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GENOTOXIC IMPURITIES IN PHARMACEUTICALS PRODUCTS

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Abstract: Genotoxins refer to agents or carriers, such as chemicals or radiation that have the ability to induce damage to DNA or the structure of chromosomes. This damage can lead to mutations, and the overall process is known as genotoxicity. It is crucial to identify and comprehend genotoxins during the early stages of drug development in order to prevent potential harm caused by these agents. Various regulatory bodies, including the International Council for Harmonization, EMEA, USFDA, and European Pharmacopeia, have provided guidelines to restrict the presence of impurities in both drug substances and drug products. These guidelines aim to ensure the safety and efficacy of pharmaceuticals. A potential genotoxic impurity (PGI) has been defined as an "Impurity that shows a Structural alert for genotoxicity but that has not been tested in an experimental test model. Here potentially relates to genotoxicity, not to the presence or absence of this Impurity".

The current study is based on analysis of data to determine the presence of genotoxic impurities in drugs and medicinal products. Start with the definition of genotoxic impurities, their sources, properties, and classification by toxicity. This article also describes various regulatory mechanisms for genotoxic impurities in pharmaceuticals. This review also describes the separation, identification and characterization of impurities and their measurement by various analytical methods.

Index Terms - Genotoxic, Impurities, Drug substances, Guidelines, Pharmaceutical drug product.

I. INTRODUCTION

Genotoxicity:

Genotoxicity refers to the detrimental impact on the genetic material (DNA and RNA) of a cell, leading to a disruption in its integrity. Genotoxins encompass mutagens, which can be radiation, chemical, or physical agents. A substance that exhibits genotoxicity is referred to as a genotoxin. Genotoxins can manifest as carcinogens, causing cancer, mutagens, inducing mutations, or teratogens, resulting in birth defects.[1]

The International Council for Harmonization, in its guideline ICH S2 (R1), provides a definition of genotoxicity as "a comprehensive term encompassing any harmful alteration in the genetic material, irrespective of the method by which the alteration is caused." On the other hand, genotoxic impurities are described as "impurities that have been proven to possess genotoxic properties through suitable genotoxicity testing models, such as the bacterial gene mutation test." Pharmaceutical drugs are invariably linked to the presence of undesirable chemicals in addition to their active components. These undesirable chemical compounds are referred to as impurities. Therefore, impurities can be defined as any substances that coexist with the drug substance, including starting materials, reagents, catalysts, raw materials, or intermediates that

arise during synthesis or develop during the storage or transportation of the drug. Impurities do not offer any advantages to the patient, but they have the potential to cause adverse effects. [2]

A potential genotoxic impurity (PGI) is characterized as an impurity that exhibits a structural alert for genotoxicity but has not undergone experimental testing in a model. The term "potentially" in this context refers to its association with genotoxicity rather than the mere presence or absence of the impurity.

Genotoxic substances can cause mutations in the genetic material by breaking chromosomes, rearranging them, binding covalently or inserting into DNA during replication. Even exposure to small amounts of these substances can lead to cancer. Therefore, it is crucial to detect genotoxic impurities in medicines and keep them under strict surveillance at low levels to ensure public safety. [3, 4]

SOURCES OF GENOTOXIC IMPURITIES:

There are several sources from which genotoxic impurities can be incorporated into drug substances. The primary source is the starting material used in the synthesis of the drug substance and its impurities. Additionally, genotoxic intermediates and by-products formed during the synthesis process may also be carried forward to the drug substance as impurities. Solvents, catalysts, and reagents used in the synthesis process can also potentially be a source of genotoxic impurities in drug substances. Furthermore, degradation products generated during storage, shipment, or exposure to light, air oxidation, or hydrolysis can contribute to the generation of impurities in drug substances. If the required drug substance is a specific isomer, then stereoisomers of the raw material and intermediate can also contribute to the generation of chiral impurities in drug substances. **Figure 1** illustrates the generation of impurities during different phases of drug synthesis. In addition to these excipients and their impurities, extractable and leachable substances can also contribute to the presence of genotoxic impurities in drug products [5, 6] **(Table 1).**

The International Council for Harmonization (ICH), the Food and Drug Administration (FDA), and the United States Pharmacopeia (USP) have established classifications for impurities. These impurities include drug-related impurities, process-related impurities (PRIs), residual solvents, and heavy metals, as shown in **Figure 2**. There are two types of impurities related to active pharmaceutical ingredients (API). The first type is caused by reactions, such as oxidation, dehydration, and carbon dioxide removal. The second type is a result of the interaction between the API and excipients, containers, or residual impurities in excipients, reagents, or solvents. It is important to note that API-related impurities have the potential to be genotoxic, mutagenic, and carcinogenic due to their structure-activity relationship. [7]

Table 1 Genotoxic compounds in drug substances

Category/Stage	Compounds
Starting material	Hydrazine, Nitroso, acrylonitrile compounds
Intermediate	Benzaldehyde, Nitro compounds
By-product	Sulphonate esters, phosgene
Reagent	Formaldehyde, epoxides, esters of phosphate & sulphonates
Solvent	Benzene, 1,2-dichloroethane
Catalyst	Toxic heavy metals, metal phosphates
Degradation product	N-oxides, aldehydes,

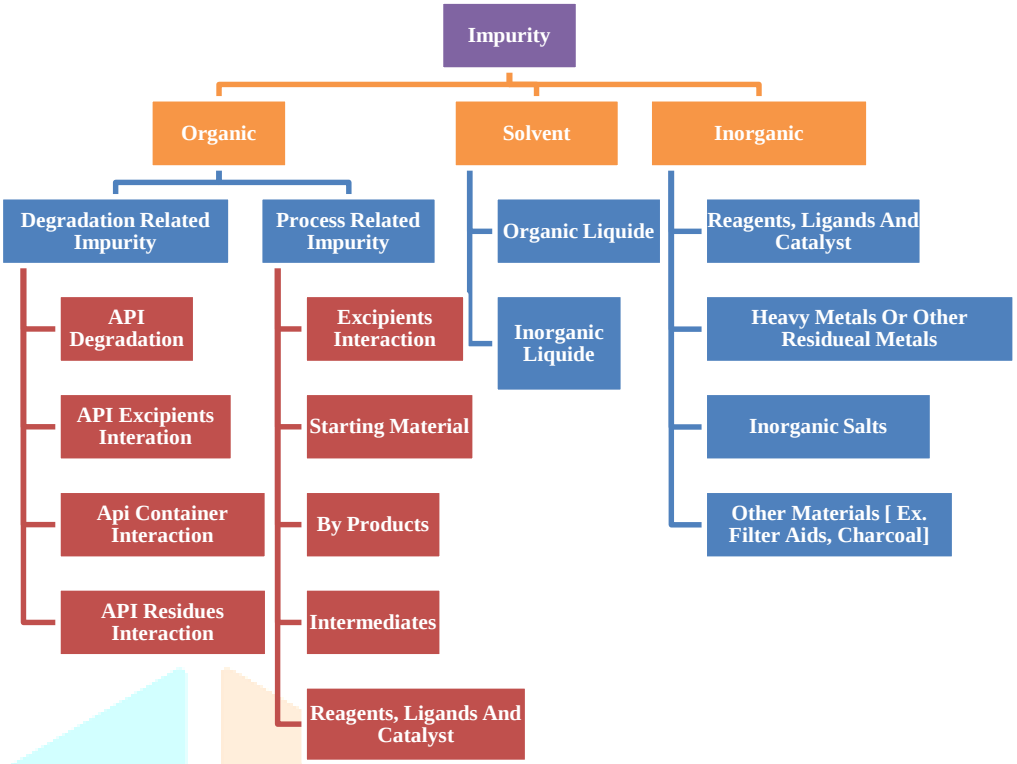

Figure 2 Classification of impurities

NEED OF GENOTOXICITY DETERMINATION:

Genotoxicity data play a crucial role in assessing the risks associated with chemicals, as well as food and feed, consumer products, human and veterinary medicines, and industrial substances. These data serve as the foundation for evaluating the potential risks posed by natural and environmental contaminants found in chemicals, food, and feed. Based on this information, numerous regulatory agencies and advisory bodies have provided recommendations on genotoxicity testing strategies. Genetic alterations in somatic and germ cells, even at low exposure levels, can have serious implications for human health. Mutations in proto-oncogenes, tumor suppressor genes, or DNA damage response genes, caused by various factors such as physical or chemical agents, can lead to a range of genetic diseases. Additionally, somatic cells with damaged DNA can

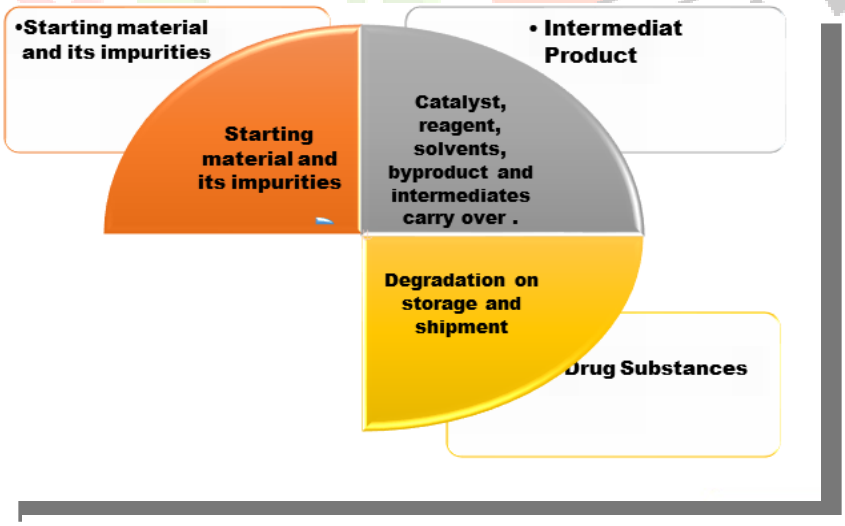

Figure 1: Sources of genotoxic impurities.

contribute to degenerative conditions like accelerated aging, immune dysfunction, cardiovascular issues, and neurodegenerative diseases (refer to **Fig. 3** for a visual representation). To mitigate the adverse effects of genetic damage on human health, assessing the mutagenic potential is a fundamental aspect of chemical risk assessment. [8]

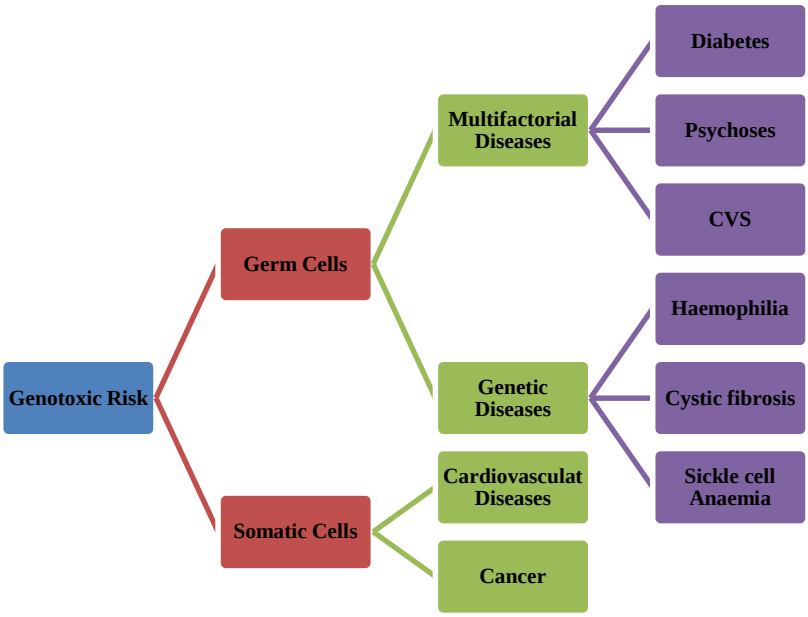

Figure 3 Genetic risk flow chart

Regulatory authorities worldwide require comprehensive data on the genotoxic potential of new drugs as part of the safety evaluation process. These data serve as documented evidence for the safety assessment of both the product and the manufacturing process. Consequently, pre-clinical studies are typically conducted to evaluate the basic toxicological data of new chemical entities (NCE). By analyzing this toxicological data, the safety and efficacy of NCE can be assessed, aiding in the determination of potential risks or benefits during the new drug application (NDA) process. Furthermore, this evaluation helps identify genotoxic hazards related to DNA damage, including cancer-causing agents, mutagens, mutation-causing agents, teratogens, and agents that cause birth defects. [9]

REGULATORY GUIDELINES: **[10, 11, 12, 13]**

ICH, EMEA, USFDA, European Pharmacopoeia guidance, and the Guidance for oncology products all offer comprehensive guidelines to restrict the presence of impurities in both drug substances and drug products.

EMEA guideline:

The EMEA guideline on the limits of genotoxic/mutagenic impurities (GTIs) divides GTIs into two categories. GTIs that have experimental data available provide clarification on their mechanism. These GTIs are specified in class 2 solvents according to ICH Q3C (R4). In cases where there is no experimental data to support the mechanism of GTIs, they can still be controlled to the lowest feasible level in accordance with the ALARP principle. The acceptable limits for GTIs take into account the therapeutic toxic concentration (TTC) value, with a maximum intake of 1.5 g/day.[14,15]

Pharmaceutical Research and Manufacturers Association (PhRMA): [16]

The Pharmaceutical Research and Manufacturers Association (PhRMA) released a set of regulations in 2006 regarding the testing, classification, qualification, and assessment of toxicological risks associated with genotoxic impurities. In this publication, PhRMA categorized genotoxic impurities into different groups based on the functional groups they possess, which have the ability to react with DNA. These functional groups were further classified into three categories: Aromatic, Alkyl/Aryl, and Heteroatomic (as shown in **Figure 4).**

Group 1-
Includes aromatic groups such as N hydroxyaryls, N-acylated amino-aryls, aza-aryl N-oxides, amino-aryls, alkylated amino-aryls, purines or pyrimidines, intercalators, PNAs, or PNAHs.

Group 2 –
consists of alkyl and aryl groups like aldehydes, N-methylols, N-nitrosamines, nitro compounds, carbamates (urethanes), epoxides, aziridines, propiolactones, propiosulfones, N or S mustards (beta-haloethyl), hydrazines, and azo compounds.

Group 3 –
is composed of hetero aromatic groups such as Michael Reactive acceptors, alkyl esters of phosphonates or sulfonates, haloalkenes, and primary halides (alkyl and aryl-CH2).

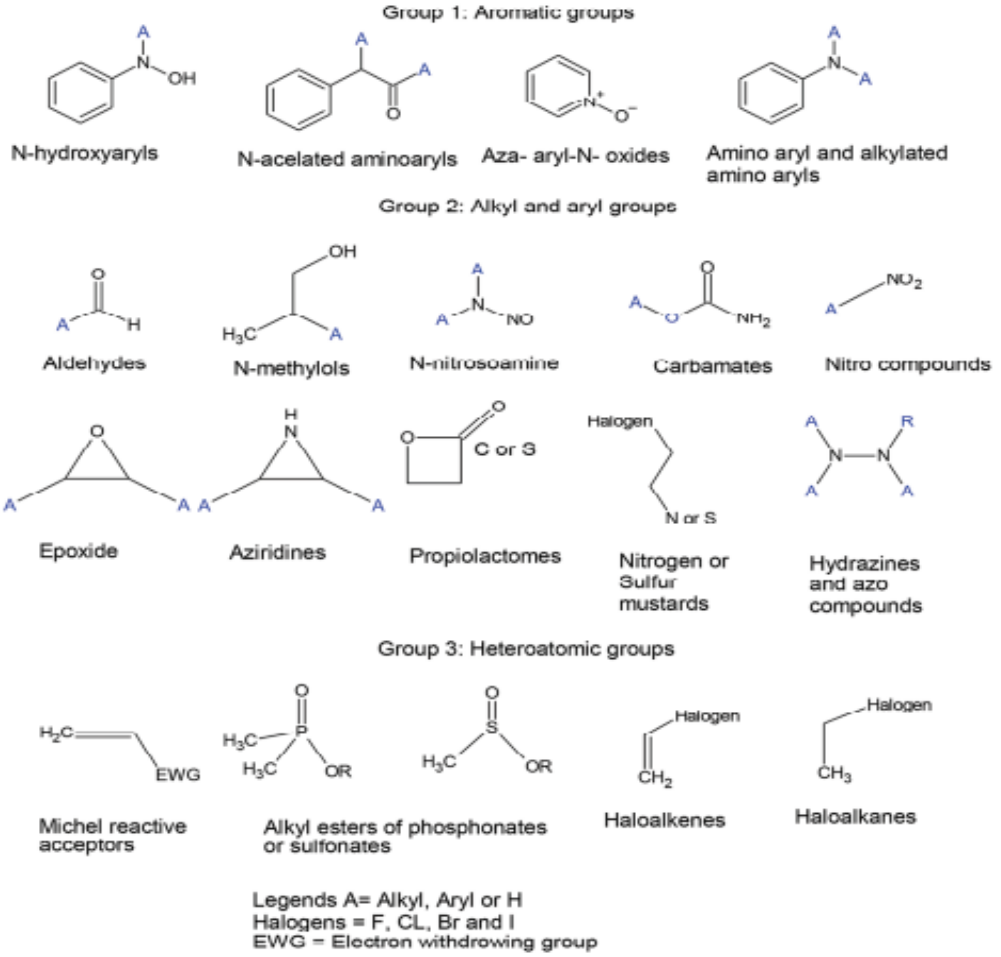

Figure 4 Structure aletring functional groups.

PhRMA categorized impurities into five classes:

Table 2 PhRMA classification of impurities: [17]

Class	Definition	Proposed action for control
C Class 1	known carcinogens with more risk of genotoxicity	C control at or below compound specific acceptable limits (TTC)
C Class 2	known mutagens with unknown carcinogenic potential	Con control at or below specific acceptable limits (appropriate TTC)
C Class 3	Drug structural alerts, not related to drug structure No genotoxic, mutagenic data	Control at or below acceptable limits (TTC) or conduct QSAR studies Non-mutagenic=Class 5 Mutagenic=Class 2
C Class 4	Alerting structures are same as that of functional groups related to drugs which are tested and found to be non mutagenic	Non-genotoxic impurity
C Class 5	No structural alerts with sufficient data, evidence indicates lack of genotoxicity or carcinogenicity	Non-genotoxic impurity

TTC: Therapeutic toxic concentration, QSAR: Quantitative structure–activity relationship.

European Medicine Agency (EMA): [18]

The European Medicine Agency (EMA) implemented the Guideline on the Limits of Genotoxic Impurities following the release of draft versions for consultation in 2002 and 2004. The final version was made available in 2006. The toxicity data pertaining to genotoxic impurities varies significantly and serves as the primary determinant for the assessment of acceptable limits. In cases where data is lacking, the use of an established risk assessment method becomes necessary. Consequently, EMA initially introduced the concept and values for the threshold of toxicological concern (TTC) to regulate genotoxic impurities. In 2010, EMA also published a set of questions and answers on the Guideline on the Limits of Genotoxic Impurities to provide further clarification and ensure consistency in the implementation of the genotoxicity guideline.

Food Drug Administration (FDA): [19]

In December 2008, the USFDA released a preliminary guidance for the pharmaceutical industry titled "Genotoxic and Carcinogenic Impurities in Drug Substances and Products." This guidance from the FDA offers detailed suggestions on how to assess the safety of impurities that are known or suspected to have genotoxic or carcinogenic properties. It outlines various methods to evaluate and minimize the risk of cancer for patients exposed to genotoxins and carcinogenic impurities. Interestingly, the approaches outlined in this guidance were comparable to those presented in the EMA guideline (**Table 2).**

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Table 3 FDA guidance steps risk minimization

Step	Action
1	Modify the synthesis or purification procedure to reduce the occurrence of the pertinent impurity and enhance its elimination.
2	Set a general objective of a maximum daily exposure limit of 1.5 µg per day for the relevant impurity.
3	Conduct a more comprehensive assessment of the genotoxic and carcinogenic hazards to provide better guidance on impurity specifications, whether for increased or decreased levels.

International Council for Harmonization (ICH): [20]

The initial guideline concerning genotoxicity was introduced by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) in July 1995. This guideline, known as S2A, provided specific recommendations and guidance for conducting regulatory genotoxicity tests for pharmaceuticals. It covered both in vitro and in vivo tests, as well as the evaluation of test results.

In 1997, the second guideline, S2B, was published. This guideline focused on establishing a standard battery of genotoxicity tests for pharmaceuticals.

In 2013, the M7 guideline was released, which offered guidance on the analysis of Structure-Activity Relationships (SAR) for genotoxicity.

Following this, the M7 (R1) guideline was published in June 2015 (step 2) and May 2017 (step 4).

Currently, the M7 (R2) guideline is being revised. This updated guideline will incorporate acceptable limits (Acceptable Intakes or Permitted Daily Exposures) for new DNA reactive (mutagenic) impurities. It will also revise the acceptable limits for impurities already listed in the Addendum, based on new data as it becomes available. The revised guideline, known as ICH M7 (R2), will be released in the future.

CLASSIFICATION OF GENOTOXIC IMPURITIES: [21]

Genotoxic impurities are categorized based on their risk assessment, which involves an initial analysis of both actual and potential impurities. This analysis is conducted by conducting searches in databases and literature to gather data on carcinogenicity and bacterial mutagenicity. Based on this data, the impurities are classified into Class 1, 2, or 5. If there is a lack of data for classification, an assessment of Structure-Activity Relationships (SAR) is performed, with a focus on bacterial mutagenicity predictions. This assessment can result in the impurities being classified into Class 3, 4, or 5.

Each class is defined as follows:

Class 1:
These impurities have established mutagenic and carcinogenic data, making them the most serious risk. It is crucial to eliminate these impurities by modifying the process. If elimination is not possible, these impurities should be limited to the "Threshold of Toxicological Concern (TTC)" as a last resort.

Class 2:
These impurities have well-established mutagenic data, but their potential to cause cancer is unknown. Therefore, these impurities should be controlled using the TTC approach.

Class 3:
These impurities have alert structures that are unrelated to the structure of the drug substances and their genotoxic potential is unknown. Their toxicity is determined based on the structure-activity relationship (SAR) and the presence of specific functional groups within their molecule.

Class 4:
These impurities have structures similar to the drug substances and may contain functional groups or moieties that have potential alerts shared with the parent structure. However, they are considered to be non-genotoxic.

Class 5:
These impurities do not have any alert structures and there is evidence indicating the absence of genotoxicity. These compounds are treated as normal impurities and controlled according to the guidelines set by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH).

RISK ASSESSMENT OF IMPURITY: [22]

The management of genotoxic impurities (GTIs) is a highly significant undertaking that is carried out for all drug substances and drug products. A crucial aspect of this process is the risk assessment, which involves utilizing in silico assessment tools effectively. In the case of a particular impurity, the first step is to identify its structure and conduct a GTI assessment. If the impurity structure does not raise any concerns, it is controlled as a regular impurity in accordance with the ICH Q3 guidelines. However, if the impurity exhibits a structure alert, it undergoes an Ames test. A negative result from the Ames test leads to controlling the impurity as per the ICH Q3 guideline. On the other hand, if the Ames test yields a positive result, the impurity is limited to a safety level based on the TTC approach. Having knowledge of the chemical structure of the impurity and its formation mechanism is crucial in evaluating its toxicological implications. This knowledge also aids in enhancing synthetic chemical processes to minimize or eliminate the impurity. (**Figure 5).**

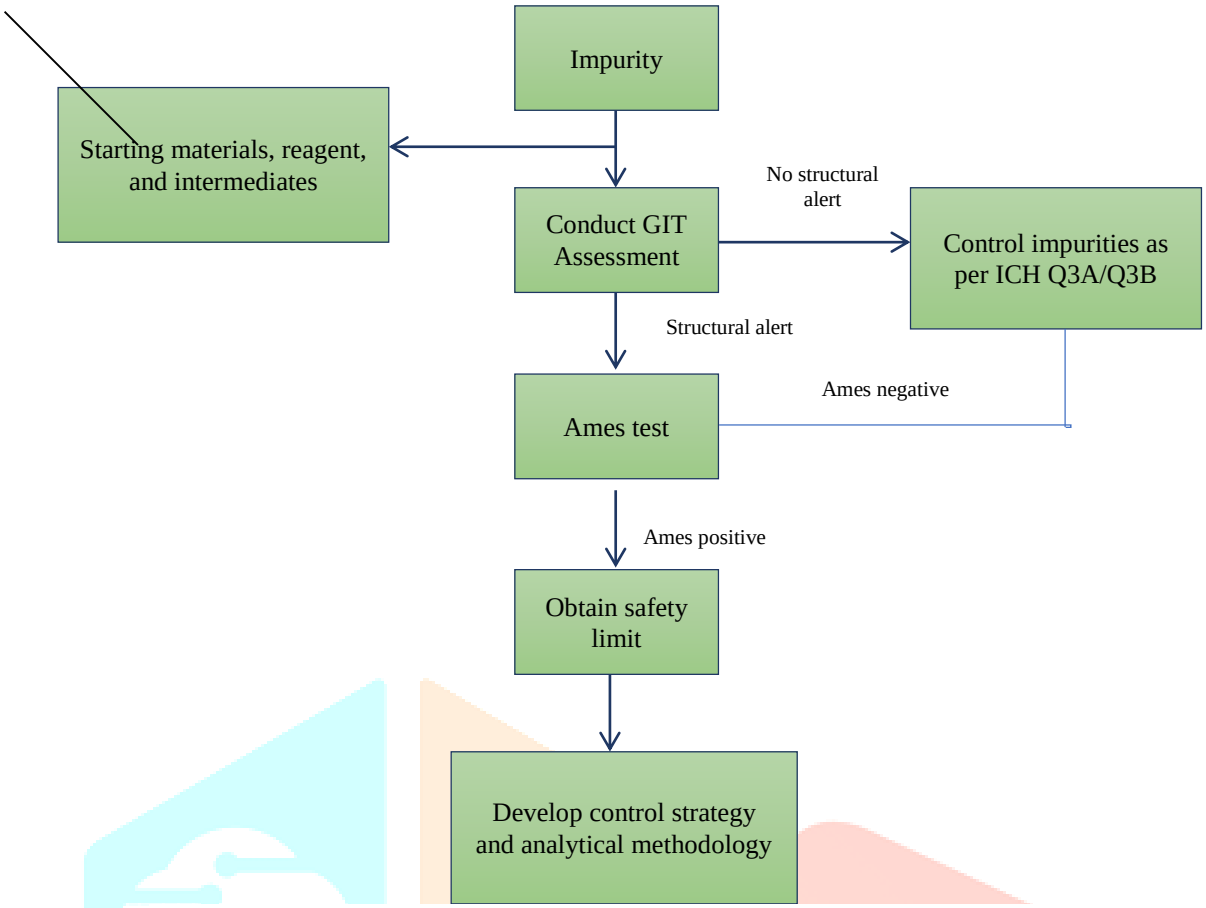

Figure 5: Genotoxic impurity assessment decision tree

Risk assessment for genotoxic and carcinogenic substances:

European Commission Health and Consumer Protection Directorate. General risk assessment methodologies and approaches for genotoxic and carcinogenic substances .

- ICH Q3A -Impurities in new drug substances
- ICH Q3B -Impurities in new drug products
- ICH Q3C- Guidance for Residual solvents.[23]

The standard test battery for genotoxicity recommends the following for genotoxicity evaluation [Table4]: [24]

Table 4 The genotoxicity evaluation or testing guidelines

TG 471	**Bacterial reverse mutation test (ames test)**
TG 472	Genetic toxicology: Escherichia coli, reverse assay
TG 473	In vitro mammalian chromosome aberration test
TG 474	Mammalian erythrocyte micronucleus test
TG 475	Mammalian bone marrow chromosome aberration test
TG 476	In vitro mammalian cell gene mutation test
TG 477	Genetic toxicology: Sex-linked recessive lethal test in drosophila melanogaster
TG 478	Genetic toxicology: Rodent dominant lethal test
TG 479	Genetic toxicology: In vitro sister chromatid exchange assay in mammalian cells
TG 480	Genetic toxicology: Saccharomyces cerevisiae, gene mutation assay
TG 481	Genetic toxicology: Saccharomyces cerevisiae, mitotic recombination assay
TG 482	Genetic toxicology: DNA damage and repair, unscheduled DNA synthesis in mammalian cells in vitro
TG 483	Mammalian spermatogonial chromosome aberration test
TG 484	Genetic toxicology: Mouse spot test

TG 485	Genetic toxicology: Mouse heritable translocation assay
TG 486	Unscheduled DNA synthesis (UDS) test with mouse liver cells in vitro
TG 487	In vitro mammalian cell micronucleus test

Tests to investigate the in vivo relevance of in vitro mutagens (positive bacterial mutagenicity (Table 5) [25]

Table 5 Tests to investigate the in vivo relevance of in vitro mutagens [positive bacterial mutagenicity]

In vivo test	Factors to justify choice of test as fit-forpurpose
Transgenic mutation assays	For any bacterial mutagenicity positive. Justify selection of assay tissue/organ
Pig-a assay (blood)	For directly acting mutagens (bacterial mutagenicity positive without S9)*
Micronucleus test (blood or bone marrow)	For directly acting mutagens (bacterial mutagenicity positive without S9) and compounds known to be clastogenic*
Rat liver unscheduled DNA synthesis (UDS) test	in particular for bacterial mutagenicity positive with S9 only responsible liver metabolite known to be generated in test species used to induce bulky adducts
Comet assay	Justification needed (chemical class specific mode of action to form alkaline labile sites or single-strand breaks as preceding DNA damage that can potentially lead to mutations justify selection of assay tissue/organ With convincing justification

*These table enlisted the mutagenicity test which wills helps to investigate the study of in-vitro mutation caused by in-vitro mutagens

TECHNIQUES TO DETECT GENOTOXIC IMPURITIES: [26]

In order to establish rational and effective control strategies for GTIs, it is crucial to employ sensitive, selective, and robust analytical methods to analyze impurities. The selection of methodology for analyzing GTIs is dependent on the target specifications and expected values for these impurities to comply with regulatory standards. Therefore, the chosen methodology should provide reliable analytical data and be suitable for routine testing. The selection of analytical techniques is based on the genotoxic and chemical properties of GTIs, such as their structures, reactivity's, and responsiveness to detection methods, as well as potential matrix effects, as outlined in Table 5.

The quantitation of GTIs at ppm levels in pharmaceuticals poses several challenges for analytical chemists, including:

- Appropriate selection of analytical techniques for method development based on the properties of the GTI, such as volatility, thermal stability, presence of a chromophore, and hydrophobicity.
- The reactive nature and stability of the GTI must also be reflected during method development to ensure requisite reproducibility and accuracy.
- Selection of a sensitive analytical method, as well as consideration of the clinical dose and duration of the study, must be taken into account during method development.

  Parameters for the analytical method to be developed include the choice of detection technique, such as ultraviolet (UV) or light scattering., electrochemical detection, mass spectrometry, and other techniques are commonly used for the analysis of GTI. However, there are challenges posed by the sample matrix and interfering components. These challenges can be overcome through various methods such as :

  1. Isolating the analyte of interest through sample preparation,
  2. Utilizing chromatographic resolution, or
  3. Using a more selective detector.

  Given the high structural diversity and complexity of the sample matrix, it is difficult to determine a single ideal method for analysis. No single approach can effectively address all the problems associated with GTI analysis. Over time, conventional separations, hyphenated techniques, and software-based in silico drug designs have become widely used in analytical laboratories.

In the pharmaceutical industry, systematic strategies are followed for GTI method development, which involve two steps.

[1] The volatility of the analyte is evaluated to select appropriate chromatographic techniques.

[2] Detection technique is chosen based on the properties of the analyte, such as the presence of a chromophore or a halogen atom within the molecule.

Table 6 Genotoxic properties and the chemical properties GTI's

Genotoxicity properties	Examples	Chemical properties	Analytical techniques
DNA reactive	Alkylating electrophiles, epoxides, azo compounds. Aromatic amines, aromatic nitro compounds	Unstable, polar, low molecular weight. Stable, moderate polarity, variable molecular weight	Derivatization followed by GC-MS or LC-MS. Direct analysis by GC-MS or LC-MS
DNA intercalation	Polyaromatic hydrocarbons, polynuclear aromatics, cytostatic molecules	Stable, low to moderate polarity, moderate to high molecular weight	Direct analysis by LC-MS

GC-MS: Gas chromatography-mass spectroscopy, LC-MS: Liquid chromatography-mass spectrometry, GTI: Genotoxic impurity.

Table 7 International guidelines outlining regulatory requirements for the control and test of IMPs/DPs in drug substances and products for human use

ICH (USA, EU, Japan)	Q3A(R2): Impurities in new drug substances Q3B(R2): Impurities in new drug products Q3C(R5): Impurities; guidelines for for residual solvents Q3D: Impurities: Guideline for metal impurities (final concept paper)

EMA (Europe)	M7: Evaluation and management of mutagenic (DNA reactive) contaminants in drugs to reduce the risk of cancer (final concept paper) EMEA/CHMP/CVMP/QWP/450653/2006 evaluation of the active ingredients in pharmaceuticals that are currently on the market or are known to be active EMEA/CHMP/QWP/251344/2006: Guidelines on the limits of genotoxic impurities and CPMP/SWP/5199/02: Control of impurities of pharmacopoeia substances QWP/CPMP/SWP/4446/00; corr: Guidelines for Metal Catalyst Residue Specification Limits EMA/CHMP/CVMP/QWP/199250/2009: Draft guidelines for specifying relevant contaminants in antibiotics
USFDA (USA)	NDAs: Suggested methodology for handling genotoxic and carcinogenic contaminants in pharmacological substances and products (draft) ANDAs: Impurities in pharmaceutical items
TPD (Canada)	Impurities in Existing Drug Substances and Products (draft)
TGA (Australia)	Guidelines for Australian regulations pertaining to prescription drugs: Appendix 18: Impurities in active components and finished pharmaceutical goods

ISOLATION OF GENOTOXIC IMPURITIES: [27].

To establish the structure and toxicity of a substance, it is crucial to isolate its impurities. Chromatographic techniques are commonly utilized in conjunction with classical methods for this purpose. The isolation of genotoxic impurities has been achieved through various methods.

a) Solid-phase extraction
b) Column chromatography
c) Flash chromatography
d) Supercritical fluid chromatography
e) Thin-layer chromatography
f) Capillary electrophoresis
g) Preparative high-pressure liquid chromatography
h) Accelerated solvent extraction,
i) Liquid-liquid extraction

Initially, the isolation of impurities should be carried out using simple extraction or partition methods. It is feasible to selectively extract impurities based on their acidity, basicity, or neutrality. The extraction process typically involves liquid-liquid extraction, where one phase is an aqueous solution and the other is a nonpolar organic phase. In the chromatographic method, the desired impurity peak or band is separated, concentrated, and isolated. This can be achieved by utilizing a basic chromatographic column prepared in the laboratory or by employing an instrument such as the Flash and a Preparative chromatograph. [12]

ANALYSIS OF IMPURITIES:

Identification of Impurity

Impurity profiling encompasses a range of activities aimed at determining the chemical structures of impurities found in drug substances or observed during stability studies beyond a specific threshold. The identification of pharmaceutical impurities can be achieved through various spectroscopic techniques, including Ultraviolet (UV), Infrared (IR), Mass (MS), and Nuclear Magnetic Resonance (NMR). On the other hand, quantitation of these impurities can be accomplished using chromatographic techniques such as High-Performance Liquid Chromatography (HPLC) equipped with a mass or UV detector, Gas Chromatography (GC) equipped with mass, Flame Ionization Detector (FID), or Electron Capture Detector (ECD), Supercritical Fluid Chromatography (SFC), and Thin Layer Chromatography (TLC/HPTLC). According to the ICH guidelines, the identification of all genotoxic impurities in a drug substance is mandatory if they are present at or above a specific limit known as the identification threshold.[28]

Analytical Method Development

The primary objective in developing an analytical method for genotoxic impurities is to create a method capable of detecting these impurities at trace levels and significantly below the Threshold of Toxicological Concern (TTC). By conducting a series of controlled experiments, the aim is to minimize variability in the developed analytical method, ensuring the production of high-quality and safe drug products. Given the increasingly stringent global regulatory requirements, it is essential for analytical methods to meet these standards for global products. Method development is an ongoing process with the ultimate goal of consistently enhancing product quality [29].

Analytical Method Validation

Validation is the process of obtaining documented evidence that provides a high level of confidence that a particular procedure, process, equipment, activity, or system will consistently produce a product that meets its predetermined specifications and quality attributes. It is a crucial aspect of analytical method development as it is closely linked to the quality of the results. All analytical methods, whether qualitative or quantitative, must be validated, with the degree of validation varying depending on the type of method and its application. In recent years, validation studies, guidelines, and procedures have primarily focused on quantitative methods of analysis. Validation is particularly important in impurities profiling, where the developed analytical method used to determine genotoxic impurities in drug substances must be validated to ensure that it is suitable for its intended purpose. Analytical methods are validated using specificity, linearity, precision, accuracy, ruggedness, robustness, and forced degradation parameters in accordance with ICH Harmonized Tripartite Guidelines [30, 31].

APPROACHES FOR GTI MITIGATION IN THE PHARMACEUTICAL INDUSTRY:

In the preceding section, it was demonstrated that the production of pharmaceutical products often necessitates the utilization of highly reactive reagents for the synthesis of APIs or their intermediates.[32] Consequently, the final API or drug product may contain trace amounts of these reagents or their byproducts as impurities. These chemically reactive impurities can possess undesirable toxicities, such as genotoxicity and carcinogenicity, thereby significantly impacting the risk assessment of the product.[33] While it is possible to avoid these impurities in certain cases, their presence in postreaction streams during API synthesis is often challenging to circumvent. To address this issue, R&D scientists must proactively identify these impurities during the process development stage, establish analytical methods, and implement synthetic processes to control and confine them. By carefully optimizing synthetic approaches (preferably preventive) or resorting to purification strategies as a last resort, GTIs can be effectively reduced to levels compliant with regulatory standards.

1. Chemical Synthetic Approaches

In the initial approach to minimize GTIs in API production, R&D chemists refrain from utilizing and producing GTIs at any stage of the synthetic route. Instead, they explore alternative chemical sequences to attain the desired API or intermediate, or enhance the efficiency of the current synthetic route.[34] In certain exceptional circumstances, this approach can be implemented without causing a substantial decrease in yield. Instances of redesigning the synthetic process to specifically avoid GTIs can be found.

However, it is often inevitable to use reagents and intermediates that are reactive and synthetically useful, which may result in their interaction with DNA. In some cases, it may not be feasible to modify the synthetic steps during development to control or minimize GTIs, especially when the process has already been scaled up. As a result, a second approach to achieving GTI-free drug products is through prevention, which involves

reducing or eliminating the concentration of GTI during the critical synthetic step. This can be accomplished by adjusting appropriate reaction conditions, such as (i) the proportions of reaction components, (ii) changing the order of addition of the reactants, and (iii) modifying the quality or preparation method of key starting materials. Additionally, a quality by design (QbD) approach can aid in better GTI control.[35]

1.1 Quality by Design (QbD):

The concept of Quality by Design (QbD) has been proposed as a means to develop synthetic routes and select conditions for API synthesis. It can also be applied to control the formation of GTI below certain threshold values. In the pharmaceutical industry, QbD aims to design and produce API formulations in such a way that the final quality is ensured from the beginning through the design of synthetic routes and the manufacturing process. QbD generally consists of four stages: (i) defining the targeted quality profile, (ii) designing the product and manufacturing process to achieve that quality, (iii) identifying and selecting quality attributes, process parameters, and sources of variability, and (iv) implementing control mechanisms to ensure quality over time. In the specific case of controlling GTI risk, maintaining GTI below threshold numbers while achieving high API yields is the goal for product quality. Regulatory agencies strongly endorse Quality by Testing (QbT) as the primary approach, leading to the development of highly effective analytical tools and rigorous screening for GTIs in raw materials, intermediates, and APIs. Quality by Design (QbD) is also utilized to reduce the likelihood of potential GTIs, such as nitroso compounds and hydroxylamine.[36] The hydrogenation of nitroaromatics was carried out using a Pd/C catalyst in a specific process. The purification process involved filtration to remove Pd, concentration of the filtrate, addition of an antisolvent for recrystallization, and filtration/drying of the solid obtained. To assess the effectiveness of impurity purging, solutions spiked with potential GTIs were used at different stages of the purification units. The process parameters, including temperature, amount of catalyst, and reaction time, were optimized using the design of experiments (DoE) approach to identify acceptable operating ranges. This led to high product yield and GTI levels below TTC values. [35]

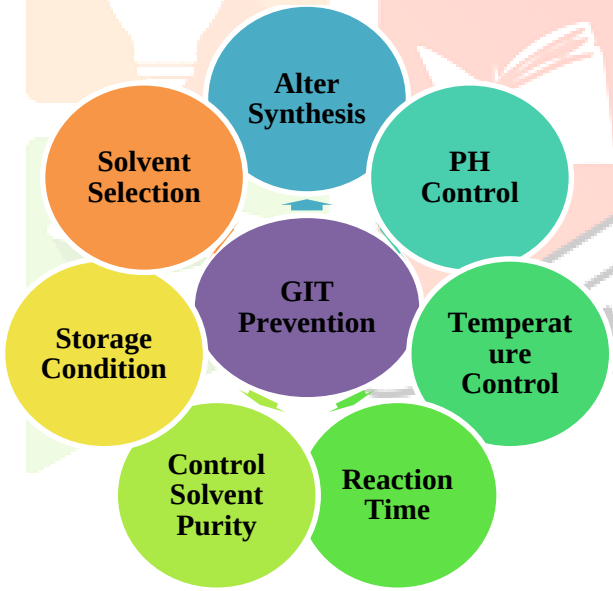

Figure 6 Quality-by-design [QbD] strategy for prevention of GTI formation.

1.2 . API Purification:

Conventional API purification techniques
1. Crystallization
2. Solvent liquid-liquid extraction
3. Precipitation
4. Distillation
5. Granular activated carbon (GAC)
6. lon exchange resins
7. Column Chromatography

Advanced API purification techniques
8. Supercritical extraction
9. Membrane separations

10. Molecular Imprinted Polymers

1.2.1. Purge Factors:

The presence of GTIs can often be avoided or reduced through innovative chemical designs or controlled reaction conditions. It is worth mentioning that purification units are already in place at various stages of API synthesis. Although these units may not be specifically designed to target GTIs, they have the capability to remove GTIs along with other impurities. Therefore, there are multiple pathways through which a specific GTI can be eliminated during the synthesis process. Previous studies have addressed the issue of purging by considering the number of synthetic steps between the appearance of a GTI and the final production step. It was recommended that if the GTI is more than four steps away from the final synthetic step, a chemical rationale should be used to determine whether GTI-specific impurity removal is necessary. However, this empirical approach is not tailored to a specific process. To address this, Teasdale et al. developed a semiquantitative "assessment purge tool" that focuses on the specific GTIs of concern and the chemical properties of the process. This tool evaluates the risk of a GTI being present in the final API. The main purge factors defined by this tool include the GTI's reactivity, solubility (in the solvents used for recrystallization), volatility (e.g., removal of GTI with solvent during distillation), ionizability (e.g., partition of GTI and API between aqueous/organic phases), and purification processes (e.g., chromatography). Each purge factor is assigned a score on a scale, as described in Table 4 , where the purge factor is defined as the ratio of GTI concentration before and after purging. [36]

Table 8 Example of Key Parameters in Purge Factors in the Tool by Teasdale et al. [36]

Physicochemical parameters	Purge factor
Reactivity	High reactivity = 100 Moderately reactivity = 10 Low/no reactivity = 1
Solubility	Freely soluble = 10 Moderately soluble = 3 Sparingly soluble = 1
Volatility	Boiling point >20 °C below that of the reaction/process solvent = 10 Boiling point ±10 °C that of the reaction/ prcess solvent = 3 Boiling point >20 °C above that of the reaction/prcess solvent = 1
Ionisability	Ionization potential of GTI significantly different
Physical processes (e.g., chromatography)	Chromatographically, GTI elutes prior to the desired product = 10 Chromatographically, GTI elutes after the desired product = 10 Others processes are evaluated on an individual basis

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SEPARATION TECHNIQUES:

Advancements in the Analysis of Dangerous Genotoxic Impurities in Pharmaceuticals using HPLC, GC, and CE:

Currently, significant advancements have been made in the field of GTIs analysis with the introduction of a wider range of analytical methods. Cronly et al. have successfully identified up to 10 and 11 nitroimidazoles in animal plasma and eggs. Various techniques such as spectrophotometer and electrophoresis have also been developed. Chromatographic methods, particularly GC and LC, coupled with conventional detectors like UV detection or mass spectrometry, remain the most commonly used methods for GTI determination. A flow diagram has been created to represent the identification, control, and determination of GTIs in drug substances.

1. Liquid Chromatography (LC):

Non-volatile GTIs are commonly examined using HPLC, with reversed phase (RP) HPLC being the most commonly used separation mode. Different stationary phases are suitable for chromatographic separation of pharmaceutical starting materials, intermediates, and final APIs. Gradient elution is used instead of isocratic

elution, and mobile phases with different compositions are used. However, binary systems with an aqueous component and a less polar organic solvent, such as acetonitrile or methanol, are still widely used. A weak buffer is used to maintain pH during gradient runs. Long-term use of an acid medium can shorten column working life and cause piston seals to corrosion. Gradient elution using water, acetonitrile, and methanol is used to avoid these issues. Hydrophilic interaction liquid chromatography (HILIC) has gained importance as a complementary technique for small molecule polar analytes. In addition to ion exchange and hydrogen bonding, additional possible separation mechanisms include the formation of a layer on the stationary phase surface by the little quantity of water, which allows polar analytes to partition. For highly polar analytes, HILIC can enhance retention; nevertheless, poor retention may occur in samples with high water content. A more modern analytical tool for GTI analysis, ultra-performance liquid chromatography (UPLC) offers greater resolution, sensitivity, and speed. It offers a substantial advantage over classical approaches with longer run times, as it has been utilized to identify veterinary medicines in meat matrices and separate nitroimidazoles and metabolites in swine kidney in 15 minutes. [37, 38, 39, 40, 41, 42]

2. Gas Chromatography (GC):

Gas chromatography (GC) is used to analyze GTIs based on their volatility, which can be categorized into volatile and non-volatile groups. Common injection methods include liquid injection and headspace sampling. However, injecting a large amount of non-volatile API can cause issues like accumulation in the injector liner or column head, affecting method performance. Headspace injection is more advantageous as it minimizes contamination by the injector or column. In headspace mode, the sample is dissolved in a high-boiling point solvent and volatile analytes partition into the vial during incubation. Headspace injection is a method that introduces volatile components and desired analytes to the injector, minimizing contamination. It also prevents non-volatile API compounds from entering the column due to partitioning. However, certain analytes for GC analysis must be injected as a solution due to potential issues with vapor pressure or high temperatures during incubation. [42, 43, 44, 45]

3. Capillary Electrophoresis (CE):

Several researchers, including Steen and Zeinab, have examined various electrically driven separation techniques such as capillary zone electrophoresis (CZE), micellar electro kinetic chromatography (MEKC), and micro emulsion micellar electro kinetic chromatography (MEEKC) to determine residual alkylating impurities in bromazepam API. Despite the widespread use of CE, it is still considered a limited instrument due to its lack of sensitivity caused by the small path length of the sample cell, even when specialized detection cells like bubble or Z-cells are utilized. Liu et al. addressed this issue by developing a palladium-modified carbon fiber array micro disk electrode for the simultaneous determination of hydrazine, methyl hydrazine, and isoniazide using CE. The modification of the electrode was necessary due to the challenging analysis of hydrazine, which exhibits a high over potential towards electron-oxidation on ordinary solid electrodes. The limit of detection (LOD) for hydrazine in this study was found to be 1.0 ppm, while the LODs for methyl hydrazine and isoniazide were 5.0 ppm, respectively, demonstrating a good linear range for all three analytes. You et al. used electrochemical chromatography (CE) to detect hydrazine, isoniazid, and methyl hydrazine using a self-assembled micro disk platinum electrode. The validation parameters were satisfactory, with linearity ranges of 0.2-400 ppm, 0.5-2000 ppm, and 0.2-400 ppm, respectively. The linearity limits (LODs) were 0.1 ppm, 0.2 ppm, and 0.1 ppm. Srinivasu et al. developed a MEKC method for celecoxib and its residual levels of 4-hydrazine benzene sulphonamide (impurity III). The LOD and LOQ for impurity III were 3500 ppm and 12,000 ppm, respectively. The recoveries for impurity III were satisfactory, ranging from 95.5% to 97.5%. CE generally has lower sensitivity compared to other separation methods. Micro-emulsion electro kinetic chromatography (MEEKC) is a highly appealing capillary electrophoretic technique that utilizes a micro emulsion as the carrier electrolyte. In this mode, the analytes have the ability to partition between the aqueous phase of the micro emulsion and the oil droplets, which function as a pseudo stationary phase. This particular mode is particularly well-suited for the separation of neutral analytes, but it can also be effectively utilized for charged analytes. Mahuzier et al. (2001) have previously reported the degradation products of rizatriptan. The method employed in their study was proven to be stability indicating and semi-quantitative, with a limit of detection (LOD) of 1000 ppm and a limit of quantitation (LOQ) of 2000 ppm (0.2%).[46, 47, 48, 49, 50, 51]

DETECTION AND IDENTIFICATION:

1. Detectors for HPLC:

HPLC detection systems, including UV detection, are commonly used in pharmaceutical analysis. However, some compounds lack a UV chromophore or have insufficient UV response at low concentrations, making UV detection unsuitable. Evaporative light scattering detectors (ELSD) have been developed to detect non-volatile samples in volatile eluents, but have limitations in sensitivity and dynamic range. Despite these limitations, low ppm limit of quantitation (LOQ) methods using ELSD are possible. Charged aerosol detectors (CAD) can detect compounds without a UV chromophore by charging aerosol particles. While some attempts have been made to develop methods using ELSD and CAD detectors, MS methods have been implemented. Chemiluminescent nitrogen detection (CLND) has been explored for low-level impurity quantitation, but its use for UV chromophore-less compounds has not been demonstrated. Atmospheric pressure ionization mass spectrometry (MS) is the most sensitive, versatile, and selective technique for trace analysis of impurities. MS detection offers high precision and a wide dynamic range for analyzing trace levels of GTIs. Recent advancements in ICP-MS instrumentation have enhanced detection sensitivity for non-metal elements like Br, S, and Cl, which can now be detected at ppb levels in solution. Each detection technique has its advantages and disadvantages, and selection should be based on desired sensitivity. MS detection is increasingly preferred by research groups due to its sensitivity and selectivity, making it the optimal choice for drug development. [52, 53, 54, 55, 56, 57]

2. Detectors for GC:

Smith and Webb's review of detection techniques in gas chromatography (GC) highlights the versatility of the flame ionization detector (FID) and electron capture detector (ECD) as primary methods for detecting volatile organic compounds. FID is preferred for detecting volatile organic compounds, while ECD is highly selective towards halogen elements, making it crucial for GTI analysis. Mass spectroscopy (MS) is also used for confirmation purposes, with traditional biological methods like ELISA or biosensors providing rapid diagnostic capabilities. The European Union relies on MS for identifying chemical residues in foodstuff. Although GC-MS is used by the Community Reference Laboratory to determine nitroimidazole, few reported applications have been made in literature. Carlin et al. used gas chromatography (GC) with electro capture (EC) detection to analyze benzalazine derivative. EC did not improve sensitivity compared to flame ionization detection (FID), but it offered increased selectivity. The method had a limit of quantification (LOQ) of 10 ppm and linearity within the 10 - 100 ppm range. The inter-day residual standard deviation (RSD) was 15% at 10 ppm, but improved at higher concentrations. Headspace injection is preferred when non-volatile active pharmaceutical ingredient (API) doesn't partition into headspace. Other detectors like nitrogen-phosphorus detector (NPD) can be used for gas-to-liquid analysis, but poor sensitivity and LODs have been reported. [37, 42, 48, 58, 59, 60, 61]

3. Detectors for CE:

CE is the preferred choice for method development in separating a drug and its main impurities due to its compatibility and simplicity with different detection modes. CE can be coupled with several detection modes, with direct UV detection being the most commonly used despite its low sensitivity caused by the restricted internal diameter of the capillary and short path length. Indirect UV detection is still employed but requires stringent separation conditions. CE equipped with an MS detector is useful not only for identification but also for structural elucidation purposes. CE methods have been applied to determine impurities in various drugs with different pharmacological activities, including cardiovascular drugs, chemotherapeutic agents, antihistaminic drugs, analgesics, antibiotics, and antiviral drugs. Nowadays, CE methods are increasingly being considered as an alternative or complement to HPLC for analyzing impurities observed during drug production or storage. [62, 63, 64]

A sensitive analytical methodology for quantifying GTIs at low levels is a technical challenge due to the need to separate interferences from APIs. Mass spectrometry (MS) detection, combined with gas chromatography (GC), high-performance liquid chromatography (HPLC), or capillary electrophoresis (CE), is crucial for determining trace amounts of GTIs in drug development. However, direct analysis is hindered by instability and lack of structural features. Analytical approaches like derivatization and coordination ion spray-MS can improve analytes' detectability. Matrix deactivation can improve analytical sensitivity and recovery rates. Combining these strategies with hyphenated mass spectrometry instrumentation will make progress in GTI analysis.

CONCLUSION

This article provides regulatory guidance on GTIs and various analytical methods for quantifying them in drug formulation. It highlights the importance of accurate, sensitive, and quantitative methods for establishing a safety regulatory framework for novel drugs, pharmaceuticals, and humans. Control strategies are summarized to ensure GTIs can be controlled during the initial stages of drug production. Analytical approaches like derivatization and coordination ion spray-MS are valuable tools for stabilizing analytes and enhancing their detectability. Matrix deactivation, a novel strategy for stabilizing reactive GTIs, can improve analytical sensitivity and recoveries. Developing sensitive analytical methodologies for quantifying GTIs at low levels is technically challenging due to the need to separate interferences. MS detection, when coupled with GC, HPLC, or CE, plays a crucial role in trace GTI determination.

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