The Challenge of Detecting Nitrosamines & Mutagenic Impurities

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Current guidelines, including ICH M7, provide an overview of assessing and evaluating limits of pharmaceutical impurities suspected or classified as mutagenic impurities. These impurities may be associated with known added agents, environmental factors, or degradation products from pharmaceutical compositions. Proposed limits for genotoxic impurities reside well below common impurities discussed in the ICH Q3A2 guidance and require analytical techniques capable of detecting and measuring ppm to ppb levels. This whitepaper provides an overview of analytical technologies for detecting mutagenic impurities.

Introduction
Investigational new drug development requires a demonstration of safety and efficacy. Over the last two decades, the safety requirements for CMC have become more clearly defined. Specifically, evaluation of impurities of actives and drug products in relation to container closures, as well as manufacturing, are covered in guidelines such as ICH, regulatory agencies, and USP. The introduction of guidelines for trace metals and mutagenic impurities suggest rigorous control of impurities. The ICH M7 guidance outlines limiting carcinogenic risk by assessing possible mutagenic impurities in new drug substances and products. The primary challenge associated in measuring mutagenic impurities is often the need for low to very low-level detection limits.

A mutagen is anything that causes a mutation or a change in the DNA of a cell. DNA changes caused by mutagens may harm cells and cause certain diseases, such as cancer. Examples of mutagens include radioactive substances, x-rays, ultraviolet radiation, and certain chemicals.

Assessing Levels of Mutagenic Impurities
Non-mutagenic impurities are typically evaluated in drug substances at levels above 0.05% weight/weight or relative peak area using standard detection techniques (ICH Q3A). Suggested threshold levels of mutagenic impurities are determined by daily intake and dose duration. These limit mutagenic impurities to less than 1.5 µg per day for a concentration of less than 10 ppm. Therefore, a detection technique of 70-fold lower may be needed, as profiled in Table 1. One way to view the introduction of mutagenic impurities is to categorize from three primary sources with the detection complexities differing based on the source of the mutagenic impurity.

Thus, it is clear from Table 1 and a little math, the mutagenic impurities to be quantitated may require much higher sensitivity than for standard Q3A impurities at the 0.05% level and at 30% Threshold of Toxicological Concern.

This paper discusses two of the three sources of mutagenic impurities: those that are added and those that may form in the matrix. Environmental MIs, also known as leachables, are not covered here, as these are typically analyzed in independently defined programs.

<table>
<thead>
<tr>
<th>Standard Impurities</th>
<th>Mutagenic Impurities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily Dose (mg)</td>
<td>Duration</td>
</tr>
<tr>
<td>Q3A ID Threshold</td>
<td>≤ 1 Mo</td>
</tr>
<tr>
<td>TTC (µg) ICH M7</td>
<td>&gt; 1-12 Mo</td>
</tr>
<tr>
<td></td>
<td>&gt;1-10 yrs</td>
</tr>
<tr>
<td></td>
<td>&gt; 10 yrs to LT</td>
</tr>
<tr>
<td>Daily Intake (µg)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>250</td>
<td>10</td>
</tr>
<tr>
<td>500</td>
<td>1.5</td>
</tr>
<tr>
<td>2001</td>
<td>TTC (µg)</td>
</tr>
<tr>
<td></td>
<td>1000.5</td>
</tr>
</tbody>
</table>

Table 1: Comparing Q3A and M7 levels
chloride was added at step 3 of a 5-step synthesis, a sample is available, and detection characteristics can be extrapolated, suggests a straightforward process of detecting the mutagenic impurity. In addition, available toxicological data simplifies the assessment.

When the assessment requires evaluating the final drug substance or intermediate for the presence of the added mutagenic impurity, a separation technique and detection technique is evaluated. This raises some questions to consider:

- Does my current analytical methodology detect the mutagenic impurity? And if yes, what is the detection limit?
- What is the desired detection or quantification limit based on TTC?
- Is the compound volatile?
- What is the expected ionization characteristic of the mutagenic impurity and its applicability to MS?
- How reactive is the mutagenic impurity and should derivatization be considered?

In general, added mutagenic impurities typically are of higher chemical reactivity, and this should be considered during method development to assess stability of these reactive species when the API is spiked into samples as part of accuracy. For example, alkyl halide mutagenic impurities are known to react with amines and have been observed in GC headspace analysis to affect accuracy in recovery studies.

**Nitrosamines-Unintentionally Added or Formed:**

There has been recent attention on residual nitrosamines found in marketed products. The carcinogenic risk associated with compounds of the N-nitroso family and the great media coverage led to one of the widest mobilizations of scientists and regulatory experts. FDA, EMA and other international agencies started an intense collaboration to share experience, data and knowledge. At first regulators had to carefully balance possible drug shortages with the need to ensure patients health; marketing of contaminated drugs with a valid clinical alternative, such as ranitidine, was suspended in the US and Europe as a precaution, although the decision does not yet seem definitive.

Where this approach was not feasible (i.e. for Sartans), strict temporary limits on levels of these impurities were introduced in the Test section of the related Ph. Eur. Monographs as well as a general chapter (2.4.36) for all active substance or in USP <1469>.

These GC/MS or HPLC/HRMS methods show adequate sensitivity. Nitrosamines can form from existing amines that are part of the manufacturing process or are present due to degradation and/or reactions of residuals. For example, dimethylformamide, a manufacturing solvent that may form dimethylamine, and if in the presence of nitrogen oxides, nitrosamines formation is possible. Thus when considering confirmatory testing, should one test for nitrogen oxides and/or secondary amines?

Nitrosamines formed in water sources as profiled by Krasner et al.3 exemplify the depth needed when assessing nitrosamines. Most critical is the control procedures needed for water sources, in particular if municipal water is part of the manufacturing process. Chloramines, amines, and nitrogen oxides are all undesirable residuals in municipal water, and due to potentially high day-to-day variability, make for complicated control strategies.

Regulatory decisions were based upon the necessity of increasingly sensitive testing methods (such as Hi-res techniques with MSMS detection) and the common opinion that an in-depth review of the manufacturing processes would be needed to mitigate the risk of contamination.

As a result, a risk-based evaluation for all drugs is required by EMA4. The extended deadline for this step is now March 2021. In a recent guidance for industry issued by FDA at the beginning of September[XI], the US agency fully aligned with Europe. Notably, biological products are now included, even though they were not initially considered due to very low risk of contamination. This change in approach aligns with the agency’s expectations to cover all marketed and newly submitted drugs.

EMA is also running a lessons learned exercise. As a result, the international guidelines that are now effective for controlling mutagenic impurities, such as ICH M7, will most likely undergo a long and complex revision cycle to avoid the repetition of similar situations in the future.

**Mutagenic Impurities as Degradation Products or Formed from Matrix or Process**

More complex than “mutagenic impurities that are added” is the discovery of degradation products that alert for mutagenicity. If the Q3A(R2) process for impurity qualification or other information finds a degradation product with toxicological concerns,
such as defined in Figure 1, additional efforts may be required. We find a subtle gap in both the Q3A(R2) decision tree and the note in the decision tree diagram stating, “Lower thresholds can be appropriate if the degradation product is unusually toxic.” This addresses toxic degradation products but at the same time does not suggest the need for identification. The decision tree suggests an option to reduce the degradation product to less than the identification threshold, thus no further action is needed. How does one assess unusual toxicity of an unidentified degradation product? Thus Q3A(R2) alone lacks rigor to assess mutagenic impurities and one should defer to ICH M7 for a mutagenic impurity evaluation.

Consider an example of a worst-case scenario:

- The M7-like assessment identifies a possible degradation product of concern in the API or the corresponding drug product contains two actives and many excipients.

Further studies may be considered such as purposeful stressing of the drug substance to identify the presence of the alerting structure, followed by insilico analysis and a bacterial assay. Additional questions to ask in addition to the above regarding “mutagenic impurities that are added” are:

- Is isolation and/or synthesis of the degradation product required to confirm absolute structure, provide analytical reference material, and provide material for in vivo studies?
- Should this degradation product be monitored or evaluated in one’s complex drug product such as part of long term stability studies?

One situation that may arise is the presence of an in silico mutagenic impurity alerting the functional group that is contained in the primary structure such as a substituted aniline. Clearly any proposed or known degradation product containing the aniline

<table>
<thead>
<tr>
<th>Detector</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>1</td>
</tr>
<tr>
<td>Diode Array (DAD)</td>
<td>7</td>
</tr>
<tr>
<td>Charged Aerosol (CAD)</td>
<td>1.5</td>
</tr>
<tr>
<td>Light Scattering (ELSD)</td>
<td>7</td>
</tr>
<tr>
<td>Refractive Index (10)</td>
<td>10</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>0.1</td>
</tr>
<tr>
<td>Conductivity</td>
<td>2</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>0.001</td>
</tr>
<tr>
<td>MS</td>
<td>1</td>
</tr>
<tr>
<td>MS Trap</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Table 2: General Sensitivity Overview of HPLC Detectors

Is the degradation product greater than identification threshold?

- **NO**: No action
- **YES**: Structure Identified

Structure Identified

- **YES**: Any known human risks?
- **NO**: Reduce to not more than identification threshold

Reduce to not more than identification threshold

- **YES**: Structure Identified
- **NO**: Greater than qualification threshold?

Greater than qualification threshold?

- **NO**: No action
- **YES**: Reduce to not more than qualification threshold

Reduce to not more than qualification threshold

- **NO**: Any clinically-relevant adverse effects?
- **YES**: Reduce to safe level

Reduce to safe level

- **YES**: Qualified
- **NO**: Consider patient population and duration of use and consider conducting:
  - Genotoxicology studies (point mutation, chromosomal aberration)
  - General toxicology studies (one species, usually 14 to 90 days)
  - Other toxicity endpoints, as appropriate

Consider patient population and duration of use and consider conducting:

- Any clinically-relevant adverse effects?
- **YES**: Reduce to safe level

Reduce to safe level

- **YES**: Qualified
- **NO**: No action

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Figure 1: A Q3B(R2) decision tree for the identification and qualification of a degradation product
substructure would give an in silico alert. It is generally accepted if the parent molecule is shown not to be mutagenic; then similar degradation products would follow this pattern. However, at least a risk assessment would be recommended. ICH suggests M7 is not applicable for advanced cancer drugs.

Detection Techniques

When we encounter the need to quantitate low level impurities, some options for detection prove more suitable than others. Table 2 profiles the general sensitivity of listed detectors where UV is arbitrarily assigned a value of 1 and the scale represents the relationship to other detectors. Thus, an electrochemical detector has a value of 0.1 or in general 10X more sensitive than UV. Note that these general sensitivities are very compound dependent.

Clearly mass spectrometry detection, as shown in Table 3, has superior sensitivity and the added advantage of identification potential. For example, a trap MS with single ion monitoring capability with instruments such as a Q Executive® Orbitrap allows for low level quantitation in a complex matrix and is very useful in both screening and/or monitoring mutagenic impurities.

When assessing and possibly quantitating mutagenic impurities, it is important to have input from synthesis, toxicology, analytical, and manufacturing experts to apply a compound-specific strategy with continual evaluation through drug development.

<table>
<thead>
<tr>
<th>GC Detection Type</th>
<th>Compounds</th>
<th>Approximate Detection Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>FID</td>
<td>Carbon compounds</td>
<td>0.1 ppm</td>
</tr>
<tr>
<td>ECD</td>
<td>Halogen, NO3</td>
<td>0.1 bbp</td>
</tr>
<tr>
<td>FPD</td>
<td>S, P</td>
<td>10 ppb</td>
</tr>
<tr>
<td>TCD</td>
<td>Most</td>
<td>10 ppm</td>
</tr>
<tr>
<td>FTD</td>
<td>Nitrogen Organics (phosphorus)</td>
<td>0.1-1 bbp</td>
</tr>
<tr>
<td>MS/SIM (EI)</td>
<td>Most</td>
<td>100 ppt</td>
</tr>
<tr>
<td>MS (EI) SCAN</td>
<td>Most</td>
<td>10 ppb</td>
</tr>
</tbody>
</table>

Table 3: GC Detector Sensitivity

References


5. ICH Q3A R2, Guidance Impurities in New Drug Substances