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A Review on Analysis of Genotoxic Impurities

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Abstract: ***Genotoxic impurity can be defined as a chemical or other agent that damages cellular DNA, resulting in mutations or cancer. Genotoxic impurities impact the genetic material by means of mutations through chromosomal breaks, rearrangements, covalent binding or insertion into the DNA during replication. These changes in the genetic material, caused by the exposure to very low levels of a genotoxic chemical, can lead to cancer. Determination of 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, requires highly sensitive analytical methodologies, which poses tremendous challenges on analytical communities in pharmaceutical R&D. Therefore, sensitive and sophisticated analytical methodologies are deemed necessary in order to be able to test and control genotoxic impurities in drug substances. Thus, it is very important to identify genotoxic impurities in drugs followed by monitoring and control at very low levels to ensure safety to the public. This review demonstrates guidelines the various regulatory guidelines to control the genotoxic impurities in drug substances and the analytical approaches reported in the literature for the analysis of the hazardous genotoxic impurities and the strategies used to enhance the sensitivity and the separation techniques for the analysis of such impurities.***

Keywords: Genotoxic impurities (GIs), Regulatory guidelines, ICH, EMEA, Threshold, Analytical approach

1. Introduction

Synthesis of Active Pharmaceutical Ingredients (APIs) is a multi-step process involving the use of reactive chemicals, reagents, solvents, catalysts, and salts. Residual levels of process-related impurities, byproducts, and degradants could have adverse health effects. To ensure patient safety, global regulatory agencies require API manufactures to reduce the levels of such compounds to safe levels. The term "genotoxic" is applied to agents that interact with DNA and/or its associated cellular components (e.g. the spindle apparatus) or enzymes (e.g. topoisomerases) (Robinson, 2010 [1]). Genotoxic compounds induce genetic mutations and/or chromosomal rearrangements and can therefore act as carcinogenic compounds (McGovern and Jacobson-Kram, 2006 [2]). These compounds cause damage to DNA by different mechanisms such as alkylation or other interactions that can lead to mutation of the genetic codes. International Council for Harmonisation in its guideline ICH S2 (R1) [3] defines genotoxicity as "a broad term that refers to any deleterious change in the genetic material, regardless of the mechanism by which the change is induced." While genotoxic impurities are defined as "Impurity that has been demonstrated to be genotoxic in an appropriate genotoxicity test model, e.g., bacterial gene mutation (Ames) test". 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". Figure 1 describes the various sources of genotoxic impurities.

The first guideline related to genotoxicity was introduced by ICH in July 1995 as S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals; this guideline provided specific guidance and recommendations for in vitro and in vivo tests and on the evaluation of test results. The second guideline was in 1997 as S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals; In 2013 M7 [4] guideline was published which offer guidance on the analysis of Structure-Activity Relationships (SAR) for genotoxicity. After which M7 (R1) guideline was released in step 2 in June 2015 and step 4 in May 2017. International Conference on Harmonization documents (ICH) Q3A(R2) [5] and ICH Q3B(R2) [6] provide guidance on limiting the majority of these less toxic impurities in new drug substances and drug products respectively. However, some reactive genotoxic impurities (GIs) even when present at very low levels could potentially bind to the DNA or proteins leading to gene mutation. The European Medicines Agency (EMEA) [7] and the Food and Drug Administration (FDA) issued guidance documents that highlight the importance of this issue and have mandated limits and controls GIs in drug substances. ICH issued draft guidance in February 2013 which provided a framework for identification, categorization, qualification, and control of potential genotoxic impurities (PGIs). Table 1 represents the various categories of genotoxic impurities present at different stages of manufacturing and figure 2 describes the structures of commonly encountered genotoxic impurities.

2. Regulatory Guidelines

1) As per ICH:

The International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use project represents the main group of guidelines with topics such as "Quality" topics and "Safety" topics. Quality topics relate to chemical and pharmaceutical quality assurance (stability testing, impurity testing, etc.) and safety topics deal with in vitro and in vivo pre-clinical studies (carcinogenicity testing, genotoxicity testing, etc.) (ICH 2008). In the guidelines, genotoxicity tests have been defined as in vitro and in vivo tests designed for detecting compounds that induce genetic damage directly or indirectly (International Conference on Harmonization, 1997). Table 2 illustrates a series of thresholds described in ICH Q3A(R) that trigger reporting, identification, and qualification

requirements. Subsequently, Table 3 depicts the thresholds for reporting, identification, and qualification of impurities in new drug products (ICH, 2006; Jacobson-Kram and McGovern, 2007).

Two options for standard test battery for genotoxicity are available in the ICH S2 (R1) guideline (ICH, 2008) [8]:

Option 1:
- A test for gene mutation in bacteria;
- A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test), or an in vitro mouse lymphoma tk gene mutation assay;
- An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

Option 2:
- A test for gene mutation in bacteria;
- An in vivo assessment of genotoxicity with two tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay.

2) As per EMEA

The European Medicines Agency (EMEA) guideline describes a general framework and practical approaches on how to deal with genotoxic impurities in new active substances. According to the guideline "The toxicological assessment of genotoxic impurities and the determination of acceptable limits for such impurities in active substances is a difficult issue and not addressed in sufficient detail in the existing ICH Q3X guidance". In addition, the EMEA guideline proposed a toxicological concern (TTC) threshold value of 1.5 µg/day intake of a genotoxic impurity which is considered to be associated with an acceptable risk (excess cancer risk of Those genotoxic compounds with sufficient evidence would be regulated according to the procedure as outlined for class 2 solvents in the "Q3C Note for Guidance on Impurities: Residual Solvents". For genotoxic compounds without sufficient evidence for a threshold related mechanism, the guideline proposes a policy of controlling levels to "as low as reasonably practicable" (ALARP) principle, where avoiding is not possible. On the other hand, this guideline provides no advice on acceptable TTCs for drugs during development, especially for trials of short duration (Jacobson-Kram and McGovern, 2006). The pharmaceutical research and manufacturing association (PhRMA) has established a procedure for the testing, classification, qualification, toxicological risk assessment, and control of impurities processing genotoxic potential in pharmaceutical products. As most medicines are given for a limited period of time, this procedure proposes a staged TTC to adjust the limits for shorter exposure time during clinical trials (Table 4). Thus, the staged TTC can be used for genotoxic compounds having genotoxicity data that are normally not suitable for a quantitative risk assessment (Muller et al., 2006) [9].

Classification of Genotoxic impurities

As per ICH M7 guidance, reagents, starting materials, intermediates, byproducts, process-related impurities, and potential degradation products in drug substances are categorized into classes 1 to 5 based on comprehensive hazard evaluations commonly known as in-silico assessments. Genotoxic impurities are classified based on their risk assessment which involves an initial analysis of actual and potential impurities by conducting database and literature searches for carcinogenicity and bacterial mutagenicity data which classify them to Class 1, 2, or 5. If data for such a classification is not available, an assessment of Structure-Activity Relationships (SAR) that focuses on bacterial mutagenicity predictions is performed. This could classify it into Class 3, 4, or 5. Each class is defined as below [10].

Class 1: These impurities have established mutagenic and carcinogenic data and are known to be the most serious risk and need to eliminate them by modifying the process. If this is not possible, these impurities are to be limited at "Threshold of Toxicological Concern (TTC)" as the last option.

Class 2: These impurities have the well-established mutagenic data, but their potential to cause carcinogen is not known. Hence, these impurities need to be controlled using the TTC approach.

Class 3: These impurities are having alert structures unrelated to the structure of the drug substances and of unknown genotoxic potential. Based on functional groups within their molecule, they can be classified as genotoxic. The toxicity of these impurities is identified based on the structure-activity relationship (SAR).

Class 4: These impurities are having structures similar to the structure of drug substances and additionally contain functional or moiety that has potentially alert shared with the parent structure and consider being non-genotoxic.

Class 5: These impurities have no alert structures, and evidence indicates the absence of genotoxicity. These compounds are to be treated as normal impurities and controlled according to the ICH guidelines.

Method Selection

The analysis of GIs taking into consideration the physicochemical properties of the compounds of interest and the required specificity and sensitivity of the analytical techniques as shown in Figures 3 and 4. Figure 3 shows the decision tree for volatile compounds and Figure 4 shows the decision tree for non-volatile compounds.

3. Analytical Approach

1) HPLC methods

In general, non-volatile GIs are analyzed by HPLC separation techniques, among which reversed phase HPLC (RPLC) is the most widely used separation mode (Elder et al., 2008) [11]. A simple isocratic RPLC method has been employed for the determination of four genotoxic alkyl benzenesulfonates (ABSs) viz. methyl, ethyl, n-propyl, and isopropyl benzenesulfonates (MBS, EBS, NPBS, and IPBS) in amlodipine besylate (ADB). The RPLC is also applicable

for sulfonate impurities with phenyl moiety such as methyl (MTs), ethyl (ETs) and isopropyl tosylates (ITs), methyl (MBs), ethyl (EBs), butyl (BBs) and isopropyl besylates (IBs) (Raman et al., 2008) [12]. Epoxides/hydroperoxides were analyzed using HPLC, and simple RPLC methods employing direct analysis (no sample preparation) were used for some of them. Yasueda et al. (2004) [13] described an HPLC method for the determination of loteprednol impurities including a minor photolytic epoxide degradation product. A rapid resolution HPLC method was used for separating and quantifying the related impurities of atorvastatin, including two epoxide impurities atorvastatin epoxy dihydroxy and atorvastatin epoxy diketone.. Kong et al. (2001) [14] determined two epoxide terpenoid impurities (actein and 27-deoxyactein) in a traditional Chinese herbal preparation (Cimicifuga foetida L.). Subsequently, they compared the HPLC results with evaporative light scattering detection (ELSD) with UV detection and found that the ELSD was significantly more sensitive. Sample pretreatment was performed prior to analysis owing to the complexity of the matrix. For the two epoxides the on-column sensitivity using UV detection was found to be 606 and 880 ng, respectively, whereas the sensitivity using ELSD was 40 and 33 ng, respectively. Using the optimized extraction procedure (methanol/water, 80/20 v/v) the levels of the two analytes were detected to be 3.44±0.02% and 1.42±0.01%, respectively. A more common method for the analysis of alkylating impurities is by RPLC and MS detection; however, HPLC/UV methods are also carried out successfully for alkylating impurities. Valvo et al. (1997) [15] reported an HPLC/UV method for the separation of 13 impurities of verapamil; this method is claimed to be superior to both the existing pharmacopoeial methods for verapamil. Using this method, the LOD and LOQ were found to be 0.01% (0.05 µg/ml) and 0.02% (1.0 µg/ml), respectively. Also, the method was found to be sensitive to pH and mobile phase composition; however, it was in contrast to the findings of previous studies insensitive to stationary phase changes. Hydrophilic interaction liquid chromatography (HILIC) seems complementary to RPLC for the retention and separation of small molecule polar analytes, and has thus gained increasing attention recently. Good retention can be achieved for more polar analytes, which is not possible on RPLC columns. In the hydrazine group, the HILIC method was used in addition to the HPLC/UV and HPLC/MS methods (Liu et al., 2010) [16]. An Indian research group reported the development and validation of a stability indicating HPLC method for the determination of the anti-tuberculosis drug, rizatriptan, and its degradation products, including a hydrazone impurity (Rao et al., 2006) [17]. Hmelnickis et al. (2008) [18] used an HILIC method with different polar stationary phases (silica, cyano, amino, and the zwitterionic sulfobetaine) to separate six polar impurities, including 1,1,1-trimethylhydrazinium bromide, and demonstrated that HILIC was a useful alternative to reverse phase or ion chromatography (IC). Elder et al. (2010) [19] reported a table summarizing the various HPLC methods that were used in the literature for a wide range of drugs is given in table 5.

2) GC methods

GC methods are commonly used for the analysis of several volatile small molecule GIs. Some examples include the liquid injection technique and the headspace sampling technique. Liquid injection is prone to contamination in which injection of a large amount of non-volatile API can accumulate in the injector liner or on the head of the GC column, which can cause a sudden deterioration in method performance. Headspace injection, on the other hand, is desirable because it minimizes potential contamination of the injector or column by avoiding the introduction of a large quantity of API (Liu et al., 2010). David et al. (2010) [20] proposed a method selection chart (Figure 5) containing GC or LC methods, both in combination with a single quadrupole mass spectrometer as detector. These methods applied for a wide range of analytes including sulphonates, alkyl halides, and epoxides.

Nassar et al. (2009) [21] developed a GC/MS method for residual levels of EMS in a mesylate salt of an API crystallized from ethanol. The method was capable of detecting EMS down to levels of 50-200 ppb. Subsequently, extraction techniques were developed for eliminating or reducing matrix related interference. Thus, Colon and Richoll (2005) [22] surveyed liquid–liquid extraction (LLE), liquid phase micro-extraction (LPME), solid phase extraction (SPE), and solid phase micro-extraction (SPME) coupled with GC/MS and single ion-monitoring (SIM). Using these approaches, they developed limit tests (5 ppm) for some alkyl aryl esters of sulfonic acids. GC methods were rarely used for the analysis of epoxides/ hydroperoxides, as compared to other impurities, owing to the size of molecule and the volatility properties within this group. Non-volatile API does not partition into the headspace and therefore does not enter the GC system; as a result, headspace injection becomes the preferred choice whenever possible.

3) TLC/HPTLC methods

In general practice, thin layer chromatography (TLC) is not preferred for the accurate determination of very low residual analyte level. However, this technique is still used for the determination of related substances in the pharmacopoeial monographs for amiodarone, bromazepam, carmustine, ifosamide, indoramin, and tolnaftate (Elder et al., 2008). Nevertheless, there are several examples of its use in association with determining levels of the epoxyl alkaloid, including scopolamine in extracts of Datura stramonium. Sass and Stutz (1981) [23] used TLC to determine residual sulfur and nitrogen mustards (beta haloethyl compounds) in a variety of substrates in which the sensitivities in the microgram range were typically achievable. High performance thin layer chromatography (HPTLC) was used for monitoring the degradation products of rifampicin, including the hydrazones (25-desacetyl rifampicin (DAR)) and rifampicin quinone (RQU).

4) Capillary electrophoresis methods

Jouyban and Kenndler (2008) [24] reviewed the applicability of capillary electrophoresis (CE) methods for the analysis of pharmaceutical impurities. In addition, they discussed the applications of these methods in various groups of compounds such as chemotherapeutic agents, central nervous system (CNS) drugs, histamine receptor and cardiovascular drugs. The main advantage of CE techniques is their selectivity; thus, they are suitable for the analysis of

complex herbal products. Bempong et al. (1993) [25] reported the separation of 13-cis and all-trans retinoic acid and their photo-degradation products (including all-trans-5, 6- epoxy retinoic acid, 13-cis-5, 6-epoxy retinoic acid) using both capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) methods. Hansen and Sheribah (2005) [26] evaluated a series of electrically driven separation techniques: CZE, MEKC, and microemulsion electrokinetic chromatography (MEEKC) for the determination of residual alkylating impurities in bromazepam API. The problem of limited sensitivity of CE methods can be solved either by the use of detection methods with sensitivity higher than UV absorption or by pre-concentration of the analytes.

5) Enhancing methods

Alternatively, the structure of the molecule as well as its properties can be altered to enhance detectability which in turn will help to achieve the desired sensitivity. This is especially true for GIs that lack structural features for sensitive detection (Bai et al., 2010) [27]. A number of general approaches could be considered, some of which are explained below:

3.1 Chemical derivatization

This method is generally used for stabilizing reactive GIs and for introducing a detection specific moiety for enhanced detection, i.e. chromophore for UV. Also, this method sometimes produces a single compound for several GIs; thus, it becomes non-specific which can be considered as an advantage in determining a group of structurally related compounds (Liu et al., 2010). Bai et al. (2010) introduced a chemical derivatization method for analyzing two alkyl halides and one epoxide. The objective of the three derivatization reactions is to generate a strong basic center by introducing an amine functional group. All three derivatization products are good candidates for electrospray ionization (ESI)-MS owing to the high proton affinity or the permanent charge.

3.2 Coordination ion spray-MS

Owing to their structural features, several analytes are not amenable to atmospheric pressure ionization methods, such as the ESI method. Alkali metal ions such as Li+, Na+, and K+ can form complexes with some organic molecules in the gas phase; this fact could be used as a solution for the analytes subjected previously (Liu et al., 2010).

3.3 Matrix deactivation

The matrix deactivation approach is a chemical approach to stabilize unstable/reactive analytes. It is based upon the hypothesis that the instability of certain GIs at trace level is caused by the reaction between the analytes and reactive species in the sample matrix. Thus, controlling the reactivity of the reactive species in the sample matrix would stabilize the unstable/reactive GI analytes (Liu et al., 2010). As an example the alkylators are reactive unknown impurities which possess mainly nucleophilic characteristics. Their reactivity can be attenuated by either protonation or scavenging approaches. Sun et al. (2010) [28] reported a matrix deactivation methodology for improving the stability of unstable and reactive GIs for their trace analysis. This approach appears to be commonly applicable to techniques like direct GC–MS and LC–MS analyses, or coupled with chemical derivatization as well.

4. Conclusion

Genotoxic impurities have become a common issue for health concerns. Thus, regulatory agencies have made several attempts to construct a systematic method for controlling and analyzing GIs. This is attributed to the need for selective and sensitive methods of analysis in order to separate the interferences such as excipients from APIs. MS detection, as a powerful tool, when coupled with GC, HPLC, or CE plays a vital role in trace GTI determination in different stages of drug development. GTIs are unstable to be analyzed directly; therefore, low recovery and poor sensitivity could be a challenge. Additionally, some analytes do not have structural features that match the commonly used detectors. Therefore, analytical approaches such as derivatization and coordination ion spray-MS are invaluable tools for stabilizing analytes and/or enhancing their detectability. A novel strategy that could be used for stabilizing the reactive GTIs is the matrix deactivation which could improve the analytical sensitivity and recoveries accordingly. Upon coupling this strategy with hyphenated MS instrumentation, GTIs analysis will lead to obvious step forward over the next few years. Simple HPLC/UV, GC/FID, or CE/UV methods should be implemented as a first approach, while more advanced LC/MS, LC/MS/MS, or CE/MS methods should be the last option as many laboratories do not have a MS detector. Additionally, sensitivity can be greatly enhanced by using SPME, SPE, or PTV with GC-MS or 2D-GC techniques. For non-volatile GIs, RPLC-UV or RPLC-MS can be investigated. Selectivity can be enhanced by performing MMC or HILIC chromatography, and sensitivity can be easily improved by stacked injections. For volatile or nonvolatile compounds, derivatization can be employed to change the physicochemical properties of the GIs to allow for suitable analysis. For extremely challenging analysis, 2D-GC or 2D-LC-MS can be used to demonstrate superior selectivity and sensitivity.

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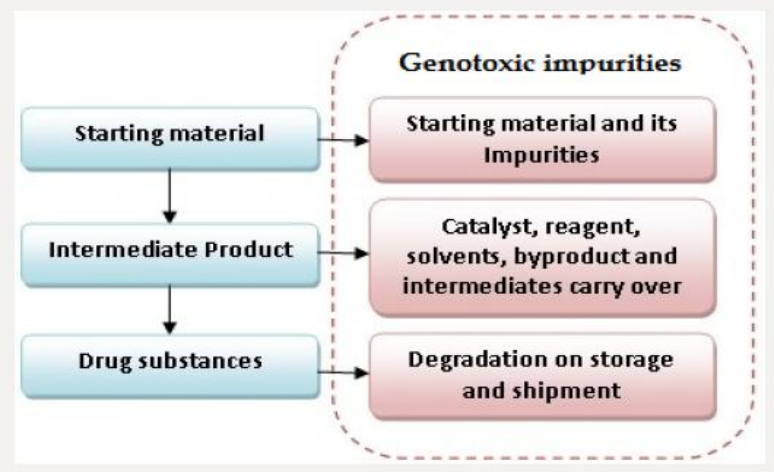

Figure 1: Sources of Genotoxic Impurities

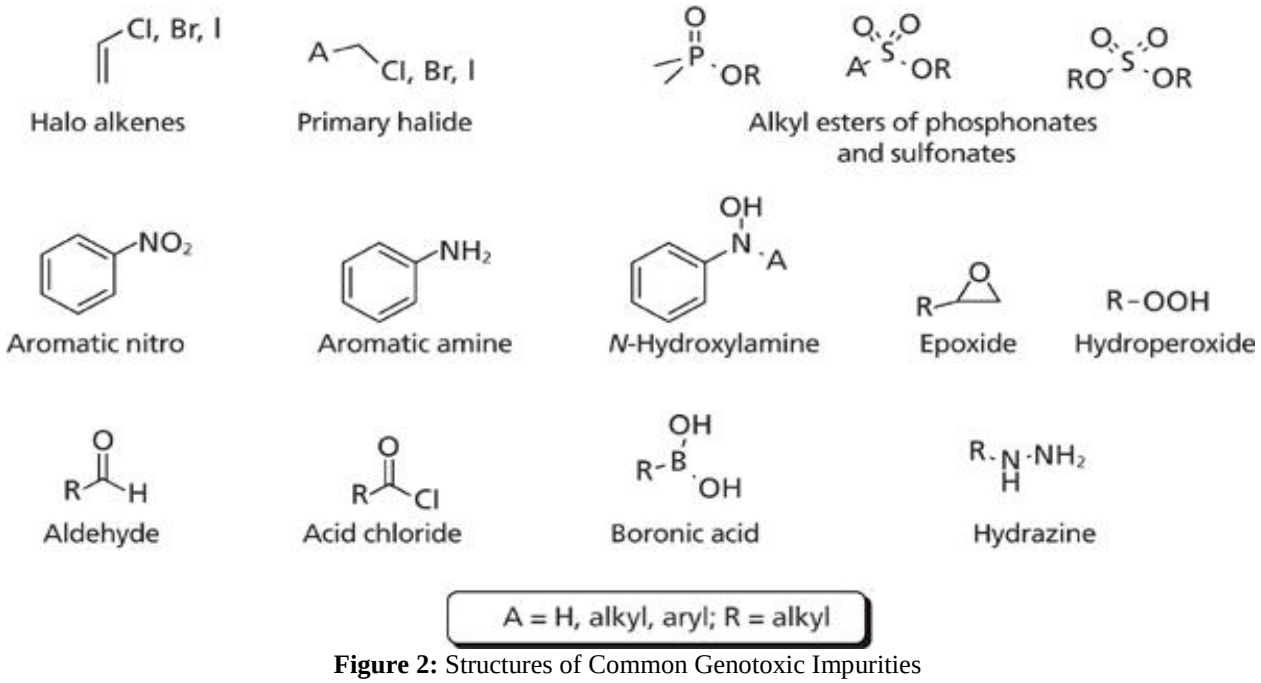

Figure 2: Structures of Common Genotoxic Impurities

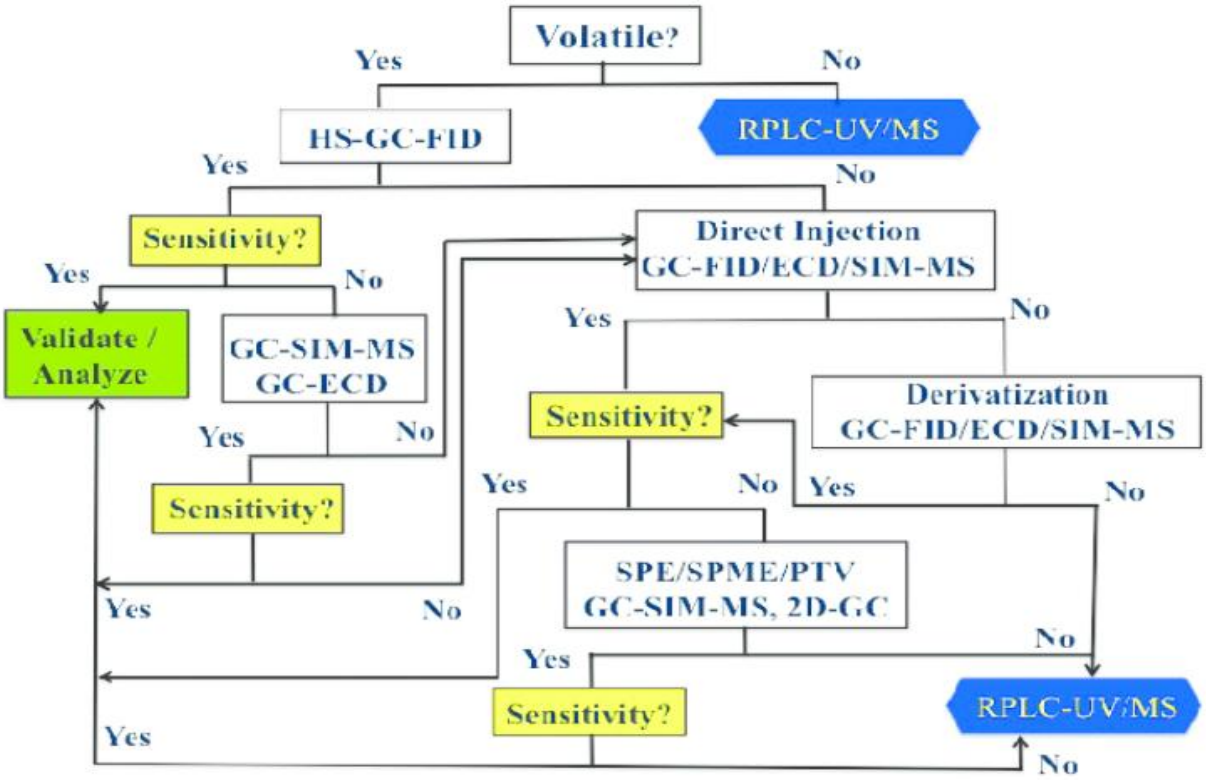

Figure 3: Decision Tree for Volatile Compounds

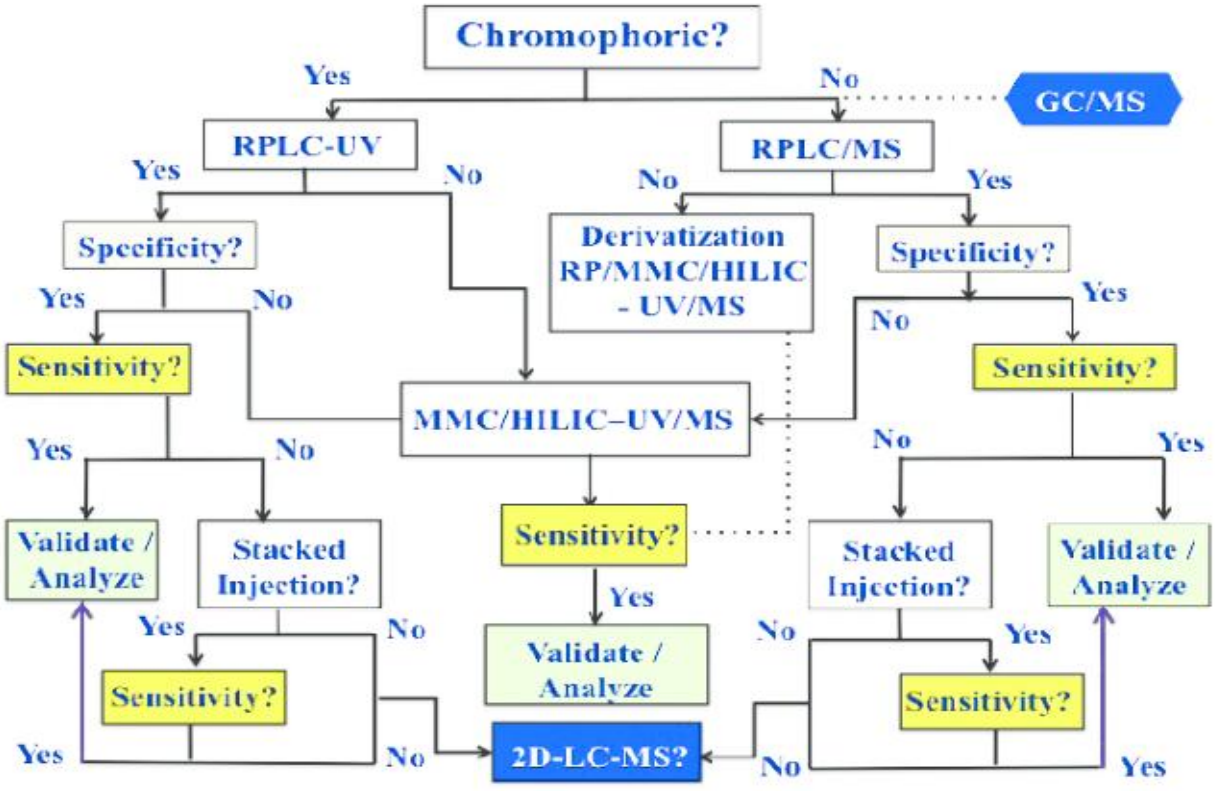

Figure 4: Decision tree for non-volatile compounds

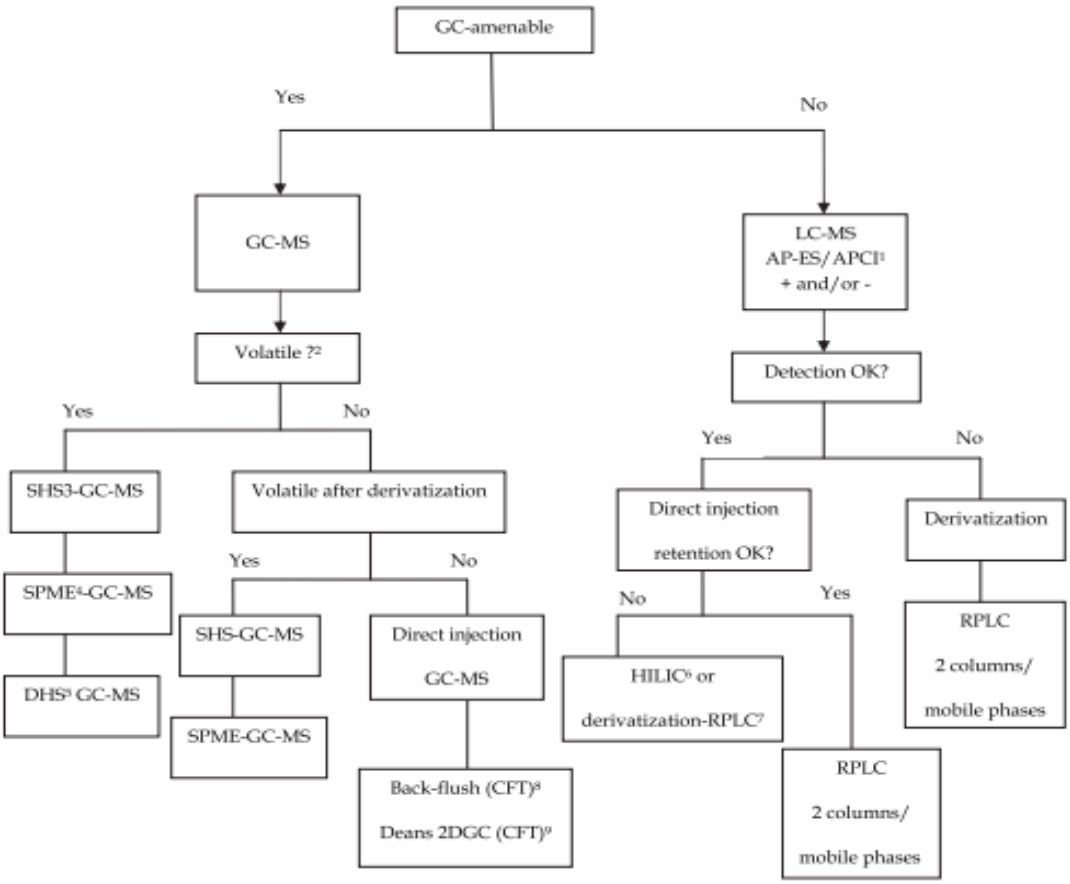

Figure 5: Method selection chart for analyzing genotoxic impurities

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,

Table 2: Threshold for APIs

Thresholds	Maximum daily dose	
	≤2 g/day	>2 g/day
Reporting threshold	0.05%	0.03%
Identification threshold	0.10% or 1.0 mg per day intake (whichever is lower)	0.05%
Qualification threshold	0.15% or 1.0 mg per day intake (whichever is lower)	0.05%

Table 3: Thresholds for degradation products in new drug products

Maximum Daily Dose[1]	Reporting Thresholds[2,3]	Identification Thresholds[2,3]	Qualification Thresholds[2,3]
≤ 1 mg		1.0% or 5 µg TDI whichever is lower	
1 – 10 mg		0.5% or 20 µg TDI whichever is lower	
10 – 100 mg			0.5% or 200 µg TDI whichever is lower
<10 mg			1.0% or 50 µg TDI whichever is lower
> 10 mg - 2 g		0.2% or 2 mg TDI whichever is lower	
> 100 mg – 2 g			0.2% or 3 mg TDI whichever is lower
≤1 g	0.1 %		
> 1 g	0.05 %		
> 2 g		0.1%	
> 2 g			0.15%

Table 4: PhRMA genotoxic impurity task force proposal – allowable daily intake (µg/day) for genotoxic impurities during clinical development using the staged TTC approach

	Duration of clinical trial exposure				
	≤ 1 month	> 1-3 month	> 3-6 month	>6-12 month	>12 month
Allowable Daily Intake (µg/day) for all phases of development	120	60	20	10	1.5
Alternative maximum level of allowable impurity based on percentage of impurity in API	0.5%	0.5%	0.5%	0.5%	0.5%

Table 5: Various HPLC methods used for a wide range of drugs

Active Pharmaceutical Ingredient (API)	Impurities	Method details
Allopurinol	Hydrazine	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5 µm cyanosilyl stationary phase (R type) at 30 °C. Mobile phase: 2-propanol/hexane (5/95, v/v). Flow rate 1. 5 ml/min; detection at 310 nm.
API (general method)	Hydrazine	(1) Derivatization using benzaldehyde. HPLC with no operating conditions reported. (2) LSE, followed by derivatization using benzaldehyde at lower temperatures. HPLC with no operating conditions reported. Detection at 190 nm.
Azelastine	Impurity A: benzohydrazide, impurity B: 1- benzoyl-2-[(4RS)-1-methylhexahydro1Hazepin-4yl] diazane	HPLC with a 10µm cyanosilyl stationary phase (R) at 30◦C. Mobile phase: pH 3.0 phosphate buffer and sodium octane sulphonic acid in water/acetonitrile (740/260, v/v). Flow rate 2.0 ml/min; detection at 210 nm.
Aryl hydrazones	E-Aryl hydrazones	(1) HPLC with a 5 µm ODS stationary phase (Merck LiChrospher) at 25◦C. Mobile phase: 1mM pH 6.0 phosphate buffer with 2 mM EDTA and methanol (40/60, v/v). Flow rate 1.0 ml/min; detection at 200–400 nm (DAD). (2) HPLC with a 5 µm phenyl hexyl stationary phase (Phenomenex Luna) at 25 °C. Mobile phase: water and acetonitrile (50/50, v/v). Flow rate 0.3 ml/min. (3) Positive and negative ion mode ESI with ion trap analyzer in SIM mode (M + H ion). Range 50–1000 m/z. Voltage 4 kV, capillary temperature 250 °C.
Carbidopa	Hydrazine	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5µm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.
Celecoxib	Intermediate I: 4- hydrazine benzene sulphonamide	HPLC with a 4 µm ODS stationary phase (NovapaK C18). Mobile phase: pH 4.8 10mM phosphate buffer and acetonitrile (450/550, v/v). Flow rate 1.0 ml/min; detection at 252 nm.
Copovidone	Hydrazine	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5µm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.
Dihydralazine sulphate	Hydrazine (impurity B)	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5µm ODS stationary phase (R type). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.
Ebifuramin	Impurity III: (+)-5- morpholino methyl3-(5- nitrofurfurylidene amino)-oxazolidin2-one	HPLC with a 5µm ODS stationary phase (Hypersil ODS). Mobile phase: acetonitrile/THF/pH 2.6 10mM dibutyl aminephosphate (15/5/80, v/v/v). Flow rate 1.5 ml/min; detection at 254 nm.
Hydralazine	Hydrazine	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5 µm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.
Isoniazid	Impurity I: 1- nicotinyl-2-lactosyl hydrazine	HPLC with a 10 µm cyanopropyl stationary phase and a mobile phase consisting of a mixture of pH 3.5 10 mM acetate buffer and acetonitrile (95/5, v/v). Flow rate and detection wavelength not specified.
Isoniazid	Hydrazine	HPLC-MS using negative electrospray ionization ESI with a Bruker Daltonics ToF. TLC with a silica gel F254 TLC plate with a water/acetone/methanol/ethylacetate (10/20/20/50, v/v) mobile phase. Visualization using dimethyl aminobenzaldehyde solution; examination

		under daylight
Nitrofural, Nitrofurazone and nitrofuroxazide	Hydrazine	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5µm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.
Povidone	Hydrazine	Derivatization using benzaldehyde, followed by LLE. HPLC with a 5 µm ODS stationary phase (Altima C18, Hypersil ODS). Mobile phase aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.
Rifampicin	Hydrazones: rifampicin quinone and 25-desacetyl rifampicin	HPTLC with a silica gel 60 TLC plate (Merck) with a chloroform/methanol/water (80/20/2.5, v/v/v) mobile phase. Examined using Scanner II (Camag) at 330nm for 25-desacetyl rifampicin and 490 nm for rifampicin quinone.
Rizatriptan	Impurity I: 1-(4-hydrazinophenyl) methyl-1,2,3-triazole	HPLC with a 5 µm nitrile stationary phase (Zorbax SBCN) at 25 °C and a gradient mobile phase consisting of varying mixtures of pH 3.4 10 mM phosphate buffer, acetonitrile, and methanol. Flow rate 1.0 ml/min; detection at 225 nm.
Vindesine sulphate	Impurity C (desacetyl vinblastine hydrazide)	HPLC with a 5 µm ODS stationary phase (R type) and a gradient mobile phase consisting of varying mixtures of pH 7.5 diethyl aminephosphate buffer and methanol. Flow rate 2.0 ml/min; detection at 270 nm.