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Review

Recent advances in trace analysis of pharmaceutical genotoxic impurities

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ABSTRACT

Genotoxic impurities (GTIs) in pharmaceuticals at trace levels are of increasing concerns to both pharmaceutical industries and regulatory agencies due to their potentials for human carcinogenesis. Determination of these impurities at ppm levels requires highly sensitive analytical methodologies, which poses tremendous challenges on analytical communities in pharmaceutical R&D. Practical guidance with respect to the analytical determination of diverse classes of GTIs is currently lacking in the literature. This article provides an industrial perspective with regard to the analysis of various structural classes of GTIs that are commonly encountered during chemical development. The recent literatures will be reviewed, and several practical approaches for enhancing analyte detectability developed in recent years will be highlighted. As such, this article is organized into the following main sections: (1) trace analysis toolbox including sample introduction, separation, and detection techniques, as well as several 'general' approaches for enhancing detectability; (2) method development: chemical structure and property-based approaches; (3) method validation considerations; and (4) testing and control strategies in process chemistry. The general approaches for enhancing detection sensitivity to be discussed include chemical derivatization, 'matrix deactivation', and 'coordination ion spray-mass spectrometry'. Leveraging the use of these general approaches in method development greatly facilitates the analysis of poorly detectable or unstable/reactive GTIs. It is the authors' intent to provide a contemporary perspective on method development and validation that can guide analytical scientists in the pharmaceutical industries.

Contents

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1. Introduction

Pharmaceutical genotoxic impurities (GTIs) may induce genetic mutations, chromosomal breaks, or chromosomal rearrangements, and have the potential to cause cancer in human [1–3]. Therefore, exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicological concern [4–6]. Therefore, it is important for process chemists to explore possible opportunities to avoid the use and generation of these genotoxic materials in the manufacturing process. However, completely eliminating the use of such chemicals or preventing the generation of DNA-reactive impurities is not always guaranteed. Although present at trace levels, GTIs can be critical in drug development [5], and if not addressed correctly, could lead to clinical holds or delayed approval from regulatory agencies. This poses an imperative challenge on analytical scientists to develop appropriate analytical methodologies to accurately measure and control the levels of GTIs in pharmaceuticals. Adequate analytical methods are not only important for ensuring patient safety but also for the development of a robust manufacture process. In addition to process impurities, certain drugs may generate GTIs via degradation during formulation or storage. For instance, oxidative degradation products such as hydroperoxides or epoxides, and hydrolytic products such as anilines are of potential genotoxicity concern. Also, components in excipients may react with API or its counter ion and form a new impurity that is of genotoxic concern (e.g. halogenated furanones) [7]. This burdens the drug development process with additional roadblocks.

The European Medicines Agency (EMEA) [8] proposes the use of a "threshold of toxicological concern" (TTC) for GTIs where TTC refers to an exposure level to compounds that does not pose a significant risk (one in 10,000 lifetime risk) for carcinogenic effects. As such, an exposure level of 1.5 μg/person/day for each impurity can be considered as an acceptable qualification threshold for supporting a marketing authorization application by EMEA and US FDA [9]. During clinical development stages, however, a staged TTC applies where greater daily intake can be allowed for shorter dosing durations as described in Table 1 [9,10]. The allowable daily intake provides a basis for estimating the analytical testing limit required for the development of an analytical method for certain GTIs. For instance, a 1.5 μg/day TTC for a given drug with a dose of 1 g/day would require an analytical testing limit of 1.5 ppm ((1.5 μg/day)/(1 g/day) = 1.5 ppm). A method capable of such a level of detection is non-routine in the context of traditional pharmaceutical analysis, where the typical level of interest is above 0.05% (equivalent to 500 ppm). This poses significant challenges for analytical method development for controlling these impurities. The challenges are increasing as regulatory agencies push for lower limits: for example, recent FDA draft guidance [9] suggests further safety factors for pediatric patients, recommending an adjustment factor of 10 for exposures for children before 2 years of age and a factor of 3 for children between 2 and <16 years of age. Furthermore, in cases where multiple structurally similar genotoxic impurities are identified and are expected to have similar mechanisms for toxicity, the TTC becomes the total exposure to all of the related compounds. These considerations push the analytical testing limit several folds lower beyond an already very low limit, rendering additional challenges to the analytical science in pharmaceutical R&D.

Ideally, conventional analytical instrumentations in pharmaceutical analysis such as HPLC with UV detection (for typical non-volatile analytes) or GC with FID detection (for volatile small molecules), should be employed as the standard first attempt for GTI analysis, but are often inadequate for accurate determination of analytes at low ppm levels, depending on properties of the analytes and sample matrices. Even if it is technically achievable in some cases, the effort required to develop such a method may overwrite the benefit, especially in modern fast-paced pharmaceutical R&D environment. Consequently, in the past few years, analytical scientists in the pharmaceutical industry have strived to develop analytical strategies to meet this challenge [11]. As a result, various types of sample introduction methodologies, different chromatographic separation tools, and various kinds of detectors have been explored and demonstrated as useful approaches [12,13]. An increasing number of publications with regard to individual genotoxic impurity or a specific class of impurities have appeared in the recent literature [12–17].

Nonetheless, practical guidance for the analytical determination of various classes of GTIs is currently lacking. The aim of this article is to review recent advances in analysis of different structural classes of GTIs that are commonly encountered during process development. This encompasses the trends in the recent literature, together with several practical 'general' approaches developed in the authors' laboratories in recent years. As such, this article is organized into the following main sections: (1) trace analysis toolbox including sample introduction, separation, and detection techniques, as well as several general approaches for enhancing detectability; (2) method development: chemical structure and property-based approaches; (3) method validation considerations; and (4) testing and control strategies in process

Table 1
US FDA and EMEA recommended acceptable qualification thresholds for genotoxic impurities in pharmaceuticals in clinical studies.

Control threshold (μg/day)	TTC limits corresponding to the duration of dosing					
	120	60	20	10	5	1.5
Allowable duration US FDA	<14 days	14 days to 1 month	1–3 months	3–6 months	6–12 months	>12 months
Allowable duration EMEA	1 day	<1 month	<3 months	<6 months	<12 months	>12 months
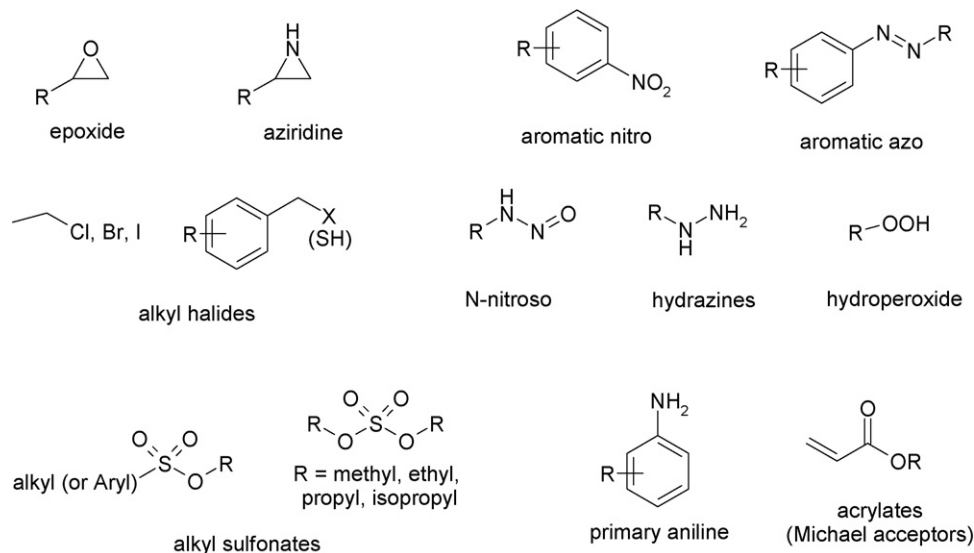
Fig. 1. Representative structures of potential genotoxic impurities.

chemistry. It is authors' intent to provide an industrial perspective with respect to method development and validation that can guide the analytical communities in the pharmaceutical industry.

2. Structures of commonly encountered GTIs

Knowledge about the chemical functional groups that can cause DNA mutation has been used to develop computer programs such as DEREK, MCase, and TOPKAT, for the prediction of potential genotoxicities [18–20]. A positive *in silico* result from a chemical structure infers potential genotoxicity. This potential alert is usually investigated by a bacterial reverse mutation test such as the "Ames test", by which DNA reactive genotoxins can be identified [21]. Conversely, a clear negative result in an appropriate genotoxicity test usually indicates the absence of genotoxicity [22]. For the purpose of deciding whether a given impurity possesses a genotoxicity risk and must be controlled at a TTC level, Mueller et al. [3] has classified impurities into groups based on their respective genotoxicity potentials: *Class 1* impurities are known to be both genotoxic and carcinogenic; *Class 2* impurities are genotoxic but with unknown carcinogenic potential; *Class 3* impurities are struc-

(a) generation of alkyl halides
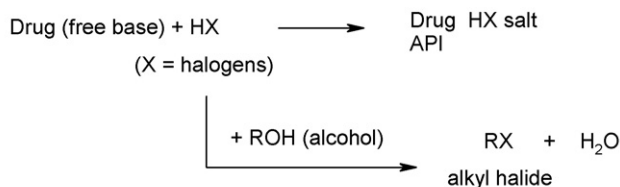
(b) generation of alkyl sulfonates
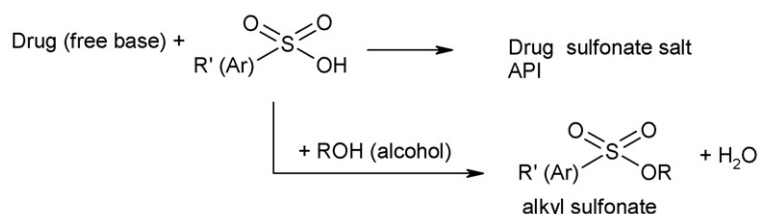
Scheme 1. Generation of (a) alkyl halides and (b) alkyl sulfonates during chemical synthesis.

tural alerts with unknown genotoxic potential (namely potential genotoxic impurities) and for which the structures are unrelated to the API structure; *Class 4* impurities are structural alerts but share the alerting structure with the API (and can be qualified via a determination of the absence of genotoxicity of the API); *Class 5* impurities are not structural alerts, thus controlled as ordinary impurities covered by ICH Q3 guidelines [23–25]. The impurities discussed in the paper mainly refer to *Classes 1–3* impurities.

Some of the commonly encountered potentially genotoxic structural motifs are exemplified in Fig. 1 [26]. A group of these are referred to alkylating agents, such as alkyl halides [16], alkyl sulfonates [17,27], and related structures. These molecules might be used as reagents or can be otherwise generated during chemical synthesis. For example, a salt counter ion of a basic molecule such as HX (X = halogens) reacts with alcohols to form an alkyl halide (Scheme 1a). Alkyl sulfonates, including alkyl esters of sulfate, methanesulfonic acid (mesylate), benzenesulfonic acid (besylate) and p-toluenesulfonic acid (tosylate), are commonly used as alkylation agents in chemical synthesis. For example, dimethyl sulfate and diethyl sulfate are commonly used as methylating and ethylating agents, respectively. In addition, certain sulfonic acids are commonly used as counter ions to form API salts. Interactions of the acids with residual alcohols may lead to the generation of alkyl sulfonates, which are potential GTIs [28], as illustrated in Scheme 1b. Aromatic amines and nitro compounds may become genotoxic after bioactivation *in vivo* [29]. Acrylates are Michael acceptors and thus susceptible to nucleophilic additions, although recent data demonstrated that ethyl acrylate is non-genotoxic in humans [30]. Epoxides [31,32] and aziridines [33] can alkylate DNA via ring opening reactions. Some hydroperoxides can result in oxidative damage to DNA, and their degradation products may react with DNA [34]. Hydrazines are known genotoxic impurities and potential human carcinogens [35]. Fig. 1 is by no means an exhaustive list of potential GTIs, but is presented to show the representative structural features of some commonly encountered GTIs that frequently require analysis during chemical synthesis of drugs.

3. Trace analysis toolbox

There are multiple challenges in the analytical determination of GTIs in pharmaceuticals at low ppm level. Firstly, as described above, there are diverse structural types, necessitating the selection and use of different analytical approaches. Secondly, many analytes are unstable or chemically reactive in nature, and thus requiring special handling techniques. Thirdly, an extremely high level of API concomitantly present with GTI analytes interferes with the analysis. Some low level impurities (known or unknown) may also interfere, especially upon exposure to reactive analytes.

The first step of method development is to select the analytical instrumentation based on the molecular structure and in the context of the analytical testing limit. The term 'analytical testing limit' is used here to describe the target testing concentration of a GTI in a specific sample, which differs from the limit of detection (of a method) or specification limit (of a drug substance batch). The actual limit of detection (or quantitation) of a method could be much more sensitive than the target testing limit. In addition, analytical testing limit is not necessarily equivalent to the specification limit. For a batch release with a proposed specification, analytical testing limit is the same as the specification limit. However, when developing method for supporting process understanding, there are no specifications proposed; therefore, analytical testing limit is more of a control limit or target level of GTI in a specific sample from analytical perspective. In early phases of clinical development, the complexity and long-term ruggedness of analytical methods are generally not a major concern. Rapid development of a sensi-

tive and specific method is usually the main objective. Therefore, more advanced instrumentation such as LC/MS and GC/MS is often chosen in order to provide quick data to the project team guiding the process development. The main advantage of using the most selective detector is to minimize issues caused by interferences in the sample matrix, and thus improve data quality. Another reason for choosing the most selective detector is that dose and duration associated with clinical trials change frequently, and the analytical testing limit becomes a moving target; thus quantitative numbers might be warranted, requiring the method to have a much lower detection limit than the actual analytical testing limit. Furthermore, on many occasions it is necessary to provide quantitative results to establish the actual levels of genotoxic impurities in intermediates and drug substances in support of process understanding; again requiring the method to have a lower detection limit than the actual analytical testing limit for successful tracking of the purging levels in various reaction stages. Generation of such data to support registration of new drugs should be considered.

The section below describes the available separation, detection and sample introduction techniques as well as some useful general approaches that enhance analyte detectability. The discussion will focus on the applicability of the techniques in the context of GTI analysis. The fundamentals of various chromatographic methods and detectors will not be discussed in detail here since comprehensive reviews are available in the literature. Commonly used analyte enrichment techniques, such as solid phase extraction and liquid–liquid extraction, are beyond the scope of this review.

3.1. Choosing the right separation techniques

3.1.1. Gas chromatography

GTIs can be generally divided into two groups based on their volatility, those that are volatile and those that are non-volatile. GC is the method of choice for analysis of many volatile small molecule GTIs. Several injection modes can be considered, and Skett has provided a comprehensive summary [12]. Commonly used approaches include liquid injection and the headspace sampling technique. Liquid injection is prone to contamination. Injection of large amount of non-volatile API can accumulate in the injector liner or on the head of the GC column which can cause deterioration in method performance rather quickly (peak tailing, recovery, sensitivity, etc.). 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. In this mode, the sample is dissolved in a high-boiling point solvent including water, dimethyl sulfoxide (DMSO), N-methylpyrrolidone (NMP), or N,N-dimethylformamide (DMF) in a closed headspace vial. Upon heating, volatile analytes partition into the vial headspace during an incubation period and the headspace vapor is sampled and injected into a gas chromatograph. The advantage of using headspace injection is that only volatile components, and the analytes of interest are injected, thus limiting the potential contamination of the injector or column. Non-volatile API does not partition into the headspace and therefore would not enter the GC system. Consequently, headspace injection becomes the preferred choice whenever possible. Nonetheless, many analytes that are amenable to GC analysis must be injected as solution because they may not have a sufficient vapor pressure to be introduced by conventional headspace injection, or may not be able to survive the high temperature incubation period.

3.1.2. Liquid chromatography

Non-volatile GTIs are generally analyzed by HPLC separation techniques, among which reversed phase (RP) HPLC is the most widely used separation mode. Many stationary phases are well established for chromatographic separation of various types of

pharmaceutical starting materials, intermediates and APIs. Selection of columns and chromatographic conditions for analyzing GTIs should follow the same principle used in drug-related impurity chromatographic methods. However, because ppm levels of GTIs are in the matrix of an extremely high level of API, a good separation of the analyte peak from the main component is critically important irrespective of what detector is to be used (see next section regarding the selectivity of various detectors).

Hydrophilic interaction liquid chromatography (HILIC) is complementary to RP HPLC for the retention and separation of small molecule polar analytes and has gained increasing attention recently [15,36,37]. In HILIC, a polar stationary phase is used with an organic mobile phase such as ACN with a small amount of water (typically less than 30%, v/v). The small amount of water is believed to form a layer on the surface of the stationary phase, into which polar analytes can partition. Additional separation mechanisms such as ion exchange and hydrogen bonding are also possible and sometimes dominating [38]. Good retention can be achieved for very polar analytes that is not possible on RP HPLC columns. It is worth noting that the sample diluent must not be too aqueous, otherwise injection of high water content sample will result in poor retention of very polar analytes. Restriction on the use of water in the sample diluent could be a limitation on the use of this separation technique, especially when high water content is required for dissolving the drug substance or formulated drug product. The use of other separation techniques including normal phase HPLC, IC, CE, and SFC for GTI analysis appears to be sporadic and is not the subject of this discussion. The readers should refer to the relevant literature if there are specific interests.

3.2. Choosing the right detector

3.2.1. Detectors for HPLC
Several types of detection techniques are available for HPLC. UV detection is the most widely used detector in pharmaceutical analysis and most accessible detector in most laboratories. Therefore, it is the preferred choice whenever feasible [39]. However, many GTIs either lack a UV chromophore or offer insufficient UV response at low ppm concentration. Furthermore, the fact that a UV detector is generally non-selective places more stringent requirements on the analytical separation. In certain cases, however, low ppm limit of quantitation (LOQ) methods are still possible as demonstrated by Yuabova et al. [13].

Evaporative light scattering detectors (ELSD) can detect compounds that lack UV chromophores, however, this detector tends to be limited in sensitivity and dynamic range. The response is highly dependent on eluent composition and analyte volatility. Furthermore volatile compounds may get lost in the interface during solvent evaporation. The charged aerosol detectors (CAD) can also detect compounds that do not have a UV chromophore. CAD relies on the charging of the aerosol particles, which is in a way analogous to an atmospheric pressure chemical ionization (APCI) source in LC/MS. However, CAD is highly dependent on analyte volatility. Unsuccessful experiences in developing a method using ELSD and CAD detectors have been reported for investigational compounds [13] and at the end an alternative MS method was implemented. Chemiluminescent nitrogen detection (CLND) has also been explored for quantitation of low level pharmaceutical impurities since most pharmaceutical compounds contain nitrogen atoms [36,40], however, its use for analysis of GTIs has yet to be demonstrated.

In contrast, atmospheric pressure ionization mass spectrometry (MS), including electrospray (ESI) and atmospheric pressure chemical ionization (APCI) in single ion monitoring (SIM) or multiple reaction monitoring (MRM) mode, has been established as the most versatile, sensitive and selective analytical technique for
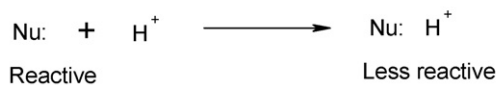
Scheme 2. 'Matrix deactivation' general strategy for enhancing analyte stability and recovery.

trace analysis. Methods using MS detection have good precision and dynamic range. An accelerating number of publications report MS as the detector for trace level analysis of GTIs, either directly or coupled with derivatization approaches.

Recent advances in the instrumentation of ICP-MS have increased the detection sensitivity for some non-metal elements, such as S, Br, and Cl, which can be detected at ppb levels in solution. Carr et al. discussed the possibility of applying non-metal LC–ICP-MS to the determination of GTIs [41]. Thus, it is reasonable to speculate that one could use LC–ICP-MS to analyze alkyl sulfonates and alkyl halides in the foreseeable future. Further improvements in the instrumentation may help achieve the detection limit required for GTI analysis.

Yuabova et al. [13] have attempted to provide a decision tree for selecting which detector to use for liquid chromatography, based largely on the simplicity or ease of use of the detector. It is evident that every detection technique has it own pros and cons. As exemplified in the literature and also in the authors' laboratories, MS is the technique of choice for the sensitivity and selectivity that is needed on many occasions. Therefore, in order to support fast-paced drug development, it is our experience to leap quickly to MS detection if UV detection is proven to be inadequate, in contrast to some laboratories where a decision tree is used systematically. Increased use of MS detection also appears to be the preferred approach of several other groups [42,43].

3.2.2. Detectors for GC
A recent review on commonly used detection techniques for GC has been provided by Skett [12]. The flame-ionization detector (FID) remains the most versatile and widely available detector for GC. Its simplicity of use (equivalent to UV for HPLC) makes it the first choice for detecting volatile organic molecules. However, selectivity and sensitivity are somewhat limited for measuring trace levels of GTIs in the presence of large quantities of API, and thus its performance is not always satisfactory.

Because a significant number of GTIs contains halogens atoms (e.g. alkyl halide), electron capture detector (ECD) has emerged as an important detector in GTI analysis because of its unique selectivity toward to halogen elements. The sensitivity increases in the order of Cl, Br, I, such that alkyl chlorides give the weakest response. It is worth noting that the presence of other electron capturing species in the sample matrix could hamper the sensitivity or selectivity. Other element specific detectors, such as nitrogen–phosphorus detector (NPD), offer an additional tool for GTI analysis because of their selectivity and sensitivity, although wide utility has not yet been demonstrated. NPD responds selectively to organic compounds containing nitrogen and/or phosphorus. Another detector reported in the literature is the thermal energy analyzer (TEA), which has been used for the analysis of environmental toxins such as volatile nitrosamines [44].

It is evident that mass spectrometry detectors including electron impact (EI) or chemical ionization (CI) operating in the SIM mode
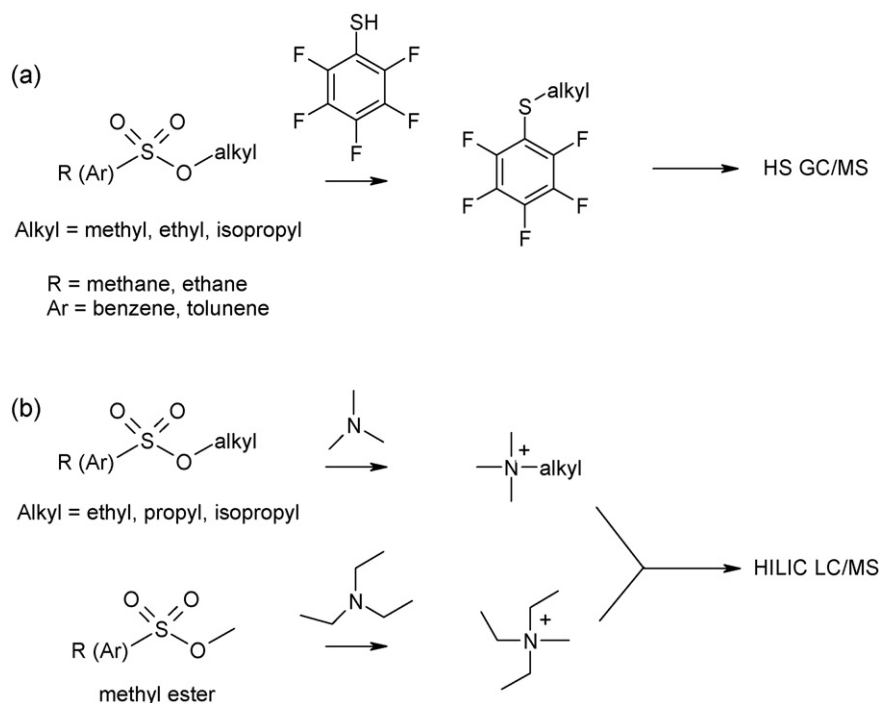
Scheme 3. Two derivatization strategies for alkyl sulfonates: (a) HS GC/MS method and (b) LC/MS method.

offer the most sensitive and selective detection in most GC methods. Because of its distinct advantage of mass selective detection (compound specific), an MS detector is much less prone to interferences compared to other methods. Due to the reduced background noise, MS detection in GC is the method of choice for trace analysis of GTIs.

3.3. General approaches for enhancing detectability

Careful selection of the separation mode and detector is critically important but may not necessarily guarantee a satisfactory method. Additional considerations of the molecule's structure and properties to enhance detectability may be required in order to achieve the desired sensitivity. Several 'general' approaches developed in the authors' laboratories are discussed below.

3.3.1. Chemical derivatization

A large number of GTIs are unstable or reactive and/or lack an appropriate structural moiety for sensitive detection at low ppm levels. Therefore, chemical derivatization becomes a general strategy that is useful in the following situations: (a) to stabilize reactive GTIs; and (b) to introduce a detection specific moiety for enhanced detection (chromophore for UV, basic nitrogen for MS, etc.). Of course, different analyte structures require specific types of derivatization approaches. General references on the selection of derivatization methods for analytical purposes are available in the literature [45,46]. Some compound-specific examples are provided in Section 4. The derivatization approach is sometimes non-specific, especially when dealing with alkylators. The reported derivatization methods for alkylators are not able to distinguish structurally related compounds [14,15,47]. For example, both ethyl sulfonates and ethyl halides can react with a nucleophilic derivatization reagent, producing the same ethyl derivatives. On the flip side, such a derivatization approach could be advantageous in determinating a group of structurally related compounds if the method is designed properly.

3.3.2. Matrix deactivation

Matrix deactivation [48] is a chemical approach to stabilize unstable/reactive analytes during sample preparation and/or chromatographic analysis. In contrast to conventional chemical derivatization where the analyte is chemically transformed into a stable and detectable species, the matrix deactivation approach chemically deactivates the reactive interfering species in the sample matrix. The matrix deactivation approach is based upon the hypothesis that the instability of certain GTIs at trace level is caused by the reaction between the analytes and reactive species in sample matrix. Thus, controlling the reactivity of the reactive species in the sample matrix would stabilize the unstable/reactive GTI analytes. As an example, electrophilic alkylators are destabilized by nucleophiles or bases via either nucleophilic substitution or elimination reactions. One way to suppress the reactivity of those nucleophiles and bases is to protonate the matrix, which is a very convenient and effective approach. A second approach is to add a nucleophile scavenger into the sample matrix to remove the nucleophiles completely. This is analogous to the use of an antioxidant and metal chelator to prevent oxidation in lipid analysis [49]. The matrix deactivation strategy is illustrated in Scheme 2.

3.3.3. Coordination ion spray-MS

HPLC coupled with MS detection appears to be a general methodology for determination of a wide range of pharmaceutical GTIs. However, many analytes do not possess structural features that are amenable to atmospheric pressure ionization methods such as ESI. In our laboratory, the coordination ion spray-MS strategy has been demonstrated to be useful for many analytes in general. It is well known that some alkali metal ions, such as Li^+, Na^+, and K^+ (also NH_4^+) are able to form complexes with some organic molecules in the gas phase through the formation of non-covalent bonds with heteroatoms including O, N, P, and S in the molecules [50]. Coordination ion spray-MS can also be used to detect compounds that can form π-complexes [51] with transition metals such as Ag^+. Through the use of coordination ion spray-MS, analytes such
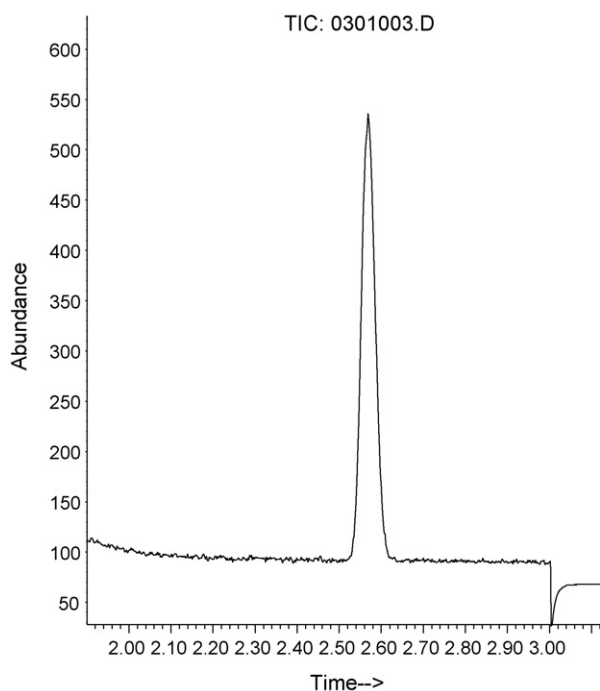
Fig. 2. GC/MS chromatogram of ethyl chloride at 100 ng/mL.

as n-propyl-p-toluenesulfonate can be analyzed effectively [52]. Selected applications are provided in Section 4.

4. Method development: chemical structure and property-based approaches

Many GTIs have specific structural features that can be utilized for designing methods for their analysis. Nonetheless, the structural characteristics that lead to potential toxicity may not necessarily be the only consideration for analytical method development. Instead, overall structural features of the analyte should be carefully examined to determine its chemical and physical properties which in turn will be used to choose the best analytical approach in terms of sample preparation, sample introduction, separation, and detection. Typical questions to be kept in mind include: Is the compound volatile? Is the analyte sufficiently stable for direct analysis? What are the detection features? How would the matrix (API structure) interfere with the analysis? In short, knowing the properties of the analyte in the context of sample matrix is the key to success in trace analysis method development. The following sections attempt to provide a summary on recent advances in methods for selected structural classes of GTIs.

4.1. Alkyl sulfonates

Alkyl sulfonates are suspected of being generated by sulfonic acid salts in the presence of alcohols, though studies have demonstrated the risk for their production is low [53,54]. They may also be directly used as reagent during manufacture processes. The recent discovery of a surprisingly high level of ethyl methanesulfonate contaminant in the prescription HIV drug Viracept led to its temporary withdrawal from the market as mandated by the EMEA [55]. This event demonstrates the severity of consequences of uncontrolled alkyl sulfonates in APIs, although subsequent research findings cast doubt on the current control limit of 1.5 μg/day for ethyl mesylate in API [56].

The evolution of analytical methodologies for analysis of alkyl sulfonates has been thoroughly reviewed by Elder et al. [17]. Sul-

fonates are very reactive electrophiles and thus relatively poor for direct analysis. Taylor et al. reported degradation of alkyl esters in the injection liner of direct analysis GC; as a result, HPLC/UV and coordination ion spray-LC/MS methods were used to detect benzenesulfonates or p-toluenesulfonates directly [52]. Their sensitivity and reproducibility were limited due to the poor stability of the analytes. Therefore, derivatization approaches were developed in order to overcome the instability of alkyl sulfonates under separation conditions and to enhance detectability. Lee et al. reported the use of sodium thiosulfate as the derivatization reagent in a reaction HS GC/MS method [47]. However, it gave a mixture of corresponding alkyl thiocyanate and alkyl isothiocyanate.

Most recently, two generic approaches have been developed independently, one involving the use of headspace GC/MS [14] and another using HILIC–LC/MS [15]. Both derivatization methods aimed to improve the detectability of the analytes and separation from API matrix interferences. Alzaga et al. [14] converted the compounds into volatile pentafluorothiobenzene derivatives (Scheme 3a). Taking advantage of the discriminating nature of headspace sampling with GC, the derivatives were easily separated from non-volatile APIs. An et al. [15], on the other hand, attempted to convert the alkyl sulfonates into very polar positively charged quaternary ammonium ions (Scheme 3b), which is ideal for ESI-MS detection. The polar nature of the quaternary ammonium derivatization products made them excellent candidates for HILIC separation. The APIs are generally not polar enough to be retained on HILIC columns, and thus there is no need to adjust chromatographic conditions for different APIs. Both methods are capable of detecting alkyl sulfonates at sub-ppm level in various APIs.

4.2. Alkyl halides

4.2.1. Volatile alkyl halides

Volatile alkyl halides are often used as reagents in chemical synthesis of APIs. Consequently a great deal of analytical attention and effort has been devoted to ensure residual levels of such reagents are either eliminated or minimized and so as to not present a significant risk to patients. A complete literature review on the analysis of organohalides was given by Elder et al. recently [16]. GC/FID was widely used historically because of instrument simplicity and is still used especially when it is desirable to determine alkyl halides in the same method as used for the quantification of the process solvents (rather than running a separate method using an ECD detector). Nonetheless, GC/ECD has become more routinely used recently because of superior selectivity and sensitivity. In order to avoid interference from non-volatile APIs, whenever possible, headspace sampling techniques should be considered in order to minimize matrix interferences. A headspace GC/ECD method has been reported [16] for the determination of 23 alky/aryl halides in various APIs. The APIs are dissolved in a 70/30 mixture of water and DMSO and under headspace conditions, volatile alkyl halides are vaporized into the headspace of sample vial during analysis.

Generally, it was found that ECD is more sensitive for iodides and bromides than chlorides. Thus, for compounds containing only a single chlorine, GC/MS might serve as a better alterative for improved sensitivity and specificity. We explored the application of headspace GC/ECD for the determination of methyl and ethyl chlorides. It was found that they were not well separated from the air peak on the popular DB-624 column (at the tail of the large air peak), thus the sensitivity of these chlorides was inevitably compromised. In fact, a DMSO solution of ethyl chloride at 400 ng/mL was not detectable by ECD. In the alternative headspace GC/MS method, however, ethyl chloride at a quarter of the concentration gave a signal/noise (S/N) of 109 when monitoring the m/z 64 ion in the SIM mode (Fig. 2).
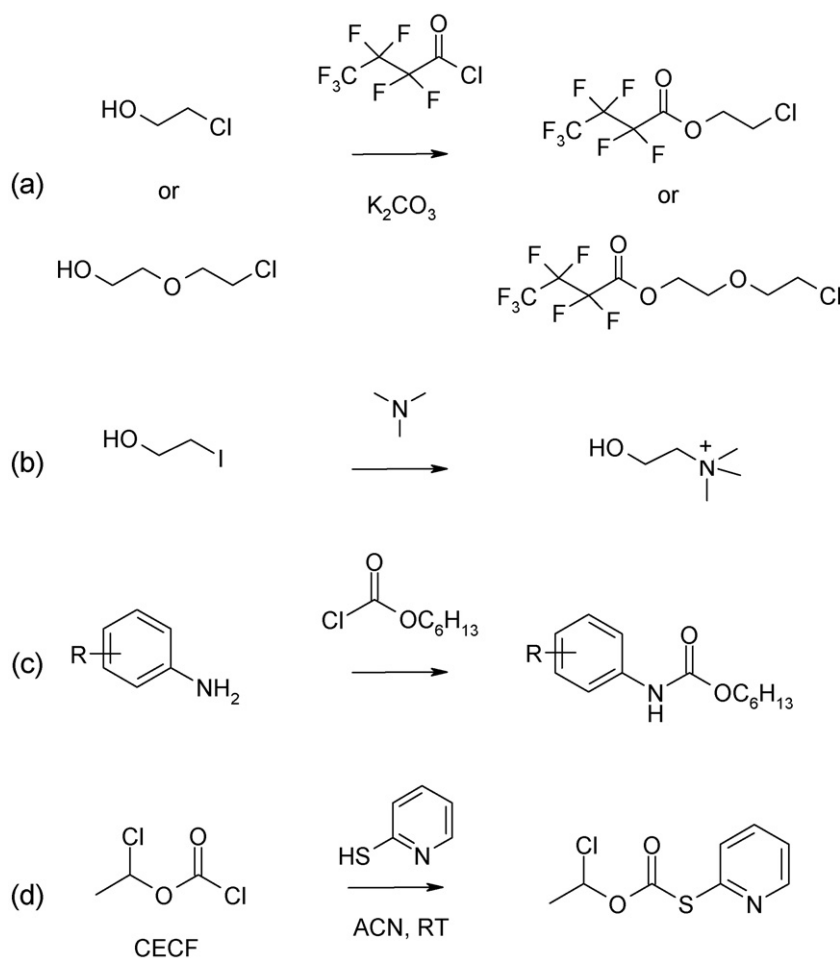
Scheme 4. Some derivatization reactions discussed in this review.

4.2.2. Non-volatile alkyl halides

Some alkyl halides are not sufficiently volatile for GC sampling techniques. Consequently, chemical derivatization is commonly used to improve their volatility. Chloroethanol and chloroethyloxyethanol, for example, are potential GTIs in an experimental drug. They are not suitable for GC/ECD because of their high boiling points. In addition, they contain only a single chlorine atom, which limits the detection sensitivity in ECD. As expected, the direct injection GC/ECD method yielded low sensitivity and recovery. The low

recovery may be attributed to undesired reactions in the GC injection port. In order to overcome the above issues, chloroethanol and chloroethyloxyethanol were derivatized with heptafluorobutyryl chloride (Scheme 4a). This led to the increase of the volatility of the analytes as well as ECD detection sensitivity due to the introduction of multiple fluorine atoms [57]. Typical chromatograms of a standard of chloroethanol and chloroethyloxyethanol at a concentration of 5 ng/mL are shown in Fig. 3a and b, respectively. The recoveries of chloroethanol and chloroethyloxyethanol in an inves-
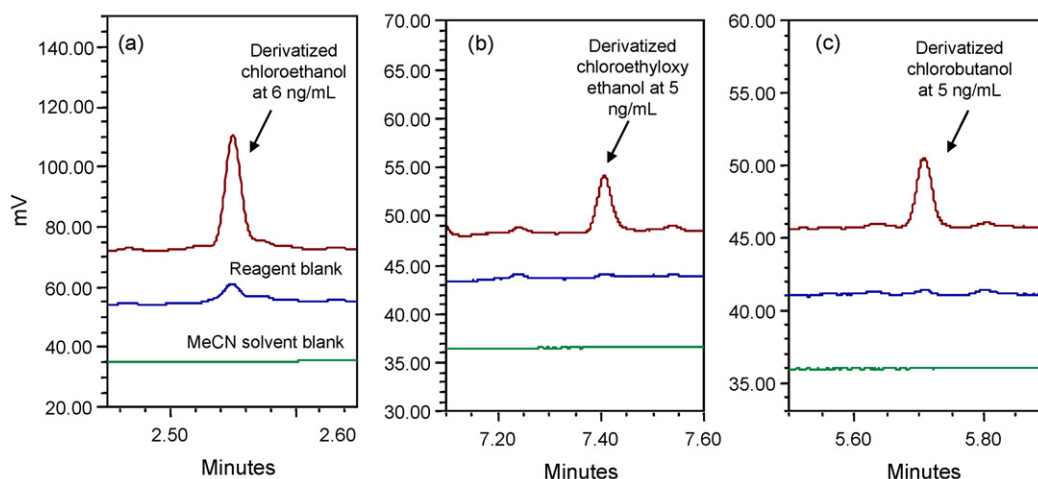
Fig. 3. Typical GC/MS chromatograms of: (a) chloroethanol, (b) chloroethyloxyethanol, and (c) chlorobutanol following heptafluorobutyryl chloride derivatization.

tigational drug were 87% and 112%, respectively. This methodology was also applicable to the analysis of chlorobutanol. An example chromatogram of chlorobutanol after derivatization is shown in Fig. 3c. The derivatization of chloroalcohols to the corresponding heptafluorobutryl esters increased the overall sensitivity by at least 20-fold, as seven fluorine atoms were incorporated into the molecules.

Alkyl halides are generally good electrophiles, therefore they can be alternatively derivatized by nucleophilic reagents such as alkyl amines. 2-Iodoethanol, as an example, can react with trimethyl amine to produce a quaternary ammonium derivative (Scheme 4b), which can be easily separated from the API and other interferences by HILIC and detected by MS [37]. This approach not only improved the sensitivity but also stabilized the analyte. A method with a detection limit as low as 0.1 ppm was achieved for an experimental drug.

4.3. Non-halogenated volatile small molecules

Some GTIs are small volatile molecules such as ethyl acrylate and benzene, that lack halogen atoms, so they cannot be analyzed by the GC/ECD generic approach. Therefore GC coupled to a FID or MS detector is the common approach for their analysis. Once again, as in the analysis of halogenated volatiles, the headspace sampling technique is preferred over direct injection since the later tends to contaminate the instrument, leading to poor method reproducibility and robustness.

GC/FID methods, if sufficiently sensitive depending on the analytical testing limit, are generally preferred because of their simplicity and the fact they can be integrated with the residual solvent methods. On the other hand, GC/MS methods are generally much more sensitive than GC/FID, where typically EI-MS SIM is used as the detection mode. For analysis of benzene, an LOQ of 7 ng/mL was demonstrated by MS detection, while an LOQ of 250 ng/mL was found for the FID method (unpublished data). Similarly, in the case of ethyl acrylate, an LOQ of 6.5 ng/mL was achieved by MS while it was as high as 400 ng/mL by FID detection (unpublished data). For both compounds, the GC/MS methods offered much greater sensitivity compared to FID detection. However, both approaches could be used to detect the analytes at low ppm levels, with the caveat that much higher concentrations of the API samples might be required for FID methods in order to achieve a lower testing limit.

4.4. Non-volatile pharmaceutical intermediates

A great number of GTIs are non-volatile starting materials, synthetic intermediates or reaction by-products. The majority of this type of analytes contains UV chromophores. If the method requirement in terms of the analytical testing limit is not so high (e.g. in the hundreds of ppm), an HPLC/UV method should be the first choice. When low or sub-ppm method sensitivity is required, it is expected that HPLC/UV detection will not be adequate, and LC/MS methods become a logical choice. From the LC/MS method development point of view, these analytes can be grouped into three empirical categories, basic, acidic or neutral, based upon their structural features. For LC/MS analysis of basic and acidic analytes, positive and negative ionization modes are used, respectively. The ionization techniques can be either ESI or APCI depending on the molecular structures. For neutral analytes, however, protonation or deprotonation of the analytes may not occur readily in gas phase, and special techniques such as ion coordination or chemical derivatization would have to be implemented. Detailed examples are given below.

4.4.1. Analytes with strong UV chromophores

GTIs that have strong UV chromophores can be analyzed by HPLC/UV provided they have sufficient aqueous/organic solubility for dissolving adequate samples. Soman et al. [39] validated an HPLC/UV method for the analysis of 4-amino-2-ethoxy-cinnamic acid and its ester together with 4-bromo-3-ethoxy-nitrobenzene in a drug substance and drug product. The detection wavelength was set at 275 nm. The method gave an LOD of 2–5 ppm when the sample concentration was 15 mg/mL. All three GTIs were completely resolved from each other and from the API peak and other related impurities within a 40 min chromatographic run. Adequate validation results in terms of method specificity, precision, recovery (accuracy), and linearity were achieved. It appears that the HPLC column had to be overloaded in order to achieve the desired sensitivity. For HPLC/UV methods, poor solubility of API in aqueous/organic diluent is very often the limiting factor for the method. Due to limited selectivity of the UV detector, separation of the analytes from API peak is also crucial to the success of method development.

4.4.2. Basic nitrogen-containing analytes

Basic analytes generally have high proton affinity, and can be analyzed by atmospheric pressure ionization MS with high sensitivity. Compound **I** (Fig. 4), as an example, contains multiple basic nitrogens, and it is an ideal molecule for electrospray ionization LC/MS. The analyte was monitored in the SIM mode at m/z 288 [M+H]$^{+}$ by ESI. An LOQ of 0.2 ppm was achieved when samples of API were prepared by dissolving 4–5 mg of solid materials in 1 mL of diluent solvents which are typically mixtures of water and acetonitrile. At the 1.7 ppm analytical testing limit, excellent accuracy (% recovery) and precision (%RSD) were achieved [58]. Borman et al. [59] reported an LC/MS/MS method using MRM detection for analysis of FMTP, (4-fluorophenyl)-1-methyl-1,2,3,6-tetrahydropyridine (**II**, Fig. 4), a neurotoxic impurity at ppb level. The method showed acceptable repeatability and linearity and was used as a limit test for the detection of FMTP at 10 ppb in paroxetine API. The paroxetine samples were prepared at a concentration of 20 mg/mL in water/ACN (75/25, v/v) spiked with 0.1% TFA.

Simple aromatic amines can be ionized readily by positive ion MS. However, these compounds are relatively polar and are not well retained on C18 columns, where high aqueous mobile phase is generally required. Vanhoenacker et al. [60] reported a generic LC/MS method for the determination of arylamines and aminopyridines in pharmaceutical products to improve the retention of the analytes. The analytes were converted to hexylcarbarmate derivatives with hexylchloroformate as the derivatization agent (Scheme 4c). Introduction of the lipophilic hexyl side chain results in increased retention on reverse phase columns. The method can generally detect the analytes at 1 ppm. Other recently reported useful approaches that aim to improve both retention and sensitivity include derivatization with fluorescence tags followed by HPLC with fluorescence detection [61], derivatization with pentafluorobenzaldehyde followed by GC/MS analysis [62], derivatization with dansyl chloride followed by LC/ESI-MS detection [63], and a combination of electrochemical oxidation and ESI-MS/MS for the analysis of (p-chlorophenyl)-aniline [64]. Also, this group of compounds can be potentially analyzed by HILIC interfaced with ESI-MS, in which polar analytes retain on column while non-polar APIs elute near the void [65].

4.4.3. Acidic analytes

Most acidic organic compounds can be detected in negative ion mode mass spectrometry via deprotonation. Weakly acidic compounds, however, may not ionize adequately in electrospray ionization whereas better sensitivity could be achieved by negative APCI. The three compounds shown in Fig. 4 are either acidic
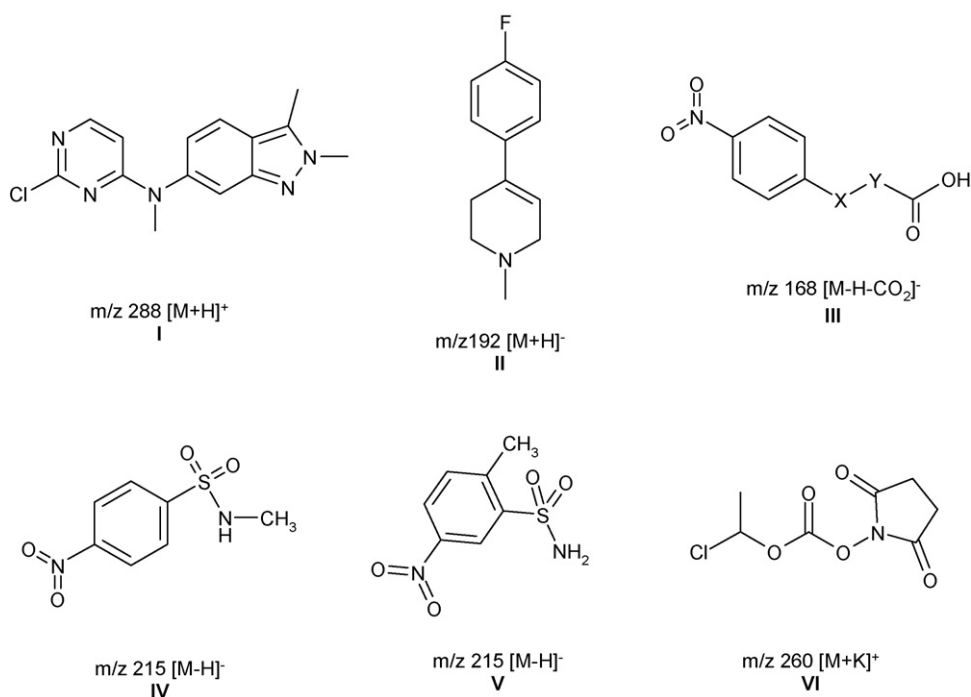
Fig. 4. Structures of some potential genotoxic impurities (**I**–**VI**) discussed in this review.

(**III**) or weakly acidic (**IV** and **V**). Aromatic nitro compound **V**, one of the starting materials in the synthesis of pazopanib HCl [58], contains a sulfonamide moiety which is slightly acidic due to the electron withdrawing aromatic nitro group. Negative ESI could not give the desired sensitivity, while both APCI and APPI (atmospheric pressure photoionization) produced an acceptable detection limit. Considering the simplicity of mobile phase, APCI was selected. The separation was achieved on a C18 column with 0.1% formic acid as the weak mobile phase and methanol as the strong mobile phase. As shown in Fig. 5, injection of 4 μL of a 6 ng/mL solution gave an S/N of 71 by monitoring the m/z 215 $[M-H]^-$ ion. A second sulfonamide **IV** gave an S/N of 192 upon injecting 4 μL of an 18 ng/mL solution (also monitoring the m/z 215 $[M-H]^-$ ion). The third analyte **III** afforded an S/N of 24 at 18 ng/mL by monitoring the $[M-H-CO_2]^-$ ion at m/z 168. If the API can be dissolved at 5 mg/mL,

it would give analytical testing limits of 3.6 and 1.2 ppm respectively. Therefore, negative APCI methodology could be widely used as a general strategy for analyzing sulfonamides if the analyte does not contain alternative detection feature other than the sulfonamide moiety.

4.4.4. Neutral analytes

Neutral molecules with low proton affinity or electron affinity, cannot be easily protonated or deprotonated in atmospheric pressure ionization mass spectrometry. Depending on the overall structure features, they may be suited for coordination ion spray-MS analysis. They can form adducts with cations including Li^+, Na^+, K^+, and NH_4^+ in the mobile phase and be detected in positive ion mass spectrometry [50]. Compound **VI** (Fig. 4), for example, is a potential GTI in the synthesis of an investigational drug [48]. It con-
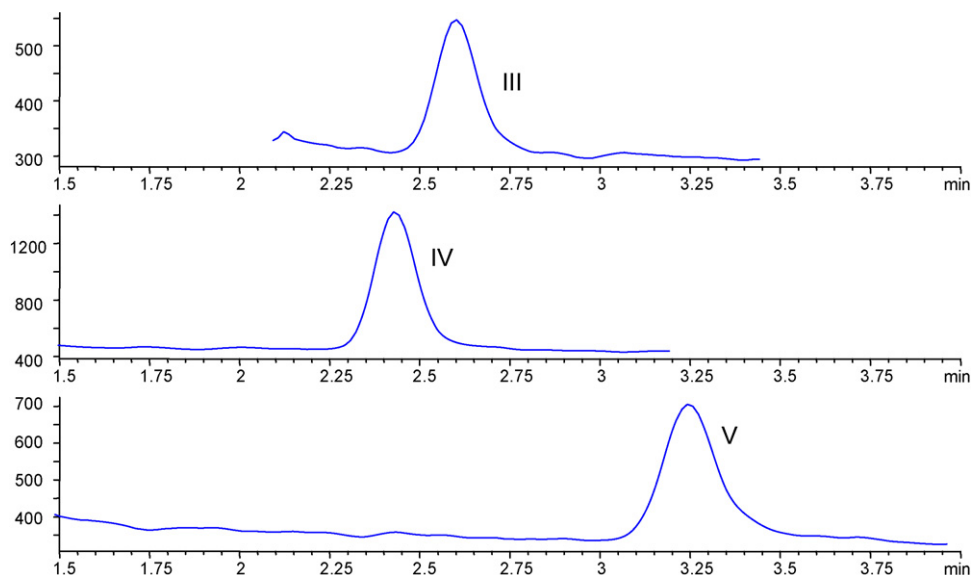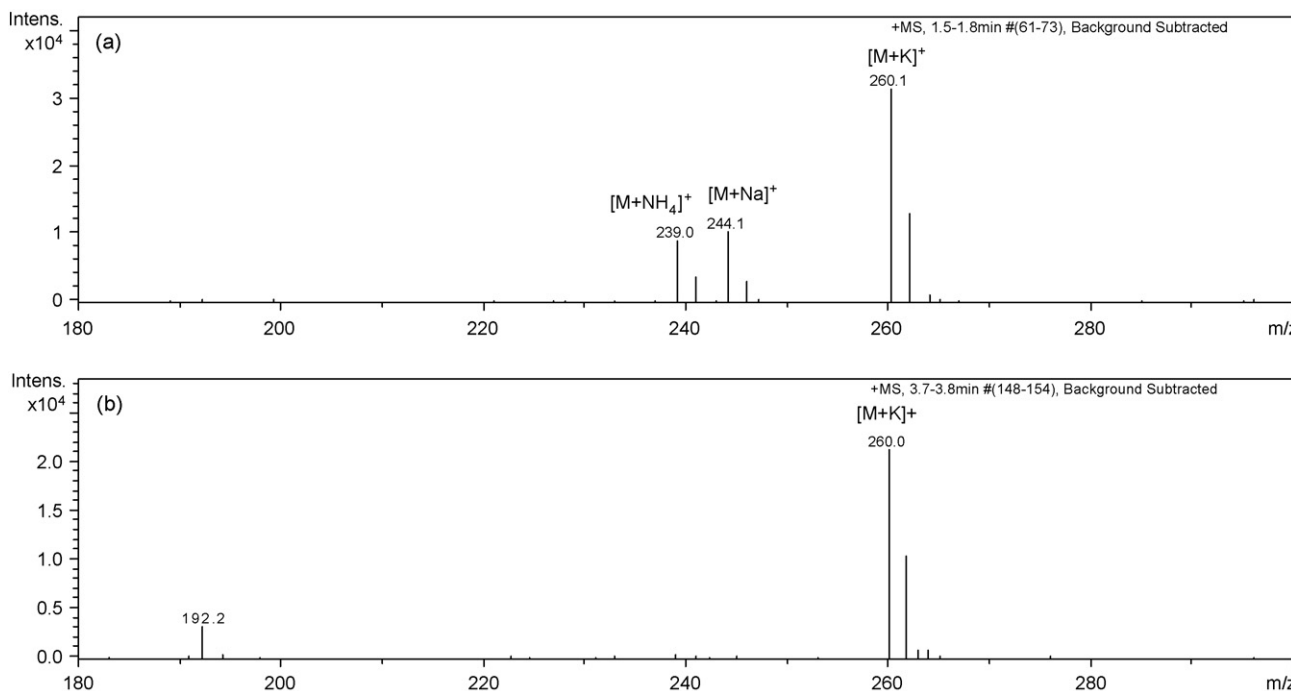

Fig. 5. Negative ion APCI LC/MS chromatograms of GTIs **III**–**V**.

Fig. 6. MS spectra of compound **VI** generated during coordination ion spray-MS method: (a) mobile phase fortified with 0.1% formic acid plus 0.1 mM NH_4^+/Na^+/K^+; (b) with 0.1% formic acid and 0.1 mM K^+.

tains multiple proximate oxygen atoms; therefore, it is expected to be a good chelator in the gas phase.

To screen the adduct ions for optimal detection, a mobile phase containing 0.1% formic acid with 0.1 mM NH_4^+, Na^+, and K^+ was used as the aqueous mobile phase while ACN was used as the organic phase. A typical spectrum obtained on an Agilent single quadrupole LC/MSD is shown in Fig. 6a. The molecule did not afford the $[M+H]^+$ ion (expected to be at m/z 222 if present) but rather it gave intense $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ adduct ions at 239, 244 and 260, respectively. As illustrated in the figure, the analyte seems to have the highest affinity toward K^+ and preferentially formed the $[M+K]^+$ adduct. Consequently, a new mobile phase containing only K^+ was chosen for the method. A typical spectrum acquired under the new conditions is illustrated in Fig. 6b, showing an intense potassium adduct ion peak at m/z 260. By SIM monitoring using an Agilent MSD, a method with an LOD of 10 ng/mL was developed and validated. Monitoring the ammonium adduct can also produce satisfactory sensitivity when higher concentration (4 mM) of ammonium formate or acetate was added to aqueous mobile phase.

4.5. Unstable/reactive and poorly detectable analytes

4.5.1. Chloroformates

Chloroformates are very reactive and moisture sensitive compounds. Initial attempts for the analysis of chloroethylchloroformate (CECF) were focused on derivatization approaches. It was observed that strong basic derivatization reagents caused decomposition of the desired derivatives. Therefore, a less basic nucleophile, 2-mercaptopyridine, which could also serve as a proton sink to drive the reaction to completion, was selected (Scheme 4d). The derivatization product can be monitored by LC/MS using the m/z 218 $[M+H]^+$ ion. However, because chloroformate is moisture sensitive, the dilution solvent acetonitrile must be dried with activated molecular sieves and the glassware must be oven dried before use in order to achieve the desired method sensitivity and reproducibility. As a result, an LOQ of 5 ng/mL was achievable (equivalent to 0.1 ppm relative to a 50 mg/mL API sample).

The above derivatization method was successfully applied to the analysis of the final API. However, when applied to the analysis of CECF in an intermediate, the method became problematic because of the presence of a large quantity of a structural analogue **VI** (Fig. 4) which also reacts with the derivatization reagent and produces the same derivative. To overcome the interferences, a direct analysis method had to be developed where the matrix deactivation strategy (described in Section 3.3.2) must be implemented to stabilize the analyte. As a result, CECF can be analyzed by direct injection GC/MS at 4 ng/mL (equivalent to 0.4 ppm relative to a 10 mg/mL sample) in methylene chloride spiked with oxalyl chloride [48].

4.5.2. Hydroperoxides

Hydroperoxides can be easily reduced to alcohols or oxidized to ketones in addition to other degradation products. The detectability of individual hydroperoxide is very much dependent upon its stability. HPLC with mercury cathode electrochemical detection has been applied to detect peroxides in lipids with excellent sensitivity [66]. More recently HPLC/ESI-MS has been used to specifically determine hydroperoxides in biological samples. Most ionization techniques can result in loss of water from the protonated molecule, as well as other fragmented products [67]. In fact, hydroperoxides can form more stable ion adducts with Li^+, Na^+, K^+, Ag^+ and NH_4^+. Thus, they have been alternatively detected by coordination ion-spray mass spectrometry as Ag^+ or ammonium adducts [68].

4.5.3. Hydrazines

Hydrazine is a very polar small analyte, which is also susceptible to oxidation. Direct analysis of hydrazine in an investigational drug was reported where ion chromatography (IC) coupled with an electrochemical detector were used. An LOQ of 100 ppm was successfully achieved [69]. Liu et al. [36] developed a HILIC method for simultaneous determination of hydrazine, 1,1-dimethylhydrazine, methyl hydrazine, and 1,2-dimethyl hydrazine using CLND detection. The LOD for hydrazine in an experimental drug was validated at 0.02% (200 ppm).
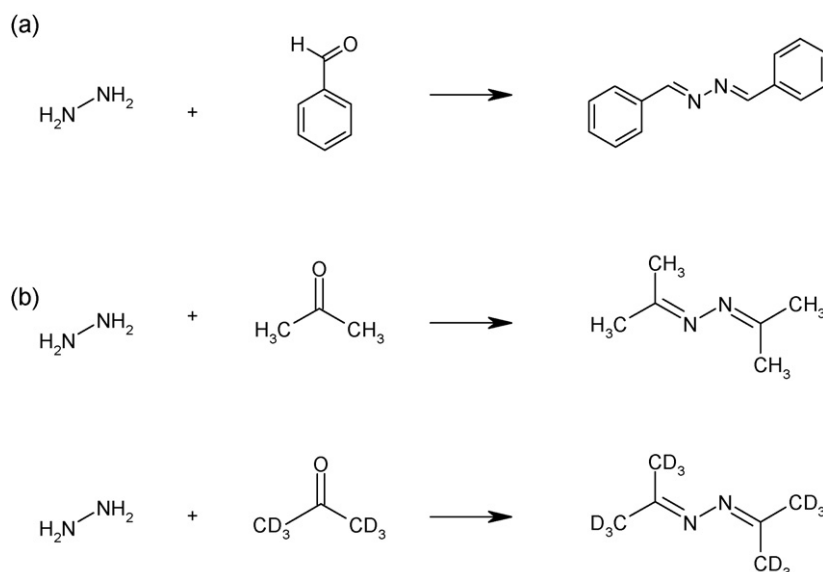
Scheme 5. Derivatization approaches for hydrazines: (a) converting to UV active derivative by reacting with benzaldehyde; (b) converting to corresponding acetone azine or acetone azine-d_{12} by reacting with acetone.

Kean et al. [35] has provided a recent review on a number of approaches for determination of hydrazines in pharmaceuticals. It was proposed that derivatization followed by LC could be a fit-for-purpose general approach. Thus, a general HPLC/UV method after benzaldehyde derivatization (forming a benzaldehyde azine) was validated (Scheme 5a). The LOQ of the method can be as low as 10 ng/mL in solution, equivalent to 0.2 ppm relative to an API sample of 50 mg/mL. Very recently, the authors' laboratory reported a general method using *in situ* derivatization-headspace GC/MS methodology for the determination of hydrazine in drug substance at low ppm levels [70]. This method uses acetone or acetone-d_6 as the derivatization reagent and the resulting acetone azine or acetone azine-d_{12} (Scheme 5b) can then be analyzed by headspace GC/MS. For the acetone derivative, the molecular ion at m/z 112 was monitored, while for the acetone-d_6 derivative, both ions at m/z 124 and m/z 106 were monitored for best results. The method gives excellent sensitivity with an LOQ as low as 0.1 ppm when the API samples were prepared at 10 mg per headspace injection vial. The spike recoveries of hydrazine at the 1 ppm level were between 79% and 117% in various APIs tested. The precision (%RSD) of six preparations of the hydrazine standards at the 1 ppm level was typically between 2.7% and 5.6%. A linear range from 0.1 to 10 ppm has been demonstrated. This general method has been tested in a number of API matrices and successfully applied to the determination of hydrazine in support of API batch releases.

4.5.4. Formaldehyde

Aldehydes are known to be DNA reactive while some are endogenous in human [71]. Snodin [72] argued that formaldehyde should not be considered as a standard GTI since, although it is genotoxic *in vitro*, it was non-carcinogenic *in vivo* in rats. Several independent experts reported that an oral Permitted Daily Exposure (PDE) of at least 10 mg/day can be determined; therefore the control limit of formaldehyde to the current default limit of 1.5 µg/day may be unnecessary. Formaldehyde is traditionally analyzed after chemical derivatization [45] since the direct analysis method was unable to achieve the desired sensitivity [73]. Multiple derivatization reagents, including hydrazines [74,75], diamines [76,77], dopamine [78], alcohol [79], hydroxylamine [80,81] and Hantzsch's reagent [82,83], have been reported. Derivatization with the Hantzsch reaction (reacting with acetyl

acetone to form 3,5-deacetyl-1,4-dihydrolutidine [83]) followed by HPLC/UV detection at 412 nm provided good selectivity for formaldehyde with an estimated LOD of 1 ppm. Small responses observed for reagent blanks prevented a lower detection limits. Spike recovery of 113% was demonstrated at 10 ppm [82]. Two headspace GC/MS methods have been developed for the analysis of formaldehyde in drug excipients using acidified ethanol [79] and O-(2,3,4,5,6-pentaflurobenzyl) hydroxylamine (PFPHA) [80,81]. The LOQs were 0.2 and 0.15 ppm for the ethanol method and the hydroxylamine method, respectively. The hydroxylamine method has been used to detect formaldehyde in solids, liquid and semi-liquid formulations. The derivatization reactions may be subject to potential interferences caused by the presence of large amounts of strong nucleophiles (e.g. amino groups in API molecules), causing low recovery in the spiked samples. Thus, a large excess of reagents are required to minimize the side reactions.

4.5.5. Epoxides

Epoxides are generally DNA mutagens, and some were classified as carcinogens [84]. Epoxides are unstable and can degrade through ring opening reactions. For example, the half-life of carcinogenic epichlorohydrin is 6.2 days in water at pH 7 at 20 °C. Therefore, derivatization is commonly used for their analysis. A direct analysis method using GC/FID experienced poor precision and accuracy issues due to the decomposition of analytes at high temperature in GC injection port [85,86]. Several methods that can detect epoxides at trace level using mass spectrometry have been reported. To facilitate their detection in water by GC/MS, small epoxides were sequentially reacted with 3,5-difluorobenzylamine (DFBA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [87]. The method could detect small epoxides at 5–10 ng/L in water following solid phase extraction. Another GC/MS method converted epichlorohydrin to an oxolane, which was than analyzed by direct injection [88]. The LOD of the method can be as low as 1 µg/L in dioxane solution. The aminolysis derivatization approach with DFBA was also explored for LC–MS/MS analysis [89]. The DFBA-epichlorohydrin derivative can be analyzed by LC/MS with an LOD of 30 ng/L. The epoxide functional group can also be converted to a disulfonic acid, which can be readily detected in negative ion mode MS. Epichlorohydrin was converted to 2-hydroxy-1,3-propane-disulfonic acid and its isomer 3-hydroxy-1,2-propane-disulfonic
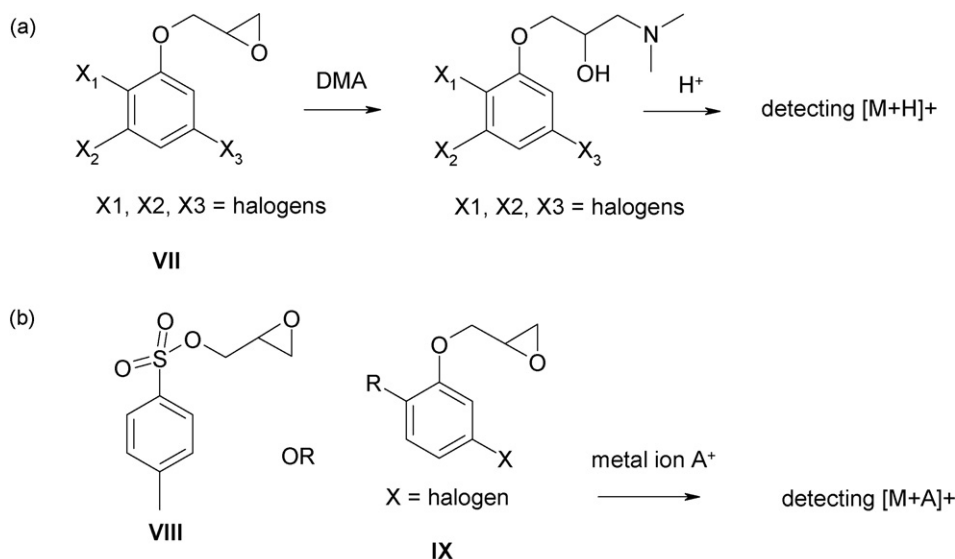
Scheme 6. (a) Chemical derivatization LC/MS and (b) coordination ion spray-LC/MS approaches for analysis of epoxides.

acid by reaction with sodium sulfite, which was analyzed by an ion chromatography (IC) APCI-MS method [90]. An LOD of 2 ng/mL was achieved. With some optimization, the above approaches could be applied to the analysis of GTIs in pharmaceuticals.

Bai et al. [91] utilized the aminolysis derivatization approach for determination of an epoxide, **VII,** which was converted to a dimethyl amine derivative (Scheme 6a). The high proton affinitive dimethyl amine derivative is an excellent candidate for ESI-MS analysis. An LOD of about 1 ng/mL was achieved. For a second epoxide **VIII**, however, multiple products could be generated following aminolysis due to its multiple electrophilic sites; thus it was not suited for the derivatization approach. Therefore, the analyte was analyzed directly despite potential stability issues. Though epoxide **VIII** (Scheme 6b) cannot be protonated or deprotonated easily, its multiple proximate oxygen atoms make it a good candidate for the coordination ion-spray MS method. Upon screening it was found that the analyte gave $[M+K]^+$ as the strongest species. As such, a method with an LOQ of 1.5 ppm (relative to a 5 mg/mL sample) was validated for an experimental drug [91]. The linearity was established from 1.5 to 20 ppm, and the precision (RSD) and recovery at 3 ppm were 1.3% and 87%, respectively. The coordination ion-spray MS approach was also applied to the analysis of another epoxide **IX** (Scheme 6b).

5. Method validation considerations

Methods for GTI analysis can be either limit tests or quantitative tests. A limit test in essence is a comparison of the concentration of an analyte to that of a known standard, and results are reported as not greater (pass) or greater than (fail) that standard. This is different from the quantitative analysis where the level or concentration of analyte is numerically reported. A disadvantage of limit tests is that they cannot support process development effectively, where usually actual levels are desired. Furthermore, for release testing, if the TTC lowered (which happens frequently due to changes in clinical dose or duration), previously generated results may not be adequate for releasing drug substance batches.

The validation of limit test methods specified in ICH guideline Q2 (R1) only requires specificity and detection limit [92]. Specificity can be demonstrated by comparison of a standard at analytical testing limit, a sample, and a blank. Blanks are used to ensure no interference. In our opinion, interferences in the blank less than 10% of the analytical testing limit are considered insignificant and acceptable. For trace analysis, the detection limit is not experimentally determined in the conventional sense (it is an assessment of the method sensitivity), but rather it is the analytical testing limit of a specific GTI acceptable in a sample. However, the acceptable method sensitivity must be demonstrated in a similar manner as for LOD where a minimum of signal/noise ratio of 3 is required for the standard at the concentration of analytical testing limit. In fact, in order to have a robust method that is less susceptible to matrix interferences, typically methods are developed to give an S/N higher than 3. The actual method LOD can then be estimated by extrapolation based on the S/N. In light of the reactive nature of many GTIs and their trace level concentration, it is empirical to demonstrate the method accuracy in terms of % recovery using multiple test samples. Also, it is important to demonstrate the recovery in each analysis since different batches of API may contain different impurities as interferences. For limit tests, reproducibility and solution stability are generally not required but can be demonstrated by replicate injections of a standard by bracketing the analysis sequence if desired,

In limit tests, typically a one-point calibration at the analytical testing limit is utilized. This works well for late phase projects in which the analytical testing limit is usually fixed. But for compounds in early phase development, it is advisable to include more than a single-point calibration, largely due to the fact that analytical testing limit is often a moving target. In such a case, we advocate a three-point calibration (i.e., semi-quantitative) approach: 'low', 'medium', and 'high' analytical testing limits. The 'high' analytical testing limit is selected as the highest limit that is the best estimate at the time based on initial prediction of the clinical dose and clinical trial duration. The 'medium' limit is intended to cover the scenario wherein the dose needs to be increased or the duration needs to be extended. Lastly, a 'low' point is included to cover the worst case scenario. The spike recoveries are demonstrated at all three levels. With the three calibration point approach, the experimental data generated the first time are mostly sufficient to cover a broader range of acceptance limits and thus minimize re-testing of the batches. Quantitative methods require more stringent validation. In addition to the method specificity, detection limit, and method accuracy (recovery) required for a limit method, additional validation requirements include precision, linearity and range, LOQ, and solution stability [92]. For quantitative methods, one time more extensive recovery test at the analytical testing limit using a minimum of five recovery samples to generate an average recovery and

relative standard deviation (RSD) is recommended. The S/N at LOQ should be greater than 10. The precision (repeatability) is established by a minimum of five injections of a standard at analytical testing limit and RSD should be less than 20% [93].

Three types of calibration methods are typically used, namely the external standard (ES), internal standard (IS), and standard addition (SA) methods. In drug metabolism and pharmacokinetics bioanalysis, structural analogues or isotopically labeled internal standards of drugs are typically used to compensate for analyte loss due to biological sample processing, ion suppression, and instrument variation. In pharmaceutical analysis of highly pure API, the use of sample extraction is not common; thus external calibration for quantitation is quite effective. This is also due to the fact that obtaining stable isotope-labeled GTIs is not always practical. In fact, we compared a GC/MS method using external calibration with the use of a stable isotope-labeled internal standard, and found that the latter did not offer superior results. However, Alzaga et al. [14] noticed that in the derivatization HS-GC/MS method for alkyl sulfonates, standard addition with internal standards was found to be the most suitable method of quantitation. Indeed, standard addition is a useful procedure in pharmaceutical analysis for determination of analytes at trace levels because it compensates for the matrix effects, especially in the case where the method recovery is problematic. In our experience, external calibration is a practical choice since the sample matrix in pharmaceutical analysis is not as complex and matrix effect is less severe; thus, rarely requires sample extraction. Also, the injection sequence is typically short leading to a decreased chance of instrument drift.

6. Testing and control strategies in process chemistry

6.1. Considerations in transferring methods to manufacturing labs: method simplification

Borman et al. [59] discussed the challenges in transferring a complex LC/MS/MS method on a triple quadrupole instrument to a production factory environment. The required linearity, repeatability and reproducibility of the method were demonstrated. The reproducibility (ruggedness) was carried out as a fully nested design, and the variation of each factor and total variation were determined. It was found that the analyst and sample preparation gave the largest sources of variation. It was concluded that it is possible to transfer this sensitive method (10 ppb) using the same approach that would be used for the transfer of any analytical method from R&D to a manufacturing environment. It was also recognized that the challenges of validating and transferring such extremely sensitive methods into a routine factory environment are quite significant. First of all, the analytical instrumentation that is common in R&D is not necessarily established in the manufacturing environment. Moreover, the instrumentation often requires specialized expert analytical scientists for operation. Thus significant investment in technology and staff in the factory becomes prerequisites.

Most recently, we have demonstrated a strategy for analytical control of GTIs in the pazopanib hydrochloride NDA (new drug application) [58]. It is our experiences that for later phase projects in preparation for commercial manufacturing, developing a testing strategy that uses simplified analytical instrumentation and higher detection limits that can be easily implemented in manufacturing sites is a fruitful exercises. This 'method simplification' must build upon sound scientific understanding of the purgeability of the GTIs of the commercial process, obtained through extensive spiking/purging studies. In the case of pazopanib HCl, five GTIs had to be controlled during clinical phases, and multiple mass spectrometry-based methods were used successfully to support the

process understanding and batch releases of the final API. Based on the process understanding, all GTIs can be controlled and tested upstream either in the starting materials or intermediates. As such, the mass spectrometry-based methods with ppm sensitivity were eliminated, and conventional HPLC/UV methods with % level sensitivity were implemented. The simplified LC/UV methods are much more robust and amenable to be transferred into manufacturing QC labs. This strategy could be generally applied to other products where GTIs can be controlled upstream of the synthetic process.

6.2. Testing and control strategy

From the process chemistry perspective, by first intent, every practical effort should be taken to prevent the formation of genotoxic or carcinogenic compounds during synthesis of drug substance or manufacturing of drug product, as recommended by US FDA guidance [9]. In cases where attempts to prevent the formation of such impurities of concern or to reduce their presence to an acceptable level are not feasible, further characterization of the genotoxic and carcinogenic potential of these impurities should be carried out. This can be accomplished by conducting genotoxicity assays to characterize the toxicological potential if not already known. Negative in vitro results qualify the impurity to become a regular ICH impurity. Positive in vitro testing results should be followed by an in vivo assay where a negative finding could override the positive in vitro results, as demonstrated by Eichenbaum et al. in the case of p-nitrophenol [94]. When genotoxicity is confirmed in vivo, the setting of analytical specification and testing according to the TTC set by the FDA guidance then becomes necessary.

Pierson et al. [42] proposed an empirical testing strategy based on the number of synthetic steps between the final API and the point at which the GTI is introduced. If the GTI is introduced in the final step, then API should be tested to show that the GTI is below TTC, meeting the specification limit. If the GTI is introduced in the penultimate step, the intermediate grade API (penultimate intermediate) should be tested as a control. Should it be already below the API specification limit, then no testing is required for the final API. In the case where the GTI enters the synthetic process 3-4 steps away from the API, testing the earliest intermediate and beyond to understand the rejection of the GTI in the following steps is recommended. The rejection cability/purgeability of the process should be established to design an appropriate control point at intermediate steps whenever possible (thus avoiding the need to test the final API). If the GTI is introduced more than 4 steps away from the API, a chemistry and process rationale to justify the rejection of the GTI could be a compelling argument especially when some GTIs are reactive in nature. In such a case, testing the API might be unnecessary. That being said, if an impurity rejection argument is not strong, testing intermediates or the API to show the effective impurity rejection must be demonstrated.

Snodin [72] argued that although it is unlikely to carryover to the final API of a reagent introduced 3–4 steps from API, some GTIs can be difficult to remove completely (for example, hydrazine). Conversely, a GTI introduced in the final step of synthesis can be removed completely depending on its reactivity or physical chemical properties and the process implemented. For instance, residual ethyl chloroformate used for N-acylation in the final stage is likely to be eliminated if aqueous workup and/or alcohol recrystallization is employed.

In the previously mentioned case of pazopanib HCl [58], however, a more systematic quality-by-design approach was taken in order to mitigate the risk of regulatory uncertainty. In this example, even the GTIs introduced more than 3 steps away from the final API were tested during the batch release to ensure no risk to patients. Extensive spiking/purging experiments were conducted to show that the much higher levels of the GTIs were rejectable

by the commercial process. Based on sound scientific data, the five GTIs are well-justified to be controlled in the starting materials or intermediate using HPLC/UV methods, and testing the final API by LC/MS methods was unnecessary. The pazopanib HCl NDA and its GTI control strategies have been approved by the US FDA recently.

7. Concluding remarks

Rapid development of extremely sensitive and robust analytical methodologies that can adequately monitor GTIs at very low levels is technically challenging. The biggest challenge lies in the need for high method sensitivity and selectivity, so that matrix interferences from APIs or excipients in the case of formulated drug products can be overcome. Although simplified separation and detection methodologies are desired in the manufacturing quality control laboratories, MS detection coupled with GC or HPLC plays a critical role in trace GTI analysis in various stages of pre-clinical and clinical drug development. This is primarily driven by the non-routine requirement of method sensitivity, specificity, and speed of method development needed for project support. Analyst expertise in operating such equipment is a non-issue for organizations where specialized teams are responsible for GTI analyses. However, this could be a concern for some organizations where individual project analysts are responsible for performing such type of analysis for their own projects. It is not surprising, in those situations, that analysts are sometimes intimidated by mass spectrometry detectors and thus tend to limit themselves to the more common UV or FID methods. Although this makes sense from the instrument simplicity perspective, the analyst may need to struggle for the sensitivity and selectivity, and the method development may take an unnecessarily longer time. It is our experience that the vast majority of the methods end up using MS detection because of the unparalleled specificity and sensitivity of this technique.

Understanding the molecular structure and properties of GTIs is the key to developing robust methods for their analysis. Being reactive in nature, many GTIs are unstable for direct analysis, so that low recovery and poor sensitivity presents a true challenge in trace analysis. In addition, some analytes do not have structural features that are amenable to common analytical detectors. Therefore, analytical strategies such as chemical derivatization and coordination ion spray-MS are invaluable tools for stabilizing analytes and/or enhancing their detectability. Furthermore, a 'matrix deactivation' sample treatment strategy has been developed to effectively quench interfering factors in the sample matrices, caused by either API itself or low level impurities and solvents. This makes it possible to perform direct analysis of some unstable/reactive GTIs. Matrix deactivation represents a novel general strategy for stabilizing reactive GTIs and thus improving analytical sensitivity and recoveries simultaneously. By coupling these and other practical strategies with hyphenated mass spectrometry instrumentation, GTI analysis will experience a major leap forward over the next few years. Nevertheless, GTI analysis still represents a non-routine task, where method troubleshooting skills are highly demanding; it is our experience that a specialized expert group in analytical R&D can deliver the most efficient and reliable GTI analysis support to projects.

The challenges of transferring highly sensitive methods developed in R&D labs using the state-of-the-art instrumentation requiring highly trained specialists into manufacturing quality control labs should not be underestimated. Therefore, when a method is to be implemented in manufacturing labs, an effective GTI control strategy should be properly designed based upon process understanding. Simple HPLC/UV or GC/FID methods should be implemented by first intent whenever possible, while more sophisticated LC/MS or LC/MS/MS methods should be the last resort.

Nevertheless, many manufacturing labs are now equipped with single quadrupole LC/MS and GC/MS instruments, and transfers of more methods that require these advanced instruments in the foreseeable future is expected.

Acknowledgements

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