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Brief Report

Toxicological Assessment and Risk Management of Pharmaceutical Impurities

Arun HS Kumar*

Stemcology, School of Veterinary Medicine, University College Dublin, Belfield, Dublin, IRELAND.

ABSTRACT

The presence of impurities in pharmaceutical products poses significant toxicological and regulatory concerns, necessitating systematic assessment and risk management strategies. Pharmaceutical impurities, which may arise from synthesis, degradation, or contamination, can compromise drug safety and efficacy. This report provides a concise overview of the principles underlying the toxicological evaluation of these impurities, emphasizing Threshold of Toxicological Concern (TTC) approaches, structure-activity relationships, and genotoxicity assessment. Regulatory frameworks, including guidelines from the International Council for Harmonisation (ICH) Q3A/B, are discussed to highlight standardised limits and reporting requirements. Risk management strategies focus on identifying, quantifying, and controlling impurities throughout the drug development and manufacturing lifecycle. Analytical techniques such as High-Performance Liquid Chromatography (HPLC), mass spectrometry, and Nuclear Magnetic Resonance (NMR) spectroscopy are crucial for accurate detection and characterisation of trace-level impurities. The integration of predictive toxicology models and *in vitro* assays aids in prioritising impurities that pose significant health risks, enabling efficient allocation of resources for a detailed investigation. The report also outlines application of risk-based impurity assessment, demonstrating how a combination of toxicological data, exposure analysis, and regulatory guidance can inform safe pharmaceutical development. The effective management of pharmaceutical impurities is essential for ensuring patient safety, regulatory compliance, and the sustained quality of therapeutic products. The report underscores the need for continued advancements in analytical methodologies and predictive toxicology to address emerging challenges in impurity risk assessment.

Keywords: Genotoxicity, Pharmaceutical impurities, Risk management, Threshold of toxicological concern, Toxicological assessment.

Correspondence:
Arun HS Kumar, DVM, PhD.,
Stemcology, School of Veterinary Medicine, University College Dublin, Belfield, Dublin-04, IRELAND.
Email: arun.kumar@ucd.ie

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INTRODUCTION

The development of safe, effective, and high-quality pharmaceutical products is a complex and highly regulated process that demands careful consideration of numerous scientific and regulatory factors. Among these, the identification, characterisation, and control of impurities represent a critical aspect of ensuring drug safety (Figure 1). One of the most concerning scenarios encountered during drug development is the detection of an unknown impurity or degradation product at levels approaching or exceeding the thresholds established by the International Council for Harmonisation (ICH).[1,2] Such findings can significantly delay development timelines, necessitate additional studies, and raise serious concerns regarding patient safety and regulatory approval. Impurities are defined as any components present in a pharmaceutical substance or product that are not the intended Active Pharmaceutical Ingredient (API) or excipients.[3,4] These substances may originate from a variety of sources, including raw materials, intermediates, by-products of synthesis, degradation during storage, or interactions with packaging components (Figure 2). Regardless of their origin, impurities are inherently undesirable because they provide no therapeutic benefit and may pose safety/toxicological risks. Their presence introduces uncertainty into the safety profile of a drug, as even trace amounts of certain impurities can lead to adverse biological effects.

The toxicological impact of impurities depends on several factors, including their chemical structure, concentration, duration of exposure, and route of administration.[3,5] Some impurities may be relatively benign at low levels, while others, such as genotoxic or carcinogenic compounds, can pose significant health risks even at very low concentrations. As a result, the overall risk associated with a pharmaceutical product is directly influenced by the number, nature, and quantity of impurities it contains. This

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makes the assessment and control of impurities a fundamental component of pharmaceutical quality assurance and risk management. Regulatory authorities worldwide (Figure 3), through frameworks such as those established by the ICH, have developed guidelines to define acceptable limits for impurities and to outline strategies for their identification and qualification.[6,7] These guidelines emphasize a science-based approach, where impurity levels are evaluated in the context of patient exposure and potential toxicity. Thresholds such as the identification threshold, qualification threshold, and reporting threshold are used to determine when an impurity must be further investigated (Figure 4).[3,8] When these thresholds are exceeded, comprehensive toxicological studies may be required to assess the safety of the impurity and justify its presence in the final product.

A particularly challenging situation arises when an impurity is unknown or insufficiently characterized. In such cases, the lack of structural and toxicological information complicates risk assessment and regulatory decision-making. Advanced analytical techniques, including chromatography and spectroscopy, are often employed to identify and quantify such impurities (Figure 5). Once identified, a range of *in silico, in vitro*, and *in vivo* methods may be used to evaluate their toxicological potential (Figure 6).[9-11] These approaches help determine whether the impurity poses a genotoxic, mutagenic, or carcinogenic risk, and whether its levels must be reduced or controlled more stringently. Impurity control is not only a regulatory requirement but also a critical component of ensuring patient safety throughout the product lifecycle. From early-stage development to commercialisation and post-marketing surveillance, continuous monitoring of impurities is necessary to maintain product quality.[12,13] Stability studies play a key role in understanding how impurities form and evolve over time, particularly under various environmental conditions such as temperature, humidity, and light exposure.[14-16] This information is essential for establishing appropriate storage conditions, shelf life, and packaging strategies. Furthermore, the concept of risk-benefit balance is central to impurity qualification. While all impurities are undesirable, their presence at low and controlled levels may be considered acceptable if the therapeutic benefits of the drug outweigh the potential risks.[17,18] This balance must be carefully evaluated, particularly for life-saving medications where some degree of impurity may be unavoidable. In such cases, a thorough toxicological assessment is required to ensure that any residual risk remains within acceptable limits.

Advancements in pharmaceutical science and technology have significantly improved the ability to detect and control impurities at increasingly lower levels (Figure 5). However, these advancements have also raised expectations from regulatory authorities regarding impurity profiling and safety assessment. As analytical methods become more sensitive, previously undetectable impurities can now be identified, necessitating additional evaluation and control measures. This underscores the importance of integrating toxicological considerations early in the drug development process to proactively address potential impurity-related risks. Impurities represent a critical challenge in pharmaceutical development due to their potential to compromise drug safety without providing any therapeutic benefit. Their presence must be carefully managed through rigorous identification, qualification, and control strategies in accordance with regulatory guidelines. A comprehensive understanding of the sources, behaviour, and toxicological implications of impurities is hence essential for ensuring that pharmaceutical products meet the highest standards of safety and quality. Ultimately, the effective management of impurities plays a vital role in protecting patient health and maintaining confidence in pharmaceutical therapies.

Classification and Control of Pharmaceutical Impurities

The classification and control of impurities are fundamental components of pharmaceutical development, directly influencing the safety, quality, and regulatory acceptability of drug substances and products. According to guidelines established by the ICH, impurities associated with drug substances are broadly categorised into three principal groups: organic impurities, inorganic (elemental) impurities, and residual solvents.[3] Each category arises from different sources and presents distinct challenges in terms of detection, evaluation, and control, necessitating a comprehensive and systematic approach to impurity management throughout the drug lifecycle (Figure 2). Organic impurities are the most common type encountered in pharmaceutical products and typically originate from the synthetic process.[13,19] These include starting materials, intermediates, by-products, reagents, and degradation products formed during manufacturing or storage. Their presence is often influenced by reaction conditions, purification processes, and the stability of the Active Pharmaceutical Ingredient (API). Because organic impurities are structurally related to the drug substance, their toxicological profiles may vary widely, requiring detailed characterisation and evaluation. Advanced analytical techniques such as High-Performance Liquid Chromatography (HPLC) and mass spectrometry are routinely employed to identify and quantify these impurities, ensuring that they remain within acceptable limits (Figure 5).[20,21]

In contrast, inorganic impurities, also referred to as elemental impurities, are typically derived from catalysts, reagents, ligands, or equipment used during the manufacturing process.[3,22] These may include metals such as lead, arsenic, cadmium, and mercury, which can be toxic even at very low concentrations. The control of inorganic impurities is governed by stringent regulatory standards due to their cumulative toxicity and potential to cause serious health effects. Their assessment often involves techniques such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS), which allows for precise detection of trace elemental

contaminants.[3,22] Residual solvents represent another significant category of impurities and are defined as volatile organic chemicals used or produced during the manufacture of drug substances or excipients.[3] These solvents may not be completely removed during processing and can remain in the final product. Depending on their toxicity, residual solvents are classified into different classes, with strict limits imposed on their permissible levels. For example, solvents known to be carcinogenic or environmentally hazardous are subject to tighter restrictions compared to those considered less harmful. Effective solvent removal techniques and process optimisation are essential to minimise their presence and ensure compliance with regulatory standards.

Within these three major categories of impurities, genotoxic impurities occupy a particularly critical position due to their potential to interact with genetic material.[3,23] These impurities can cause mutations in DNA, which may lead to carcinogenesis or heritable genetic damage. Even at extremely low concentrations, genotoxic impurities pose significant safety risks, making their identification and control a top priority in pharmaceutical development.[24,25] Unlike other impurities, which may be tolerated at certain levels based on toxicological thresholds, genotoxic impurities often require much stricter limits due to their irreversible and potentially severe biological effects. The regulatory framework for impurity classification and control is further supported by specific guidelines such as ICH Q3A, which provides detailed recommendations for impurities in new drug substances.[17,26] This guideline establishes threshold values for reporting, identification, and qualification of impurities, based on the maximum daily dose of the drug (Figure 4). The reporting threshold defines the level at which an impurity must be reported, the identification threshold specifies when structural characterization is required, and the qualification threshold indicates when toxicological evaluation is necessary. These thresholds ensure a consistent and science-based approach to impurity assessment, enabling regulatory authorities and pharmaceutical developers to make informed decisions regarding product safety.

While classification provides a framework for understanding impurities, the goal should always be their effective reduction and control. The first and most critical step in impurity management is prevention, particularly in the case of genotoxic impurities. Pharmaceutical developers are strongly encouraged to design synthetic routes and manufacturing processes that avoid the formation of such hazardous compounds altogether. This proactive approach not only enhances product safety but also simplifies regulatory compliance and reduces the need for extensive toxicological studies. Regulatory agencies (Figure 3), emphasize the importance of minimising or eliminating genotoxic and carcinogenic impurities during drug development.[3,17,25] Developers should make every feasible effort to ensure that these impurities are not generated during the synthesis of the drug substance or the manufacture of the final product. This may involve selecting alternative reagents, modifying reaction conditions, or implementing additional purification steps to prevent impurity formation at the source. However, in certain cases, the complete avoidance of genotoxic impurities may not be technically feasible. When this occurs, it becomes necessary to implement strategies to reduce their levels to acceptable limits. One widely accepted principle in this context is the concept of keeping impurity levels "As Low As Reasonably Practicable" (ALAR).[18,27] This approach recognizes that while zero presence may not always be achievable, every effort must be made to minimize exposure to the lowest possible level without compromising the feasibility of the manufacturing process.

Impurities reduction strategies may include process optimisation, improved purification techniques, and enhanced control of raw materials and intermediates. For example, refining reaction conditions can minimize the formation of unwanted by-products, while advanced purification methods such as crystallisation, distillation, or chromatography can effectively remove impurities from the final product.[3,15] Additionally, implementing robust quality control systems ensures consistent monitoring and maintenance of impurity levels within acceptable limits (Figure 1). Another important aspect of impurity reduction is the application of risk assessment methodologies.[28,29] These approaches involve evaluating the potential toxicity, exposure level, and duration of contact associated with a given impurity. By integrating toxicological data with process knowledge, pharmaceutical scientists can prioritise control measures and allocate resources more effectively.[28,29] This risk-based approach aligns with modern regulatory expectations and supports the development of safer pharmaceutical products. Also, continuous monitoring and lifecycle management play a crucial role in maintaining impurity control. Even after a drug product has been approved, ongoing stability studies and quality assessments are necessary to ensure that impurity levels remain within specified limits throughout the product's shelf life. Any changes in manufacturing processes, raw materials, or storage conditions must be carefully evaluated to prevent unintended increases in impurity levels.[30,31] The classification and reduction of impurities are interdependent aspects of pharmaceutical quality assurance. The categorisation of impurities (Figure 2) into organic, inorganic, and residual solvent groups provides a structured framework for their evaluation, while the identification of genotoxic impurities highlights the need for continued vigilance due to their high-risk nature. Regulatory guidelines such as ICH Q3A establish clear thresholds and expectations for impurity management, ensuring consistency and scientific rigor in safety assessments. The effective control of impurities through prevention, reduction, and continuous monitoring is essential for safeguarding patient health and ensuring the successful development of pharmaceutical products.

Risk Assessment Approach for Genotoxic Impurities

The presence of genotoxic impurities in pharmaceutical products represents a significant toxicological concern due to their potential to interact with genetic material and induce mutations that may lead to cancer or heritable defects. In an ideal scenario, such impurities would be completely avoided during the design and manufacture of drug substances. However, in practice, it is not always possible to entirely prevent their formation or achieve their total removal. In such cases, a scientifically justified risk assessment approach becomes essential to ensure that any residual levels of genotoxic impurities do not pose a meaningful threat to human health. A fundamental principle of risk assessment for genotoxic impurities is the estimation of acceptable daily exposure levels that correspond to a negligible risk. This approach is based on the concept that there may be a threshold of exposure below which the probability of adverse effects, such as carcinogenesis, is extremely low.[25,27] Regulatory frameworks recommend the use of conservative assumptions to establish such limits, ensuring that patient safety is not compromised even under worst-case scenarios. The assessment typically involves calculating the daily intake of the impurity based on its concentration in the drug product and the maximum daily dose of the medication. Ideally, the determination of acceptable intake levels for genotoxic impurities should rely on comprehensive toxicological data, including results from long-term carcinogenicity studies conducted in animals (Figure 7). These studies provide valuable insights into the relationship between dose and tumour formation, enabling the identification of exposure levels associated with minimal or no observable risk. From such data, parameters such as the No-Observed-Effect Level (NOEL) or benchmark dose can be derived and used to establish safe exposure limits for humans through appropriate extrapolation and safety factors.[32,33] However, such extensive data are rarely available for individual impurities, particularly those that are newly identified or present at very low concentrations. Conducting long-term carcinogenicity studies for each impurity is often impractical due to the significant time, cost, and resource requirements involved. As a result, toxicological assessments of genotoxic impurities frequently rely on limited datasets, primarily derived from *in vitro* studies (Figure 6). Commonly used assays include bacterial reverse mutation tests, such as the Ames test, and chromosomal aberration assays, which provide initial indications of a compound's genotoxic potential.

While *in vitro* assays are valuable screening tools, they have inherent limitations when it comes to quantitative risk assessment. These tests are designed to detect the presence or absence of genotoxic activity rather than to establish dose-response relationships suitable for determining safe exposure levels. Consequently, the direct calculation of acceptable limits based on *in vitro* data alone is not considered scientifically robust.[33,34] In particular, the use of safety multiples derived from such data, where exposure limits are set by applying arbitrary factors to observed effect levels, is generally regarded as inappropriate and unreliable for regulatory decision-making. Another limitation arises from the testing of genotoxic impurities within the context of the drug substance itself.[35,36] In some cases, studies may be conducted using the final drug product containing trace levels of the impurity, often in the parts-per-million (ppm) range. While negative results in such studies may appear reassuring, they do not provide sufficient confidence in the safety of the impurity. This is primarily due to the limited sensitivity of these testing approaches, which may not be capable of detecting the effects of highly potent mutagens present at very low concentrations. As a result, the absence of observed genotoxic or carcinogenic effects in such studies cannot be interpreted as definitive evidence of safety. Indeed, even highly potent genotoxic carcinogens may go undetected when tested as minor components within a complex mixture, particularly when exposure levels are extremely low. This underscores the importance of adopting a cautious and conservative approach to risk assessment, recognising the limitations of available data and the potential for false-negative results. Rather than relying solely on experimental findings, risk assessment strategies often incorporate additional tools such as Structure-Activity Relationship (SAR) analysis and *in silico* modelling to predict the genotoxic potential of impurities based on their chemical structure (Figure 6).[34,36]

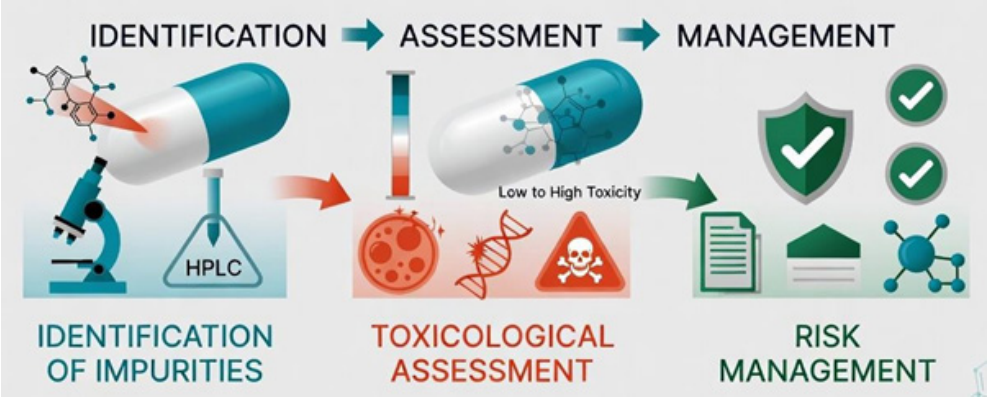

Figure 1: Workflow for assessing the risks associated with impurities in pharmaceutical products.

A widely accepted approach in the absence of compound-specific carcinogenicity data is the use of the Threshold of Toxicological Concern (TTC) concept. This method establishes a generic exposure limit for genotoxic impurities based on an extensive database of known carcinogens.[37,38] The TTC represents a level of daily exposure that is considered to pose a negligible risk of cancer over a lifetime, typically corresponding to an excess lifetime cancer risk of one in 100,000 or lower. By applying this conservative threshold, regulatory authorities can ensure a high level of protection for patients even in the absence of detailed toxicological data. The implementation of a risk assessment approach also involves consideration of treatment duration and patient population. For example, higher exposure levels may be considered acceptable for short-term therapies compared to chronic treatments, as the cumulative risk of adverse effects is lower. Similarly, the acceptable risk may vary depending on the severity of the disease being treated, with greater flexibility allowed for life-threatening conditions where the benefits of treatment outweigh potential risks. In addition to establishing acceptable intake levels, risk assessment plays a crucial role in guiding impurity control strategies.[13,17] By identifying impurities of concern and evaluating their potential impact, pharmaceutical developers can prioritise efforts to reduce or eliminate these substances through process optimisation and improved manufacturing practices. This risk-based approach ensures that resources are focused on the most critical safety issues, enhancing both efficiency and effectiveness in impurity management. It is also important to recognise that risk assessment is not a one-time activity but an ongoing process that continues throughout the lifecycle of a pharmaceutical product.[39,40] As new data become available, whether from additional studies, post-marketing surveillance, or advances in scientific understanding, risk assessments may need to be updated to reflect the latest evidence. This dynamic approach ensures that safety evaluations remain current and relevant, providing continued protection for patients. The risk assessment of genotoxic impurities is a complex but essential component of pharmaceutical development. When the complete avoidance or removal of such impurities is not feasible, a scientifically grounded and conservative approach is required to estimate acceptable exposure levels and ensure patient safety. Although the ideal scenario would involve comprehensive carcinogenicity data, practical limitations often necessitate reliance on limited *in vitro* data and predictive models. Recognising the limitations of these approaches, regulatory frameworks emphasise caution, discouraging the use of simplistic calculations and highlighting the need for robust, evidence-based methodologies.

Quantitative Structure Activity Relationships (QSAR) and *in silico* Models in Impurity Assessment

The evaluation of impurities in Active Pharmaceutical Ingredients (APIs) increasingly relies on computational toxicology approaches, particularly Quantitative Structure-Activity Relationships (QSAR) and *in silico* modelling.[41,42] These methods provide a rapid, cost-effective, and scientifically robust means of predicting the toxicological properties of chemical entities, especially when experimental data are limited or unavailable (Figure 6). One of the earliest and most critical steps in impurity assessment involves understanding the Structure Activity Relationship (SAR), which establishes a link between the chemical structure

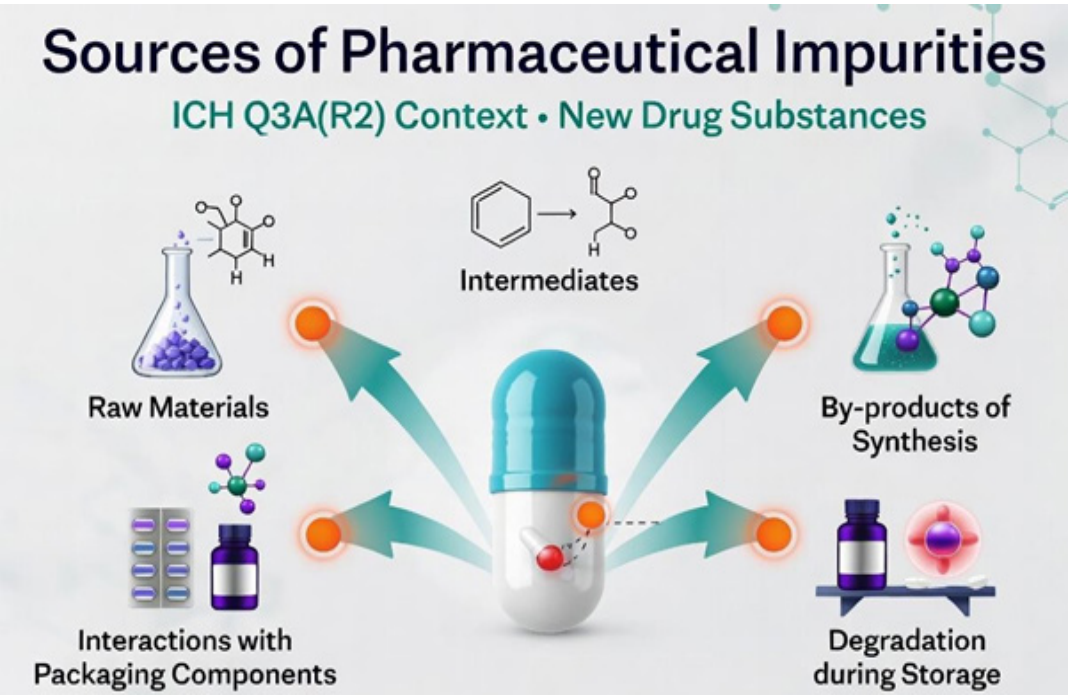

Figure 2: Potential sources of impurities in pharmaceutical products.

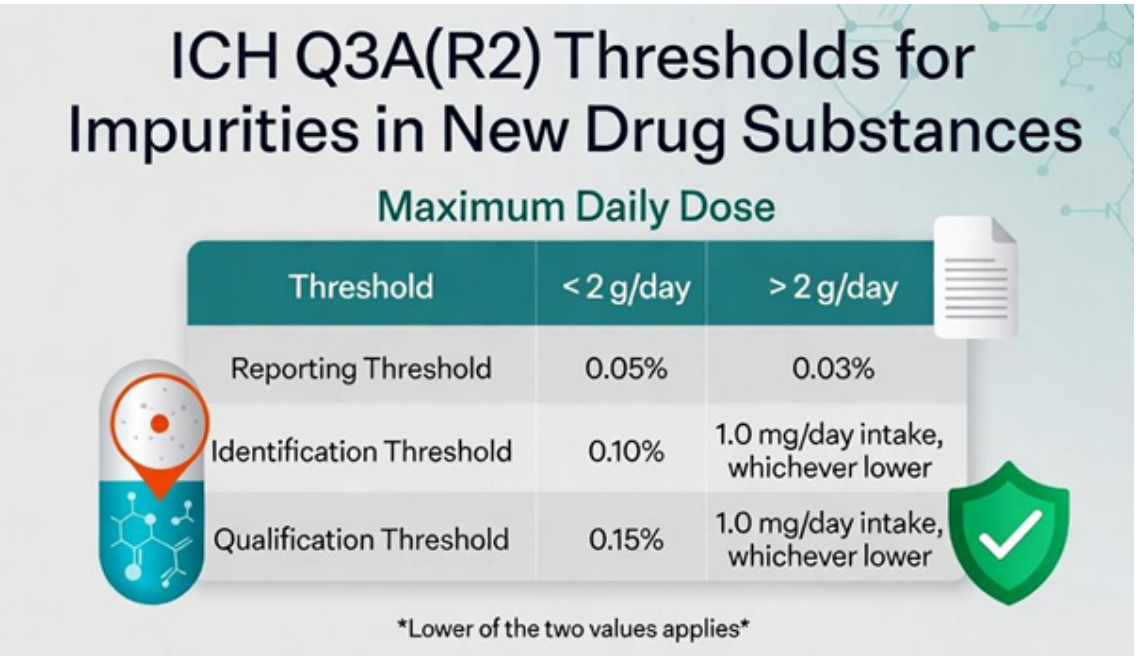

Figure 4: Thresholds for impurities in pharmaceutical products defined by the ICHQ3A guidelines.

of a compound and its biological or toxicological activity.[43] This approach enables scientists to make informed predictions about the potential hazards associated with impurities based on their molecular features. A central concept within SAR analysis is the identification of "Structural Alerts" (SAs). Structural alerts are specific molecular substructures, functional groups, or chemical motifs that are known to be associated with toxicological effects, particularly carcinogenicity and mutagenicity.[44,45] These alerts are derived from empirical observations and extensive toxicological databases that document the behaviour of various chemical classes. For example, certain electrophilic groups, aromatic amines, nitro compounds, and epoxides are well-recognised structural alerts due to their ability to interact with biological macromolecules. When such features are present in a chemical structure, they signal a potential risk and warrant further investigation. The relevance of structural alerts is closely tied to the mechanisms by which many carcinogens exert their effects. A significant number of carcinogenic compounds act through genotoxic mechanisms, meaning they interact directly with DNA, causing damage that can lead to mutations and ultimately cancer.[46,47] These interactions often involve covalent binding to DNA, resulting in the formation of DNA adducts or structural modifications that disrupt normal cellular processes. Since mutagenicity is a key step in the carcinogenic process, structural alerts identified for carcinogenicity are often equally applicable to predicting mutagenic potential. Consequently, the presence of a structural alert in a chemical impurity typically leads to its classification as potentially genotoxic, even in the absence of experimental data.

In practical terms, a compound that contains one or more recognised structural alerts is predicted to be positive for genotoxicity, indicating a potential safety concern. Conversely, if a compound lacks any known structural alerts, it is generally predicted to be negative, suggesting a lower likelihood of genotoxic risk. This binary classification provides a useful initial screening tool for prioritising impurities for further evaluation. However, it is important to note that SAR-based predictions are not infallible and must be interpreted within the broader context of available data and scientific judgment. To enhance the predictive power and consistency of SAR analysis, a variety of *in silico* expert systems have been developed.[48-50] These systems utilize computational algorithms and curated knowledge bases to analyse chemical structures and predict their toxicological properties (Figure 6). Broadly, *in silico* models used in impurity assessment can be categorized into three main types: rule-based systems, statistical or machine-learning models, and hybrid systems that integrate elements of both approaches. Rule-based systems are among the earliest and most widely used *in silico* tools for toxicological prediction. These systems rely on predefined rules derived from expert knowledge and empirical data to identify structural alerts and assess their relevance. Examples of such systems include Toxtree and Derek.[51,52] These tools operate by comparing the chemical structure of an impurity against a database of known toxicophores, structural features associated with specific toxicological endpoints. When a match is identified, the system generates an alert along with a rationale explaining the potential mechanism of toxicity. Rule-based systems are valued for their transparency and interpretability, as they provide clear explanations for their predictions, allowing users to understand the underlying reasoning. In contrast, statistical

and machine-learning models take a data-driven approach to toxicity prediction.[53,54] These models are built using large datasets of chemical structures and associated biological activities, from which they derive mathematical relationships between molecular descriptors and toxicological outcomes. Examples of such systems include TOPKAT, MultiCASE, and CAESAR. These tools employ techniques such as regression analysis, neural networks, and pattern recognition to generate predictions. While they may offer higher predictive accuracy in some cases, their outputs are often less interpretable than those of rule-based systems, as the underlying models can be complex and not easily understood by users. Hybrid systems represent a more recent development in *in silico* toxicology, combining the strengths of both rule-based and statistical approaches. These systems integrate expert knowledge with data-driven modelling to provide more comprehensive and reliable predictions.[52,53,55,56] By using multiple sources of information, hybrid models can improve both the sensitivity and specificity of toxicity assessments, reducing the likelihood of false positives and false negatives. This integrated approach is particularly valuable in regulatory contexts, where robust and defensible predictions are essential. The application of QSAR and *in silico* models in impurity assessment is supported by regulatory guidelines, which recognise their value as part of a weight-of-evidence approach to toxicological evaluation. These methods are especially useful in the early stages of drug development, where rapid screening of multiple impurities is required. By identifying potentially hazardous compounds early on, developers can prioritise resources and implement appropriate control strategies before advancing to more costly and time-consuming experimental studies.

An important consideration in the use of QSAR models is the concept of applicability domain. Each model is developed based on a specific set of chemical structures and may only be valid for compounds that fall within this domain.[57,58] Predictions made for chemicals outside the model's applicability domain may be less reliable and should be interpreted with caution. Therefore, it is essential to ensure that the impurity being evaluated is adequately represented within the model's training dataset. Another key aspect of QSAR-based assessment is the use of multiple complementary models to increase confidence in predictions.[59,60] Regulatory guidance often recommends the use of at least two independent *in silico* methods, typically one rule-based and one statistical, to evaluate genotoxic potential. Concordant results from different models strengthen the overall assessment, while conflicting predictions may indicate the need for additional investigation or experimental testing. The absence of structural alerts in a chemical impurity is generally considered a strong indicator of low genotoxic risk. In many cases, this finding may be sufficient to justify the decision not to conduct further toxicological qualification studies, particularly when supported by consistent predictions from multiple *in silico* models. This approach can significantly reduce the need for animal testing and expedite the drug development process, aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement) in scientific research. However, the interpretation of *in silico* results requires careful consideration of their limitations. Factors such as model quality, data availability, and the complexity of chemical structures can influence the accuracy of predictions.[58,61] As such, QSAR and *in silico* models are best used as part of an integrated assessment strategy that combines computational predictions with experimental data, chemical knowledge, and expert judgment (Figure 6). In the context of impurity qualification,

Figure 3: Major regulatory authorities tasked with overseeing approval of pharmaceutical products.

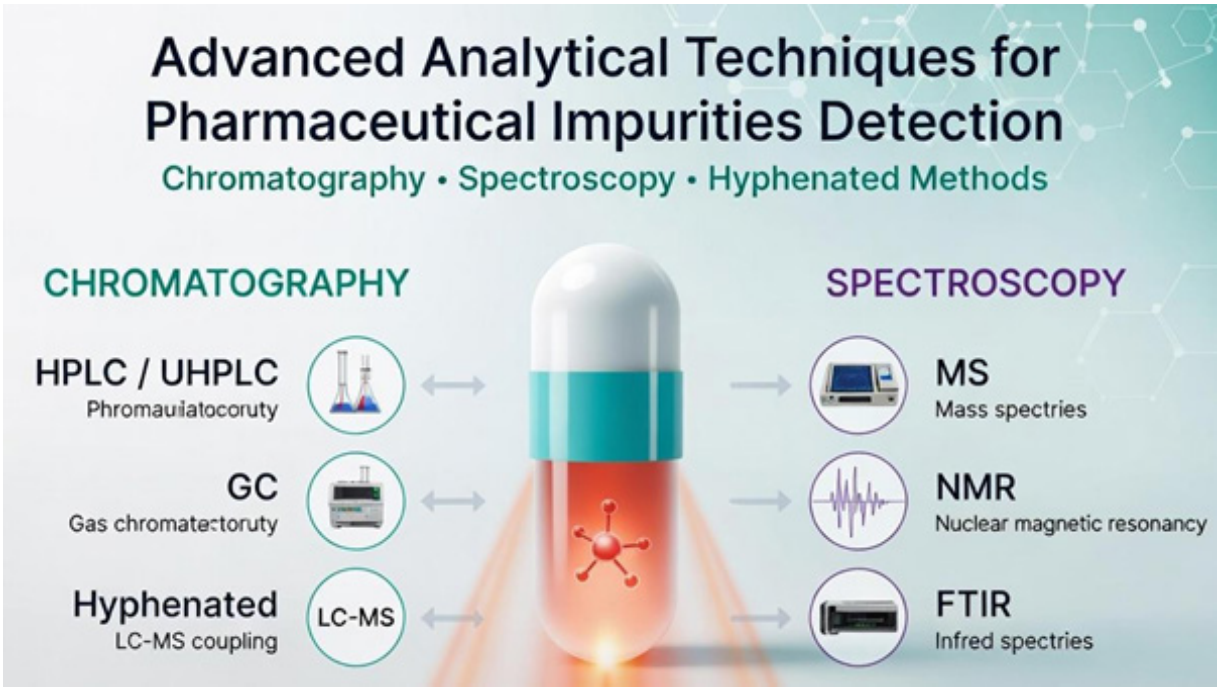

Figure 5: Various analytical techniques used for the quantification of impurities in pharmaceutical products.

QSAR and *in silico* approaches have become indispensable tools for evaluating genotoxic risk. Their ability to rapidly screen and prioritize impurities, identify structural alerts, and provide mechanistic insights has transformed the way toxicological assessments are conducted in the pharmaceutical industry. By enabling more efficient and informed decision-making, these methods contribute to the development of safer and more effective drug products while minimising reliance on traditional testing methods.

Potential Genotoxic Impurities (PGIs) and Their Relationship with Carcinogenicity

In the context of pharmaceutical impurity assessment, the identification and evaluation of genotoxic risks are of paramount importance due to their potential long-term consequences on human health. Among the various categories of impurities, those that raise concerns regarding genetic toxicity are classified as Potential Genotoxic Impurities (PGIs). A PGI is typically defined as an impurity that contains a recognised structural alert associated with genotoxicity, but for which definitive experimental or computational evidence confirming its DNA-reactive potential is not yet available. In essence, the designation of a compound as a PGI represents a precautionary classification, signalling the need for further investigation to clarify its toxicological profile.

The concept of structural alerts plays a central role in identifying PGIs. As previously discussed, structural alerts are specific molecular features or functional groups that have been associated with genotoxic or carcinogenic activity based on empirical data and mechanistic understanding.[59,62] When such an alert is identified within the chemical structure of an impurity, it raises an initial concern regarding its potential to interact with DNA and induce mutations. However, it is important to recognise that the presence of a structural alert does not guarantee genotoxicity or carcinogenicity. Instead, it indicates a possibility that must be evaluated through additional testing and analysis. The relationship between structural alerts and carcinogenic potential is complex and often uncertain. While structural alerts are useful for flagging compounds that may pose a genotoxic risk, their predictive value for carcinogenicity is less robust.[63,64] This is because carcinogenesis is a multifactorial process that may involve both genotoxic and non-genotoxic mechanisms. Consequently, the link between a structural alert and actual carcinogenic activity is highly dependent on the specific chemical context. Factors such as metabolic activation, chemical reactivity, exposure level, and biological environment can all influence whether a structurally alerting compound ultimately exhibits carcinogenic effects. Given this uncertainty, impurities identified as PGIs require further evaluation through experimental or computational methods to determine their true genotoxic potential.[27,65] One of the most widely used and regulatory-accepted assays for this purpose is the Ames test, a bacterial reverse mutation assay that detects the ability of a compound to induce genetic mutations in specific strains of bacteria (Figure 6). Once an impurity is classified as being of toxicological concern, it is generally mandatory to perform at least an Ames test to assess its mutagenic potential. Depending on the results and the level of concern, additional assays, such as *in vitro* mammalian cell tests or *in vivo* genotoxicity studies may also be conducted to provide a more comprehensive evaluation. The outcome of the Ames test plays a critical role in determining the regulatory and toxicological handling of a PGI.[24,66] A negative result in the Ames assay can significantly reduce the level of

concern associated with a structural alert. In many cases, a negative Ames test is considered sufficient to override the initial alert, provided that the impurity is present at levels below the thresholds established by relevant regulatory guidelines, such as those outlined in ICH Q3A and Q3B. This reflects the regulatory confidence in the Ames test as a reliable indicator of genotoxic potential, particularly for DNA-reactive compounds. However, while the Ames test is a powerful and widely accepted tool, it is not without limitations.[67,68] Its predictive accuracy is not absolute, and it is important to interpret its results within the context of its known performance characteristics. The assay is reported to have a positive predictivity of approximately 80%, meaning that a significant proportion of compounds that test positive in the Ames assay are indeed genotoxic and potentially carcinogenic. This relatively high positive predictivity supports the cautious approach of treating Ames-positive impurities as potential carcinogens unless proven otherwise. In contrast, the negative predictivity of the Ames test is considerably lower (<50%). This indicates that a substantial number of compounds that test negative in the assay may still exhibit carcinogenic effects through mechanisms that are not detected by the test. These so-called non-genotoxic carcinogens do not directly interact with DNA but may promote cancer development through alternative pathways, such as hormonal disruption, chronic inflammation, or epigenetic modifications.[47,69] As a result, a negative Ames test does not eliminate the possibility of carcinogenic risk, although it significantly reduces the likelihood of DNA-reactive genotoxicity.

The discrepancy between genotoxicity and carcinogenicity is further illustrated by findings from long-term carcinogenicity studies. These studies, typically conducted in rodents, often involve administering high doses of a test compound over extended periods to assess its tumorigenic potential. In many cases, the doses used correspond to the Maximum Tolerated Dose (MTD), which is the highest dose that does not cause unacceptable levels of toxicity. While this approach increases the sensitivity of the assay, it can also introduce confounding factors such as cytotoxicity, tissue damage, and metabolic overload. These effects may lead to tumour formation through mechanisms unrelated to the intrinsic genotoxicity of the compound, thereby complicating the interpretation of results.[70,71] Consequently, some compounds that are Ames-negative may still produce tumours in carcinogenicity studies due to these non-genotoxic mechanisms. Conversely, there are also instances where compounds that are mutagenic in the Ames test do not exhibit carcinogenic activity in long-term studies. This apparent inconsistency highlights the complexity of the relationship between genotoxicity and carcinogenicity and underscores the importance of using a weight-of-evidence approach in toxicological assessment. Despite these complexities, it is generally considered prudent to assume that an Ames-positive impurity may possess carcinogenic potential, particularly in the absence of additional data to the contrary. This conservative approach is aligned with the precautionary principles that underpin regulatory decision-making in the pharmaceutical industry. By treating mutagenic impurities as potential carcinogens, developers can implement appropriate control measures to minimise patient exposure and ensure a high level of safety.[17,18] At the same time, it is important to recognise that not all mutagenic compounds will necessarily lead to cancer, and the presence of a positive Ames result does not automatically preclude the use of a drug product.[67] Instead, the level of risk must be carefully evaluated in the context of factors such as exposure duration, therapeutic benefit, and available alternatives.[72,73] In some cases, higher levels of a genotoxic impurity may be deemed acceptable for short-term treatments or for drugs used in life-threatening conditions,

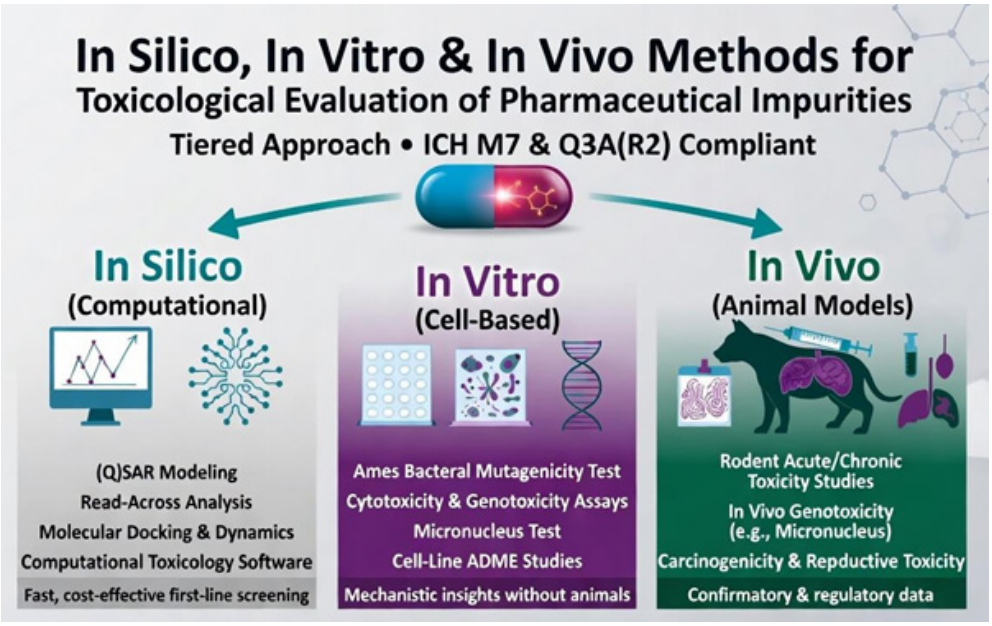

Figure 6: Various methods used for the toxicological evaluations of impurities in pharmaceutical products.

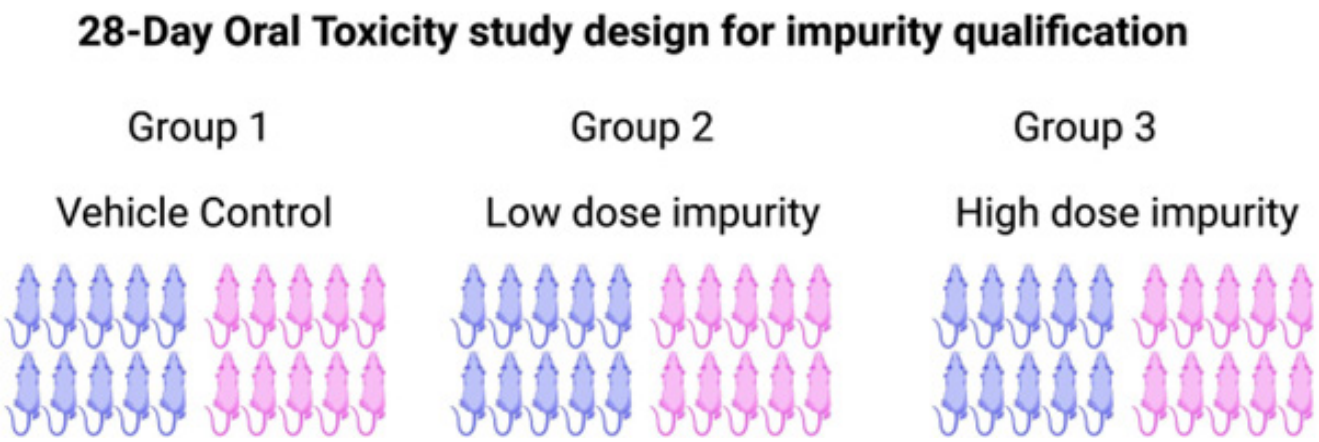

Figure 7: Outline of a 28-day oral toxicity study design for impurity qualification in rats.

where the benefits outweigh the potential risks. The classification of impurities as PGIs and their subsequent evaluation through assays such as the Ames test represent critical steps in the overall framework of impurity risk assessment. These processes enable the identification of potentially hazardous compounds, guide the selection of appropriate testing strategies, and inform decisions on impurity control and regulatory compliance. By integrating structural analysis, experimental data, and scientific judgment, pharmaceutical developers can effectively manage the risks associated with genotoxic impurities and ensure the safety of drug products intended for clinical use.

Qualification of Impurities in Active Pharmaceutical Ingredients (APIs)

Impurity qualification is a critical component of pharmaceutical development, focusing on the establishment of the biological safety of impurities present in APIs and finished drug products. Qualification refers to the systematic process of acquiring, analysing, and interpreting data that demonstrate that a specific impurity, degradation product, or metabolite is safe at the levels at which it is present (Figure 2). This process is essential to ensure that unintended substances within a drug do not pose unacceptable risks to patients, even when present in small quantities. At its core, impurity qualification is a science-based evaluation that integrates toxicological, pharmacological, and exposure data.[17,74] The goal is to determine whether an impurity can be considered safe within defined limits, based on evidence derived from nonclinical (animal) studies, clinical trials, or other relevant sources. This evaluation becomes particularly important when impurity levels exceed regulatory thresholds (Figure 4) for identification or qualification, as outlined by guidelines from the International Council for Harmonisation.

One of the key principles in impurity qualification is that an impurity may be considered qualified if it has been adequately tested as part of the drug substance used in nonclinical safety studies or clinical trials.[3,17] In such cases, the impurity is already present in the material administered to test subjects, and its safety is indirectly assessed through the overall toxicological evaluation of the drug product. This approach relies on the assumption that any adverse effects observed during testing would reflect the combined influence of the active ingredient and its associated impurities. A crucial step in this process is the accurate quantification of impurities present in the batches of drug substance used during nonclinical safety studies. Without precise knowledge of impurity levels in these studies, it is difficult to establish a reliable link between exposure and safety outcomes.[18,30] Analytical characterization of these batches allows scientists to determine the extent to which each impurity has been tested and to assess whether its presence falls within acceptable safety margins. In many cases, impurities that are also significant human metabolites are considered qualified if they are observed in animal studies.[3,25] This is based on the understanding that metabolites formed in the human body as part of normal drug metabolism are likely to have been evaluated during toxicological testing in animals. If an impurity corresponds to such a metabolite and is present at comparable or lower levels, it is generally assumed to have an acceptable safety profile, reducing the need for additional qualification studies. Another important consideration in impurity qualification is the comparison between the levels of impurities tested in nonclinical studies and those expected in clinical use.[3,17] Often, nonclinical safety studies are conducted at dose levels significantly higher than the intended clinical dose to maximize the likelihood of detecting potential toxic effects. As a result, both the active pharmaceutical ingredient and its associated impurities are administered at elevated levels during these studies.[3,13,75] This provides an opportunity to evaluate the safety of impurities under worst-case exposure conditions. By comparing the doses used in nonclinical studies with the proposed clinical dose, scientists can establish a margin of safety for each impurity. If the exposure to an impurity in nonclinical studies is substantially higher than that expected in patients, and no adverse effects are observed, this margin may be considered sufficient to justify the impurity's presence at lower levels in the final product. This approach is particularly

useful for impurities present at low relative concentrations, where the likelihood of acute or single-dose toxicity is minimal. The concept of safety margins is central to impurity qualification, as it provides a quantitative basis for risk assessment. A large margin between the tested dose and the clinical exposure suggests a lower risk, while a smaller margin may indicate the need for additional studies or tighter control of impurity levels. This comparative approach allows for a rational and evidence-based determination of acceptable impurity limits.

It is also important to recognise that impurity qualification is not limited to a single stage of drug development but is an ongoing process that evolves as new data become available.[3,13,17,75] Changes in manufacturing processes, formulation, or storage conditions may alter the impurity profile of a drug product, necessitating re-evaluation of previously qualified impurities. Continuous monitoring and re-assessment ensure that impurity levels remain within safe and acceptable limits throughout the product lifecycle.[10,26,76] Regulatory guidelines play a crucial role in defining the thresholds and requirements for impurity qualification. ICH provides detailed guidance on reporting, identification, and qualification thresholds based on the maximum daily dose of the drug. These thresholds help determine when additional data are required and ensure a consistent approach to impurity evaluation across different regulatory jurisdictions (Figure 3). Overall, impurity qualification represents a comprehensive and structured approach to ensuring the safety of pharmaceutical products. By integrating analytical data, toxicological evidence, and exposure assessments, this process enables the identification of acceptable impurity levels that do not compromise patient health. It reflects a balance between scientific rigor and practical feasibility, ensuring that drug products meet stringent safety standards while remaining accessible and effective for clinical use.

Toxicity Studies and Design Strategies for Impurity Qualification

Toxicity studies play a central role in the qualification of impurities, particularly those with suspected or confirmed genotoxic potential.[3,77] These studies are designed to evaluate the biological and toxicological effects of impurities to ensure that their presence in pharmaceutical products does not pose unacceptable risks to human health. The selection and design of appropriate toxicity studies depend on the nature of the impurity, the level of exposure, and the availability of existing data. A structured and stepwise approach is typically employed, beginning with standard genotoxicity assays and progressing to more comprehensive studies if necessary (Figure 6).[33,78] For impurities with potential genotoxic properties, regulatory frameworks recommend a standard battery of tests to assess their ability to induce genetic damage. The first and most widely used test in this battery is the bacterial gene mutation assay, commonly known as the Ames test.[67,79] This assay evaluates the capacity of a compound to cause mutations in specific strains of bacteria, providing a rapid and sensitive indication of mutagenic potential. Due to its reliability and extensive validation, the Ames test is considered a cornerstone of genotoxicity testing and is often the initial step in impurity evaluation. In addition to the Ames test, *in vitro* cytogenetic assays are conducted to assess chromosomal damage in mammalian cells.[80,81] These tests include evaluations of structural and numerical chromosomal aberrations, which can provide insight into the clastogenic or aneugenic effects of a compound.[82,83] Another commonly used assay is the *in vitro* mouse lymphoma Thymidine Kinase (TK) assay, which detects gene mutations at the thymidine kinase locus in cultured mammalian cells.[84,85] Together, these assays form a comprehensive screening battery that addresses different mechanisms of genetic damage, enhancing the overall reliability of the assessment. When the results of these standard genotoxicity tests are negative, the impurity is generally considered to have a low likelihood of causing genetic damage. In such cases, no further genotoxicity studies are typically required, and the impurity may be regarded as sufficiently qualified with respect to its genotoxic potential. This outcome allows developers to proceed with confidence, as the absence of genotoxic effects significantly reduces concerns about long-term risks such as carcinogenicity. However, the situation becomes more complex when one or more of the genotoxicity assays yield positive results, or when other data suggest a potential carcinogenic risk. Positive findings indicate that the impurity may interact with genetic material, necessitating further investigation to clarify the extent and relevance of the risk. In such cases, additional studies may be required, including *in vivo* genotoxicity assays or longer-term toxicity studies, to determine whether the observed effects are reproducible and relevant under physiological conditions.

In instances where the results of initial testing are inconclusive or where the potential risk is considered higher than acceptable, general toxicity studies are recommended. These studies are typically conducted in one animal species, most commonly the rat, and are designed to evaluate systemic toxicity over a defined period. The duration of these studies can range from 14 to 90 days, depending on the level of concern and the intended duration of clinical exposure.[86,87] A commonly employed design is the 28-day repeated-dose toxicity study (Figure 7), which provides a balance between depth of evaluation and practical feasibility. The design and execution of such studies generally follow internationally recognised guidelines, such as the OECD 407 guideline for repeated-dose toxicity studies in rodents. These guidelines outline the parameters to be monitored, including clinical observations, body weight, food consumption, haematology, clinical chemistry, organ weights, and histopathological examination of tissues.[88,89] By systematically assessing these endpoints, researchers can identify potential target organs, dose-response relationships, and any adverse effects associated with the impurity. Beyond the selection of appropriate toxicity tests, the design of impurity evaluation studies can take two primary forms, each with its own

advantages and limitations. The first approach involves testing the drug substance or product containing the impurity at levels that are equal to or higher than those expected in the final formulation. In this method, the impurity is evaluated within the context of the complete pharmaceutical matrix, allowing for a realistic assessment of its effects in combination with the active ingredient and other components. This approach offers several practical advantages. It enables the simultaneous evaluation of multiple impurities, which can be particularly useful when dealing with complex impurity profiles. Additionally, the preparation of test samples is often straightforward, as impurities can be generated through forced degradation or by modifying manufacturing conditions. This makes the approach relatively efficient and cost-effective, especially in the early stages of development. However, testing impurities within the drug product matrix also presents certain challenges. Because the sample contains multiple chemical species, it may be difficult to attribute observed toxic effects to a specific impurity. There is also the potential for interactions between impurities or between impurities and the active ingredient, which may lead to synergistic or antagonistic effects. These interactions can complicate the interpretation of results and may result in false-positive/negative findings that do not accurately reflect the risk associated with an individual impurity. The second approach involves isolating the impurity and evaluating it as a single, pure compound. This method provides a more precise assessment of the impurity's toxicological properties, as it eliminates the influence of other components in the formulation. By focusing on a single substance, researchers can obtain clearer and more definitive data regarding its safety profile, facilitating more accurate risk assessment and regulatory decision-making. Despite its advantages, the isolation approach is often more challenging to implement. Obtaining enough of a pure impurity for toxicological testing can be technically demanding, particularly when the impurity is present at very low levels. The process may require complex synthesis or purification steps, which can be time-consuming and expensive. These practical constraints must be carefully considered when selecting the most appropriate study design. In practice, the choice between these two approaches depends on a variety of factors, including the nature and level of the impurity, the stage of drug development, and the resources available. In some cases, a combination of both approaches may be employed to provide a more comprehensive evaluation. For example, initial screening may be conducted using the drug product matrix, followed by targeted studies on isolated impurities to confirm specific findings. Overall, toxicity studies and their design are integral to the qualification of impurities, ensuring that any potential risks are thoroughly evaluated and appropriately managed. By employing a combination of standardised test batteries, regulatory guidelines, and strategic study designs, pharmaceutical developers can generate robust data to support the safe use of drug products. These efforts ultimately contribute to the protection of patient health and the successful development of high-quality pharmaceutical therapies.

CONCLUSION

In conclusion, the toxicological assessment and risk management of pharmaceutical impurities demand a rigorous, science-driven framework that integrates classification, predictive modelling, and experimental validation. Effective control begins with the systematic identification and categorisation of impurities, followed by a robust risk assessment approach, particularly for genotoxic impurities grounded in conservative safety thresholds and regulatory guidance. The increasing reliance on QSAR and *in silico* tools has enhanced early hazard identification, enabled more efficient prioritisation while reducing dependence on animal testing. Understanding the link between potential genotoxic impurities and carcinogenicity remains central to safeguarding patient health, reinforcing the need for cautious evaluation and control strategies. Equally critical is the qualification of impurities in APIs through well-designed toxicity studies that align with intended exposure levels and clinical context. Altogether, a multidisciplinary and adaptive approach, combining computational, analytical, and toxicological expertise ensures that impurity risks are effectively managed throughout the drug development lifecycle, ultimately supporting the delivery of safe, high-quality pharmaceutical products.

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ABBREVIATIONS

API: Active Pharmaceutical Ingredient; **ICH:** International Council for Harmonisation; **TTC:** Threshold of Toxicological Concern; **HPLC:** High-Performance Liquid Chromatography; **NMR:** Nuclear Magnetic Resonance; **ICP-MS:** Inductively Coupled Plasma Mass Spectrometry; **QSAR:** Quantitative Structure-Activity Relationship; **SAR:** Structure-Activity Relationship; **PGIs:** Potential Genotoxic Impurities; **NOEL:** No-Observed-Effect Level; **DNA:** Deoxyribonucleic Acid; **SAR:** Structure Activity Relationship; **SAs:** Structural Alerts; **TK:** Thymidine Kinase; **OECD:** Organisation for Economic Co-operation and Development; **ALAR:** As Low As Reasonably Practicable.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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