New Challenges in Impurity Profiling: Focus on Unknown Impurities and Nitrosamines

With the presence of impurities being a serious concern during the development and distribution of pharmaceutical products, what are the benefits of characterising unknown impurities and how can this be achieved?

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According to the United States Pharmacopeia (USP), an impurity is defined as any component that is neither the chemical entity that is defined as the drug substance, nor a drug substance or an excipient in the drug product.[1] The presence of impurities can be a serious concern in the production and release of pharmaceutical products, since they can affect efficacy and safety. Impurities are mainly classified in three categories, addressed by specific International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) quality guidelines:

1. Organic impurities (ICH-Q3A and Q3B)
2. Inorganic impurities (ICH-Q3D)
3. Residual solvents (ICH-Q3C).[2]

Among listed impurities, important attention must be given to unknown impurities and nitrosamines. Unknown impurities are by definition molecules for which structural characterisations have not been achieved and that are identified solely by qualitative analytical properties, thus representing a high concern for the safety and control of the risks of parent drug substance or product. Unknowns are nowadays a daily issue to be addressed by pharmaceutical manufacturers, since with the increased sensitivity of analytical techniques, new unwanted species are being detected even for well-established synthetic and manufacturing processes.

Nitrosamines in pharmaceutical products are quite novel impurities that came on the scene after 2018, when the antihypertensive agent Valsartan, manufactured in China, was found to be contaminated by nitrosamine N-nitrosodimethylamine (NDMA), belonging to N-nitroso compounds that are among the structural groups of high potency mutagenic carcinogens.[1,3] This finding triggered the detection of other nitrosamines in many other drug products (which were even recalled from the market) and pharmaceutical synthetic routes, greatly increasing concerns about the presence of these molecules in pharmaceutical scenarios and paving the way for new regulatory requirements and qualitative standards to be fulfilled. The aim of this article is to offer a technical focus on the analytical possibilities related to the characterisation of unknown impurities and nitrosamines testing.

Characterisation of Unknown Impurities

As mentioned, unknown impurities can be expected to be present in pharmaceutical products at very low concentrations, but the related toxicity is unknown as well. When high concentrations of unknowns are found, the identification of the species becomes compulsory.[2] Generally, unspecified compounds are found within routine analytical activities, including assay and impurities chromatographic tests of the drug substance or product.

The investigation process for the identification of an unknown impurity finds its starting point in the analytical activity that revealed its presence and it proceeds by consequential steps, designed as best fit on a case-by-case basis. Given below is a summary of the possible analytical steps that can be followed

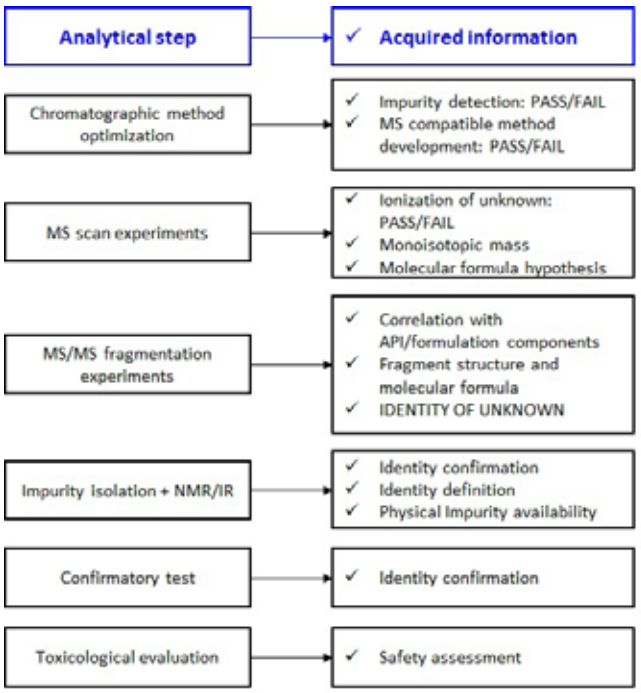


Figure 1: Schematic steps of an unknown impurity identification project

when a comprehensive indentification is reported, including most commonly used instrumental techniques and relative achievable information.

Impurity peak enhancement

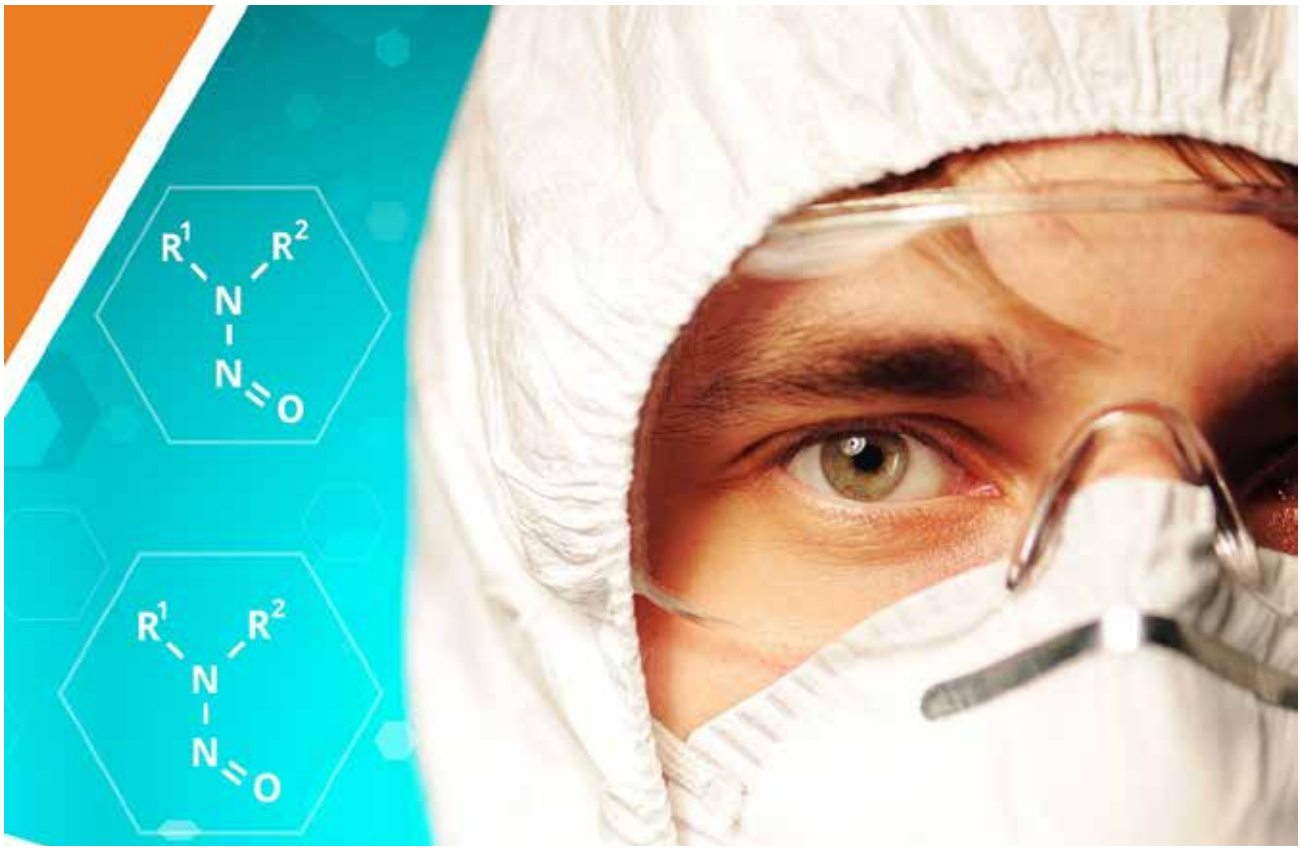
Sometimes unknown impurities are detected as a consequence of a specific step of drug manufacturing or within stability studies. The first tip to facilitate the investigation process is in the expression 'the worse quality sample is the best analytical sample': stress or forced degradation conditions can be implemented to replicate impurity formation and increase the corresponding chromatographic peak.

High resolution LC/MS studies

Liquid chromatography equipped with a high resolution mass spectrometry (LC/HRMS) detector is the technique of choice for identification studies. The untargeted impurity detection requires a MS-compatible chromatographic method; thus, it may first be necessary to adapt the initial method, maintaining the control over unknown impurity peak. MS scan experiments are then performed to detect any ionisable species present under the peak of interest: all the relevant detected m/z ratios are designated as putative candidates and corresponding molecular formulas are extracted and critically revised. Fragmentation experiments are then performed on selected parent ions, with different ionisation modes and/or fragmentation energies. Native and fragmentations spectra are compared and evaluated in order to restrict the number of possible candidates, to suggest a putative molecular structure and to find possible correlation between the impurity and formulation components.

Impurity isolation and structural identification

Impurity isolation may be of great interest when the synthesis of impurity standards represents a cost-effective step, or when spectroscopic characterisation is needed to



identify the molecular structure (eg, when LC/MS does not lead to a complete structural identification). Preparative high-performance liquid chromatography (HPLC) is used to isolate an appropriate amount of the molecule of interest, which is then analysed with nuclear magnetic resonance (NMR) and/or infrared (IR), either for structural identification – if the impurity is still unknown – or for a comparative test with an analytical standard – in case of confirmation of the putative structure.

Confirmatory experiments

The putative identity of a recognised impurity (either after LC/MS studies or isolation process) can be assayed in confirmatory studies, using commercially available standards and a customised analytical method.

Toxicological evaluation

Once the molecular identity of the investigated impurity is discovered, a toxicological evaluation may be conducted to check the correlated risk, according to the administration characteristics of the parent drug substance or product. This final evaluation permits the definition of a new specification limit that must be complied with in order to assure the safety of the pharmaceutical product.

Nitrosamine Testing

As mentioned, regulatory agencies (eg, European Medicines Agency (EMA) and Food and Drug Administration (FDA)) quite recently provided requirements and qualitative standards to be fulfilled for nitrosamines in pharmaceutical products, advising Market Authorisation Holders (MAHs) that the presence of these impurities in their products was to be monitored and avoided.[4,5] These documents include a list of nitrosamines of particular concern, the acceptable limits for their presence and the documental and analytical approach to be followed for their determination and monitoring.

Considering the toxicity of nitrosamines, the acceptable limits and the requests in terms of sensitivity and specificity for testing are definitely challenging. Furthermore, due to the quite recent emergence of the topic, guidelines themselves are under a continuous improvement and modification process, constantly generating new requests and challenges. For example, the nitrosamines-of-concern list is still updated on a regular basis to add new molecules, and the first analytical procedures suggested by the authorities were published in nitrosamines-dedicated chapters within both the European and United States' Pharmacopoeias only in the last couple of years.[4,6,7]

The investigation process prescribed to MAHs is a three-step approach: 1. Risk assessment; 2. Confirmatory testing; 3. Remediation.[4] During Step 1, the nitrosamines' presence/formation risk is investigated for each product; during Step 2, the presence and amount of nitrosamines is confirmed with a sensitive and appropriately validated analytical method; during Step 3, the modifications identified as needed within the process are applied, to mitigate the risk of nitrosamines forming. Focusing on Step 2, given below is a technical summary of analytical steps that can be followed to reach an appropriate validated analytical method for nitrosamine testing to confirm their presence.

Analytical techniques

Nitrosamines are, in general, very small and highly polar, with very few chromophore molecules. Given their toxicity, it is important that the method is sufficiently sensitive to detect very low levels (fraction of ppb) and highly specific, to be able to exclude any possible interference that might affect the compound identification (eg, dimethylformamide (DMF) vs NDMA). For these reasons tandem mass spectrometry (MS/MS) and high-resolution mass spectrometry (HRMS) are actually widely recognised as techniques of election to be used in this field of testing.[6,7]

Within the mass spectrometry applications, the subsequent choice is between liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS). LC-MS/MS' most relevant advantage is that it works at ambient temperature, minimising any risk of thermal degradation (which for example affects NMBA) and/or in situ nitrosamine formation triggered by the high temperature, typical of GC analysis (which, for example, affects ranitidine). GC-MS/MS works at high temperatures but it also allows organic solvents usage, not applicable in LC, greatly widening the sample preparation options. LC-MS/MS or GC-MS/MS are therefore interchangeable in some cases and the choice should relate to the detection of nitrosamines and the characteristics of the sample to be treated.

Sample preparation

Regulatory requirements apply to MAHs of all human medicinal products.[4] The presence of nitrosamines is therefore to be tested across a wide range of presentations: powders, granules, tablets, capsules, liquids, emulsions and ointments, among others. Each analyte has its own regulatory limit and therefore its own sensitivity requests.[4]

Considering the techniques involved and the working concentrations generally needed, sample preparation should aim to purify the samples as much as possible (from matrix, excipients, coating, etc) in order to maximise the reached sensitivity. When a chromatographic exclusion is not enough (eg, applying a gradient table to separate nitrosamines from matrix-provided interferent), extractive procedures may be chosen (liquid-liquid, solid-liquid, solid-phase extractions) and internal standards usage could also be evaluated.

Reference materials and reagents

Considering the health risk carried by nitrosamines, the quantitative impurities method validation approach is

generally followed for these determinations to obtain punctual and precise amount values for the samples. To appropriately validate such a method, certified reference materials for quantitative usage are needed for each nitrosamine to be determined. External standard quantification is generally applied, but a calibration curve at different concentrations may also be evaluated.

In case of deuterated nitrosamine usage as Internal Standard (eg, d6-NDMA), not considered as mandatory, its most relevant advantage is to improve method accuracy, but only a few molecules are commercially available and materials of the appropriate quality should be chosen.

For the same reason cited above, reagents also have to be carefully chosen for these kinds of analytical testing, to minimise and avoid artefacts and solvent-mediated nitrosamine contamination as much as possible. In general, LC-MS quality solvents and water are considered as mandatory and are the only commercially available materials able to reach the needed purity level to detect nitrosamines with the requested sensitivity.

Conclusion

The monitoring of nitrosamines of concern is constantly and rapidly improving, as is the sensitivity requirements prescribed by agencies to detect them. Both the factors continue to raise new and more difficult analytical challenges. MS/MS is widely recognised as the technique of choice for nitrosamines testing, for its sensitivity and unchallenged specificity.

To successfully validate a method for quantitative nitrosamine determination, which fulfils regulatory requirements (primarily in terms of sensitivity), reference materials for quantitative usage and reagents of the appropriate quality are needed. In terms of sample preparation, a procedure that involves extraction and/or purification may be also required, considered the sensitivity levels to be reached for the involved molecules.

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