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Liquid chromatography–mass spectrometry using the hydrogen/deuterium exchange reaction as a tool for impurity identification in pharmaceutical process development

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Abstract

HPLC–MS employing deuterium oxide and common MS-compatible deuterated additives in the mobile phase with electrospray ionization is shown to be a viable approach for the structural elucidation of impurities in pharmaceutically active agents following initial studies with protic mobile phases. This approach incorporates the hydrogen/deuterium (H/D) exchange reaction where deuterium is substituted for hydrogen at labile sites. Some developmental compounds studied include an amide, amine, lipopeptide, indole and methyl sulfone. H/D exchange is rapid and the chromatographic performance using deuterated mobile phases is comparable to protic counterparts.

Keywords: LC–MS; Hydrogen/Deuterium exchange; Structural determination

1. Introduction

The exchange of hydrogen by deuterium (H/D exchange) has been shown to be a simple yet powerful approach for structural analysis when coupled with mass spectrometry. Functional groups having a labile hydrogen amenable to this transformation include alcohols, phenols, carboxylic acids, amines, amides, thiols and compounds having an active methylene or methyne moiety [1–4]. Essentially the exchange is an atomic derivatization rather than the usual molecular one. Some recent examples of this strategy include the MS study of peptide and protein conformational and folding solution dynamics [5–7], gas phase nucleotide conformation [8], elucidation of the structure and collision-induced dissociation of peptides, antibiotics, saccharides, natural products and small organic molecules [9–12] and investigation of drug–drug non-covalent complexation [13].

Hypenated techniques integrating mass spectrometry with a chromatographic technique such as liquid chromatography have extended the utilization of mass spectrometry as a means of measurement. A particularly useful combination is liquid chromatography with hydroorganic mobile phases, often referred to as reversed-phase HPLC (RPLC). The selectivity and versatility offered by RPLC has made it a method of choice for the analysis of complex mixtures of a variety of pharmaceutically important compounds such as reversed transcriptase inhibitors, antibiotics and antidepressants [14–16]. An example of the versatility of RPLC is the wide array of mobile phase components which can be employed to influence separation variables such as retention and resolution. A popular strategy is the investigation of the effect of the organic component, methanol and acetonitrile being popular organic modifiers, on the separation [17]. However, an interesting alternative is the substitution of deuterium oxide for water as the aqueous mobile phase component, and the effect of D_2O upon selectivity for separations of alkyl benzenes and aromatic hydrocarbons has been reported [18]. When coupled with MS detection, RPLC with D_2O as a mobile phase component allows either for dynamic H/D exchange on column or retention of H/D exchange if the compound of interest has been equilibrated in a deuterated

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medium prior to chromatography. The measured increase in mass shift relative to that observed in a protic environment can then be used for structural elucidation. The thermospray interface was used to demonstrate this approach for steroids, prostaglandins and nucleosides [19]. Subsequent investigations using popular atmospheric pressure ionization interfaces such as chemical ionization (APCI) and electrospray (ESI) showed the most facile and efficient method of H/D exchange could be accomplished dynamically on-column compared to post column addition of D_2O to a protic mobile phase or flow injection of a deuterated medium into a protic carrier [20,21]. H/D exchange with either LC–MS or LC–tandem MS has been used for compound identification in forced degradation studies of the antibiotic amoxicillin [22], stability studies of an investigational substance P antagonist receptor [23] and a pharamaceutical formulation of hydralazine [20] and in drug metabolism studies to identify metabolites of denopamine, promethazine, epithilone B, an investigational α-amino amide, a calcium channel antagonist and a matrix metalloprotease [21,24–27].

Process chemical research plays an important role in drug development activities by developing cost-effective, efficient, scaleable synthetic routes to drug candidates for the support of pre-clinical and clinical programs. Regulatory agencies require that these investigational materials be rigorously characterized for purity as well as impurity levels so that drug metabolism, safety and clinical studies may not be jeopardized by compound variability. The identification of impurities in drug substances by LC–MS is a common practice, and in many ways more difficult than metabolite identification in drug metabolism studies. The human body has limits on the types of metabolic transformations it can perform which translates to a smaller array of potential metabolites such as oxidation, sulfonation, glucuronidation and demethylation products to name a few. Multi-step syntheses, on the other hand, can generate a wider array of potential side products, since side products generated at any step in a process can carry forward or be modified in subsequent steps. This in turn makes interpretation of LC–MS and MS/MS impurity profiles of drug substances challenging.

This study describes the use of D_2O and deuterated acids and salts as mobile phase additives in RPLC in conjunction with API-MS and MS/MS as an enhancement for structural elucidation of impurities in a wide array of drug study compounds in support of process chemical development.

2. Experimental

2.1. Chemicals

HPLC solvents acetonitrile and water were Optima grade and were purchased from Fisher Scientific (Bridgewater, NJ. USA). Trifluoroacetic acid, formic acid, ammonium acetate, trifluoroacetic acid-d (99.5 atom.%), acetic-d_3 acid-d, (99.9 atom.%), ammonium-d_4 acetate-d_3 (99 atom.%) and deuterium oxide (99.9 atom.%) were from Aldrich (Milwaukee, WI, USA). Investigational compounds were supplied by the Process Research Department of Merck Research Laboratories.

2.2. Equipment and operating conditions

Chromatographic separations were performed using a Hewlett-Packard model 1100 HPLC equipped with a photodiode array detector. The injection volume was 25 μL.

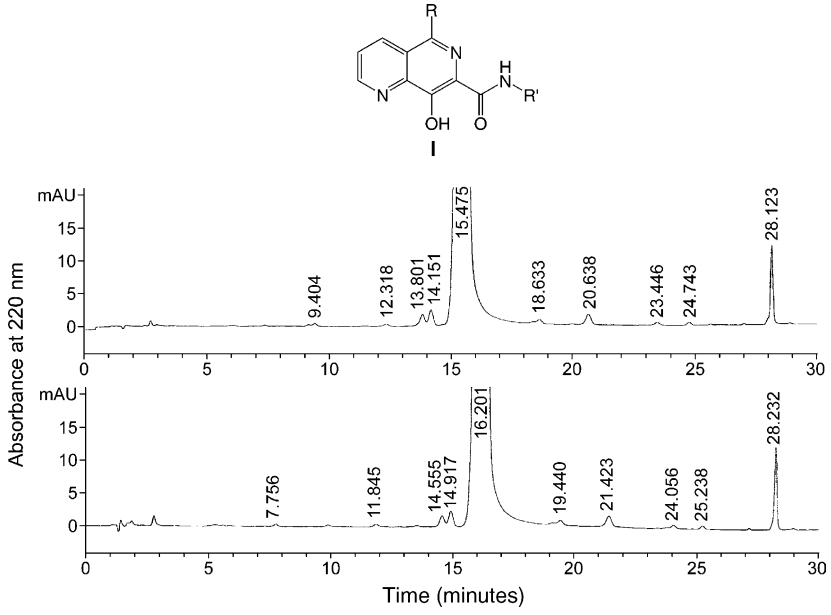

Fig. 1. Chromatograms of the hydroxyamide, **1**, using a protic mobile phase (top) and deuterated mobile phase (bottom). The separations were monitored by UV detection using a wavelength of 220 nm.

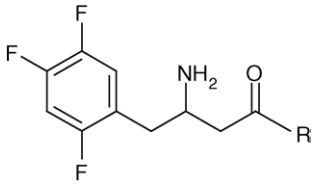

Fig. 2. Structure of the amine, **2**, MW 407, used as a model compound to investigate the rate of H/D exchange within the chromatographic system.

Separations were performed using a flow rate of 1 mL/min. The columns used were either C_8 or C_{18} from various manufacturers, 15 or 25 cm length and 4.6 mm internal diameter. Gradient elution was used in all cases at either ambient or 40 °C. For studies using a protic mobile phase, the aqueous component was 0.02 vol.% acid or 10 mM ammonium acetate and the organic component was 0.02 vol.% acid in acetonitrile or acetonitrile only when using ammonium acetate as the aqueous salt. For studies using deuterium exchange, the deuterated analogs were substituted for the protic components in D_2O or acetonitrile. The separations were monitored at 220 nm.

The mass spectrometer was a Finnigan TSQ 7000 equipped with an electrospray interface connected in series after the diode array detector. The electrospray voltage was 4.5 kV. The sheath and auxilliary gases were nitrogen at 60 psi and 30 units (rotameter), respectively. The interface capillary was operated at 250 °C and the manifold temperature was 70 °C. In full scan mode, the mass ranges were from 0.5 to 2.5 times the mass of the compound of interest at an acquisition time of 2 s/spectrum. In MS/MS mode, argon was used as the collision gas at 2.0 mTorr. The collision energy was adjusted to give 10% relative intensity of the parent ion in the product ion spectrum. The resolution in the first quadrupole, Q_1, was decreased resulting in a parent ion transmission of 4.0 amu FWHH. The product ion spectrum was also acquired at the rate of 2 s/spectrum. The interface capillary and tube lens voltages were optimized by injection of the compound of interest into the protic or deuterated mobile phase carrier. All MS measurements were made in the positive ion mode.

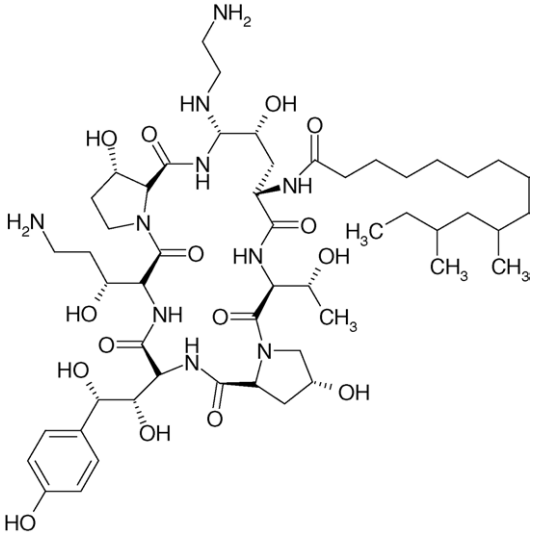

Fig. 3. Structure of caspofungin, **3**, MW 1092.6.

2.3. Sample preparation

Solid samples were dissolved in either 50:50 water:acetonitrile for experiments using protic mobile phases or 50:50 deuterium oxide:acetonitrile for experiments using deuterated mobile phases at a concentration of 1 mg/mL. Samples were injected immediately after dilution. Solution samples were used without any dilution.

3. Results and discussion

3.1. Chromatography

The effects of D_2O upon chromatographic performance, specifically retention and selectivity, has been investigated for the separations of benzene, monodeutero and perdeuterobenzene, alkylbenzenes, aromatics and degradation products of amoxicillin [18,22,28]. D_2O appeared to offer little selectivity advantages for separations of benzene from its deuterated

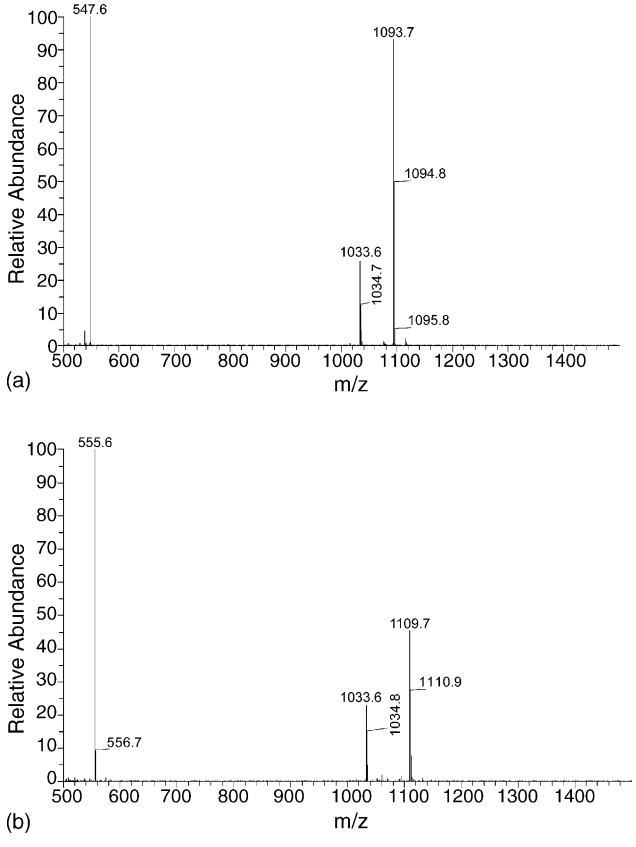

Fig. 4. Full scan LCMS spectra of caspofungin (a) and oxidized impurity (b) observed using a protic mobile phase.

analogs, from alkyl benzenes having aliphatic chain lengths of 1–5 and for amoxicillin degradates. Some slight enhancement in selectivity was noted for less polar aromatic species. This may be attributable to more interaction of the less polar analytes with the stationary phase, since D_2O is slightly more polar than H_2O [29].

The chromatographic performance in protic and deuterated mobile phases of an investigational hydroxyamide, **1**, was compared using TFA and trifluoroacetic acid-d as the acidic additive under gradient elution conditions. The gradient conditions were from 65:35 TFA (0.02%) in H_2O (or 0.02% TFA-d in D_2O):0.02% TFA in acetonitrile (or 0.02% TFA-d in acetonitrile) to 55:45 aqueous:organic in 20 min followed by another gradient ramp to 25:75 aqueous:organic at 30 min. The comparative separations are shown in Fig. 1 and the relevant chromatographic parameters listed in Table 1. The results indicate no appreciable difference in selectivity or efficiency. The only effect of the deuterated mobile phase is increased retention, presumably a result of the increased viscosity of D_2O relative to H_2O. This is consistent with the results of other studies performed in this laboratory where the only discernible difference noted in deuterated systems was an increase in k'.

Table 1
Retention factor, k', and selectivity factor, a, for adjacent components observed in the hydroxyamide, **1**, using protic (H_2O) and deuterated (D_2O) mobile phases

k' H_2O	k' D_2O	α H_2O	α D_2O
7.62	8.06		
7.81	8.31	1.02	1.03
8.62	9.12	1.10	1.10
10.6	11.1	1.23	1.22
11.6	12.3	1.12	1.11
13.6	14.0	1.15	1.14
14.4	14.8	1.06	1.05
15.6	16.6	1.15	1.13

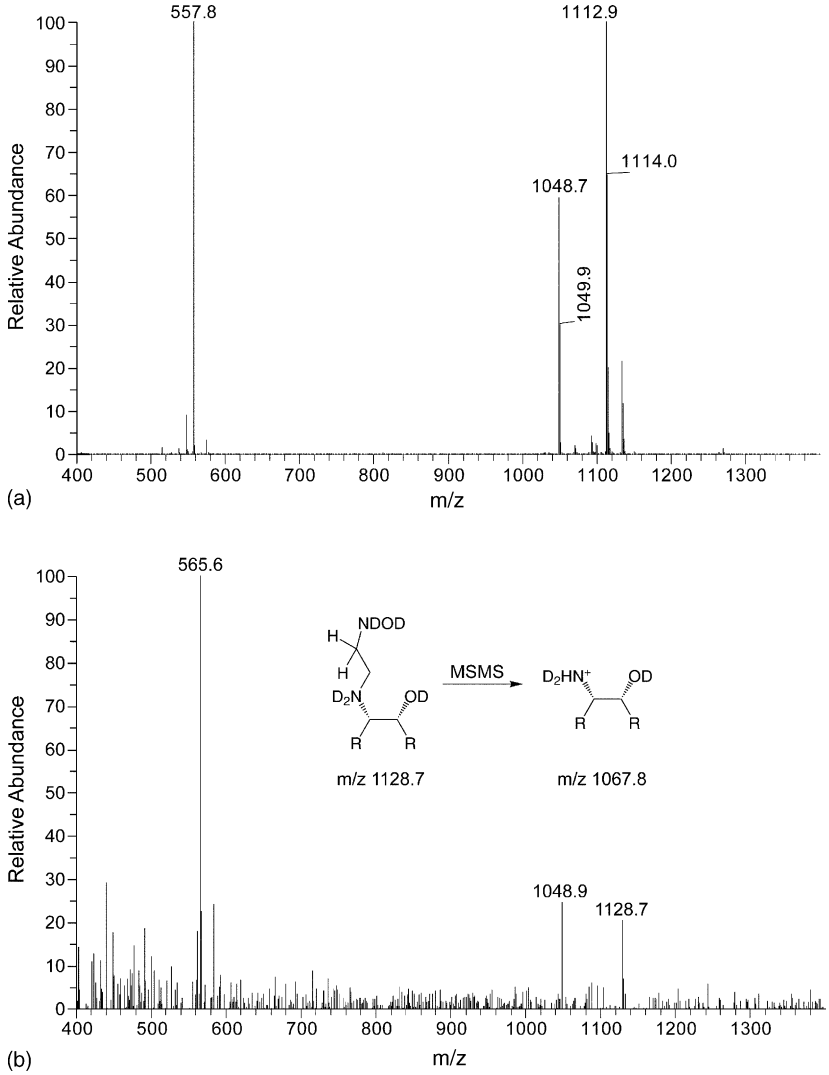

Fig. 5. Full scan LCMS spectra of caspofungin (a) and oxidized impurity (b) observed using a deuterated mobile phase. The inset shows a proposed MSMS fragmentation pathway accounting for a fragment consistent with primary amine oxidation.

3.2. Chromatographic exchange rate

The rate at which deuterium replaces hydrogen within the chromatographic system is dependent not only upon the intrinsic volume and exchange sites within the column but also the associated dead volume prior to the column head. This has been investigated by monitoring the total time required for complete exchange for probe analytes after switching from a protic to deuterated mobile phase [19,22]. Full exchange has been reported as 2.3–2.5 times the system breakthrough volume.

To investigate the rate of exchange the investigational amine, **2**, MW 407, shown in Fig. 2 having two exchangeable protons, was chosen as a probe. In a protic mobile phase of 65:35 water:acetonitrile and a 250 mm × 4.6 mm C_{18} column, the observed retention time of **2**, after dissolution in water, was 3.5 min and the observed *m*/*z* was 408, consistent with $(M + H)^+$. The same result was observed when an equilibrated solution of **2** in D_2O was injected, indicating complete back exchange within the chromatographic system. The column was disconnected from the system, and the system flushed with 65:35 D_2O:acetonitrile for 10 min. The column was reconnected, and the deuterated mobile phase introduced concurrent with an injection of a freshly prepared solution of **2** in D_2O. At the expected retention time of **2**, *m*/*z* 411 was observed with no *m*/*z* 408 noted, consistent with complete exchange of both amino hydrogens as $(M_D + D)^+$. A solution of **2**, freshly prepared in water was injected and *m*/*z* 411 was observed with no *m*/*z* 408 noted. Finally, the column was removed and the system flushed with 65:35 water acetonitrile for 5 min. The column was reattached and an injection of **2** dissolved in D_2O was made. An *m*/*z* of 408 was observed at the expected retention time of **2** with no *m*/*z* 411 observed.

These results show that complete H/D exchange is possible at 1.8 times the column dead volume, which was approximately 2 mL, even in the absence of sample preequilibration in a deuterated solvent, and also that a rapid cycle time from a protic to a deuterated system is possible, providing that external column volume effects are not great.

3.3. Structural elucidation

Caspofungin, **3** (Fig. 3) having a MW of 1092.6, is a member of the class of antifungal agents that inhibits the synthesis of β (1,3)-D-glucan, an integral component of the fungal cell wall. It is a semisyntheric lipopeptide synthesized from a fermentation product of Glarea lozoyensis. During process development of **3**, the identification of an impurity observed under gradient HPLC conditions at 0.1% was required. When using TFA as an additive to both mobile phase components, water and acetonitrile, the impurity was observed at a k' of 2.3.

The observed protonated molecule of the parent, shown in Fig. 4a, under these mobile phase conditions $(M_H + H)^+$ when using electrospray ionization was 1093.7, with other

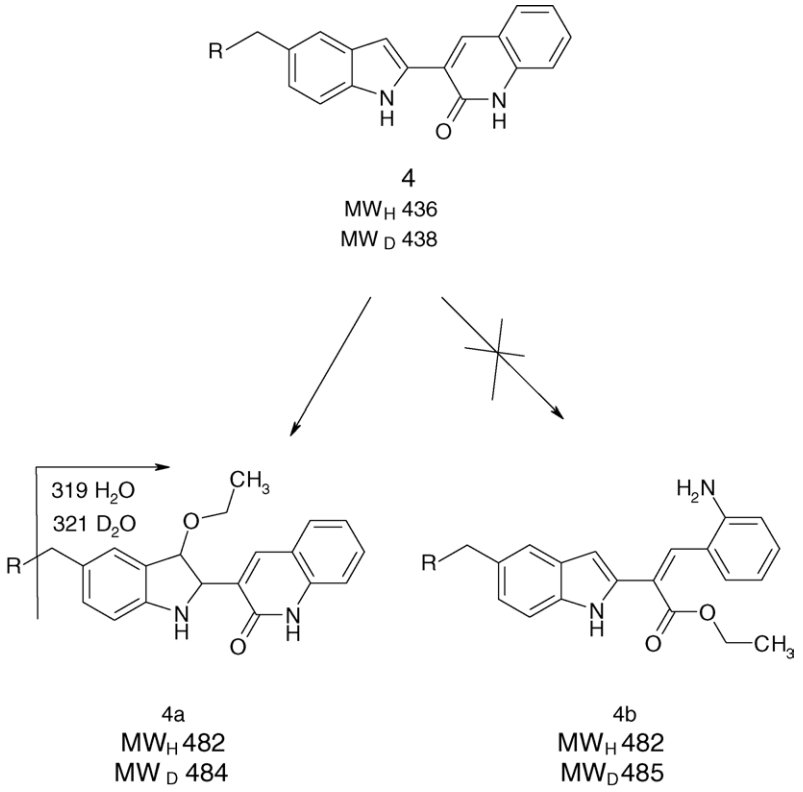

Fig. 6. Structure of the indole, **4**, MW 436, and possible impurities **4a** and **4b** observed after crystallization with aqueous ethanol.

ions observed at *m*/*z* 1033.6 and *m*/*z* 547.6. The *m*/*z* 1033.6 ion was attributed to in-source fragmentation and is indicative of the loss of the ethylene diamine sidechain moiety, while *m*/*z* 547.6 is attributable to the doubly charged parent, $(M_H + 2H^+)/2$ [30]. The observed protonated molecule of the impurity of interest, shown in Fig. 4b, was 1109.7, with ions at *m*/*z* 1033.6 and *m*/*z* 555.6. The presence of the *m*/*z* 1033.6 ion suggests the mass difference lies in the ethylene diamine side chain, and both the protonated molecule, *m*/*z* 1109.7 and doubly charged ion, *m*/*z* 555.6, suggest +16 amu addition of oxygen within the ethylene diamine moiety.

Upon dissolution of **3** in D_2O:acetonitrile and chromatography using 0.02% TFA-d in both D_2O and acetonitrile, Fig. 5a shows that **3** was observed as *m*/*z* 1112.9, $(M_D + D)^+$ indicating complete exchange of all 18 active hydrogens, with a doubly charged ion, $(M_D + 2D^+)/2$, observed at *m*/*z* 557.8. The loss of the deuterated ethylene diamine moiety was observed as *m*/*z* 1048.7, consistent with replacement of 15 active hydrogens within the resulting fragment with deuterium. The impurity of interest eluted at k' of 2.2, and the observed *m*/*z*, shown in Fig. 5b, was found to be 1128.7 with a doubly charged ion at *m*/*z* 565.6 and fragment ion at *m*/*z* 1048.9. This shows the impurity of interest has 18 exchangeable hydrogens and that oxygen addition within the ethylenediamine side chain cannot be at an aliphatic carbon for this kind of addition would show 19 exchangeable hydrogens. Oxygen addition must be at either of the two amine moieties to give a hydroxylamine analog and further MS/MS experiments on the deuterated impurity gave a key fragment at *m*/*z* 1067.8. The proposed mechanism accounting for this fragment, shown in the inset of Fig. 5b, is addition of D to the secondary nitrogen followed by a 1,3 H migration with loss of CH_2=CHNDOD. This supports the structure of the impurity having oxygen addition at the pri-

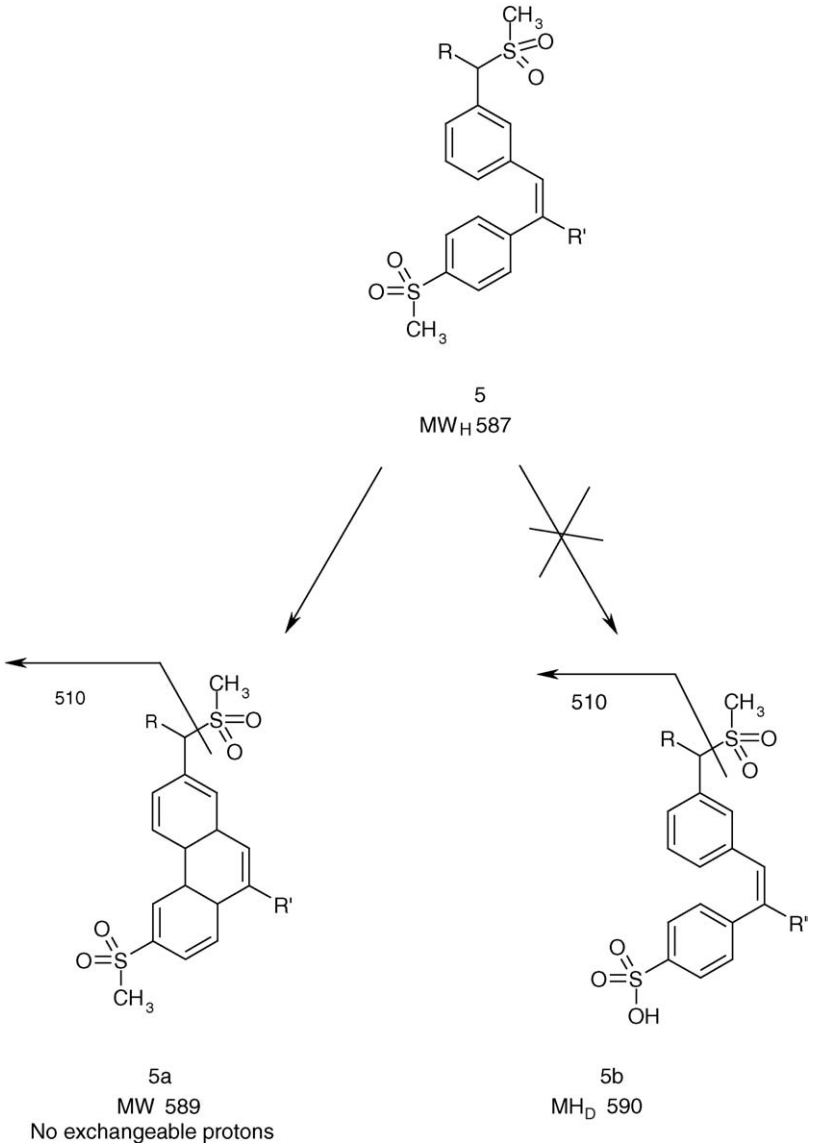

Fig. 7. Structure of the sulfone, **5**, MW 587 and possible sulfone **5a**, and sulfonic acid **5b**, analog impurities having MW_H 589.

mary amino functionality. Careful elimination of sources of oxidation during processing inhibited formation of the impurity.

The purification procedure in the isolation of a pharmaceutical intermediate or drug substance typically involves crystallization. The hope is for a high yield of the desired compound with minimal amounts of undesired impurities. In the development of an isolation procedure for the indole, **4**, MW 436 (Fig. 6) an impurity was observed at the 0.3% level using a mixture of ethanol and water as a crystallization solvent. The observed molecular weight, 482 amu, obtained from full scan LC–MS experiments in a protic mobile phase system suggested the ethanolysis product, **4b**, as the impurity. However, full scan LC–MS experiments using a deuterated mobile phase with ammonium-d_4 acetate-d_3 showed a molecular weight as a result of exchange of 484 and MS/MS data of a key fragment showed *m*/*z* 321 for the deuterated compound versus *m*/*z* 319 for the proteo compound, suggesting two exchangeable hydrogens in the unknown. The ethanolysis proposal on the basis of full scan experiments using a protic mobile phase only is inconsistent with the deuterated experiments as 3 exchanges would be expected. An alternative structure involving ethanol addition to the indole, compound **4a**, was consistent with the full scan and product ion experiments.

An early eluting impurity at 0.2% observed in compound **5**, MW 587 (Fig. 7) a sulfone entering safety studies, was investigated using a combination of LC–MS and LC–MS/MS with protic solvents and LCMS with deuterated solvents in order to elucidate a structure. The impurity was found to have a MW of 589, an increase of +2 amu relative to the parent. The observed retention time which was less than that of the parent, **5**, taken together with an MS/MS experiment generating a major fragment at *m*/*z* 510, and process chemistry, suggested a sulfonic acid analog of the parent, **5**b, where the methyl sulfone is replaced by a sulfonic acid, thus giving the correct mass differential. However, upon performing the separation in either acetic-d_3 acid-d or ammonium-d_4 acetate-d_3 with D_2O, the observed $(MW + D)^+$ was *m*/*z* 591 and not *m*/*z* 592, expected for **5**b. An alternate structure shown in Fig. 7 was proposed, the ring closed compound **5**a, which was subsequently confirmed by ^1H NMR. This example shows that the lack of exchange can help in compound identification.

An impurity observed in a process development sample at 0.l% in the amine **2** (Fig. 2) warranted investigation since the impurity was not observed in previous batches of material used in pre clinical development studies. The observed MW of the impurity was found to be 421 amu using full scan MS. The difference of 14 amu relative to the parent molecular weight of 407 initially suggested an oxidation within the parent had occurred. However, the observed retention of the impurity at a k' greater than that of the parent was inconsistent with an oxidation, since oxidation would give rise to a more polar compound with an expected retention time less than that of the parent. An alternative possibility to account for the mass increase is methylation. The results of an

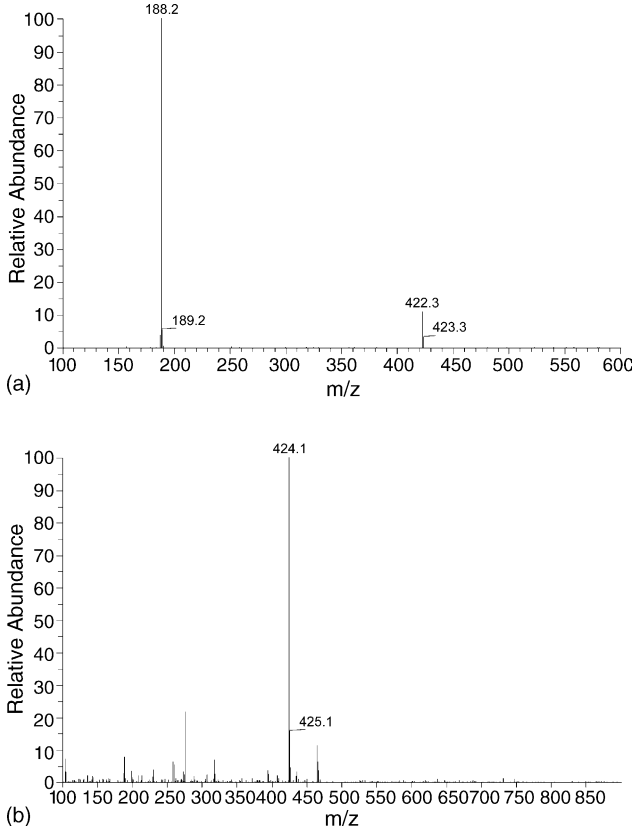

Fig. 8. Product ion (MSMS) spectrum using a protic mobile phase (a) and full scan MS spectrum using a deuterated mobile phase (b) of the impurity **6b**, MW 421, observed in the amine, **2**.

LC–MS/MS experiment for the impurity of interest, shown in Fig. 8a, performed with a protic mobile phase was not conclusive to establish oxidation or methylation assignments, since the key diagnostic fragment, *m*/*z* 188, is consistent with either possibility. However, a full scan spectrum of an LC–MS experiment using a deuterated mobile phase with trifluoroacetic acid-d, shown in Fig. 8b, gave a $(MW_d + D)^+$ of 424. This indicated a single hydrogen available for exchange and established unequivocally that the correct assignment of the impurity, shown in Fig. 9, is the *N*-methyl analog of the parent, **6**b, and not the oxidation product, **6**a, which would have given a MW_d of 423.

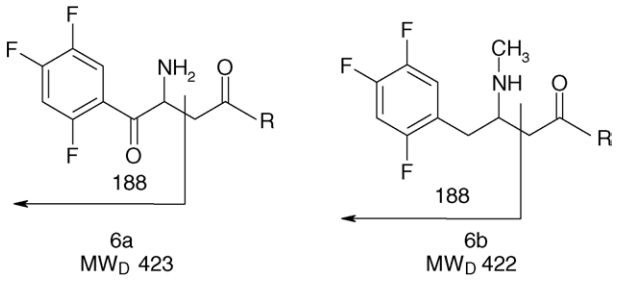

Fig. 9. Possible assigments, the oxidation product **6a** or the *N*-methyl analog, **6b**, of an impurity, MW 421, observed in the amine, **2**.

4. Conclusions

LC–MS using D_2O and deuterated additives following experiments with protic mobile phases is a facile approach for impurity identification in support of pharmaceutical process developmental efforts. For the wide variety of compounds used in this study, the chromatographic performance was similar to those obtained with protic solvents. Changeover from protic to deuterated mobile phases did not require an inordinate amount of time, typically about 15 min was required. The combined approach allowed for the initial generation of reasonable possibilities, then subsequent arrival at the correct structure by elimination of possibilities inconsistent with the exchange data.

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