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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 320–329
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Review

On-line H/D exchange LC–MS strategy for structural elucidation of pharmaceutical impurities

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Received 20 October 2006; received in revised form 8 January 2007; accepted 9 January 2007
Available online 19 January 2007

Abstract

Structural elucidation of pharmaceutical impurities in drug substances and drug products is an important task in pharmaceutical analysis in various phases of drug development. Liquid chromatography–mass spectrometry (LC–MS) technologies play a key role in this task owing to their general attributes of superior selectivity, sensitivity and speed. Full scan and product ion scan analysis, providing molecular weight information and fragmentation data, respectively, offer rich structural information and allow proposal of candidate structures rather quickly. However, these proposed structures often lack certainty especially when dealing with structural isomers. On-line hydrogen/deuterium (H/D) exchange by LC–MS using D_2O as the mobile phase component is a powerful tool for identifying active hydrogen atoms, thus constituting a simple strategy for distinguishing between isomeric structures which are sometimes difficult by product ion spectral data or accurate mass data. This review describes the typical experimental setup we use routinely in the laboratories for performing H/D exchange LC–MS experiments in conjunction with representative applications of the strategy in structural elucidation of pharmaceutical impurities.

Keywords: LC–MS; Mass spectrometry; Structural elucidation; On-line (or on-column) H/D exchange; Active hydrogen; Exchangeable hydrogen; Deuterated mobile phase; Deuterium oxide; Pharmaceutical impurities

Contents

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doi:10.1016/j.jpba.2007.01.019

1. Introduction

Pharmaceutical impurities consist of reaction by-products generated during synthesis of drug substances (namely, active pharmaceutical ingredients) and degradation products formed during formulation manufacturing process and/or storage of drug substances or formulated products. Pharmaceutical impurities, also referred to as 'related substances', can often have pharmacological or toxicological relevance. Therefore, the presence of such impurities and their levels in drug products are indicative of product quality which can impose a risk to patient safety. Although these impurities are usually present at very low levels, it is imperative to characterize their identities in order to make proactive decisions with respect to synthetic routes optimization and formulation development. Early understanding of the nature and mechanism of impurity formation enables scientists to incorporate a control strategy into the manufacturing process.

Liquid chromatography–mass spectrometry (LC–MS) technologies have become primary tools for identification of low-level pharmaceutical impurities in drug substances and drug products [1,2]. The attributes that have made them the methods of choice for such tasks are their superior sensitivity, selectivity and speed. Mass spectrometry coupled with modern high performance liquid chromatography (HPLC) allows trace components in complex mixtures to be studied directly with no prior preparative purification or fractionation to enrich the impurities. More importantly, automation such as data-dependant multi-stage MS analysis (MS^n) allows comprehensive structural information of multiple impurity peaks to be collected 'on-the-fly' during chromatographic separation [3] despite their structural similarity to the main component. From product ion scan data, tentative structures can be proposed rather quickly. With the impurity structures and their formation mechanisms understood, chemists can then make real time decisions to modify reactions in an effort to control impurity levels in the final drug substances if they are synthetic impurities or formulators can design a process to prevent breakdown of drug molecules if the impurities are degradation products.

Pharmaceutical compounds usually contain one or more exchangeable hydrogen atoms and determination of such exchangeable hydrogens facilitates structural elucidation. Conventionally, hydrogen/deuterium (H/D) exchange experiments are performed by infusion where generally a pure sample is required. With the strategy of using deuterium oxide as the LC–MS mobile phase, H/D exchange experiments can be performed on-line or on-column. Thus, H/D exchange can be performed directly on mixtures, and the labor-intensive purification of individual components is not required. Prior to the advent of atmospheric pressure ionization (API) LC–MS, on-column H/D exchange using deuterium oxide as the LC–MS mobile phase had been demonstrated with various ionization techniques including chemical ionization (CI), thermospray, and fast atom bombardment (FAB) [3]. However, these ionization modes are no longer the method of choice for pharmaceutical analysis due to their technical limitations, in particular their poor performance when interfaced with regular flow reversed-phase liquid chromatography. During the past decade, API techniques including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have become the primary tool for pharmaceutical analysis. By using ESI interface, the application of deuterium oxide as a chromatographic LC–MS mobile phase for conducting H/D exchange experiments has recently been demonstrated for a number of drug metabolism studies [4–7] as well as for identification of pharmaceutical impurities [8–10]. The intention of this manuscript is to provide an overview of the premise of on-line H/D exchange LC–MS experimental setup and its applications in identification of pharmaceutical impurities including both literature data and the data collected in the authors' laboratories.

2. Functional groups containing exchangeable hydrogen atoms

'Exchangeable' hydrogen atoms, also called 'active,' 'acidic,' 'labile,' or 'mobile' hydrogens, are usually those hydrogen atoms attached to heteroatoms such as oxygen (O), nitrogen (N), and sulfur (S). Some commonly encountered functional groups containing exchangeable hydrogens are summarized in Table 1. It is worth mentioning that it is not uncommon for an individual pharmaceutical compound to contain more than one type of functional group possessing exchangeable hydrogen atoms. Furthermore, some hydrogen atoms attached to the carbon (C) of an active methylene or methyne group may also undergo exchange with deuterium if they exhibit sufficient acidity (typically those adjacent to strong electron-withdrawing atoms and functional groups). On the other hand, if an active H in a structure forms a strong intramolecular H-bond, it may be only partially exchangeable. Caution should be taken when interpreting H/D exchange data for these types of structures since they may generate "overly" exchanged or "under" exchanged MS spectra that complicate the isotopic distribution patterns.

When organic molecules are exposed to D_2O, heteroatom-bonded labile hydrogens (H) will be replaced by deuterium (D) in the presence of a high concentration of D. This can be described as $R–XH + D_2O \rightarrow R–XD + DOH$ (where R: partial organic molecule structure; X: N, O, or S; H: hydrogen; D: deuterium). When n labile hydrogens of a given molecule are exchanged with n deuterium atoms, the molecular mass increases by n since deuterium has a nominal mass of 2 Da (Dalton), one Da higher than hydrogen. In other words, the molecular mass increase corresponds to the number of exchangeable

Table 1
Functional groups containing exchangeable hydrogen atoms

Functional groups	Examples
Hydroxyl (—OH)	Alcohol (R—OH), glycol, phenol (Ar—OH), hydroxylamine (N—OH), hemiacetal, etc.
Amine (—NH or $—NH_2$)	Amine, amide, imine, amidine, urea, thiourea, sulfonamide, etc.
Thiol (—SH)	R—SH
Acid	R—COOH, $R—SO_nH$ ($n = 1$, 2, or 3), $R—PO_3H$, hydroxamic acid, etc.

R: either aliphatic or aromatic side chains; Ar: aromatic.

hydrogen atoms in the structure and this resulting mass increase can be readily measured by mass spectrometry. Therefore, the number of exchangeable hydrogens can be obtained by determining the molecular mass before and after H/D exchange using a mass spectrometer. Protonation of the analyte generates an $[M_H + H]^+$ ion in the positive ion mode; therefore, if D_2O is used as the mobile phase the *m*/*z* increase is one unit greater than the number of exchangeable hydrogen atoms in the neutral molecule since $[M_D + D]^+$ ions are formed (the additional mass unit is contributed by the deuteron charge). In the negative ion mode, on the other hand, the use of D_2O leads to an *m*/*z* increase that is one unit less than the number of exchangeable hydrogen atoms in the neutral molecule since $[M_D - D]^-$ ions are formed. Conventionally, H/D exchange experiments are performed using pure samples. By using LC–MS, however, H/D exchange experiments can be carried out on-line; mixtures (i.e., impure samples) can be directly analyzed without prior purification. This offers a major opportunity for wider usage of the H/D exchange technique for aiding structural elucidation.

3. On-line H/D exchange LC–MS experimental setup

With respect to the setup of on-line H/D exchange experiments, two types of configurations have been demonstrated in recent literature. The first setup uses 'post-column addition' of deuterium oxide. In this configuration, D_2O can be introduced post-column as sheath liquid [11,12], via a Tee connector (into the LC effluent) using an infusion pump [13,14], or using a dual-sprayer source as demonstrated by Wolff and Laures recently [15]. Due to the presence of high level of H, post-column addition has the shortcoming of generating incomplete exchange mass spectra that are usually more complex to interpret. One strategy to increase the completeness of exchange is to lower the LC effluent post-column by splitting in order to increase the D_2O/H_2O ratio. One caveat of this setup, however, is that it often compromises the detection sensitivity due to splitting [13] and/or dilution. For these reasons, post-column addition setup may offer limited applications and is used primarily when low consumption of D_2O is crucial. One useful application of such a setup, however, is for normal phase method where D_2O-containing mobile phase is generally not employed (see Section 7).

The second setup of on-line H/D exchange LC–MS directly uses deuterium oxide as the LC–MS mobile phase component, which can be referred to as "on-column" H/D exchange. On-column H/D exchange is advantageous since it generally produces completely-exchanged mass spectra. The current discussion focuses primarily on ESI LC–MS, a soft ionization technique that is a preferred choice for structural analysis. The use of deuterium oxide as a mobile phase component for ESI LC–MS takes advantage of a dynamic H/D exchange reaction on-column, leading to a high yield of fully deuterated compound owing to adequate mixing time of the analytes with the deuterated mobile phase. The early use of deuterium oxide as a mobile phase for ESI LC–MS was demonstrated by Karlsson using a microbore column with μL per min flow rates [14]. The use of D_2O as the mobile phase for regular HPLC at a flow rate 0.2–1.0 mL/min has been demonstrated in several recent publications [4–10].

In our laboratories, on-column H/D exchange experiment is set up on a typical LC–MS system using three mobile phase reservoirs, A_1, A_2 and B_1. Two aqueous mobile reservoirs, A_1 and A_2, are filled with H_2O- and D_2O-containing mobile phases, respectively. HPLC grade H_2O and commercially available D_2O of >99% purity are used as the aqueous mobile phase either directly or with the addition of modifiers such as 0.01–0.1% trifluoroacetic acid (TFA), acetic acid, or formic acid, or 5–20 mM ammonium formate or acetate. It is worth mentioning that adding high concentrations of protonated modifiers such as ammonium formate or acetate may affect the completeness of exchange of labile hydrogens, especially when there are increased numbers of exchangeable hydrogens present in the structures. It is the authors' experience that 0.05% TFA serves as a good choice among acidic modifiers in terms of maintaining the chromatographic integrity and/or ionization efficiency in the positive ion mode if a pure D_2O/ACN (acetonitrile) system is inadequate. One severe drawback of adding TFA, however, is its limitation for use in the negative ion mode. In such cases, acetic or formic acids are a preferable choice. The single organic mobile phase reservoir B_1 is typically filled with ACN either used directly or with a low level of the same modifier present in the aqueous mobile phase. Acetonitrile is an ideal choice as the organic phase since it lacks active hydrogens that can compromise the completeness of H/D exchange. With this instrumental setup, both regular LC–MS analysis using a gradient of A_1 and B_1 and on-column H/D exchange using a gradient of A_2 and B_1, can be run consecutively (sequence setup) with no need to physically switch the aqueous solvent line between the H_2O and D_2O supplies. The change of mobile phase from regular H_2O to D_2O can be accomplished rapidly with a single intervening blank injection. Otherwise, replacing the H_2O reservoir with a D_2O reservoir and using the same solvent line usually require purging of the tubing, which would increase the consumption of D_2O. Therefore, a versatile LC–MS run sequence can be set up in such a way that the first part of the sequence involves regular analysis using A_1/B_1 gradient elution, and the second part of the sequence involves the H/D exchange experiment employing the A_2/B_1 gradient. Comparable chromatograms are usually generated owing to the use of the same gradient and instrument setup for both methods differing only in aqueous mobile phase composition (H_2O versus D_2O). The mass spectrometric source parameters should also be the same for both methods. When fragmentation data (product ion spectra) are desired, the mass spectrometer can be set up in such a way that mass spectra are collected in a "data-dependent" fashion. Readers should refer to relevant literature for details on this subject.

4. Differentiate isobaric structural isomers by on-line H/D exchange LC–MS

One of the major challenges during chemical synthesis is to minimize the many undesired reaction by-products. Due to the complex nature of chemical reactions involving catalysts, multiple reagents and solvents, and starting materials with var-

ious impurities present, structures of pharmaceutical impurities can be difficult to predict. Regular LC–MS analysis generating molecular weight information is usually insufficient to assign specific structures and often alternative isobaric structural isomers can be proposed. One attractive application arising from determination of the number of exchangeable hydrogens using on-line H/D exchange LC–MS is to differentiate between such structural isomers, which serves as a complementary tool to tandem MS analysis and accurate mass measurement.

4.1. Distinguishing between oxidation (ketone) and methylation structures

Oxidation is one of the most commonly seen undesired side-reactions and methylation is another occurring when methyl donors are present either as a solvent or reaction by-product. Oxidation to a ketone or methylation will both result in an increase of 14 Da in molecular weight (m_w). During the synthesis of **1** (Table 2), an impurity with an *m/z* of 14 higher than that of **1** was present at a level as high as 2%, and it could not be removed by re-crystallization. Therefore, characterization of its structure by LC–MS was requested in order to understand the nature of its formation. The desired product **1** gave a protonated molecule $[M+H]^+$ at *m/z* 433 ($m_w = 432$), while the impurity gave an *m/z* of 447 ($m_w = 446$) (Table 2; Fig. 1(a)). Based upon the molecular weight information (+ 14 Da) and taking into consideration that methylation was performed in the previous reaction, one of the plausible proposals for the impurity structure is a methylation structure, **1b** (Table 2). However, modification of the reaction accordingly in order to minimize the methyl donor could not eliminate the impurity. Therefore, on-column H/D exchange LC–MS was performed for structural elucidation of this impurity. When analyzed in D_2O mobile phase, the deuterated molecule $[M_D + D]^+$ of the impurity was detected at *m/z* 448 (Fig. 1(b)), which was 1 higher than that obtained in regular H_2O, indicating no exchangeable H was present in the neutral molecule. If the quaternary methyl structure were correct, the molecular ion would remain unchanged since it is already in an ionic form. Therefore, an oxidation structure **1a** was proposed as shown in Table 2. Unambiguous identification of this impurity was critical for the chemist in order to understand that oxygen was the root cause of the impurity formation. Based on this finding, exclusion of oxygen from the reaction vessel was adopted as an appropriate measure to successfully control the impurity level in the final product.

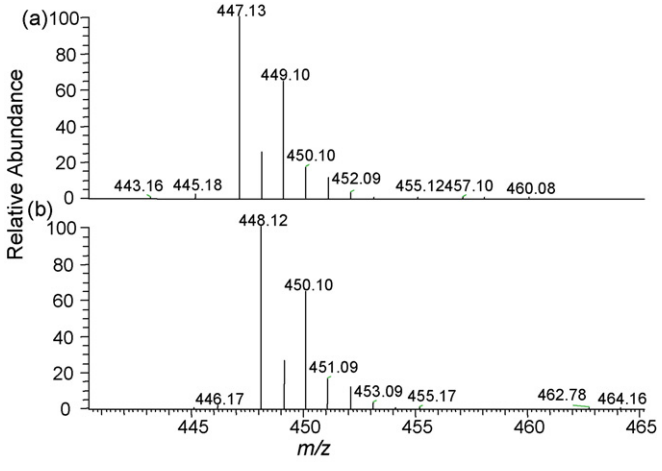


Fig. 1. Full scan MS spectra of impurity **1a** in (a) H_2O and (b) D_2O in ESI positive ion mode.

A similar case was demonstrated by Novak et al. [10]. A process impurity of an amine, compound **2**, gave an *m/z* of 422 ($m_w = 421$), which was 14 higher than that of **2** (*m/z* 408). Similarly, two possible structures could be postulated, methylation or oxidation forming a ketone, respectively (Table 2). Addition of 14 was associated with the same fragment ion, thus, MS/MS experiment performed in the regular H_2O mobile phase could not distinguish between the two proposed structures. A full scan on-line H/D exchange LC–MS experiment using D_2O mobile phase afforded $[M_D + D]^+$ at *m/z* 424 (an increase of 2 from *m/z* 422), suggesting only one hydrogen available for exchange in the neutral molecule. (*Note*: for consistency, the number of exchangeable hydrogens in this manuscript refers to those on the neutral molecules; therefore the protonated molecules should have one extra active hydrogen if the analysis is performed in the positive ion mode.) This allowed the unequivocal establishment of the methylation structure **2a**. The oxidation structure **2b**, on the other hand, would give an *m/z* of 425, an increase of 3 from *m/z* 422, in corresponding to the presence of an additional exchangeable H.

4.2. Distinguishing between N- (or S-) oxidation and C-hydroxylation structures

As in drug metabolism where oxidation is one of the most frequently encountered biotransformation reactions, drug substances are susceptible to oxidative degradation during the manufacturing process and storage. Pharmaceutical molecules containing heteroatoms such as nitrogen or sulfur atoms are often susceptible to oxidation, leading to the formation of N-oxides and S-oxides, respectively. However, both hydroxylation and N-oxidation (or S-oxidation) give rise to structures with molecular weights increased by 16 Da and this 16 mass unit increase is often associated with the same fragments in the product ion spectrum. Determination of the number of exchangeable hydrogens is a convenient methodology to distinguish between these two types of oxidation products as has been demonstrated in a number of drug metabolism studies recently [4,5,14]. Using imipramine (**3**) as an example, imipramine N-oxide (**3a**) could be a metabolite [13] or a degradation product of imipramine (Table 2). An on-line H/D exchange LC–MS experiment would allow facile differentiation between the N-oxide structure (**3a**) and the hydroxylation structure, 10-hydroxyimipramine (**3b**). Both N-oxidation (**3a**) and hydroxylation (**3b**) products would give an *m/z* of 297 ($m_w = 296$) by LC–MS analysis when regular H_2O was used as the mobile phase. When analyzed in D_2O mobile phase, however, the N-oxide (**3a**) would afford an *m/z* of 298 and the hydroxylation structure (**3b**) would give *m/z* 299 since the former has one less exchangeable H.

Table 2
Structures and *m/z* value of protonated or deuterated compounds **1–6**, **10** and their corresponding proposed impurity structures

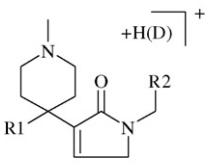
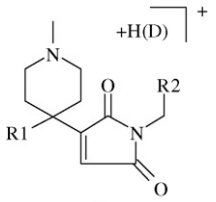
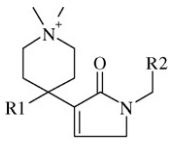
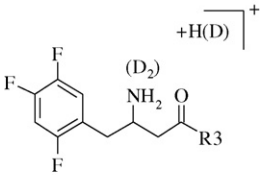
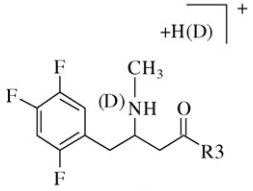
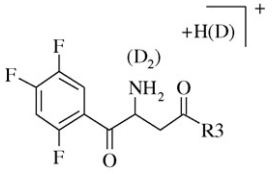
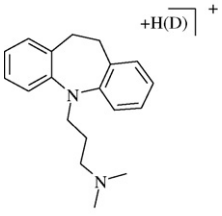
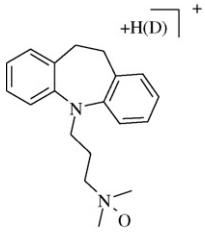
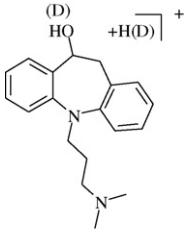
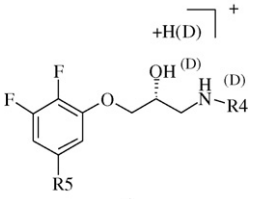
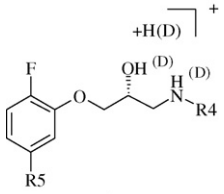
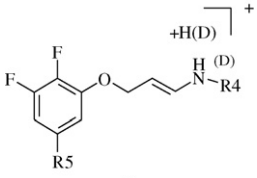
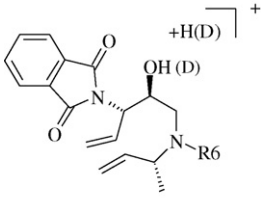
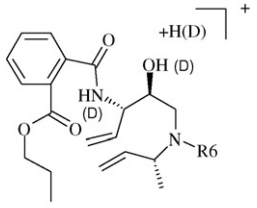
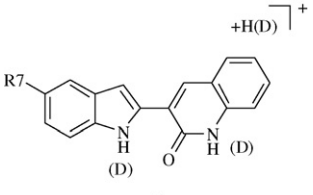
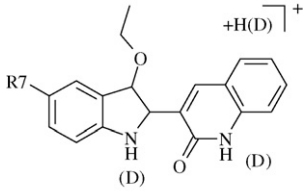
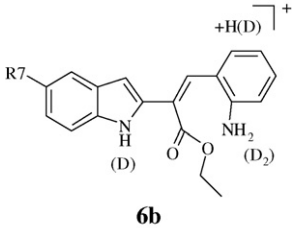
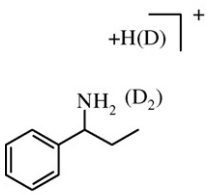
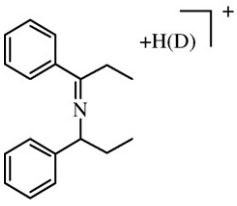
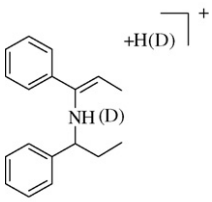
Pharmaceutical compound	Proposed impurity structure	Alternative structure
 **1** *m/z* 433 (434)	 **1a** *m/z* 447 (448)	 **1b** *m/z* 447 (447)
 **2** *m/z* 408 (411)	 **2a** *m/z* 422 (424)	 **2b** *m/z* 422 (425)
 **3** *m/z* 281 (282)	 **3a** *m/z* 297 (298)	 **3b** *m/z* 297 (299)
 **4** *m/z* 476 (479)	 **4a** *m/z* 458 (461)	 **4b** *m/z* 458 (460)
 **5** *m/z* 442 (444)	 **5a** *m/z* 502 (505)	
 **6** *m/z* 437 (440)	 **6a** *m/z* 483 (486)	 **6b** *m/z* 483 (487)

Table 2 (*Continued*)

Pharmaceutical compound	Proposed impurity structure	Alternative structure
 **10** *m/z* 136 (139)	 **10a** *m/z* 252 (253)	 **10b** *m/z* 252 (254)

The *m/z* values preceding and within the parenthesis represent $[M+H]^+$ and $[M_D+D]^+$, respectively.

4.3. Distinguishing between desfluoro and dehydration structures

An investigational compound, **4** (Table 2), gave an *m/z* of 476 in the MS spectrum (not shown). LC–MS analysis revealed that a low-level process impurity of **4** had an *m/z* of 458 (Fig. 2(a)), 18 less than that of **4**. Based on the structural features of fluorine substitution and a hydroxyl functional group, the 18 mass units could come from either desfluorination or dehydration (elimination of a molecule of H_2O). If available, accurate mass spectrometry could be used to different between the two structural isomers. However, on-line H/D exchange LC–MS is a simpler method especially when accurate mass is not readily available. When the sample was analyzed by LC–MS using D_2O mobile phase, it gave an *m/z* of 461 (Fig. 2(b)) indicating two exchangeable hydrogens in the neutral molecule. As a result, the desfluoro structure **4a** was proven to be a more reasonable structure. Otherwise, if the impurity were **4b**, it would possess one less exchangeable hydrogen atoms and would give an *m/z* of 460 in the H/D exchange MS spectrum.

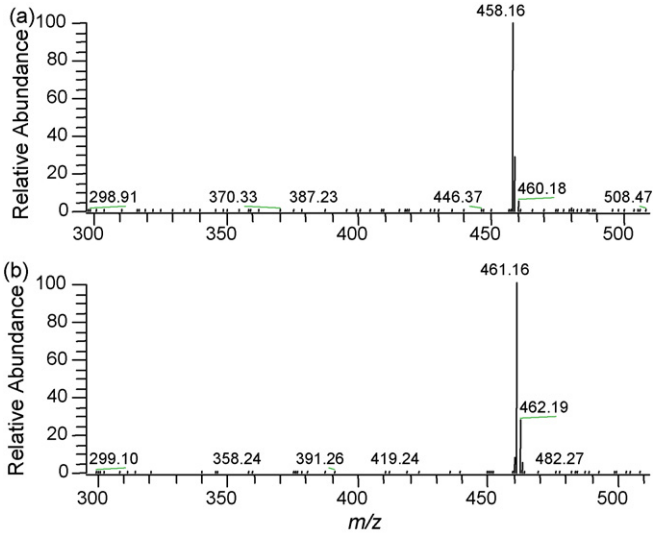


Fig. 2. Full scan MS spectra of impurity **4a** in (a) H_2O and (b) D_2O in ESI positive ion mode.

4.4. Distinguishing between alcoholysis and Michael addition structures

Alcohols are commonly used reaction solvents in organic synthesis. During the route development of compound **5** (Table 2), an impurity was found to have an *m/z* of 502 (Fig. 3(a)), 60 higher than that of desired compound **5** (*m/z* 442). Based on the molecular weight information and the fact that isopropyl alcohol was used as a reaction solvent, an isopropyl adduct was suspected. On-line H/D exchange LC–MS was employed as a convenient tool for determination of the site of modification. When analyzed by LC–MS using the D_2O mobile phase, impurity **5a** gave a $[M_D+D]^+$ at *m/z* 505, in addition to a $[M_D+Na]^+$ at *m/z* 526 (Fig. 3(b)). This suggests that in the neutral molecule of **5a**, there are two exchangeable hydrogens. Based on the fact that an additional exchangeable H was introduced into the impurity structure and addition of 60 Da in molecular weight, the structure based on alcoholysis of the amide bond (cleavage

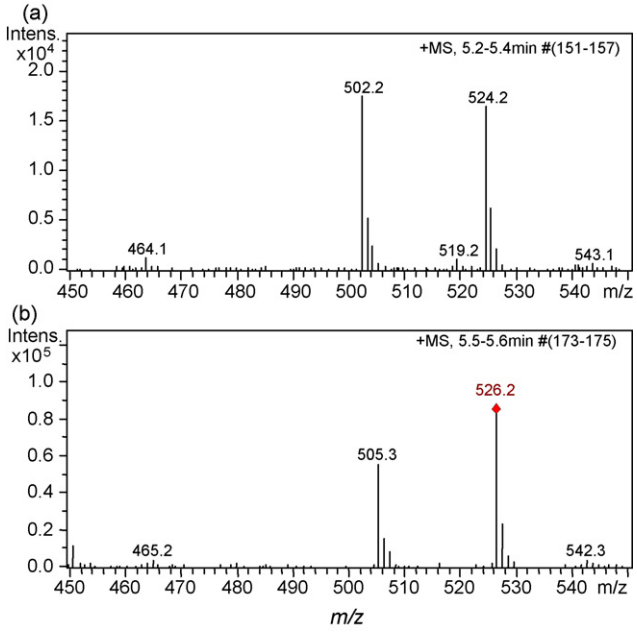


Fig. 3. Full scan MS spectra of impurity **5a** in (a) H_2O and (b) D_2O in ESI positive ion mode.

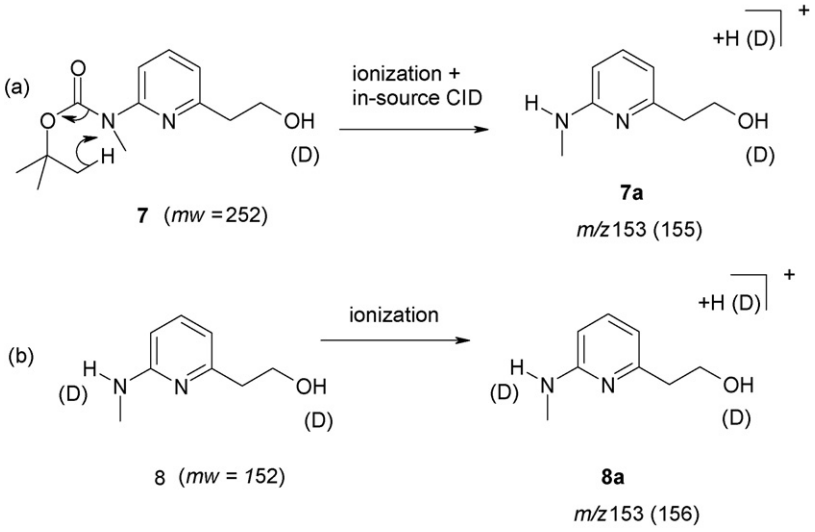


Fig. 4. (a) Ionization and collision-induced in-source fragmentation of **7** containing a *t*-Boc protecting group showing transfer of one H via McLafferty rearrangement in the presence of D_2O and (b) ionization and H/D exchange of the corresponding free amine **8**.

of the phthalamide ring) that gives rise to an isopropyl ester of phthalamidic acid was proposed. Otherwise, if the impurity was generated by Michael addition of an isopropyl alcohol to a carbon-carbon double bond, one would expect to observe one less exchangeable H and detect an *m/z* of 504 instead.

Alcohol solvents are also widely used as crystallization solvents in preparation of drug substances or intermediates, serving as an efficient approach to product purification. Novak et al. [10] reported identification of an ethanol solvent adduct impurity present at the 0.3% level generated during recrystalization of a desired reaction product, **6** (Table 2), when a mixture of ethanol and water was used as the recrystalization solvent. LC–MS analysis of the impurity using regular H_2O mobile phase gave an *m/z* of 483, which was 46 higher than that of **6** (*m/z* 437). Ethanolysis of the lactam leading to the formation of the ethyl ester (**6b**) seems to be a logical proposal as seen in the example above. If this is true, one would expect to detect *m/z* 487 corresponding to 3 exchangeable H in the molecule. The on-line H/D experiment, however, gave an *m/z* of 486 indicating that only 2 active H in its structure. Therefore, the structure resulting from Michael addition of ethanol to the indole ring, **6a**, was proposed, which was supported by the product ion spectral data [10].

4.5. Determining presence or absence of t-Boc protecting groups

The *t*-butyloxycarbonyl (*t*-Boc) protecting group is widely used in organic synthesis of pharmaceutical compounds. This warrants the development of a convenient method for the fast characterization of its absence or presence in reaction mixtures [9]. LC–MS has become the primary method for such mixture analyses. However, *t*-Boc protected compounds, unless further stablilized by forming a Na^+ adduct, readily undergo McLafferty rearrangement in the MS source resulting in the formation of ions with 56 and 100 lower than the protonated molecules of the original compounds. Sometimes in-source fragmentation of *t*-Boc protected compounds is so severe that protonated molecules are absent and only ions corresponding to the molecules without the *t*-Boc moiety are observed. Therefore, the presence or absence of a *t*-Boc group could occasionally produce essentially the same ion in the MS source when regular H_2O mobile phased is used for LC–MS analysis. Wolf et al. [9] reported the use of the on-line H/D exchange LC–MS strategy for fast evaluation of the absence or presence of *t*-Boc. For instance, compound **7** (Fig. 4) contains a *t*-Boc protecting group on the secondary amine and has a m_w of 252, but it gave primarily *m/z* 153 ion $[M+H-100]^+$ (**7a**) in the ESI MS spectrum, in addition to a dehydration fragment ion at *m/z* 135. The $[M+H-100]^+$ ion at *m/z* 153 was indistinguishable from that of the original unprotected amine, **8**, which also gave an *m/z* of 153 when analyzed using regular H_2O mobile phase. On-line H/D exchange LC–MS was then employed to differentiate between the two structures. Upon collision induced in-source fragmentation, elimination of *t*-Boc via rearrangement resulted in the transfer of a hydrogen from a methyl group of the *t*-Boc to the nitrogen (Fig. 4(a)); thus, when analyzed in D_2O mobile phase, the *t*-Boc protection of amine prevented incorporation of a deuterium into the amino group and gave $[M_D+D]^+$ at *m/z* 155. The H of the free amine (**8**), on the other hand, would exchange with D when encountering D_2O in the source and give rise to a $[M_D+D]^+$ at *m/z* 156. Therefore, *t*-Boc protection affords ions 1 lower than that of free amines when D_2O is used as the LC–MS mobile phase. This could be used as a convenient strategy for examining the presence or absence of *t*-Boc protection groups in organic synthesis.

5. H/D exchange LC–MS for probing gas-phase reaction ion structures

The combination of ACN and H_2O is a commonly used solvent system in atmospheric pressure ionization mass spectrometry coupled with reverse-phase HPLC. Nevertheless, it has been shown that gas-phase reduction of ACN in the presence of

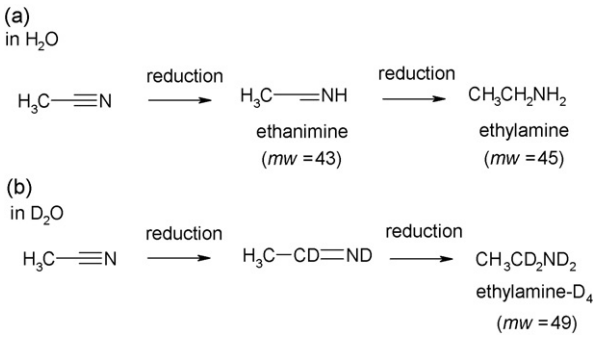


Fig. 5. Gas-phase reduction of acetonitrile in source in ESI positive ion mode generates ethylamine (a) in the presence of H_2O and (b) in the presence of D_2O showing incorporation of 4 D into ethylamine.

water leads to formation of ethanimine and ethylamine (Fig. 5) which could then form adduct ions with analytes of interest giving rise to ethanimine and ethylamine adducts at $[M+44]^+$ and $[M+46]^+$, respectively [16,17]. Data interpretation of these unusual ions may require additional experimental data while H/D exchange methodologies can be applied to confirm these gas-phase reaction products. An investigational compound, **9**, a mono-substituted sulfonamide, has a m_w of 447 thus giving the protonated molecule $[M+H]^+$ at m/z 448 (Fig. 6(a)). In addition, the $[M+Na]^+$ and $[M+K]^+$ ions were detected at m/z 470 and m/z 486, respectively. One unusual adduct ion was observed at m/z 493 $[M+46]^+$, which appeared to be consistent with an ethylamine adduct $[M+CH_3CH_2NH_2+H]^+$. To confirm this hypothesis, an on-line H/D exchange LC–MS experiment was performed using D_2O as the aqueous mobile phase. The ions corresponding to $[M_D+D]^+$, $[M_D+Na]^+$, $[M_D+K]^+$ and $[M_D+CH_3CD_2ND_2+D]^+$ were detected at m/z 450, 471, 487 and 499, respectively (Fig. 6(b)). Based upon the assignment, the unusual adduct ion at m/z 493 appeared to increase by 6 after H/D exchange. This suggests the incorporation of 5 deuterium atoms into the neutral molecule which concurs with the assignment of an ethylamine adduct. The H/D exchange experiment has demonstrated that 4 deuterium atoms from D_2O

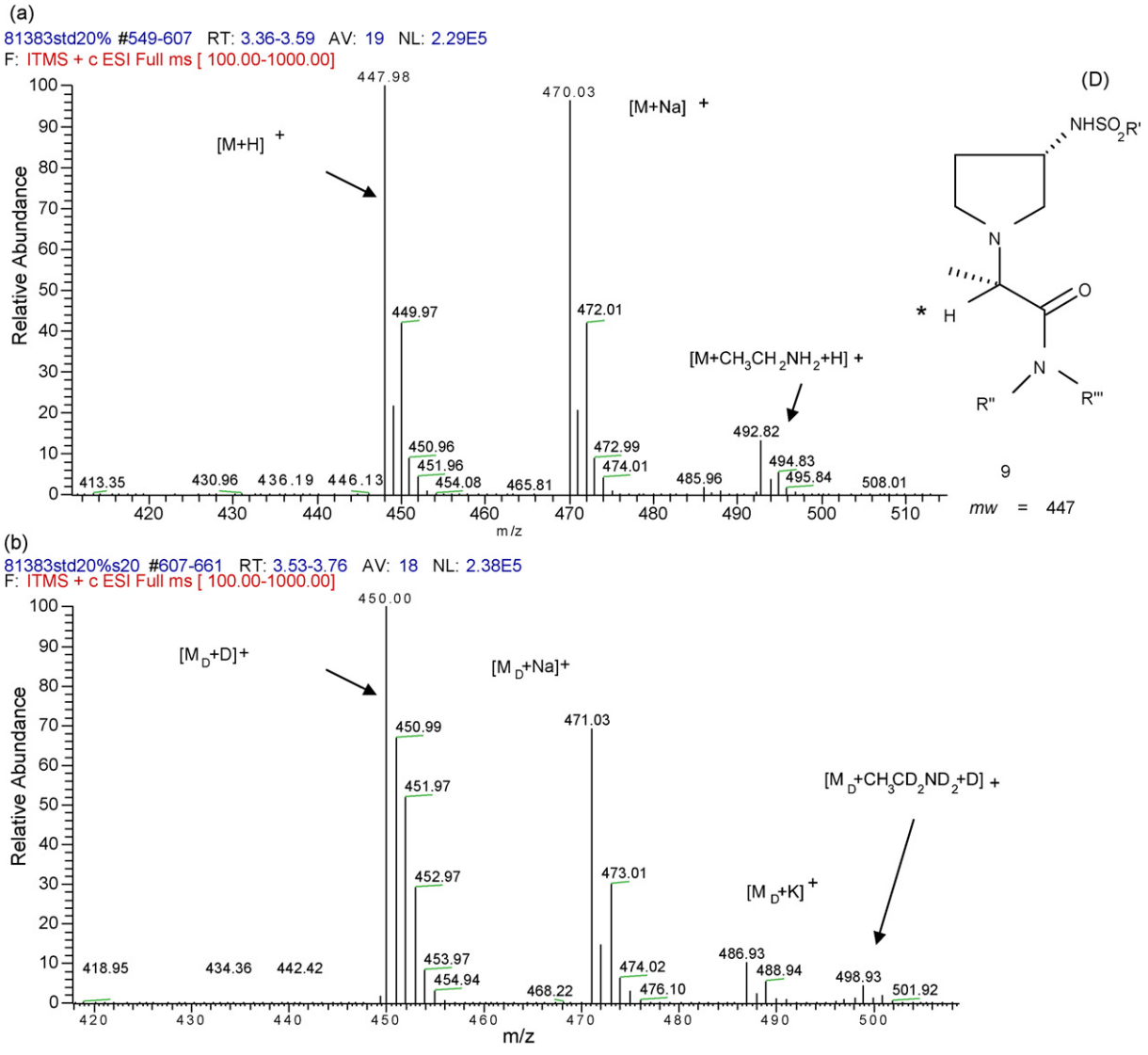


Fig. 6. Partial structure of compound **9** and its full scan MS spectra in (a) H_2O and (b) D_2O in ESI positive ion mode with gas-phase adduct ions assigned. The symbol (*) shows H is acidic which may have contributed to the higher than expected intensity of m/z 451 ion in (b).

were incorporated into acetonitrile and ethylamine-D_4 was generated during the reduction of acetonitrile in the gas phase (Fig. 5(b)). The intensity of the *m/z* 451 ion (Fig. 6(b)) was noticeably higher than expected for C-13 isotopes. This was presumably contributed by the partially exchangeable methyne H indicated with an asterisk in structure **9** (Fig. 6).

6. H/D exchange MS/MS experiment for studying fragmentation mechanisms

Even though accurate mass measurements for establishing the elemental composition and multistage MS^n analysis for assigning the origin of product ions are often adequate for proposing tentative structures and fragmentation mechanisms, H/D exchange MS/MS is another weapon in the arsenal that could help establish definitive assignments. The use of H/D exchange MS/MS data for assigning structures of fragmentation ions and studying fragmentation mechanisms has been demonstrated in literature [18–23]. Vazquez and Truscott [18] reported the use of product ion scans of deuterated kynurenine enabling the elucidation of structural rearrangements that were not evident in the spectra of the native compound. Although the original experiment was performed by dissolving the compound in D_2O followed by infusion into the MS source (since the pure sample was available), an on-line experiment would produce the same results when analyzing impure samples. Infusion of pure sample does offer advantages in terms of flexibility in collecting multi-stage MS^n data of multiple ions when needed. Diaz Sierra, et al. [21] performed H/D exchange MS/MS experiments to acquire additional evidence in support of a proposed fragmentation pathway with respect to dehydration processes that occurred within azaspiracid structures. These compounds have several functional groups that contain exchangeable hydrogens. The deuterated water (D_2O) loss of 20 Da was informative, establishing the fragmentation pathway involving the epoxide formation at the C_{20}–C_{21} diol [21]. Based on the H/D exchange data, it became evident that epoxide formation was the initial step of the fragmentation process of azaspiracids. In short, H/D exchange MS/MS is a useful means for aiding assignment of fragmentation mechanisms of pharmaceutical compounds. It is worth mentioning, however, that caution should be taken when assigning H/D exchange MS/MS data because both D_2O and ND_3 give loss of 20 when D_2O is used as the mobile phase. In such cases, H/D exchange in conjunction with accurate mass measurement would facilitate data interpretation. Also, during collision-induced dissociation, deuterium back-exchange or isotope scrambling may occur adding a degree of complexity to the H/D exchange MS/MS data interpretation [20].

7. Conduct on-line H/D exchange for normal phase HPLC

Normal-phase HPLC, though not widely used, is still sometimes a preferred method for analysis of non-polar pharmaceutical starting materials or intermediates and for characterization of aqueous-labile compounds. In addition, many chiral HPLC methods use normal phase conditions. Since normal phase HPLC methods are usually free of water as a mobile phase (major source of H for deuterium back-exchange), post-column infusion of D_2O is a useful setup for conducting on-line H/D exchange LC–MS, as long as the mobile phases are miscible with D_2O itself. A typical setup is illustrated in Fig. 7 where D_2O or other deuteron carrying solvents were delivered at a constant flow rate by a syringe pump or an LC pump via a Tee joint. The actual optimal flow rate of D_2O would depend upon the HPLC mobile phase composition and would have to be determined experimentally. A flow rate as low as 20 μL per min has been shown to give complete exchange as demonstrated in the example described below. It is anticipated that post-column splitting to reduce LC effluent flow before meeting with deuterated solvents will increase the ratio deuteron/proton and drive the H/D exchange reaction to completion. In case the LC mobile phases are incompatible with D_2O, infusion of an alternate deuterated solvent such as deuterated alcohols CH_3OD or CH_3CH_2OD could be used depending on the nature of the organic mobile phases.

Ethylbenzylamine, compound **10** (Table 2), was a starting material for the synthesis of a pharmaceutical compound. An impurity of **10** was observed by an HPLC–UV method which employed normal phase chromatographic conditions using a YMC Polyamine II column (4.6 mm × 250 mm) with isocratic 100% ACN elution at a flow rate of 1 mL/min. In order to reproduce the same LC chromatogram, the same normal phase conditions were directly adopted for LC–MS analysis. Based on

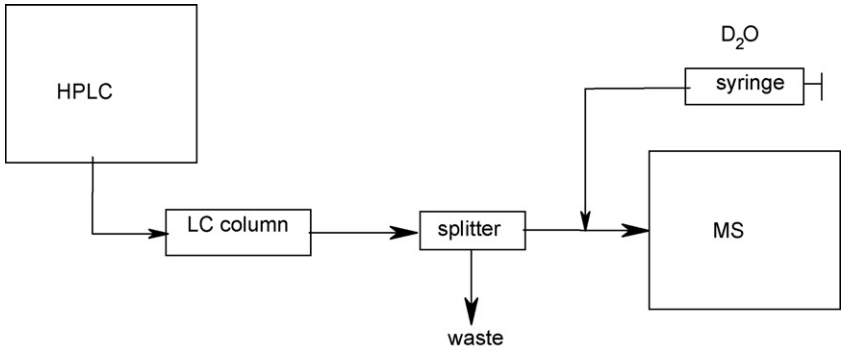


Fig. 7. A schematic diagram showing a typical post-column infusion setup for on-line H/D exchange LC–MS in the absence of aqueous mobile phase (e.g., normal phase HPLC).

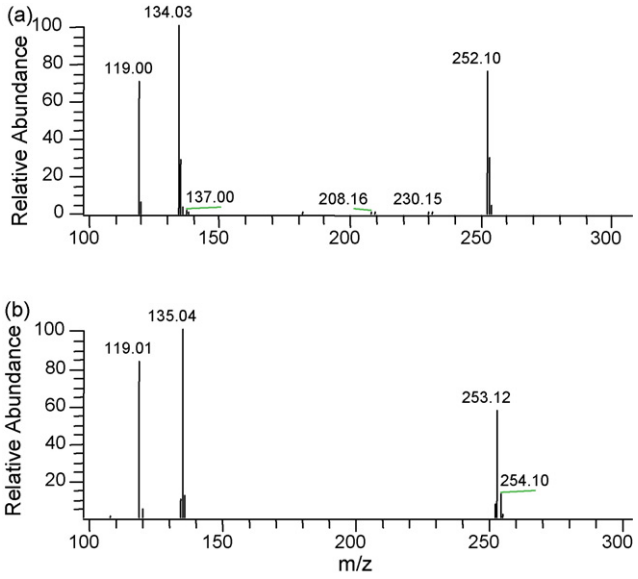


Fig. 8. Full scan MS spectra of impurity **10a** in (a) absence or (b) presence of D_2O introduced by post-column infusion into the normal phase LC effluent.

the LC–MS analysis in the ESI positive ion mode, the impurity peak exhibited a protonated molecule at *m/z* 252 ($m_w = 251$) (Fig. 8(a)). Therefore, dimeric structures could be proposed as shown in Table 2 with uncertainty as to the location of the double bond. In order to determine whether it has the imine structure **10a**, an H/D exchange experiment was performed. Since the existing chromatographic conditions do not use aqueous mobile phases, the post-column infusion setup (described above) was used. It was fortunate that the LC mobile phase was miscible with D_2O. To a 200 μL/min flow resulting from 1:4 split of the total LC effluent at 1 mL/min, 20 μL/min of D_2O was infused via a Peek Tee. Complete exchange was achieved, and the $[M_D + D]^+$ was detected at *m/z* 253 (Fig. 8), indicating no exchangeable H in the impurity molecule. This data allowed structure **10b** which possesses one exchangeable H to be ruled out.

8. Conclusions

LC–MS has become the primary strategy for identification of low-level pharmaceutical impurities in mixture samples generated during synthesis or as a result of degradation of pharmaceutical compounds. By employing D_2O as the LC–MS mobile phase, H/D exchange experiments can be performed on-line unattended. There has been an increasing trend toward using the on-line H/D exchange LC–MS approach for investigating drug metabolites in literature. However, the application of this tool in pharmaceutical analysis has only been recently demonstrated. Determining the number of active H by on-line H/D exchange experiments provides invaluable structural information for identification of unknown impurities in mixtures. Particularly noteworthy is its power to differentiate between isobaric structural isomers as demonstrated in Section 4 above. Nevertheless, it is worth mentioning that attention should be given when an acidic methyne H is present or a structural feature prone to keto-enol isomerization which may be fully or partially exchangeable. In addition, intramolecular H-bonding may prevent complete exchange of such active H obscuring the isotopic profiles. Caution should be exercised when examining H/D exchange data of such structures. When H/D exchange experiments are coupled with product ion analysis, an additional dimension of structural information can be generated to help reveal the nature of gas-phase rearrangement structures that are normally indistinguishable when analyzed in regular H_2O because of their isobaric nature [18]. H/D exchange MS/MS provides a convenient means for elucidation of fragmentation mechanisms in tandem mass spectrometry that is complementary to accurate mass measurement.

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