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A REVIEW ON ADVANCED LC-MS/MS AND GC-MS/MS METHODOLOGIES FOR GENOTOXIC IMPUIRITY PROFILING IN A SMALL MOLECULES ACTIVE PHARAMACEUTICAL INGRIDENTS

1.K. Malleswari, 2.Dr.D.Rama Brahma Reddy 3. K. Harichandana

Associate professor,Professor,Student

Pharmaceutics

Nalanda institute of pharmaceutical sciences,Kantepudi,Guntur,India,A.P

Abstract:*Genotoxins* are agents/carriers such as chemical or radiation that can cause the damage to DNA or chromosomal structure, thereby causing mutations and the process are called as genotoxicity. Identification and understanding of genotoxins at a primary stage of drug development would enable us to prevent the potential damage that can be caused by these genotoxic agents. Various regulatory agencies such as International Council for Harmonization and EMEA, USFDA, European Pharmacopeia guidance, guidance for oncology products provide guidelines to limits the level of impurities in drug substances and drug products. Nowadays, conventional protocol of isolation, various spectral analysis high-performance liquid chromatography (LC), Fourier transform infrared to on-line analysis using modern, sophisticated hyphenated tools, like gas chromatography-mass spectroscopy, LC-MS so on, as well as modern software based *in silico* drug designs are extensively used *9+633… by industry, research, and development areas and also there is tremendous increase in publications in the literature involving their use. Our review article focused on the various regulatory guidelines, application of hyphenated tools, and *in silico* techniques in genotoxic impurity and degradation product profiling of small molecules. A brief explanation is made on possible pitfalls in the experimentation and data interpretation. From this review, it concluded that there are various countries having their own regulatory agencies and regulatory guidelines for drug approvals, which may be followed by applying new chemical entities the new drug application title (NDA) in new drug application as well as there are various conventional to modern software based on techniques to quantification of genotoxic impurities.

Keywords-Genotoxic impurities (GTIs), Regulatory guidelines, Drug substances, Analytical techniques, Safety, and quality assurance.

INTRODUCTION :

Methods for the detection, identification, characterization, and quantification of chemical substances are referred to as analytical chemistry techniques. These techniques are frequently used in biology for pharmaceutical product research, development, and quality control. Drug substances must be qualified and analysed for their potency, purity, and related impurities(such as process and degradant impurities), which can be done using HPLC or GC and then qualify using LCMS or GCMS. According to ICH M7 drug substances must be assessed in light of risk assessment that could result in the generation of probable genotoxic impurities, and these impurities must then be qualified and quantified in the drug substances In recent years, genotoxic impurities (GTI) in pharmaceutical goods have drawn a lot of attention for assessment and regulation. Molecules with functional groups that makeprecursors, synthetic intermediates, and reactive building blocks useful for their genotoxicity may also be caused by small molecules. It is critical to comprehend the numerous Irrelated concerns and how they can be resolved for the clinical and commercial phases of development as they represent a possible safety hazard. During the development, it is proved that these impurities are kept hat safe levels and must be gathered. This article will brief about predicted impurities in a therapeutic substance's synthetic route (also known as an active pharmaceutical ingredient, or API), as well as how GTIs are found during the early stages of chemical process development. For establishing control of GTIs, a risk-based method that complies with regulatory standards is provided. The strategy provides information on impurity rejection, process design issues, and the appropriate use of specifications. Considering analytical factors for determining GTIs at low levels are also discussed

Over the years, significant advancements have been made in analytical techniques aimed at effectively detecting and measuring Genotoxic impurities (GTIs) and Potential genotoxic impurities (PGIs) are identified by structural alerts relationship, If a structure is not part of the cohort of concern, the existence of impurity structural warnings alone is not thought to be sufficient to trigger follow-up actions, The results of a bacterial mutagenicity assay should be predicted using (Q)SAR techniques in a computational toxicology evaluation, It is best to use two complementary (Q)SAR prediction approaches. Two approaches, one based on expert rules and the other on statistics, should be used. The Organisation for Economic Cooperation and Development (OECD) has produced wide validation requirements for (Q)SAR models utilizing multiple prediction methodologies.

Some structural groups have been proven to be so strong that intakes even below the TTC could theoretically be related to considerable cancer risk. This "cohort of concern" of highly potent mutagenesis carcinogens includes aflatoxin-like, N-nitroso, and alkyl-azoxy compounds. [1]

CLASSIFICATION OF GENOTOXIC IMPURITIES:

1.ALKYI HALIDES

In the industrial setting, methyl, ethyl, and propyl halides are frequently utilised as alkylating agents. They have been demonstrated to directly alkylate essential biologically active macromolecules like proteins and DNA, even though the exact mechanism underlying their toxicity is still not entirely known. In addition to the direct use of alkyl halides, the side reactions between alcoholic solvents and hydrogen halides or the quaternization of ammonium salts can also be sources of alkyl halides in API streams.

2.DIALKYL SULPHATE

The methyl and ethyl derivatives of dialkyl sulphates are most frequently utilised in the pharmaceutical formulation, the latter has been used as a chemical weapon . Dimethyl sulphate (DMS), a potent methylating agent, is used to attach a methyl group to atoms with unshared electron pairs, including those of oxygen, nitrogen, carbon, sulphur, phosphorus, and some metals. DMS is preferable to the alkyl halide-type methylating agents because of its faster reaction rate and decreased risk of byproduct formation.

3.EPOXIDE

Epoxides, which have three-ring atoms, are the most basic cyclic ethers. Epoxides are extremely reactive compounds and are frequently utilised as reagents in API synthesis because of the substantial ring strain associated with the three-membered ring. With alcohols, amines, halides, organometallics, cyanides, sulphides, aromatic compounds, and active methylene groups, they take part in epoxide-ring-opening reactions with ease. The extreme reactivity of these substances, however, renders them genotoxic because their two electrophilic carbon atoms can interact with the nucleophilic centres of DNA to produce alkylated products. Epichlorohydrin, 1,2-epoxy-3-butene, 2,3-epoxy propanol, and other substituted epoxides are frequently employed as building blocks during the synthesis of APIs. Due to their bifunctionality and frequent exposure to epoxide-ring-opening events, they typically serve as linking agents or can form heterocycles.

4.HYDRAZINE

production of carbocations, carbon-centred radicals, and oxygen-centred radicals, which are regarded as extremely reactive The species, is thought to be known as "alkylating agents" because they can add alkyl residues to the reactive .

nucleophile sites of DNA bases either directly or indirectly through metabolic activation the cause of the toxicity of hydrazine and its derivatives. DNA alkylation and other DNA damage have been documented for these reactive intermediates.

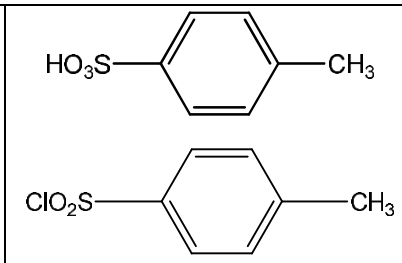
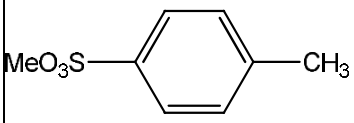
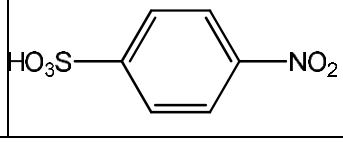
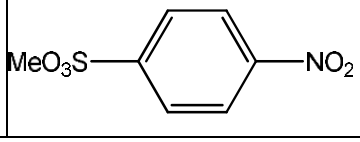
5.AROMATIC AMINE

Although aromatic amines are typically not genotoxic by nature, electrophilic species are produced during metabolic activation. The primary method of metabolism for aromatic amines is oxidation, which results in the creation of an N-hydroxy molecule that is then conjugated to an acetate, sulphate, or glucuronide. A nitrenium ion (ArN+H), which is thought to be the active genotoxin that binds to DNA, is produced by further deconjugation.

6.SULPHONATES AND ESTERS PRECAUSORS

Alkylating agents, a group of potentially genotoxic substances, include sulfonate esters. They are known as "alkylating agents" because they can add alkyl residues to the reactive nucleophile sites of DNA bases either directly or indirectly through metabolic activation. [2]

Sr. No.	Group	GTI Precursor	Genotoxic Sulphonic ester
01	Mesylate (Me)	Me-SO_3H Me-SO_2Cl	Me-SO_3Me
02	Triflate (Tf)	CF_3-SO_3H CF_3-SO_2Cl	CF_3-SO_3Me

03	Toluate (Ts)		
04	Nosylate (Ns)		

Limit of detection (LOD) and Limit of quantification (LOQ) for USFDA and ANSM methods

Analytical Technique	**LOD for the method**	**LOQ for method**	**Genotoxic impurity**	**Method Reference**
HPLC-UV	0.1ppm	0.3 ppm	NDMA in Valsartan	ANSM Method reference no 18A0399-02 [19].
LC-MS/MS	0.01ppm	0.033 – 3.33 ppm	NDMA in Ranitidine method	US Food and Drug Administration (2019). LC-MS/MS method for determining NDMA in ranitidine drug substance and drug product [20].
LC-HRMS	0.01ppm	0.03 to 0.1ppm	NDMA in ARB Drugs	US FDA. - LC-HRMS NDMA detection method in metformin drug material and drug product. (2020). [21].
LC-ESI-HRMS	0.005ppm	0.01 to 0.1ppm	NDMA in Metformin	US FDA. LC-ESI-HRMS method for the determination of
				Nitrosoamines impurities in metformin drug substance and drug product." (2020). [22].
GC-MS/MS	0.005ppm	0.008ppm	NDMA in Valsartan	US FDA method for Impurity Assay by GCMS/MS of Direct Injection N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), N-Nitrosoethylisopropylamine (NEIPA), N-Nitrosodiisopropylamine (NDIPA), and NNitrosodibutylamine (NDBA), (2019). [23].

GC-MS-HS	0.05ppm	0.3ppm	NDMA in Valsartan	US FDA. GC/MS Headspace Method for NDMA Detection in Valsartan Drug Substances and Drug Products. [24].

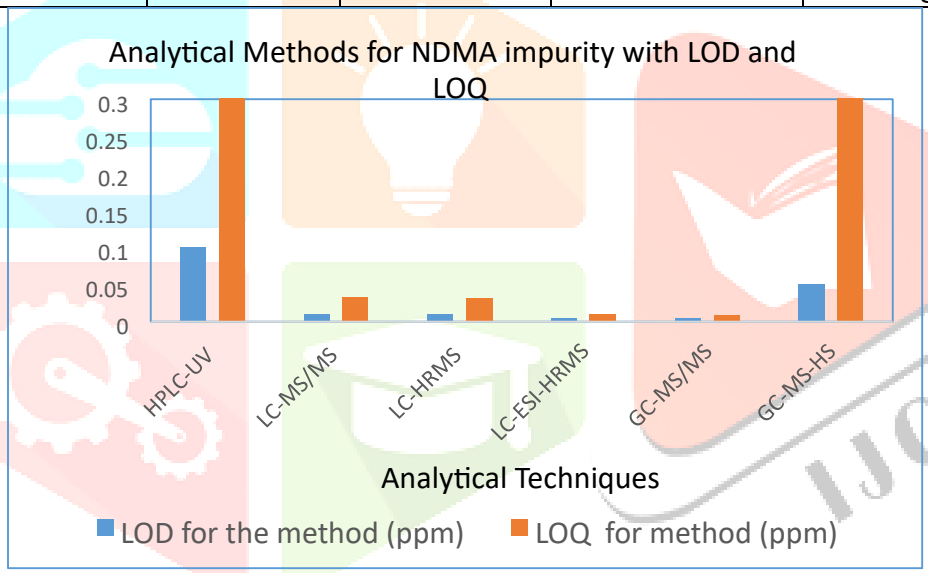


Figure : 1 Analytical methods for NDMA impurity with LOD and LOQ

Sources of Genotoxic impurities:

Recent advances in analysis of hazardous genotoxic impurities in pharmaceuticals by HPLC, GC, and CE

Nowadays, genotoxic impurities in pharmaceuticals at lower levels are of increasing concerns not only to pharmaceutical industries but also for the regulatory agencies due to their risks for human carcinogenesis and, thus, requiring manufacturers to pay extra attention for their analysis and control. The need to determine these impurities at trace levels, based on the threshold of toxicological and daily dose, taking into consideration the often reactive and labile nature of genotoxic impurities, which poses significant analytical challenges. Therefore, sensitive and sophisticated analytical methodologies are deemed necessary in order to be able to test and control genotoxic impurities in drug substances. This review demonstrates the approaches reported in the literature for the analysis of the hazardous genotoxic impurities and the strategies used to enhance the sensitivity such as using ion spray-mass spectrometry and the separation techniques for the analysis of such impurities.[3]

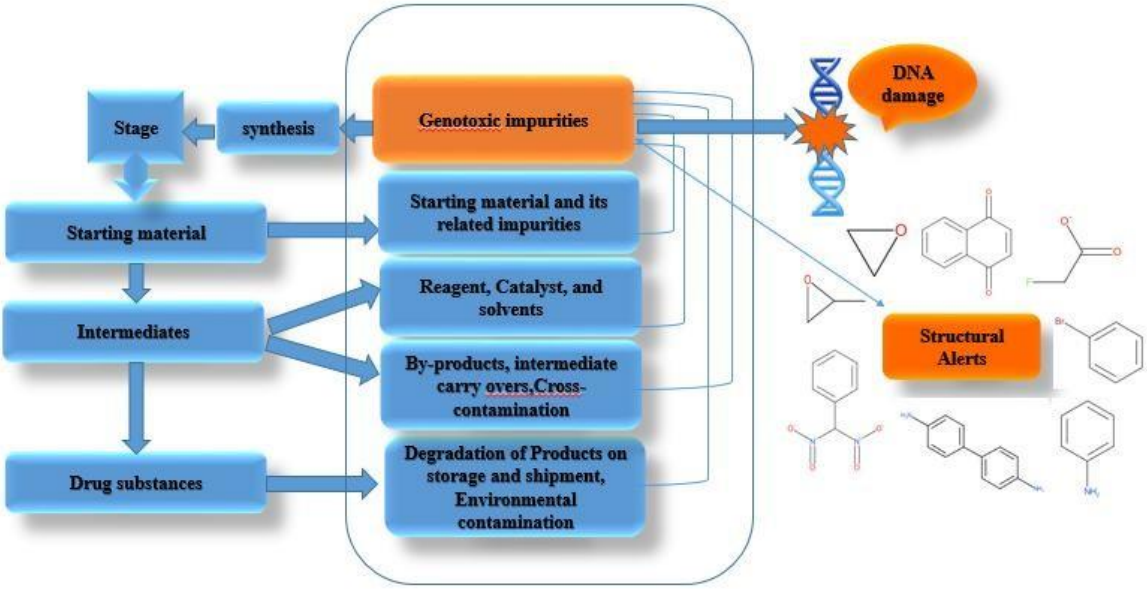
Analysis of Genotoxic Impurities: Review of Approaches Genotoxic Impurities, 249, 2010

The issue of genotoxic impurities (GIs) in active pharmaceutical ingredients (API) has over recent years, become of considerable concern to the pharmaceutical industry. Control of GIs is addressed in the recent CHMP (Committee for Medicinal Products for Human Use) guideline 1 and draft FDA (Food and Drug Administration) guideline. 2 Both indicate that a TTC (threshold of toxicological concern) of 1.5 μ g/day is appropriate for long term use, and that a staged TTC is appropriate during the clinical development phase.

As a consequence of these TTC controls at the μ g/day level, there is a parallel need for stringent analytical control measures. These measures, which often necessitate control of these GI analytes at the low ppm level relative to the API, pose very significant analytical challenges. The need for specific and sensitive trace analytical methodologies, combined with the high chemical reactivity of the analytes concerned, present significant analytical challenges, especially in sample preparation. This in turn often necessitates both the application of sensitive, analytical techniques, for example gas chromatography-mass spectroscopy (GC-MS), high performance liquid chromatography-mass spectroscopy (HPLC-MS), and of sample preparation steps prior to analysis to address both selectivity and stability issues. Ironically, the same chemical reactivity issues that bedevil the analytical procedures may also ensure that many of the GIs are unlikely to survive intact the chemical synthetic clean-up procedures used in API manufacture, and even less likely to survive the aqueous-based biological environment. Such issues are addressed in detail elsewhere within this book

Genotoxic impurities (GTIs) have gained considerable attention from health authorities as well as from the pharmaceutical industry in recent years. Analysis and control of these impurities in pharmaceutical compounds pose a significant challenge, often requiring selective, sensitive, and robust trace-level methods for analysis. This article focuses on the method development strategy and associated technologies for the analysis of GTIs. GTIs typically have a wide range of physicochemical properties such as volatility, stability, presence of UV chromophores, ionizable groups, and derivatizable functional groups. Various separation and detection technologies are available and can be used to identify and analyze. Choosing the appropriate analytical technique depends on the GTI physicochemical properties, required sensitivity, and the consideration of the matrix interference.[4]

Genotoxic impurities (GTIs) belong to a class of compounds that interact with DNA and induce genetic mutations. These compounds add significant risk without adding any benefit to patients



An overview of hyphenated techniques used for the identification of genotoxic impurities in pharmaceutical products: Current status and future perspectives

Genotoxin is a chemical that has the potential to cause DNA damage, which can result in germline and somatic mutations leading to malignant transformation and cause cancer. The assessment and control of GTIs in pharmaceuticals at trace levels are raising concerns to both the pharmaceutical and regulatory agencies due to this possibility for human carcinogens. Identifying these GTIs at trace levels requires a sophisticated approach, which poses significant challenges for analysts in pharmaceutical research and development. Therefore, it is crucial to understand the various issues related to GTIs and find the potential ways to address them. This article provides a brief overview of the various sources of potential impurities in drug components, such as active pharmaceutical ingredients (APIs), GTIs in food and cosmetics, identification of GTIs using advanced techniques like high-performance liquid chromatography-mass spectroscopy (LC-MS) and gas chromatography-mass spectroscopy (GC–MS). In addition to the sources and identification of GTIs in drug components, the article covers APIs that contain GTIs and their determination using analytical techniques and explores the optimized results obtained from this determination.[5]

Analytical technologies for genotoxic impurities in pharmaceutical compounds

Genotoxic impurities (GTIs) have gained considerable attention from health authorities as well as from the pharmaceutical industry in recent years. Analysis and control of these impurities in pharmaceutical compounds pose a significant challenge, often requiring selective, sensitive, and robust trace-level methods for analysis. This article focuses on the method development strategy and associated technologies for the analysis of GTIs. GTIs typically have a wide range of physicochemical properties such as volatility, stability, presence of UV chromophores, ionizable groups, and derivatizable functional groups. Various separation and detection technologies are available and can be used to identify and analyze. Choosing the appropriate analytical technique depends on the GTI physicochemical properties, required sensitivity, and the consideration of the matrix interference.

Genotoxic impurities (GTIs) belong to a class of compounds that interact with DNA and induce genetic mutations. These compounds add significant risk without adding any benefit to patients and are also known as mutagenic impurities. Therefore, exposure to even low levels of such impurities may be of significant toxicological concern.[6]

Comprehensive Review of Genotoxic Impurities: Current Trend, Challenges and Control Strategies for Pharmaceutical Drug Products

Genotoxic impurities (GIs) in pharmaceuticals pose a significant risk due to their potential to induce DNA damage, mutations, and carcinogenesis even at trace levels. Regulatory frameworks, including ICH M7 (R1), FDA, and EMA guidelines, have established stringent impurity assessment and control strategies based on the Threshold of Toxicological Concern (TTC) approach. This review explores the sources of GIs, including synthetic process-related byproducts, degradation products, excipient interactions, and environmental contaminants. The mechanisms of genotoxicity, encompassing DNA alkylation, chromosomal aberrations, and oxidative stress, are discussed alongside structural alerts for impurity risk prediction. Advanced analytical techniques such as LC-MS/MS, GC-MS[7]

Impurity Characterization Across Drug Development Stages: Analytical Methodologies and Regulatory Perspectives

The rigorous identification, quantification, and control of impurities are fundamental to ensuring the safety, efficacy, and quality of pharmaceutical products throughout the drug development lifecycle. As unavoidable byproducts of complex synthesis and manufacturing processes, impurities span a diverse range of compounds—including organic, inorganic, residual solvent, and genotoxic species—all of which require meticulous characterization using a suite of analytical techniques. This review synthesizes best practices for impurity classification, source mapping, and alignment of control strategies with global regulations (ICH Q3A/B, Q3C, Q3D, and M7), distinguishing phase-appropriate profiling, stress testing, and fully validated, stability-indicating methods. Advanced structural-elucidation tools (LC–MS/MS, HRMS, NMR) are surveyed alongside key strategies for lifecycle change management. Special emphasis is placed on mutagenic impurities, especially nitrosamines, highlighting how recent industry experience and regulatory actions have reshaped risk management and analytical sensitivity requirements. The discussion extends to emerging trends—green analytical chemistry, AI-driven prediction and interpretation, and real-time monitoring via Process Analytical Technology (PAT) and Real-Time Release Testing (RTRT)—that are redefining impurity control from reactive detection to proactive prevention. Collectively, this review provides scientists, regulators, and quality professionals with actionable frameworks for robust impurity management, facilitating compliance and accelerating pharmaceutical development. [8]

Analysis of Genotoxic Impurities: Review of Approaches

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Liquid -Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS is primarily used for the analysis of non-volatile, polar, and thermally labile impurities in small molecule APIs

ADVANTAGES:

Versatility:

Accommodates a wide range of structurally diverse and high-molecular-weight GTIs without the need for thermal vaporization

Sensitivity & Selectivity:

Utilizing Multiple Reaction Monitoring (MRM) reduces background noise, achieving accurate quantification at the ppb to ppm levels

Flexibility:

Compatible with various advanced sample-introduction techniques, including mixed-mode chromatography for highly ionic compounds

Disadvantages;

Matrix Effects: Co-eluting API matrices can cause ion suppression or enhancement, necessitating thorough sample clean-up.

Polarity Limitations:

Highly polar impurities are poorly retained on standard reversed-phase columns, sometimes requiring specialized HILIC (Hydrophilic Interaction Liquid Chromatography) or derivatization.[10]

Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS)

GC-MS/MS is the premier analytical technique for separating and quantifying volatile and semi-volatile genotoxins, such as alkyl mesylates, alkyl halides, and nitrosamines

Advantages:

Volatile Analysis:

Ideal for volatile organics and residual solvents that are difficult to retain or analyze using liquid chromatography.

Automation & Cleanliness:

Can be paired with headspace sampling (SHS) and Solid-Phase Microextraction (SPME), which physically isolates analytes from the heavy API matrix.

Spectral Libraries:

High-quality electron ionization (EI) spectra make GC-MS excellent for identifying unknown impurities by comparison to commercial databases[11]

Disadvantages:

Thermal Instability:

High inlet and column temperatures can degrade or breakdown thermolabile impurities giving false profiles.

Derivatization Needs:

Highly polar or non-volatile compounds must be chemically derivatized before injection, adding steps to the analytical workflow.

Recent Trends & Best Practices

Modern workflows integrate both techniques alongside High-Resolution Mass Spectrometry (HRMS) for comprehensive profiling. When analyzing complex or novel drug formulations, labs frequently validate complementary GC-MS/MS and LC-MS/MS methods to cover all bases[12]

CONCLUSION:

Genotoxic impurities are highly reactive compounds that can damage DNA and increase the risk of cancer, even at very low concentrations. Therefore, their identification, monitoring, and control are essential to ensure the safety and quality of pharmaceutical products. Advanced analytical techniques such as LC–MS/MS and GC–MS/MS provide highly sensitive, selective, and accurate detection of these impurities at trace levels. Combined with ICH M7 guidelines and a risk-based approach, these methods help pharmaceutical manufacturers develop safe, effective, and high-quality active pharmaceutical ingredients (APIs). Continued advancements in analytical technologies will further improve genotoxic impurity profiling and strengthen patient safety.[13]

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