Thermally Induced N-to-O Rearrangement of tert-N-Oxides in Atmospheric Pressure Chemical Ionization and Atmospheric Pressure Photoionization Mass Spectrometry: Differentiation of N-Oxidation from Hydroxylation and Potential Determination of N-Oxidation Site

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N-Oxides are known to undergo deoxygenation during atmospheric pressure chemical ionization (Ramanathan, R.; Su, A.-D.; Alvarez, N.; Blumenkrantz, N.; Chowdhury, S. K.; Alton, K.; Patrick, J. Anal. Chem. 2000, 72, 1352–1359) resulting from thermal energy activation at the vaporizer of the APCI source. In addition to deoxygenation, tert-N-oxides containing an alkyl or benzyl group on the N-oxide nitrogen also undergo an N–R to O–R rearrangement (Meisenheimer arrangement, where R = alkyl or benzyl), followed by elimination of an aldehyde (or a ketone) through an internal hydrogen transfer. This has been observed under both atmospheric pressure chemical ionization and atmospheric pressure photoionization conditions. These fragment ions were not observed in the product ion spectra from the protonated molecules of the corresponding N-oxides. The elimination of an aldehyde or a ketone, thus, results from thermal energy activation at the vaporizer and is not induced by collisional activation. These fragmentations not only distinguish N-oxides from isomeric hydroxylated metabolites but also provide a potential way to determine the position of N-oxidation when a metabolite (or molecule) contains multiple N-oxidation sites that are in different chemical environments.

The metabolism of a drug by test animals or humans can be extremely complex, may involve multiple enzymatic pathways, and often results in a large number of compounds of varying amount. Oxidation of a tertiary amino group to form an N-oxide is an important biotransformation pathway for many drugs and xenobiotics. N-Oxide metabolites have the same elemental composition as those metabolites resulting from hydroxylation. Differentiation by mass spectrometry is a challenging task because these analytes exhibit the same m/z values for their protonated or deprotonated molecules. Their product ion spectra are usually very similar particularly when the oxygen atom resides in the same fragment ion. N-Oxide metabolites have been shown to undergo thermal deoxygenation during GC/MS analysis. N-Deoxygenation also occurs due to thermal energy activation in the vaporizer of the atmospheric pressure chemical ionization (APCI) source and does not occur during electrospray ionization (ESI), which usually is a softer ionization process unless thermally activated. Furthermore, it has been shown that the deoxygenation of N-oxides does not occur by collisional activation experienced during MS/MS experiments. Deoxygenation of N-oxides during APCI represents a potential way to differentiate N-oxides from hydroxylated metabolites because the latter usually do not undergo thermal deoxygenation. More recently, hydrogen/deuterium (H/D) exchange coupled with mass spectrometry was also used to determine the nature of biotransformation-mediated oxidation in a molecule.

Both H/D exchange mass spectrometry and the simple deoxygenation of N-oxides in APCI can distinguish between N-oxides and hydroxylated metabolite isomers. Since the exact location of the N-oxidation cannot be determined by these experiments alone, further information about the chemical environment of an N-oxide moiety is often required when there is potential for N-oxidation at multiple sites in a molecule.

In this paper, we report findings from additional investigations which examine how the chemical environment surrounding the...
N-oxide moiety can affect the fragmentation following the thermal energy activation in APCI and atmospheric pressure photoionization (APPI). These results are compared to spectra obtained following ESI, an ionization mode that lacks substantial thermal energy activation. The mass spectra from a selection of N-oxides derived from aromatic amines and tertiary amines substituted with alkyl or benzyl groups have been compared. The potential for identifying site(s) of N-oxidation when multiple sites for N-oxidation of different chemical environment are present is discussed.

EXPERIMENTAL SECTION

Chemicals. Clozapine N-oxide, (-)-hyoscyamine N-oxide, 4-(dimethylamino)azobenzene N-oxide, tribenzylamine N-oxide, 2-(methylsulfonylmethyl)pyridine N-oxide, and triethylamine N-oxide (Figure 1) were purchased from Aldrich (St. Louis, MO). Caffeine and verapamil were obtained from Sigma (St. Louis, MO). HPLC grade methanol and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI). Deionized water was processed using a Millipore Milli-Q water purification system (Bedford, MA). Glacial acetic acid was obtained from Fisher Scientific (Fair Lawn, NJ). A methanolic solution of each N-oxide (10 ng/µL) was infused (200 µL/min) with mobile phase consisting of a 1:1 mixture of 10 mM ammonium acetate (pH 5.0 adjusted by acetic acid) and acetonitrile (0.1% acetic acid).

Mass Spectrometry. All mass spectrometry experiments were performed in positive ion mode on a PE Sciex QSTAR Pulsar mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a TurboIonSpray, an APCI, or an APPI source. Nitrogen was used as the nebulizer gas (gas 1), auxiliary gas (gas 2), curtain gas, and collision gas. The parameter settings for various ion optic elements included declustering potential (DP; the potential difference between skimmer and orifice) at 45 V, focusing potential (the potential difference between skimmer and focusing ring) at 240 V, and DP2 (declustering potential 2, the potential difference between Q0 and skimmer) at 20 V. Typical operating conditions of API sources are summarized in Table 1. The calibration of time-of-flight mass analyzer was performed with ions at mass-to-charge ratios (m/z) 195.0876 (protonated caffeine) and 455.2904 (protonated verapamil) from a standard mixture consisting of caffeine (50 ng/µL) and verapamil (1 ng/µL) in methanol/H2O/formic acid (v/v, 49:5:49:5:1.0). m/z are reported as thomson (Th), where Th = 1 atomic mass per unit positive charge.

RESULTS AND DISCUSSION

Clozapine N-oxide is a major metabolite of clozapine, an antipsychotic drug. APCI, APPI, and ESI mass spectra from clozapine N-oxide are presented in Figure 2 and illustrate that all

Table 1. Typical API Source Operational Conditions

<table>
<thead>
<tr>
<th>parameter</th>
<th>ESI</th>
<th>APCI</th>
<th>APPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ion spray voltage (kV)</td>
<td>5</td>
<td>na²</td>
<td>1.3</td>
</tr>
<tr>
<td>needle current (µA)</td>
<td>na</td>
<td>2</td>
<td>na²</td>
</tr>
<tr>
<td>turbo heater temp (°C)</td>
<td>250</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>heated nebulizer (vaporizer)</td>
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<td>250</td>
<td>25</td>
</tr>
<tr>
<td>curtain gas setting</td>
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<td>20</td>
<td>20</td>
</tr>
<tr>
<td>gas 1 (nebulizer gas)</td>
<td>250</td>
<td>na²</td>
<td>na²</td>
</tr>
<tr>
<td>auxiliary gas for APCI and APPI⁶</td>
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<td>60</td>
<td>60</td>
</tr>
<tr>
<td>photoenergy of krypton discharge lamp (hν)</td>
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<td>na</td>
<td>10 eV</td>
</tr>
<tr>
<td>lamp gas (N2) flow (L/min)</td>
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<td>na²</td>
<td>1</td>
</tr>
<tr>
<td>dopant</td>
<td>na</td>
<td>na²</td>
<td>toluene³</td>
</tr>
<tr>
<td>dopant (µL/min)</td>
<td>na</td>
<td>na²</td>
<td>20</td>
</tr>
</tbody>
</table>

² na = not applicable. ³ Nitrogen was used for curtain gas, nebulizer gas, and auxiliary gas. ⁶ All gas flow settings represent arbitrary units. The potential difference between the photon energy and IP of toluene is 1.17 eV.

Figure 1. Chemical structures of model N-oxides.
three ionization methods produced protonated molecules (MH+) and many fragment ions. In general, the abundance of fragment ions when normalized to that of the protonated molecule was higher in APCI and APPI than that observed with ESI. Notably, two major fragment ions at m/z 313 and 327 observed in the APCI and APPI mass spectra were not detected in the ESI mass spectrum (Figure 2). As previously reported, the fragment ion at m/z 327 (MH+ - O) arises from thermal energy activation in the vaporizer of the APCI and APPI sources. Clozapine N-oxide has been shown to undergo significant decomposition by loss of an oxygen atom and conversion back to the parent drug, clozapine, during GC analysis. Similar to thermal activation in APCI and APPI ion sources, the high inlet temperature required to volatilize analytes prior to GC separation is responsible for deoxygenation.

The most prominent fragment ion (m/z 313) resulted from the loss of formaldehyde and is attributed to thermal energy from the APCI or APPI vaporizer. We propose that this fragment ion resulted from a thermally induced Meisenheimer rearrangement with migration of the N-methyl group to the corresponding N-alkylamine, followed by elimination of formaldehyde through an internal hydrogen transfer (Scheme 1). However, this fragmentation would not be expected from hydroxylated metabolites and has not been seen in the APCI mass spectra of any hydroxylated metabolites characterized in our laboratory. Following Meisenheimer rearrangement, this fragment distinguishes not only N-oxides from isomeric hydroxylated metabolites but potentially the exact site of N-oxidation in a setting where multiple sites of differing chemical environment are present. The fragment ion at m/z 313 in APCI and APPI indicates that the position of N-oxygenation is at the nitrogen atom of the piperazine ring where the methyl group is attached, and not in the diazepine ring.

The contribution of collisional activation leading to deoxygenation and aldehyde elimination following Meisenheimer rearrangement of tert-N-oxides was evaluated from an MS/MS experiment with clozapine N-oxide (Figure 3). Although a large

Figure 2. (a) APCI, (b) APPI, and (c) ESI mass spectra of clozapine N-oxide.

Scheme 1. Proposed Mechanism of Aldehyde or Ketone Elimination Following Meisenheimer Rearrangement of tert-Amine N-Oxides

Figure 3. APCI-product ion spectrum of protonated clozapine N-oxide showing the absence of thermally induced fragment ions at m/z 327 and 313. The collision gas (N₂) was set at 5 (arbitrary unit) and collision energy was set at 25 eV.

Figure 4. (a) APCI, (b) APPI, and (c) ESI mass spectra of (-)-hyoscyamine N-oxide.
number of fragment ions were detected, the fragment ions at m/z 327 and 313 are conspicuously absent even with increase in collision energy, thereby providing strong evidence that these fragment ions result from thermal energy activation and not collisional activation.

Similarly, fragment ions resulting from the loss of formaldehyde were also observed as the most prominent ions in the APCI and APPI mass spectra of (−)-hyoscyamine N-oxide (Figure 4) and 4-(dimethylamino)azobenzene N-oxide (Figure 5), whereas these fragment ions were not observed in the ESI mass spectra.

Scheme 2. Thermally Induced Reactions of Triethylamine N-Oxide

Figure 5. (a) APCI, (b) APPI, and (c) ESI mass spectra of 4-(dimethylamino)azobenzene N-oxide.
The fragment ion at \( m/z \) 226 observed in the ESI mass spectrum of 4-(dimethylamino)azobenzene N-oxide (Figure 5c) is not due to the loss of an oxygen atom of the N-oxide moiety; instead, this ion is attributed to the \(^{13}\text{C}\) isotopic peak of \( m/z \) 225. This is consistent with two lines of evidence: (i) the calculated relative abundance of \(^{13}\text{C}/^{2}\text{H}^{15}\text{N}\) isotopic components of \( m/z \) 225 is 16.8\%, which is in a good agreement with that from the experiment (17.7\%), and (ii) the calculated \( m/z \) value for the \(^{13}\text{C}\) isotopic peak of \( m/z \) 225 is 226.1290, while that of the ion from the loss of an oxygen atom from the N-oxide is 226.1338. The measured \( m/z \) value of 226.1287 confirmed that this ion is due primarily to \(^{13}\text{C}\) isotopic peak of \( m/z \) 225.

In addition to alkyl groups, the benzyl group in tribenzylamine N-oxide was also observed to undergo N-to-O Meisenheimer rearrangement prior to fragmentation in APCI and APPI (Figure 6). The fragment ion at \( m/z \) 198 resulting from benzylaldehyde loss was only observed in APCI and APPI mode. This fragmentation required migration of the benzyl group to the corresponding N-benzyloxylamine, followed by elimination of benzylaldehyde.

Meisenheimer rearrangement is not expected for aromatic N-oxides. As shown in the APCI and APPI mass spectra of 2-(methylsulfonylmethyl)pyridine N-oxide (Figure 7), only a deoxygenated ion at \( m/z \) 172 was observed. This information can potentially be used to differentiate the N-oxides of tertiary amines from the N-oxygenated derivatives of primary, secondary, and aromatic amines. Note that the loss of an oxygen atom is from the N-oxide moiety, not from the sulfonyl group. This was confirmed from the product ion spectrum of \( m/z \) 172 (data not shown), in which the major fragment ion observed was at \( m/z \) 93 that resulted from the loss of methylsulfonyl radical, indicating the oxygens on the sulfur were intact.

The tertiary amine N-oxides with a \( \beta \)-hydrogen in the alkyl-substituted group may also undergo Cope elimination with the formation of a hydroxylamine and an olefin.\(^{19,20}\) As seen in the APCI mass spectrum of triethylamine N-oxide, all three main thermally induced reactions were observed (Scheme 2): (i) Meisenheimer rearrangement followed by loss of acetaldehyde, (ii) deoxygenation, and (iii) Cope elimination leading to the formation of diethylhydroxylamine and ethylene. When the

temperature of the heated nebulizer was incrementally elevated (250, 300, 350, 400, and 450 °C), corresponding increases in all three thermal reaction products were observed.

**CONCLUSIONS**

*N*-Oxides undergo deoxygenation resulting from thermal energy activation at the vaporizer of an APCI or APPI source. However, tert-*N*-oxides containing an alkyl or benzyl group also undergo thermally induced Meisenheimer rearrangement under APCI or APPI conditions. The fragmentation reaction that follows Meisenheimer rearrangement results in the loss of an aldehyde or a ketone. This can not only distinguish the *N*-oxides from isomeric hydroxylated metabolites but also provide a fingerprint for the position of *N*-oxidation if there are multiple sites in different chemical environments.

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