and the determined ratios, the shifted peak area can be corrected.

Microcolumn liquid chromatography

Isocratic elution was applied throughout using a Phoenix 20 CU pump operated in the constant-pressure mode. The injector was a Valco (Houston, TX, USA) CI4W (200 nl), which was used in the time split mode to introduce about 100 nl of the sample. A second injector (Rheodyne Model 7010), equipped with a 500-μl loop, was placed between the pump and the sample injector. This injector served as the mobile phase reservoir in experiments with deuterium oxide. Columns of 200–250 μm I.D. were prepared

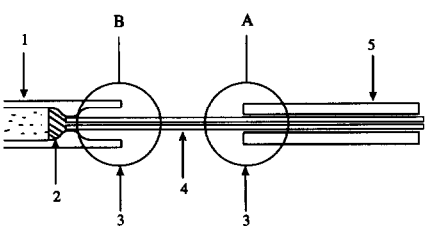

Fig. 1. (A) Sheath liquid and (B) postcolumn addition of deuterium oxide. 1 = chromatographic column; 2 = porous Teflon; 3 = Swagelock tee; 4 = fused-silica tubing; 5 = stainless-steel capillary.

from empty fused-silica tubes (Polymicro Technologies, Phoenix, AZ, USA) as described previously [24]. The packing material was 5-μm Kromasil C_8 (EKA Nobel, Surte, Sweden), held in place by a 0.2-mm long section of glass-wool ("frit") as described previously [25]. The column was connected to the electrospray needle using a 300 mm × 50 μm I.D. × 150 μm O.D. empty fused-silica tube.

Electrospray mass spectrometry

Experiments were carried out on a Finnigan TSQ 700 triple quadrupole mass spectrometer equipped with an electrospray interface (Analytica of Branford, Branford, CT, USA). All spectra were obtained using unit mass resolution. The electrospray needle was made in house using a 150 mm × 160 μm I.D. stainless-steel capillary. The fused-silica capillary from the column ended about 0.5 mm outside the needle.

RESULTS AND DISCUSSION

Addition of deuterium oxide

Two different modes of introduction of deuterium oxide were used; sheath liquid introduction (Fig. 1A) and what will be referred to as postcolumn introduction (Fig. 1B). In the latter configuration, some extra time is provided for mixing in the capillary tube. If the target molecules contain exchangeable hydrogen atoms, a fraction of the molecules will be converted into the deuterated form. The extent of this exchange will be determined by the equilibrium constants and the kinetics of the reaction. When the two liquid streams meet, a complex mixture will be formed, consisting of acetonitrile, water, deuterium oxide and H^2HO in addition to several charged species due to autoprotolysis. In the absence of a strict mathematical model for proton transfer in the electrospray process, a simple equation based on relative rates of formation for the competing ions was used:

$$\frac{I_{A^2H^+}}{I_{AH^+}} = K \cdot \frac{F^2_{H_2O}}{F_{H_2O}} \quad (1)$$

where $I_{A^2H^+}$ and I_{AH^+} represent the ion intensities for the deuterated and the corresponding protonated analyte (corrected for the heavy isotope effect) and $F^2_{H_2O}$ and F_{H_2O} are the flow-rates of the solution of deuterium oxide and water, respectively. For molecules containing exchangeable hydrogen atoms, the exchange reaction is assumed to reach an equilibrium before charging takes place, *i.e.*, in the transfer capillary. A plot based on eqn. 1 is shown in Fig. 2, for four different compounds (Fig. 3). If there is no difference between water and deuterium

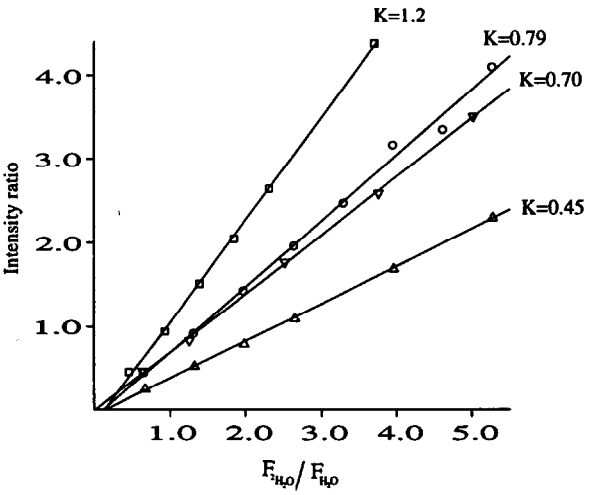

Fig. 2. Evaluation of relative formation rate constants. □ = N-methylhomatropine; ○ = N-methylomeprazole; ▽ = scopolamine; △ = omeprazole. D = deuterium.

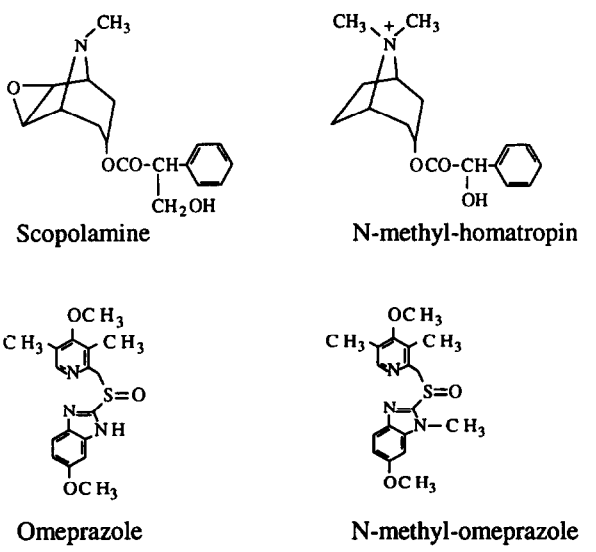

Fig. 3. Compounds studied in postcolumn experiments.

oxide systems with respect to the charging process, one would expect from statistical considerations that $K \approx 1$ for molecules that accept either a deuteron or a proton. The highest conversion efficiency ($K = 1.2$) was obtained for a permanently charged quaternary ammonium ion (N-methylhomatropine), where only one proton was exchanged. The large positive deviation ($K > 1$) seen for this compound could be due to several factors. Many species in the solution like such as $^2H_2HO^+$ and $^2HH_2O^+$ might favour the deuteron in the charging process. An additional complication is that back-exchange reactions can occur in the gas phase by ion–molecule reactions. The overall result could therefore be different for different types of molecules. This could possibly explain why the corresponding constant for N-methylomeprazole ($K = 0.8$) was found to be slightly less than unity. In such a case, one has to assume that a deuterium atom bound in a hydroxyl group (N-methylhomatropine) is bonded more strongly than an adduct deuteron ($M + D^+$) formed in the spray process and therefore less sensitive to a back-exchange reaction.

For compounds that can accept two deuterium atoms (omeprazole, scopolamine), one would expect a K value of about 0.5. This seems to be the case for omeprazole ($K = 0.45$). To summarize, the K values are higher than expected for N-methylhomatropine and scopolamine and slightly less than expected for the other two compounds.

In experiments using the liquid sheath method (Fig. 1A), linear relationships were also observed but the slopes of the lines (K) were about 10–15% lower, possibly indicating less efficient mixing of the two liquid streams.

The relative recovery (comparing only the intensities measured) of the fully deuterated compounds can be calculated using a rearranged form of eqn. 1 and will be about 83% for N-

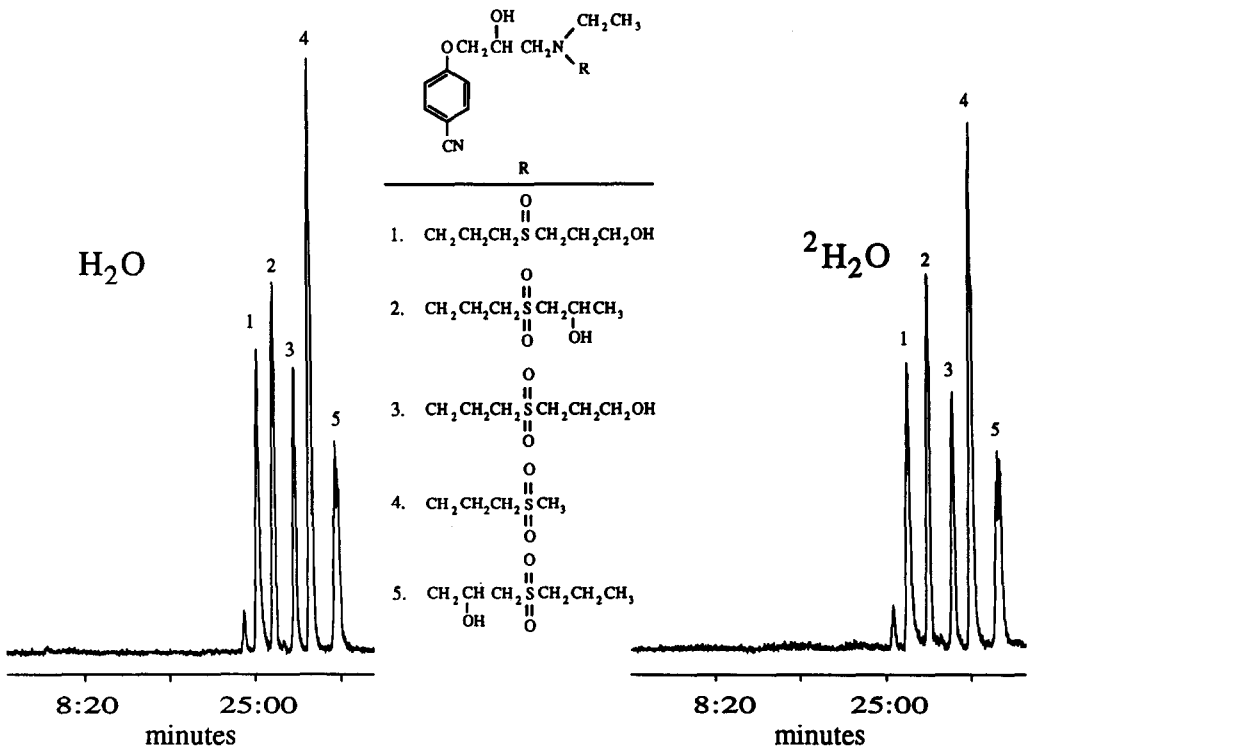

Fig. 4. Microcolumn separation using (left) water or (right) deuterium oxide in the mobile phase. Reconstructed total ion current trace using a scan range from 200 to 400 u at 0.7 s per scan. The amount injected ranged from 20 to 30 pmol. The column was 900 mm × 200 μm I.D., packed with 5-μm Kromasil C_8. The mobile phase was acetonitrile–5 m*M* ammonium acetate in water or deuterium oxide (55:45). Flow-rate, 0.95 μl/min.

methylhomatropine but only 64% for omeprazole using the constants given in Fig. 2 at a flow ratio of 4. A higher efficiency for the conversion will be necessary when the number of exchangeable hydrogen atoms increases, otherwise an envelope of ions will result. A possibility is to increase the percentage of deuterium oxide added postcolumn. A disadvantage with this approach is that the sensitivity will decrease with increasing deuterium oxide content owing to increasing surface tension. Another possibility is to decrease the flow of water by drastically reducing the column flow-rate via a reduction of the column diameter or a change to CZE or open-tubular LC.

Deuterium oxide as a mobile phase component

Although the techniques described above are fairly easy to use, difficulties arise when several exchangeable hydrogen atoms are present in the analyte. A postcolumn addition of deuterium oxide can be regarded as a batchwise operation where there always will be some water present to decrease the yield of the fully deuterated analyte. A change from a static system (fixed concentration ratio of ^2H_2O to H_2O) to a dynamic system can be done by using a chromatographic column and replacing mobile phase deuterium oxide with water. Hydrogen atoms will be exchanged and the equilibrium below forced to the right as water will migrate faster ($k' \approx 0$) on the column than a zone containing the sample ($k' > 0$):

$$RH_n + 0.5n^2H_2O \rightleftharpoons R^2H_n + 0.5nH_2O$$

In a chromatographic system there will be adequate mixing and enough time available to give a high recovery of the fully deuterated compound. Sources of protons that will decrease the recovery are to be found in the organic modifier, deuterium oxide and various buffer components.

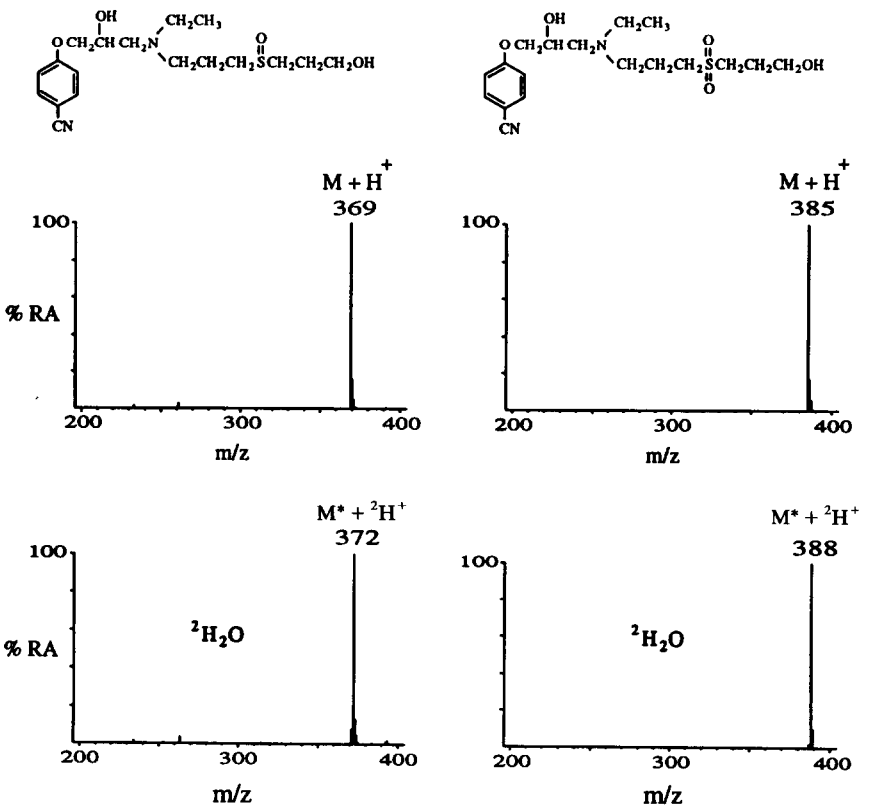

Fig. 5. Mass spectra obtained using (top) water or (bottom) deuterium oxide in the mobile phase.

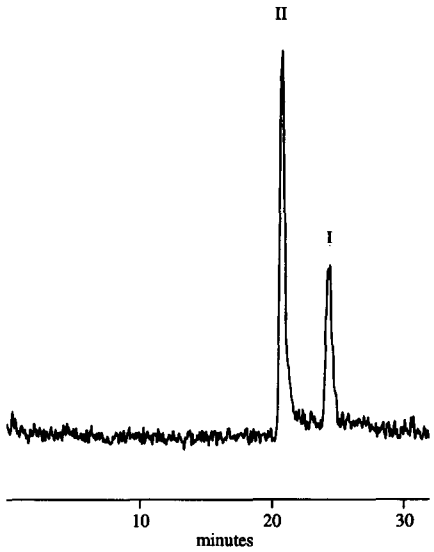

Fig. 6. Microcolumn separation of angiotensin I and II. Column, 900 mm × 0.25 mm I.D., packed with 5-μm Kromasil C_8; mobile phase, acetonitrile–5 m*M* ammonium acetate solution in deuterium oxide (40:60) at a flow-rate of 1.1 μl/min. Total ion current trace obtained by scanning from 500 to 1320 u in 2 s.

Fig. 4 shows a separation of some amino alcohols using acetonitrile–water (50:50) (left) and acetonitrile–deuterium oxide (50:50) (right) as mobile phases. A retention time a few minutes longer is observed using deuterium oxide instead of water in the mobile phase. This can probably be explained by the higher viscosity (*ca.* 20%) for deuterium oxide. No change was observed that relates to selectivity or efficiency. The latter could be degraded if there is a slow hydrogen–deuterium exchange resulting in a mixture of compounds with varying degrees of substitution. Molecules containing deuterium instead of hydrogen are differently retained [26].

Fig. 5 shows a comparison of some ESI mass spectra obtained in water and deuterium oxide systems, respectively. These compounds are metabolites of a drug ($[M + H]^+ = 353$) that lacks a hydroxyl group in the carbon chain attached to the sulphur atom (Fig. 5, top left). The peak shift of 3 u for the compound in the top left corner reveals that two exchangeable hydrogen atoms are present; the third one is responsible for the charge ($[M + {}^2H]^+$). As the molecular mass is increased by 16 u (*m/z* 353→369), a hydroxylated metabolite could be expected. Another metabolic pathway for the parent drug is oxidation of the sulphur atom to the corresponding sulphone, again an increase of 16 u. This could be easily ruled out as the peak shift would then be only 2 u. The top right spectrum corner shows that the molecular mass

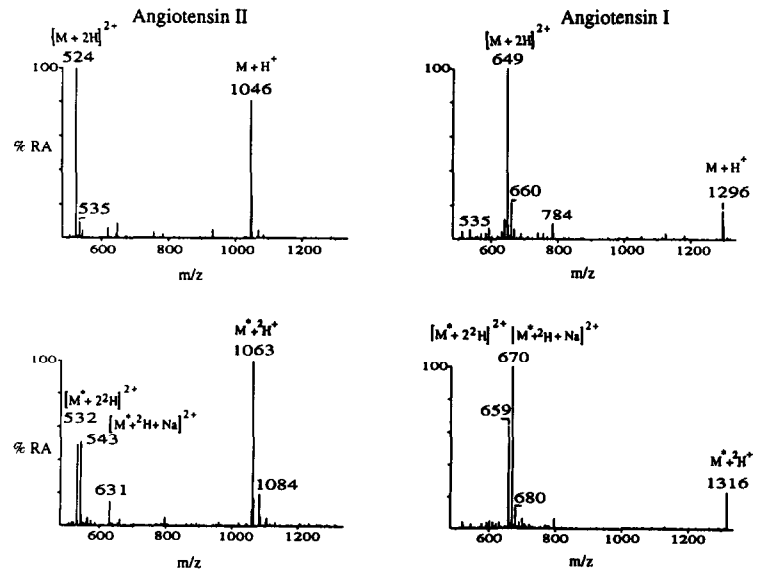

Fig. 7. Mass spectra of angiotensins I and II obtained using (top) water and (bottom) deuterium oxide in the mobile phase.

for this metabolite has increased by 32 u. The peak shift was only 3 u, which suggests that only one hydroxyl group has been formed, and the other oxygen atom should therefore be bound to sulphur.

An area that has attracted considerable interest in the last few years is peptide mapping using mass spectrometry [7,27,28]. The molecular masses of the peptides, formed by tryptic digest of a protein, are determined using either FAB or ESI methods. Sequence information on the individual peptides can be obtained in MS–MS experiments [7]. However, when only small amounts of material are available, the latter technique may not give a definite answer. Additional information can be obtained without using a higher sample load by modifying the mobile phase using deuterium oxide. Fig. 6 shows an isocratic separation of a synthetic mixture of angiotensin I (15 pmol) and II (18 pmol) using acetonitrile–5 m*M* ammonium acetate solution in ^2H_2O (40:60). The mass spectra obtained before and after changing the mobile phase are shown in Fig. 7. The small amount of ammonium acetate used as buffer obviously did not cause any problem in locating the molecular ion. Both peptides show the protonated molecular ion at *m/z* 1046 and 1296 together with the doubly charged ions ($[M + 2H]^{2+}$) at *m/z* 524 and 649, respectively. In the deuterium oxide system, the molecular ions have shifted to *m/z* 1063 and 1316, respectively. The peak shift obtained for angiotensin II is 17 u and that for angiotensin I is 20 u. These values correspond exactly to the number of exchangeable hydrogen atoms in both molecules, including the proton responsible for the charge. Knowing the exact number of heteroatoms carrying a proton in an unknown peptide will make it easier to determine which amino acid residues are present. An interesting detail here is that in the ^2H_2O system, other doubly charged molecular ions appear, carrying both a deuteron and a sodium ion. The relative intensities of these ions are consistently larger than those of the corresponding ions in the water system.

Additional information that can be obtained that will be of value concerns the presence of impurities, *e.g.*, compounds that are not peptides, especially in the very complex mixture obtained after an enzymatic digestion. For an unknown compound to be a peptide, the mass shift must be at least 5 u for a dipeptide; three hydrogen atoms from the N- and C-termini and one from the peptide bond in addition to the proton carrying the charge.

The peak shift technique was also used to help identify an unknown degradation compound found in a pharmaceutical formulation. The tablets contained hydralazine, metoprolol and hydrochlorothiazide. The mass spectra obtained with and without deuterium oxide are shown in Fig. 8. The protonated molecular ion (*m/z* 485) suggests a molecular mass much larger than that

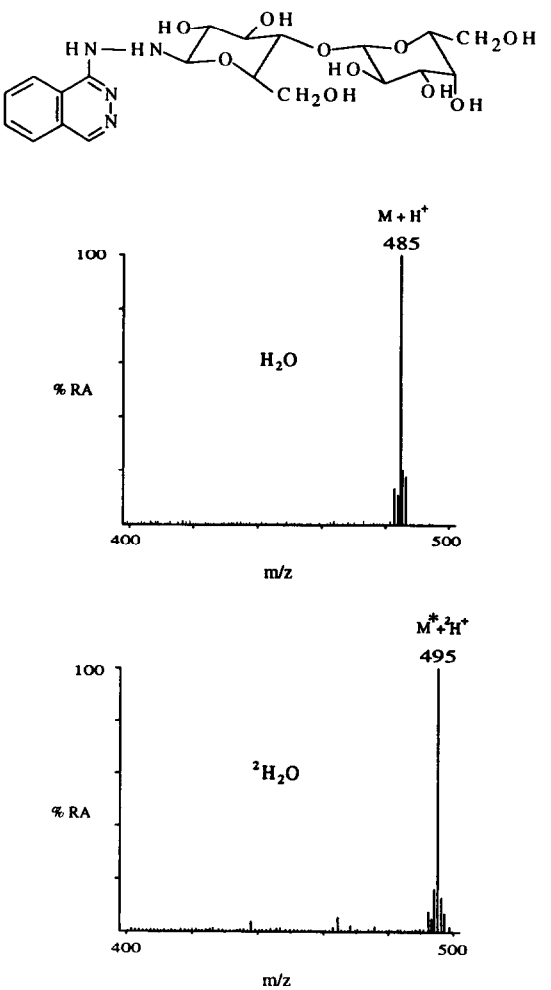

Fig. 8. Mass spectra obtained for an "unknown" degradation compound. Column and mobile phase as in Fig. 6.

of any of the active ingredients. The large peak shift of 10 u focused attention on lactose, an "inactive" ingredient in the tablets. The structure given in Fig. 8 was finally verified using NMR spectroscopy. It can be seen that all the hydrogen atoms on the sugar moiety and also on the nitrogen atoms have been exchanged.

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