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Approaches to Assessment, Testing Decisions, and Analytical Determination of Genotoxic Impurities in Drug Substances

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Abstract:

The assessment and control of genotoxic impurities (GTI) in pharmaceutical products has received considerable attention in recent years. Molecular functional groups that render starting materials and synthetic intermediates useful as reactive building blocks for small molecules may also be responsible for their genotoxicity. As a potential safety concern, it is important to understand the various issues related to GTIs and how they can be addressed for clinical and commercial phases of development. Justification that these impurities are controlled to safe levels must be obtained during development. This article will briefly discuss the multiple sources of anticipated impurities in a drug substance (also known as active pharmaceutical ingredient or API) synthetic route and how they are identified as GTIs in early chemical process development. A risk-based approach consistent with regulatory expectations is described for establishing control of GTIs. The approach includes process design considerations, impurity rejection information, and appropriate application of specifications. Analytical considerations for determination of GTIs at low levels are also discussed.

Introduction

The assessment and control of genotoxic impurities (GTIs) in chemical process development is a topic requiring input from a multidisciplinary team represented by chemical process, analytical, toxicology and regulatory functions. While avoidance of GTIs as reagents, starting materials, synthetic intermediates and byproducts in chemical processing is an important consideration, it is not always feasible, or desirable. Functional groups that render starting materials and synthetic intermediates useful as reactive building blocks may also be responsible for their genotoxicity. Avoidance of mesylate or tosylate salt isolations (to avoid potential mesylate and tosylate ester GTIs) may limit opportunities for optimal purification, physical properties, stability or bioavailability of an active pharmaceutical ingredient (API). The alternative to avoiding GTIs is to assess and manage potential risk through appropriate application of chemical process design and analytical testing.

The timing for GTI assessment and testing must also be considered. In early phases of development leading up to and including initial clinical trials, drug candidate attrition is significant. Adding to the challenge of addressing GTIs is the fact that the chemical synthesis may be rapidly changing as it progresses toward a commercial synthetic route.

Regulatory guidance related to the control of impurities to safe levels is available. The International Conference on Harmonization (ICH), Section Q3A, provides guidance on Impurities in New Drug Substances.[1] It states that lower reporting thresholds can be appropriate if the impurity is unusually toxic. Also, impurities with specific safety concerns such as genotoxicity, should be limited to levels far below those recommended for ordinary drug-related impurities. The European Medicines Agency (EMEA) issued guidelines for GTI limits and included the concept of threshold of toxicological concern (TTC) to define acceptable risk for new active substances.[2] This guideline acknowledges that it is impossible to define a zero risk for genotoxic carcinogens without a threshold, and the realization that complete elimination is often unachievable. A TTC of 1.5 μg/day is given as a level at which exposure will not pose a significant carcinogenic risk. The EMEA guidance also indicates that the TTC may be raised for short-term exposures or for known impurities which have greater potential for exposure from other sources. In 2005, the Pharmaceutical Research and Manufacturers Association (PhRMA) drafted guidance in the form of a GTI Task Force White Paper that extended the scope to all phases of clinical development.[3] Most importantly, the PhRMA paper introduces a staged TTC concept for limited exposure that balances duration of clinical trials, availability of analytical methods, maturity of synthetic route, and potential risk. In June 2008, the EMEA responded to a number of questions regarding GTI limits.[4] Significantly, the CHMP Committee agreed with the use of a staged TTC concept during clinical development, although at more conservative limits (factor of 2) compared to the PhRMA paper. Other references also discuss the issue of genotoxic impurities.[5−14]

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(1) International Conference on Harmonisation (ICH). *Guideline Q3A(R): Impurities in New Drug Substances*; February 2002.
(2) European Medicines Agency (EMEA), Committee for Medicinal Products for Human Use (CHMP). *Guideline on the limits of genotoxic impurities, CPMP/SWP/5199/02*; London, U.K., June 2006.
(3) Muller, L.; Mauthe, R. J.; Riley, C. M.; Andino, M. M.; De Antonis, D.; Beels, C.; DeGeorge, J.; De Knaep, A. G. M.; Ellison, D.; Fagerland, J. A.; Frank, R.; Fritschel, B.; Galloway, S.; Harpur, E.; Humfrey, C. D. N.; Jacks, A. S.; Jagota, N.; Mackinnon, J.; Mohan, G.; Ness, D. K.; O'Donovan, M. R.; Smith, M.; Vudathala, G.; Yotti, L. *Regul. Toxicol. Pharmacol.* **2006**, *44*, 198–211.
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(5) Jacobson-Kram, D.; McGovern, T. *Adv. Drug Delivery Rev.* **2007**, *59*, 38–42.
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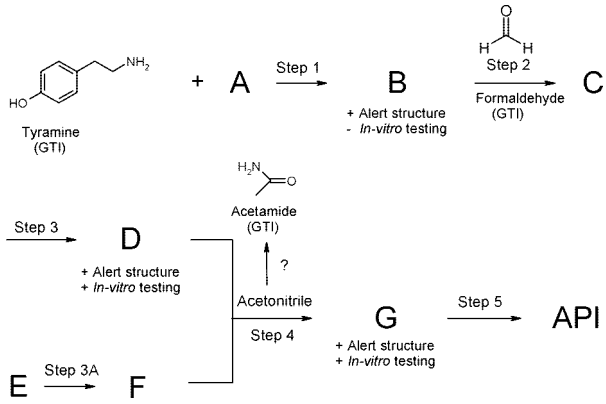

Figure 1. **Simulated synthetic route: potential sources of genotoxic impurities include starting materials, reagents, intermediates, side reactions, impurities.**

The following sections describe strategies that we have adopted for the identification of GTIs in drug substances and for establishing acceptable process and analytical controls to ensure patient safety. The strategies evolve as compounds move from early development to commercial supply but represent a risk-based approach consistent with providing a high-quality product. A related topic involves the assessment of API degradation products as potential GTIs. While a similar approach as described for reaction byproducts in this paper may be utilized, the key question is whether or not the impurity is likely to form, especially when the potential impurity has been identified through stress degradation studies. This is an ongoing issue that merits further examination.

Toxicology Assessment

Prior to the manufacture of clinical trial material, a toxicology assessment is needed to identify which compounds in a given synthetic route are genotoxic and to provide guidance on limits that are consistent with safety and regulatory expectations.[15] The synthetic route with chemical structures, as simulated in Figure 1, is submitted for toxicological assessment. In addition, the synthetic route is reviewed by a team of process chemists, analytical chemists, and toxicologists to identify likely reaction byproducts and the potential for carry-through to the API. Utilizing *in silico* evaluation and expert opinion, GTI alert structures are identified among the compounds for which no data are available. Examples of commercial software applications used for this purpose include MultiCASE[16] and DEREK.[17] With this assessment, compounds representing potential impurities can be classified according to risk potential. Muller et al. proposed the following five classes of impurities for this purpose: [3]

Class 1: Impurities known to be both genotoxic and carcinogenic

Class 2: Impurities known to be genotoxic

Class 3: Alerting structure, unrelated to API, and of unknown genotoxic potential

Class 4: Alerting structure related to the API

Class 5: No alerting structure

As shown in Figure 1, GTIs may be introduced as starting materials and reagents, or synthesized in the form of an intermediate or reaction byproduct. Following the identification of alert structures, different approaches may be considered. Additional considerations for risk assessment include whether the GTI is commonly found at considerable levels in foods. For example, tyramine, a bacterial mutagen, can be found in soy sauce at levels approaching 1 mg/mL. Patient populations and drug indication may also factor into the assessment.

Compounds that yield negative results in the alert assessment are placed in Class 5, and no additional action beyond normal impurity monitoring is needed. For any positive result (Classes 3 and 4), samples are submitted for *in vitro* mutagenicity testing, usually with the Ames or mini-Ames test.[18] If the mutagenicity test is negative, this over-rides the alert assessment, and the compound is placed in Class 5. Compounds giving a positive mutagenicity result are placed in Class 2, and a toxicology limit[19] is established on the basis of the intended clinical use. Not all compounds for which a limit is provided require analytical testing. Process and analytical chemists utilize the following guidance to determine the approach needed for a given route.

The process described is used for starting materials, intermediates, and reagents that are part of the synthetic route. For byproducts that are known to be genotoxic, a toxicology limit is obtained regarding acceptable levels in the API. For byproducts that have alerting structures, isolation or preparation of material may be required for mini-Ames testing. The need for toxicology assessment depends on where the GTI is introduced in the process and opportunities for removal (see below). In general, theoretical byproducts that are not anticipated to be formed are not assessed, nor is special testing conducted to look for them. This is consistent with EMEA guidance recommending that assessment of genotoxicity be limited to those impurities that might reasonably be expected on the basis of the chemical reactions and conditions involved.[2] Scientific judgment is required to balance the potential for impurity formation and carry-through with consideration of safety risk that would be caused by the presence of the impurity in the API.

GTI Testing Strategy

Toxicology assessment identifies genotoxic compounds in a route that need to be addressed. A "Decision Tree", as shown

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(16) *MultiCASE expert system software*; http://www.multicase.com; accessed October 2008.
(17) *DEREK expert knowledge based software*; http://lhasalimited.org; accessed October 2008.
(18) Flamand, N.; Meunier, J.-R.; Meunier, P.-A.; Agapakis-Causse, C. *Toxicol. Vitro* **2001**, *15*, 105–114.
(19) The toxicology limit for a GTI is the level in the API at which exposure poses a negligible cancer risk over background at the maximal therapeutic dose.

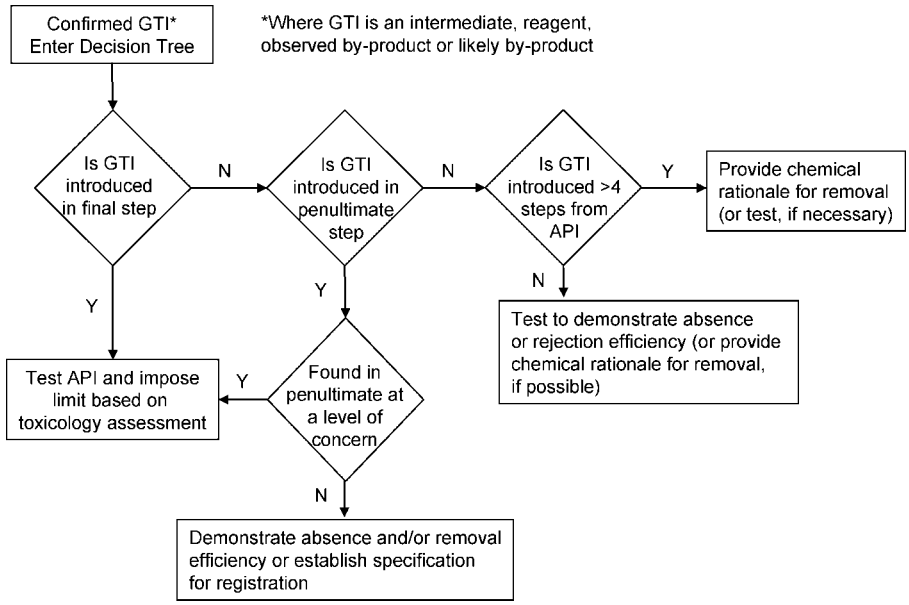

Figure 2. **Decision tree: control strategy for genotoxic impurities.**

in Figure 2, illustrates how genotoxic impurities are addressed as a function of where they enter the synthetic scheme. A chemistry-based rationale and/or data to support the GTI testing program or decisions not to perform testing are peer reviewed and documented.

The decision tree for testing incorporates a risk-based approach, depending on where the GTI is introduced in proximity to the final API. In other words, there is less risk of the GTI being present in the API when it is introduced early in the synthetic process. Introduction of a GTI less than three to four steps back from the API is often considered the point at which the risk needs to be more carefully assessed. The designation of four steps from the API as a point before which chemical rationale should suffice in most cases is based on the following consideration. If a GTI is introduced in step 1 of a four-step process and appears at 0.1% in the first intermediate followed by three additional steps with a 10-fold reduction at each step, the GTI would be in the API at 1 ppm. These conservative assumptions would lead to a negligible level of the GTI in the API, but a case-by-case assessment of reactivity and purification opportunities should be performed to determine whether testing is warranted. Later in development, the need for analytical data to support the chemical rationale may be reassessed for a marketing approval filing. A decision not to test when the GTI is introduced within three to four steps of the API could also be made, but the burden on the justification becomes greater.

The main approach for sample testing to demonstrate the absence of GTIs or process rejection efficiency is to test in the process intermediate step after the GTI has been introduced. Spiking studies can also be performed to demonstrate the capability of the process to reject an impurity. If an impurity is present at a level of concern, the next intermediate can be tested and so on to support decisions on the level of control needed for clinical trial materials and eventually, the commercial process control strategy. Although the designation of where the GTI is introduced refers to synthetic steps, the number of processing and/or purification steps present in the process that could remove the GTI should be considered when evaluating the potential for impurity presence in API. The decision tree and following discussion are utilized as guidelines. Good scientific judgment rather than arbitrary rules is needed throughout the process and may result in different decisions being taken, depending on the specific situation.

Decision Process for Testing. A detailed explanation of steps involved in the testing decision tree is provided below along with illustrative example situations.

GTI Is Introduced in the Final Step. A specification should be applied in most cases for the GTI in the API on the basis of the toxicology assessment. If data are generated to show that a GTI potentially introduced in the last step is not actually present or is efficiently rejected, it may be possible to omit a regulatory specification. For example, APIs are sometimes isolated as salts of methane sulfonic acid. When the final reaction step involves the use of alcohols, genotoxic esters such as methyl methanesulfonate (methyl mesylate) may form. In these cases it is necessary to demonstrate that even if such an ester is formed, it is rejected by the process. This is consistent with the European Pharmacopoeia production requirement for marketed sulfonic acid salts and may prevent the need for routine testing.

GTI Is Introduced in the Penultimate Step. If the GTI is shown to be below the toxicology limit for API in the penultimate intermediate, no testing is required for the API. The need for a specification limit at the penultimate intermediate should be considered on the basis of the stage of development. In a recent project, a substituted benzyl bromide was formed during the synthesis of the penultimate intermediate and was identified as a suspect genotoxic impurity. A toxicology-derived limit of 20 ppm was issued for this impurity in the API. Testing at the penultimate intermediate showed impurity levels of 2 ppm and precluded the need for a specification and testing at the API. If the GTI is present at a level of concern in the penultimate

intermediate, a specification is applied to the API to verify adequate removal in the last step.

If the GTI Is Introduced Three to Four Steps from the API. Data are needed to demonstrate rejection of the GTI to acceptable levels in laboratory trials at the earliest intermediate step possible. If the GTI is not present and/or removal is shown to be efficient, e.g. through spiking studies, no special testing/control is required for scale-up of intermediates used in production of API for clinical trials. If rejection is not adequate at an intermediate step, a specification limit for the API will be necessary. Chemical rationale for GTI removal may also be considered but must take into account the proximity of the GTI to the final API.

GTI Is Introduced Greater than Four Steps back from API. Consider developing a chemical rationale when the probability of the GTI carrying through to the API is negligible. This can be based on reactivity of the GTI, number of purification steps it will encounter, etc. If such a rationale can be provided, no testing for the impurity is required. As an example, consider a process in which one equivalent of the carcinogen formaldehyde is generated in step 5 of a 10-step synthetic route. Introduction of formaldehyde in the process was followed by four crystallizations and one reslurry purification step prior to formation of the drug substance. In this case, the rationale for not requiring analytical testing to control formaldehyde was based on the number of opportunities for removal. Formaldehyde was soluble in the aqueous extractions utilized in steps 5 and 6 and would likely be removed. Formaldehyde boils at −19 °C and would be removed during solvent exchange. Furthermore, step 6 utilized a reducing agent that would reduce any residual formaldehyde. Overall, the potential for formaldehyde in the API at any level of concern was very low.

If an impurity rejection rationale is not compelling, rejection to acceptable levels must be demonstrated in laboratory trials. Impurity rejection can be confirmed by testing intermediates prepared in the manufacture of material for clinical trials.

Ongoing Strategy Evaluation

Genotoxic impurities need to be addressed on an ongoing basis during development. Since the dose and duration associated with clinical trials will change, a revised toxicology assessment is needed. Therefore, as changes occur during development, a number of items must be addressed. If the route has changed, new intermediate compounds must be assessed. If the clinical trial dose and/or duration has changed, the toxicology limit should be reassessed. If the acceptable toxicology limit has changed, the capabilities of the process and analytical methods for control at the new level need to be assessed. If the acceptable level has changed, previously manufactured API must be assessed for suitability prior to use in subsequent clinical trials. Finally, generation of additional data on impurity rejection to support registration should be considered.

Considerations for Analytical Testing. Testing for GTIs in pharmaceutical intermediates and APIs must address a number of challenges common to trace analysis. Genotoxic analytes, by their nature, may be reactive or unstable, causing problems with reproducibility and recovery. While this complicates analysis, it could also provide justification for using a chemical rationale in place of testing. The most significant analytical challenges are introduced by the sample matrix itself, as selectivity and/or sensitivity can be compromised by poor sample solubility or chromatographic interferences from the main component, other impurities, or degradation products. Even if analytical methodology exists for a given genotoxic impurity, each new sample matrix, e.g. different intermediate or drug substance, presents new challenges requiring additional development. A number of approaches need to be considered for optimizing analytical sensitivity and selectivity. Examples of these approaches as they relate to instrumentation and sample preparation are listed in Table 1.

Table 1. **Approaches for optimizing sensitivity and selectivity for GTI Testing**

Instrument/Technique-Based
Choice of Technique
chromatographic
spectroscopic
Operating Conditions
resolution
peak shape
desired retention order
Detection Technique
element specific-detection
mass spectrometry
Sample Preparation-Based
Preconcentration of Analyte
Analyte Derivatization
improved detection
improved chromatography
Matrix Elimination
split injection
headspace GC
extraction

Analytical testing and approaches to GTI control must also be "phase appropriate", employing an appropriate level of rigor that supports the intended use of the data. In early phases of clinical development, information provided to guide process development is often generated on sophisticated HPLC/MS or GC/MS instrumentation. The main reason for this approach is to minimize issues caused by interferences that might hamper more common HPLC/UV or GC/FID methods. Methods are characterized in terms of specificity, linearity, spike recovery, solution stability, and sensitivity. It is necessary to provide quantitative results in order to track process development progress. As development proceeds, methods are optimized for specificity and sensitivity in order to generate a knowledge base from which to make decisions on necessary specification control points.

Testing intended for the control of impurities and release of material for forward processing in early phases of development is also typically performed on HPLC/MS or GC/MS instrumentation. A limit test approach is often used at the toxicology limit, or lower, as dictated by the ALARP principle[2] (as low as reasonably practical) considerations. In later clinical phases, testing may evolve toward methods and instrumentation that

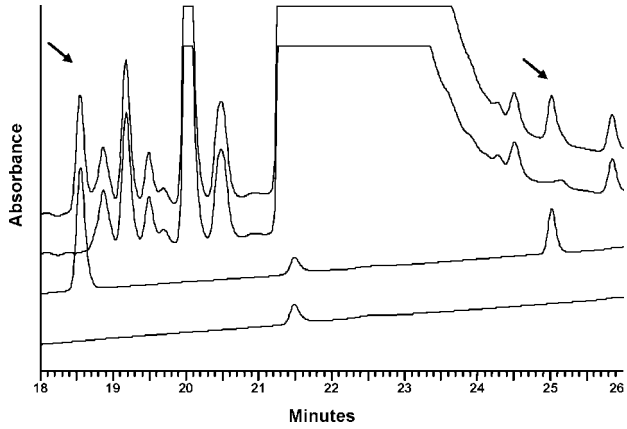

Figure 3. **Limit test approach (HPLC/UV). From bottom to top: blank injection, standard solution corresponding to 40 ppm limit concentration of each of two GTIs, sample solution, and sample spiked with standard solution to confirm retention time and recovery. Retention times for the two GTIs are approximately 18.5 and 25 min as indicated by arrows.**

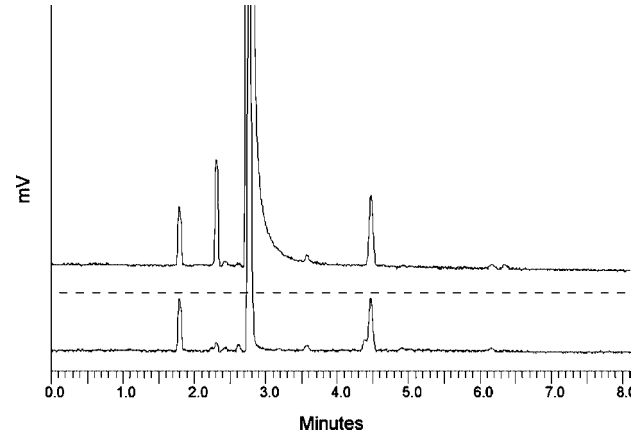

Figure 4. **Volatile GTI, headspace GC/FID approach. From bottom to top: sample solution at 20 mg/mL and standard solution at 1 μg methyl chloride/mL (corresponding to a 50 ppm level in the sample). Limit of quantitation for methyl chloride peak at 2.3 min is approximately 5 ppm.**

are compatible with quality control laboratory capabilities. Robust methods to monitor levels for commercial dose-based limits are needed for lifetime product support.

There are several examples in the literature of analytical methods for determination of trace-level GTIs. These are often for alkylating agents such as sulfonic acid esters.[20−24] All methods must address the analytical challenges of sensitivity and specificity. Examples of various approaches to deal with these issues are given below.

Limit Test Approach. In the limit test approach, a "one point calibration" at the toxicology limit is utilized. A pass/fail result is reported rather than a quantitative value for the impurity. Demonstration that the method is suitable for its intended use focuses on selectivity (chromatographic and/or spectroscopic) and adequate sensitivity and recovery of a spiked sample. A typical example is shown in Figure 3, where an HPLC/UV method was utilized for an application that did not require extremely high sensitivity. A blank injection ensures that there is no interference from the sample solvent. Injection of a standard solution prepared at the toxicology limit, accounting for sample dilution, is used to determine retention time for the analyte(s). By comparing an injection of the sample to that of the sample prepared in the standard solution (spike), the sample can be assigned a pass/fail result. Demonstration of reproducibility and solution stability is accomplished through replicate injections. One disadvantage of this nonquantitative approach is that process development progress cannot be tracked. In addition, if the toxicology limit is lowered, previously generated limit test results would not be adequate for determining acceptability.

The example in Figure 3 illustrates the complexity that may be observed for chromatographic impurity profiles at trace levels when nonspecific detection is utilized. If interferences are suspected, i.e., other components with the same retention time, a more selective method is needed. This may be accomplished by chromatographically resolving the interference or using a more selective detector, as described below.

Matrix Elimination. In cases where the GTI is a volatile species, headspace gas chromatography with flame ionization detection (FID) can be used to improve sensitivity for the analyte and minimize interference from the sample matrix. For the example illustrated in Figure 4, methyl chloride was a potential byproduct of a reaction employing methanol in the presence of hydrogen chloride. In this case, development data were generated to demonstrate that methyl chloride, if formed, was eliminated at an intermediate step in the process. This type of method could be applied to other sample matrices with adequate solubility.

Extraction of an analyte from the sample is another form of matrix elimination. This approach has been used to look for polar GTIs in synthetic intermediates prior to analysis by GC/MS. In this case, sensitivity was improved by dissolving the sample in methylene chloride and extracting the analyte from the nonpolar matrix with water.

Derivatization and Selective Detection. In some cases the analyte can be derivatized to form a species that is more stable, more amenable to chromatographic separation, and possibly easier to detect. A method for hydrazine involves the derivatization with benzaldehyde at room temperature to form 1,2-dibenzylidenehydrazine.[25] This approach was used to support a process in which hydrazine was used as a synthetic reagent. While this method led to good sensitivity for standard solutions of hydrazine, the sample matrix contained interfering peaks when HPLC/UV was used for quantitation as shown in Figure 5. By using HPLC/MS with electrospray ionization and selected ion monitoring at *m*/*z* 209.1, hydrazine could be measured in

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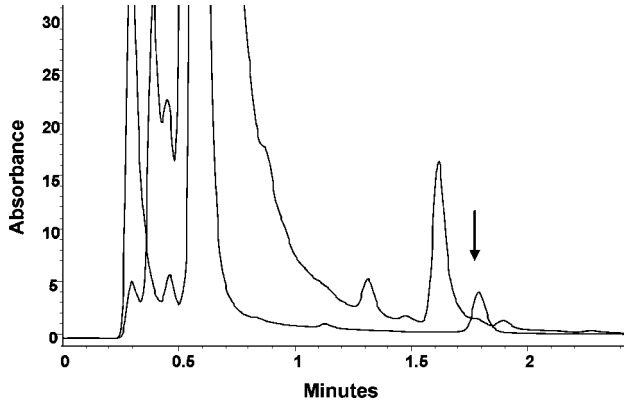


Figure 5. **Derivatization to improve sensitivity (HPLC/UV). Derivatization of hydrazine with benzaldehyde to increase detection sensitivity at 313 nm. Derivative peak at 1.8 min as indicated by arrow. Bottom to top: derivatized hydrazine standard and derivatized sample containing 0.3 ppm hydrazine.**

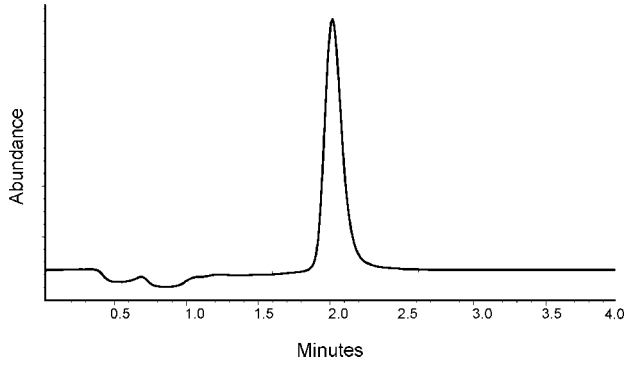


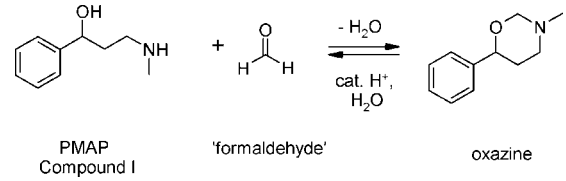
Figure 6. **Application of mass spectroscopic detection to improve sensitivity and selectivity (HPLC/MS). Same derivatized sample as in Figure 5. Electrospray ionization and selected ion monitoring at *m*/*z* 209.1.**

derivatized samples at well below 1 ppm due to enhanced signal/noise (see Figure 6).

Demonstration of Impurity Rejection. Showing the capability of a synthetic process to remove GTIs can be an effective means of demonstrating impurity control and can justify lack of routine testing for the impurity. This can be done by spiking the impurity into the process at levels well above those reasonably expected and showing that the impurity is effectively removed.

Formaldehyde may be used in the manufacture of phenylmethylamino propanol (PMAP), a starting material for atomoxetine hydrochloride and fluoxetine hydrochloride.[26,27] The PMAP starting material was used within four steps of the API, so the presence of formaldehyde in PMAP and the capability of the process to reject formaldehyde were investigated. A toxicology limit was obtained which stated that not more than 16 ppm formaldehyde should be present in the drug substance. The International Agency for Research on Cancer (IARC) classifies formaldehyde as a known human carcinogen,[28] associated mainly with inhalation exposure. For oral exposure, carcinogenicity may not be as much of a concern as evidenced by World Health Organization (WHO) drinking water standards[29] and communications from the FDA.[30] Formaldehyde also occurs naturally in foods and is a metabolic byproduct. While there may be some uncertainty regarding a limit, the following rejection studies were performed in response to a regulatory agency question.

Scheme 1. **Reversible reaction of formaldehyde with PMAP**



The investigation was complicated by the realization that formaldehyde could react with PMAP to form a cyclic phenyl oxazine as shown in Scheme 1. This reaction is reversible and could liberate formaldehyde in downstream chemistry. Method development included the goal of detecting formaldehyde present as free formaldehyde, paraformaldehyde, or the phenyl oxazine.

The basis for the analytical method was derivatization of formaldehyde with acetylacetone in the presence of excess ammonium acetate to form 3,5-deacetyl-1,4-dihydrolutidine.[31] The derivative was separated from the reagent and other sample components by reversed-phase HPLC and detected at 412 nm. The detection limit for formaldehyde was approximately 1 ppm in PMAP and in the process intermediate prepared from PMAP. Recovery of formaldehyde from each source (formaldehyde and the phenyl oxazine) spiked into PMAP and the first downstream intermediate at 10 ppm ranged from 91−113%.

Analysis of multiple batches of PMAP showed that no formaldehyde was detectable. Levels of formaldehyde (free and masked as the phenyl oxazine) as high as 5000 ppm were spiked into the synthetic step that utilizes PMAP as a starting material. Samples of the intermediate produced from the reactions contained less than 10 ppm formaldehyde, thereby establishing the impurity rejection capability of the process. The lack of detectable levels of formaldehyde in the PMAP starting material combined with the capability of the process to remove high levels provided justification for lack of formal specification controls on formaldehdye applied to PMAP.

Conclusions

Assessment and control of GTIs in chemical process development is challenging, owing to the evolving nature

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(31) Zurek, G.; Karst, U. *J. Chromatogr., A* **1999**, *864*, 191–197.

of the synthetic process, variable points of entry of GTIs in the process, and the need for analytical measurements with adequate selectivity and sensitivity. A systematic approach that is consistent with patient safety and regulatory guidelines has been presented to meet these challenges. Application of the approach results in process knowledge and controls that ensure the quality of drug substances throughout development. Although not described in detail here, degradation products in API and drug product also need to be assessed.

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