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Matrix deactivation: A general approach to improve stability of unstable and reactive pharmaceutical genotoxic impurities for trace analysis

Mingjiang Sun*, Lin Bai, Gerald J. Terfloth, David Q. Liu, Alireza S. Kord

Analytical Sciences, GlaxoSmithKline Pharmaceutical R&D, 709 Swedeland Road, King of Prussia, PA 19406, USA

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ABSTRACT

Trace analysis of unstable and reactive pharmaceutical genotoxic impurities (GTIs) is a challenging task in pharmaceutical analysis. Many method issues such as insufficient sensitivity, poor precision, and unusual (too high/low) spiking recovery are often directly related to analytes' instability. We report herein a matrix deactivation approach that chemically stabilizes these analytes for analytical method development. In contrast to the conventional chemical derivatization approach where the analytes are transformed into stable detectable species, the matrix deactivation approach chemically deactivates the hypothetical reactive species in the sample matrix. The matrix deactivation approach was developed on the premise that the instability of certain analytes at trace level is caused by reactions between the analytes and low level reactive species in the sample matrix. Thus, quenching the reactivity of the reactive species would be a key to stabilizing the unstable and reactive analytes. For example, electrophilic alkylators could be destabilized by nucleophiles or bases through either nucleophilic substitution or elimination reactions. One way to mask those reactive species is via protonation by adding acids to the diluent. Alternatively, one can use nucleophile scavengers to deplete reactive unknown species in the sample matrix completely, in analogy to the use of antioxidants and metal chelators to prevent oxidation in the analysis of compounds liable to oxidation. This paper reports the application of the matrix deactivation to the analyses of unstable and reactive pharmaceutical genotoxic impurities. Some of the methods have been used to support development of manufacturing processes for drug substances and a recent regulatory filing.

1. Introduction

Pharmaceutical genotoxic impurities (GTIs) could be chemical reagents, starting materials, reaction intermediates, or side reaction products of drug substances, introduced or generated during the manufacture process [1]. GTIs are potentially carcinogenic and may pose additional cancer risk to patients, and are thus garnering increased scrutiny from regulatory agencies and the pharmaceutical industry [2,3]. The acceptable additional risk to a patient posed by a certain GTI in a commercial drug should not be greater than 10^{-5} in a life time. For genotoxins without human toxicological data, this translates to a "threshold of toxicological concern" (TTC) of 1.5 μg daily intake per person. Most recently, regulatory agencies advocate the implementation of this TTC to the total of a group of GTIs that may have the same mode of action or similar structure [3,4]. As such, the TTC of the individual GTI would be much lower. If the daily dose of a drug is 1 g per day, then the level of a particular GTI should not be greater than 1.5 ppm and could be much lower if the grouping is applied. The low-ppm or even sub-ppm limits of the GTIs in pharmaceutical products pose great challenges to the pharmaceutical industry with regard to both the design of a robust manufacture process that controls the GTIs and the analytical methods that are adequate for accurate determination of trace level GTIs [5–8].

Reactive and unstable alkylators including aziridines, epoxides, alkyl halides, and alkyl sulfonates have structural features with genotoxic concerns. Even though it has been advocated to exclude certain reactive compounds based on genotoxic risk assessments [9–11], regulatory agencies often require them to be tested. Recently, an unstable alkylator, ethyl methanesulfonate, was found at surprisingly high levels in a commercial drug product, Viracept®, which led to a temporary product withdrawal from the market [12]. Therefore, it is prudent to monitor these reactive and unstable GTIs, at least initially, to ensure patient safety. Developing analytical methods for these unstable compounds is extremely challenging due to their instability, which can cause low sensitivity, poor precision and abnormal (too high/low) spiking recovery. Typically, these compounds have been detected after chemical derivatization, where derivatization reagents are added to the standards and samples immediately after preparation [13,14]. In

* Corresponding author at: GlaxoSmithKline UW2960, P.O. Box 1539, 709 Swedeland Road, King of Prussia, PA 19406, USA. Tel.: +1 610 270 6407; fax: +1 610 270 6727.
E-mail address: mingjiang.2.sun@gsk.com (M. Sun).

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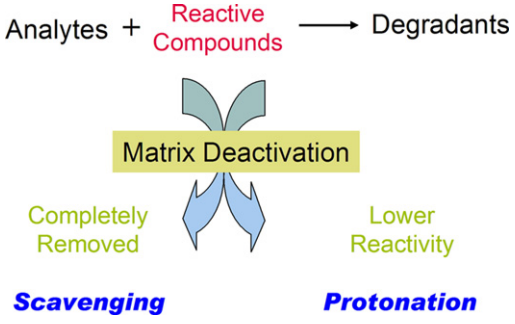
addition, these derivatization reactions are not always specific, and they may react with multiple structurally related compounds in the sample to give the same derivatization products [13,14]. From the manufacture process understanding perspective, it is imperative for a method to be selective and to be able to detect the compounds specifically. Therefore, analyte-specific methods for direct analysis (without derivatization) of these compounds are desired. Nonetheless, because of their instability, direct analysis methods of these analytes often suffer from issues such as low sensitivity, low stability, and abnormal recovery [15].

Solution stability issues are not limited to trace analysis, and have been studied for other chromatographic analysis [16]. Physical measures, including cooling and light-proofing sample solutions, have been commonly used to improve analytes' solution stability. However, the stability issues are magnified for trace analysis because some analytes become kinetically unstable at low concentration (ng/mL range). For instance, isopropyl tosylate is stable for 3 weeks at 0.5 mg/mL in acetonitrile at room temperature, while it is stable for only 30 min (less than 5% degradation) at 100 ng/mL in aqueous acetonitrile solution [15]. For chromatographic analysis, "inert" (non-reactive with analytes) diluents are usually used. However, when the concentrations of analytes in solution are at the ng/mL (ppb) level, the solvents may become apparently "reactive" because of the presence of trace reactive impurities in the "inert" solvents. When analytes are present at high concentration, the low level reactive impurities in solvent are depleted by reaction with analytes without detectable change of the analytes' concentrations. When the concentrations of analytes are very low, the effects of the reactions could become significant and cause sensitivity and precision issues for the analytical method. The reactive impurities are not necessarily from solvents, and may be impurities in drug substance or the drug substance itself. In the latter scenario, the spiking recovery of analytes will be significantly affected.

Based on the above hypothesis, suppressing the reactivity or removing trace reactive impurities in the sample matrix (solvents and/or drug substances) may improve solution stability of unstable/reactive GTIs. The "matrix deactivation" is achieved by chemical modification of the sample matrices. For instance, for alkylators, the reactive unknown impurities are mainly nucleophiles. Their reactivity can be attenuated by either protonation or scavenging approaches (Scheme 1). The current paper reports this novel strategy and its applications to improve analyte stability and subsequently solve sensitivity and spiking recovery issues encountered during method development for the trace analysis of reactive and unstable alkylators.



Scheme 1. Matrix deactivation approaches including protonation and scavenging can effectively improve the solution stability of alkylators. Reactive compounds in sample matrix could be trace nucleophiles, bases, oxygen, transition metals, and API.

2. Experimental

2.1. Reagents

GTIs 1–3 (Table 1), all drug substances and intermediates were prepared in-house with better than 98% assigned purity. Only partial structures are disclosed for some of the compounds discussed in this paper. Benzoic acid (99.5%), acetic acid (HPLC grade), formic acid (98%), acetyl chloride (98%), propionyl chloride (98%), oxalyl chloride (99%), dimethylamine (40% in water), bis(2-chloroethylamine) hydrochloride (GTI 4, 98%), and 1-chloroethyl chlorofomate (GTI 5, 98%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). N-methyl-2-pyrrolidinone (NMP, HPLC grade) and acetonitrile (HPLC grade) were purchased from Burdick & Jackson (Morristown, NJ, USA). Water used in the experiment was purified by an in-house Milli-Q system (Millipore, Billerica, MA, USA). Helium (99.996%) and nitrogen (99.999%) were obtained from PRAXAIR (Bethlehem, PA, USA).

2.2. GC/MS

GC/MS analyses were carried out using an Agilent GC/MS system (Palo Alto, CA, USA) consisting of a 6890A GC, a 5973N mass detector and a CTC Combi-Pal autosampler, in either headspace or liquid injection mode. The headspace autosampler conditions are: incubation oven temperature 100 °C; incubation time 10 min; headspace syringe temperature 110 °C; agitation speed 500 rpm; agitation 'on' time 18 s; agitation 'off' time 2 s; injection volume 500 μL; fill speed 100 μL/s; syringe pull-up delay 300 ms; injection speed 1 mL/s; pre-injection delay 2 s; post-injection delay 100 ms; syringe flush 5 min with nitrogen. The injector temperature was kept at 200 °C in split mode. The mass detector was operated in the electron impact mode (70 eV). The source temperature and quadruple temperature were set to 230 °C and 150 °C, respectively. The MSD transfer line temperature was set at 230 °C. Helium was used as the GC carrier gas. For liquid injection analysis, the injection needle and syringe were washed before and after each injection as programmed using wash vial A of acetonitrile/water (50/50) and B of acetonitrile: pre-injection, 6 times of B; post-injection, 6 times of A; then 6 times of B.

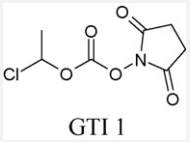
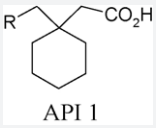
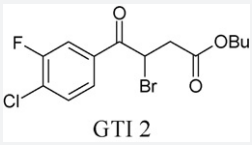
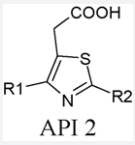
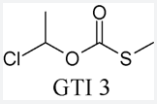
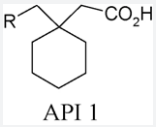
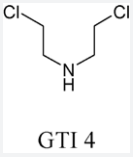
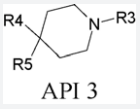
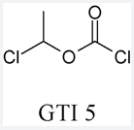
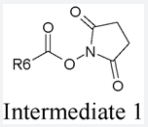
2.3. LC/MS

An Agilent 1100 LC/MSD system (Santa Clara, CA, USA) was operated in the electrospray ionization (ESI) positive ion mode with the capillary voltage set to 3 kV. Other conditions were individually optimized for each analyte. The fragmentor (cone) voltage was set to 40 V or 70 V. The drying gas flow was set to 10–13 L/min with a temperature of 350 °C. Nebulizer pressure was set in the range from 25 psi to 45 psi.

2.4. Derivatization procedures for GTI 4

An aqueous solution of dimethylamine (40% v/v) was used as the derivatization reagent for GTI 4. The derivatization reaction was carried out by adding 25 μL of derivatization reagent into 2 mL HPLC vials that contain standards or samples in 1 mL diluent (1% acetic acid in acetonitrile). All vials were capped tightly, vortexed, and then heated at 60 °C for 60 min. Upon completion of the reaction, the vials containing the corresponding quaternary ammonium derivatization products were subject to LC/MS analysis directly. A typical injection volume of 5 μL was used and can be increased to improve the method sensitivity if desired.

Table 1
Structures of GTIs 1–5 and the analytical methods.

Analyte	API	Diluent	Chromatographic conditions
 GTI 1	 API 1	5% acetic acid in acetonitrile	Column: LUNA C18(2) 100 mm × 2.0 mm, 3 μm. Column temperature: 45 °C. MA[a]: aqueous formic acid (0.1%) and potassium acetate (0.1 mM). MB[b]: acetonitrile. Gradient: 20% to 36% B in 8 min at 0.4 mL/min, followed by washing. Injection volume: 4 (L. LC–MS: SIM ion: m/z 260 $[M+K]^+$.
 GTI 2	 API 2	0.1% formic acid in acetonitril.	Column: LUNA C18(2) 100 mm × 2.0 mm, 3 (m. Column temperature: 40 °C. MA: aqueous formic acid (0.1%) and potassium acetate (0.2 mM). MB: acetonitrile. Eluent: 60% B isocratic for 8 min at 0.5 mL/min. Injection volume: 10 (L. LC-MS: SIM ion: m/z 403 $[M+K]^+$.
 GTI 3	 API 1	5% benzoic acid in N-methyl pyrrolidone (NMP)	Column: DB-624, 25 m × 0.2 mm, 1.12 (m film. Oven temperature: 105 °C isothermal for 12 min, then 30 °C /min to 230 °C and hold for 3 min. Helium Flow: constant flow 1.0 mL/min. Splitless. Injection volume: 1 mL. HS-GC/MS: SIM ion: m/z 75.
 GTI 4	 API 3	1% acetic acid in acetonitrile	Column: Atlantis, HILIC Silica 50 mm × 2.1 mm, 3 (m. Column temperature: 35 °C. MA: aqueous formic acid (0.1%) and ammonium formate (50 mM). MB: acetonitrile. Eluent: 70% B isocratic for 7 min at 0.3 mL/min. Injection volume: 5 (L. LC–MS: SIM ion of derivative: m/z 115 of 1,1-dimethylpiperazinium derivative.
 GTI 5	 Intermediate 1	1% oxalyl chloride in methylene chloride	Column: RTX-200, 30 m × 0.25 mm, 0.25 (m film. Oven temperature: 55 °C isothermal for 5 min, then 60 °C /min to 250 °C and hold for 3 min. Helium Flow: constant pressure 30 psi. Split: 1:1. Injection volume: 1 (L. GC/MS: SIM ion: m/z 107.

[a] Mobile phase A.
[b] Mobile phase B.

3. Results and discussion

3.1. Matrix deactivation, a simple sample preparation strategy that may be used to improve analyte solution stability

Analyte solution stability is one of the critical attributes of chromatographic methods [17–19]. The solution stability issues are even more pronounced for trace analysis of reactive analytes, because they become kinetically unstable at low concentration. Sometimes, the instability can be inferred from unexpected low sensitivity at lower concentration, poor injection precision, and/or abnormal spiking recovery. Improving analyte solution stability is a sensible approach to rectify the method sensitivity and spiking recovery issues. Some of the degradation pathways of these analytes require other reagents or catalysts. At trace level, the reactive impurities in the sample matrix may serve the purpose. Matrix deactivation is a general approach to chemically attenuate the reactivity of the reactive species. The selection of the matrix deactivation reagents should be based on the reactivity of the analytes and their potential degradation mechanisms. The matrix deactivation reagents can be simply doped into the diluents, and no other procedure is involved. Therefore, matrix deactivation is a simple sample preparation strategy that can be used to improve analyte solution stability. This can be achieved via either protonation by acids or scavenging as described in detail below. The examples in the paper demonstrate the application of the approach in the analysis of alkylators, a major group of GTIs.

3.2. Protonation

We hypothesized that the instability of some alkylators in solution at trace level may be a result of their nucleophilic substitution and/or elimination reactions. The reactions were promoted by trace nucleophiles and/or bases in the sample solution or matrix. Thus, regulating the reactivity of the reactive impurities may improve solution stability of alkylators. The proton is an effective reagent for masking basic functionalities in terms of atom efficiency and operational simplicity. Woodward exemplified the use of this kind of methodology to protect a phosphorous ylide in penicillin synthesis over 30 years ago [20]. It can be adopted as an effective strategy to deactivate the sample matrix. The protonation approach generally does not affect the composition of the components in the sample. Nonetheless, the acids must be carefully selected to avoid potential interferences that may be introduced by the acid itself. Generally organic acids are preferred because of their mild acidity. The physical properties of the acids must be able to match with the intended analytical technique. For example, clean volatile low molecular weight acids are good choices for HPLC related methods, while non-volatile acids are preferred for headspace GC related methods. The usefulness of the protonation strategy is demonstrated in the analytical method development for GTIs 1–4 (Table 1).

The utility of the protonation approach is not limited to matrix deactivation for trace analysis of GTIs. It is equally applicable for stabilizing target analytes through protonation of analytes themselves in ordinary RP-HPLC analysis. Certain analytes are not stable

under neutral or basic conditions, while stable in acidic conditions. Beaver stabilized aflatoxins in acetonitrile/water solution with the addition of acetic acid [16].

3.2.1. Protonation – application in LC–MS analysis

GTI 1 (Table 1), which must be controlled to a level below 0.4 ppm in the final API 1, is an excellent candidate for coordination ion spray-mass spectrometry [21] analysis due to its five approximate oxygen atoms. Experiments demonstrate that GTI 1 can form adducts with K^+, Na^+, and NH_4^+. When the mobile phase is spiked with all three cations at 0.1 mM, the K^+ adduct is dominant. Forty-fold higher NH_4^+ concentration was required to achieve sensitivity similar to that obtained with 0.1 mM K^+. The K^+ adduct appeared more stable than the NH_4^+ adduct. Thus, K^+ was selected as the mobile phase modifier, and GTI 1 was monitored at m/z 260 $[M+K]^+$.

GTI 1, a hydroxysuccinamide ester, is inherently unstable in aqueous solution, though the compound is stable enough to survive HPLC conditions. Therefore, pure acetonitrile was selected as the diluent initially to prevent possible hydrolysis. The initial injection of the standard at 40 ng/mL (0.4 ppm relative to API at 100 mg/mL) gave a reasonable size peak which decreased rather quickly in subsequent injections. The peak could not be detected in the repeat injection after 1 h. The results suggest that GTI 1 is not stable in acetonitrile. However, when the standard is spiked into the API sample solution, it produces better response than standard solution. Further experiments demonstrated that GTI 1 spiked in API 1 solution is stable for at least 64 h at 40, 20, and 10 ng/mL. It appears that the API molecule, an acid with a p*K*a of 4.7, stabilizes GTI 1 in solution. We suspected that the API actually protonated the unknown nucleophiles (interferences) that could cause degradation of GTI 1, and consequently attenuates their nucleophilicity. To simulate the observation and confirm the hypothesis, 5% glacial acetic acid (p*K*a, 4.72) was used to modify the diluent. As predicted, GTI 1 is stable in the new diluent for at least 64 h. With the modified diluent, the method (Table 1) was validated with a sensitivity of an acceptable S/N at 0.4 ppm (Table 2). Typical chromatograms of GTI 1 at different levels in the diluent and in the spiked samples are shown in Fig. 1.

Similarly, the protonation strategy was applied to improve the stability of GTI 2 (Table 1). GTI 2, which was monitored by SIM of the $[M+K]^+$ ion at m/z 403 in ESI/MS analysis, was not stable in solution. GTI 2 may readily undergo either nucleophilic substitution in the presence of nucleophiles or nucleophilic elimination in the presence of bases. Protonation of these hypothetical bases or nucleophiles by acids will suppress such degradation reactions. Thus, 0.1% formic acid was added into the diluent, which was able to stabilize the standard solution of GTI 2 for at least 31 h. With the modified diluent, the method was validated with acceptable sensitivity at 4 ppm and the validation data are listed in Table 2. Fig. 2 demonstrates the typical responses of GTI 2 in the standard solution and spike recovery sample at a concentration of 4 ppm.

3.2.2. Protonation – application in headspace GC–MS analysis

GTI 3 (Table 1), a synthetic intermediate of API 1, was controlled at 0.4 ppm. The compound has a boiling point of about 120 °C (760 mmHg). Thus, it is potentially suitable for headspace GC/MS analysis. The headspace incubation temperature was set to 105 °C, and N-methylpyrrolidone (NMP, bp. 204.3 °C) was selected as the diluent because of its higher boiling point. The effects of diluent volume on sensitivity of GTI 3 were studied by fixing the amount of GTI 3 in each vial at 20 ng/mL, equivalent to 0.2 ppm relative to 100 mg of API (Fig. 3). As expected, reducing the volume of diluent in each injection vial improved the S/N of GTI 3 peak. When the volume of NMP diluent was 20 μL, the S/N was better than 25, which is adequate for the desired method. The API

Table 2
Summary of validation results of the five methods upon using the matrix deactivation approach.

	Testing Limit (ppm)		Issues without matrix deactivation	Diluent modifier	Key method attributes after matrix deactivation				
					Linearity	Inj. Prec. (%RSD)	Sol. Stab. (hr)[a]	S/N at limit	Spiking recovery (%)
GTI 1	0.4		Unstable in acetonitrile as diluent	10% acetic acid	R^2 = 1.0000 (From 0.1 to 400 ppm, relative to 100 mg/ml API)	5.0	60	23	90
GTI 2	4.0		Unstable with acetonitrile as diluent	0.1% formic acid	R^2 = 0.9990 (From 4 to 32 ppm, relative to 4 mg/ml API)	2.0	36	44	107
GTI 3	0.4		Higher recovery with NMP as diluent	5% benzoic acid	R^2 = 0.9999 (From 0.05 to 0.4 ppm, relative to 100 mg/vial API)	5.4	–	102	122
GTI 4	1.3		Unstable with acetonitrile as diluent	1% acetic acid	R^2 = 0.9997 (From 1 to 40 ppm, relative to 5 mg/ml API)	1.6	–	43	97
GTI 5	2.0		Low sensitivity and recovery with methylene chloride as diluent	1% oxalyl chloride	R^2 = 0.9996 (From 0.2 to 25 ppm, relative to 10 mg/ml API)	9.7	48	44	103

[a] Less than 10% degradation.

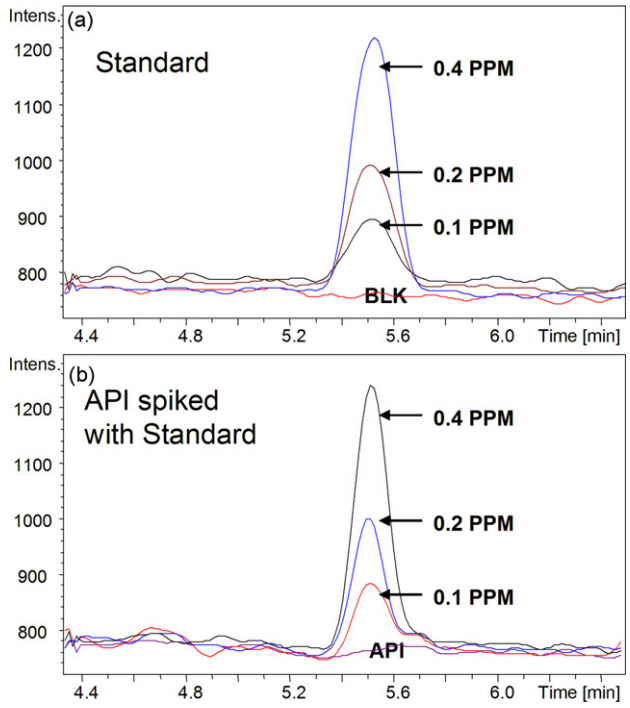


Fig. 1. Chromatograms of GTI 1 in (a) standard solution and (b) spiked recovery samples at 0.1 ppm, 0.2 ppm, and 0.4 ppm (10 ng/mL, 20 ng/mL, and 40 ng/mL relative to 100 mg/mL API).

solution was clear at the incubation temperature and believed to be homogenous. The optimized method conditions are given in Table 1.

As illustrated in Fig. 3, it was noticed that the response of GTI 3 spiked into API 1 (100 mg/vial) affords a much stronger signal than the standard solution prepared in NMP. Also, the signal enhancement effects seem to increase with decreased diluent volume. When a volume of 20 μL of diluent was used, the signal response in the spiked sample almost doubled compared to that of the standard solution at the same concentration. Since no GTI 3 was detected in the API, it was thought that the response of GTI 3 was enhanced in the presence of API. Considering the acidity of API 1 (p*K*a, 4.7), experiments were designed to determine whether the solution acidity played a role. Therefore, neutral dioctyl phthalate (DP) and benzoic acid (BA, p*K*a, 4.21) were tested against the API as the sample matrices respectively, and their effects on the response of GTI 3 were studied. A volume of 20 μL of the standard solution at 2 μg/mL was added to each vial, to which varied amounts of the three matrices were added as solid respectively. The data point was valid if only a clear solution could be obtained during incubation. The relative response of GTI 3 compared to standard

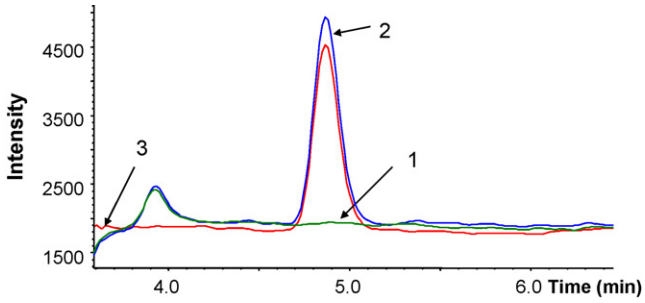


Fig. 2. Chromatograms of GTI 2 at 4 ppm (16 ng/mL relative to 4 mg/mL API 2) obtained after modifying the diluent with formic acid: (1) blank; (2) standard; and (3) spike recovery sample.

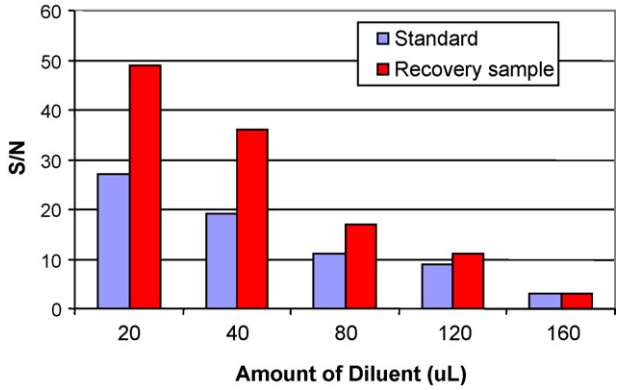


Fig. 3. Effects of the amount of diluent (NMP) on the signal response of GTI 3 at 20 ng/vial with or without 100 mg/vial API 1. S/N is calculated by peak to peak.

solution at the same concentration in these samples was plotted against the concentration of the matrices as shown in Fig. 4. The neutral dioctyl phthalate seems to have little effect on the response of GTI 3, while the two acidic compounds (BA and API 1) exhibit signal enhancing effects on GTI 3. The relative response of GTI 3 is maximized at 250% when about 0.1 mmole of the two compounds is added. The results clearly indicate that the acidity of API 1 is responsible for the signal enhancing effects during the analysis of GTI 3. Consequently, in order to improve the method sensitivity and to normalize the spike recovery of GTI 3, the diluent was modified to include 5% benzoic acid in NMP. Upon optimization, the method was validated. The final method is summarized in Table 1. Overlaid chromatograms of GTI 3 standard at 0.4 ppm, API 1, and API 1 spiked with GTI 3 are shown in Fig. 5. No interfering peak is observed in API 1. The S/N of the standard at 0.4 ppm was improved to 102 from 52, and the recovery of GTI 3 was normalized to 122% from 188% as a result of the addition of 5% benzoic acid (Table 2). The acids may improve the analyte response by stabilizing the analyte via protonation of unknown impurities in the diluent (matrix deactivation). However, we cannot rule out the possibility that the volatility of analyte is improved during headspace incubation by the ionic strength change due to the presence of acid.

3.2.3. Protonation – application in LC–MS analysis coupled with derivatization

GTI 4 (Table 1), bis(2-chloroethylamine), which is unstable in aqueous solution [22], must be controlled at low-ppm level in API 3. To improve its stability, GTI 4, was converted to 1,1-

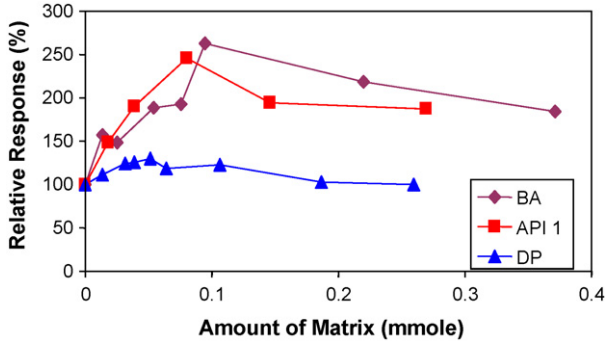


Fig. 4. The relative response of GTI 3 in three different matrices benzoic acid (BA), dioctyl phthalate, and API 1. The relative response is defined as the ratio of the response of the analyte in sample matrix to that of the standard in diluent at the same concentration. The response is affected by acidic compounds but not the neutral compound.

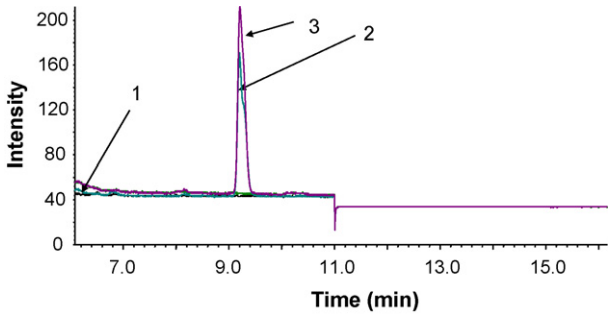


Fig. 5. Chromatograms of GTI 3 at 0.4 ppm (40 ng/vial relative to 100 mg/vial API 1) obtained after modifying the diluent with benzoic acid: (1) blank; (2) standard; and (3) spike recovery sample. The mass spectrometry detection was turned off after 11 min.

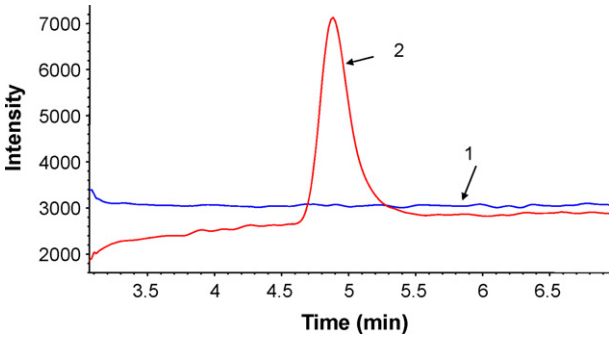


Fig. 6. Chromatograms of GTI 4 at 1 ppm (5 ng/mL relative to 5 mg/mL API 3) obtained after fortifying the diluent with acetic acid: (1) blank and (2) standard solution.

dimethylpiperazinium by reacting with dimethylamine. Initially, the standard solutions of GTI 4 were prepared in acetonitrile and the linearity from 5 ng/mL to 200 ng/mL was evaluated. Unfortunately the responses of GTI 4 were non-linear in the range. It was noticed that the response factor at low concentration was much lower than that at higher concentration. Considering the high reactivity of the compound, the observation seemed to suggest an instability issue at low concentration. Chua et al. studied the stability of GTI 4 in aqueous solution and proposed the underlying degradation pathways [22]. In the first step, the intramolecular nucleophilic substitution reaction of the amino group forms an aziridine intermediate after eliminating a chloride ion. In the second step, other nucleophiles open the aziridine ring, producing more stable derivatives. Both of the degradation steps can be slowed down by attenuating the nucleophilicity of the nitrogen of GTI 4 or any unknown nucleophiles through protonation. Therefore, two acetonitrile diluents modified with either 1% or 10% acetic acid were examined. The derivatization reaction becomes much slower at room temperature, thus a reaction temperature of 50 °C was used to accelerate the reaction. For acidified diluents, after 2 h heating, better signal responses are observed at 10 ng/mL compared to that without the acid. Eventually, the diluent containing 1% acetic acid was selected, and the reaction conditions were optimized to 1 h at 60 °C in order to drive the reaction to completion. The method was validated based on the modified derivatization conditions. Fig. 6 demonstrates the chromatogram of a standard at 5 ng/mL (equivalent to 1 ppm relative to a 5 mg/mL API sample).

3.3. Scavenging

Scavenging is another approach to improve the stability of the analytes without modifying the target analytes, by adding a scavenger to the diluent during sample preparation to deplete the hypothetical reactive species in the sample matrix that may react with the target analytes. This approach is analogous to the classical usage of antioxidants and metal chelators to prevent oxidation during analysis of many compounds [23–25], also to the masking technique, which is used to prevent interference in titration analysis [26]. The scavenger should be carefully chosen so that it does not react with target analytes itself. However, it must be at least as reactive as the target analytes toward the hypothetic reactive species present in the sample solution. In essence, scavengers act as competing reagents of the target analytes to react with (or deplete) the reactive species. The hypothesis is that when the scavenger is added in large excess, the target analyte will be spared from the degradation reactions. It is worth noting that the added scavengers may react with any of the reactive species in the sample, causing a change of composition. Thus, the scavengers must be experimentally selected to avoid generating any interference that co-elutes with the target analytes.

3.3.1. Scavenging – application in GC–MS analysis

GTI 5 (Table 1), 1-chloroethylchlorofomate, is one of the precursors of API 1 synthesis. We demonstrated the absence of GTI 5 in the intermediate 1, the penultimate product. Because of the instability of the analytes, a chemical derivatization method appeared to be a logical choice. However, a synthetic impurity, GTI 1, may also be present in the intermediate 1. The two impurities are structurally related, and may both react with nucleophilic derivatization reagents producing the same derivative. Thus, derivatization would not be able to distinguish the two GTIs. Furthermore, intermediate 1 may also react with any nucleophilic derivatization reagent, consequently reducing the effectiveness of the reagents. Most of all, it was of the interest to be able to determine the two impurities separately. After complete assessment of the available technologies and the molecule properties, direct injection GC/MS method seemed to be the best way forward to detect the compound at 2 ppm level in the intermediate.

Initially, methylene chloride was used as the diluent and electron impact MS was attempted. The m/z 107 was selected over m/z 63 in the SIM analysis due to better S/N. A standard of GTI 5 at 50 ppm (relative to 10 mg/mL API) affords good signal to noise ratio, however, the recovery in intermediate 1 is only 25%. Based on this preliminary data, it seemed very difficult to develop a method with 2 ppm detection sensitivity with acceptable recovery. We hypothesized that the low recovery was due to the high reactivity of GTI 5 that may react with the any unknown nucleophiles in intermediate 1. Therefore, attenuation of the reactivity of the unknown reactive impurities was the approach chosen to improve the recovery. Considering the high reactivity of GTI 5, protonation was not considered an option. Consequently, the addition of a nucleophile scavenger was developed, where a structural analog of the analyte is added to compete with GTI 5 to react with the unknown impurities. As such, the nucleophile scavenger deactivates the matrix interferences by depleting the nucleophilic species. In this context, acetyl chloride, propionyl chloride, and oxalyl chloride were screened. It was found that only oxalyl chloride does not interfere with the GTI 5 peak chromatographically (Fig. 7). The reagent also effectively removes moisture and other reactive species in the diluent. Thus GTI 5 is stabilized for improved detection. With the use of oxalyl chloride in the diluent, GTI 5 can be determined at 2 ppm relative to a 10 mg/mL sample with a S/N of 44 (Table 2).

Since direction injection GC/MS tends to contaminate the injection syringe and needle quickly due to the introduction of large amounts of API matrix, a two-stage needle wash program using

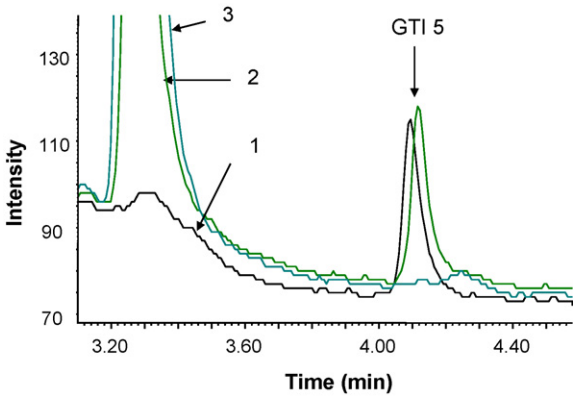


Fig. 7. Chromatograms of GTI 5 at 2 ppm (20 ng/mL relative to 10 mg/mL intermediate 1) obtained after adding oxalyl chloride to the diluent: (1) standard solution; (2) sample; and (3) spiked sample.

water and acetonitrile was implemented to improve the robustness of the method. The GC/MS system was able to run for 24 h with no need for off-line cleaning.

4. Conclusion

A matrix deactivation methodology to improve the stability of unstable and reactive GTIs for their trace analysis has been developed. This approach appears to be generally applicable to techniques like direct GC–MS and LC–MS analysis, or even coupled with chemical derivatization. Matrix deactivation agents could be acids or scavengers depending on the analyte structure and molecular property. With the use of protonation of the matrix or addition of nucleophile scavenger, reactive species in the sample matrix can be quenched effectively. As such, method sensitivity and spike recovery can be improved drastically for the trace analysis of pharmaceutical genotoxic impurities, especially alkylators. Implementation of the matrix deactivation approach makes it possible to analyze some unstable alkylators directly. Several methods developed based on the strategy have been successfully applied to support both late phase and early phase drug development programs.

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References

[1] D.Q. Liu, T.K. Chen, M.A. McGuire, A.S. Kord, Analytical control of genotoxic impurities in the pazopanib hydrochloride manufacturing process, J. Pharm. Biomed. Anal. 50 (2009) 144–150.
[2] EMEA–CHMP, Guideline on the Limit of Genotoxic Impurities CPMP/SWP/5199/02, EMEA/CHMP/QMP/251344/2006, 28 June 2006.
[3] US FDA, Guidance for industry: genotoxic and carcinogenic impurities in drug substances and products: recommended approaches (draft), December 2008, http://www.fda.gov/cder/guidance.
[4] EMEA Q&A, EMEA/CHMP/SWP/431994/2007, http://www.emea.europa.eu/pdfs/human/swp/43199407en.pdf.
[5] E.J. Delaney, An impact analysis of the application of the threshold of toxicological concern concept to pharmaceuticals, Regul. Toxicol. Pharmacol. 49 (2007) 107–124.
[6] A.G. Dragan, O.L. Cinteza, H. Weisssieker, P. Schaff, Impurities in pharmaceutical products: an overview of regulatory guidelines, emerging detection methods, residual solvents and genotoxic aspects, Fresenius Environ. Bull. 18 (2009) 3–11.
[7] D.P. Elder, D.J. Snodin, Drug substances presented as sulfonic acid salts: overview of utility, safety and regulation, J. Pharm. Pharmacol. 61 (2009) 269–278.
[8] H.J. Federsel, Chemical process research and development in the 21st century: challenges, strategies, and solutions from a pharmaceutical industry perspective, Acc. Chem. Res. 42 (2009) 671–680.
[9] L. Mueller, R.J. Mauthe, C.M. Riley, M.M. Andino, D. De Antonis, C. Beels, J. De George, A.G.M. De Knaep, D. Ellison, J.A. Fagerland, R. Frank, B. Fritschel, S. Galloway, E. Harpur, C.D.N. Humfrey, A.S. Jacks, N. Jagota, J. Mackinnon, G. Mohan, D.K. Ness, M.R. O'Donovan, M.D. Smith, G. Vudathala, L. Yotti, A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity, Regul. Toxicol. Pharmacol. 44 (2006) 198–211.
[10] D.J. Snodin, Residues of genotoxic alkyl mesylates in mesylate salt drug substances: real or imaginary problems? Regul. Toxicol. Pharmacol. 45 (2006) 79–90.
[11] D.J. Snodin, Genotoxic impurities in drug substances, Org. Process Res. Develop. 13 (2009) 409.
[12] C. Gerber, H.G. Toelle, What happened: the chemistry side of the incident with EMS contamination in viracept tablets, Toxicol. Lett. 190 (2009) 248–253.
[13] R. Alzaga, R.W. Ryan, K. Taylor-Worth, A.M. Lipczynski, R. Szucs, P. Sandra, A generic approach for the determination of residues of alkylating agents in active pharmaceutical ingredients by in situ derivatization-headspace gas chromatography–mass spectrometry, J. Pharm. Biomed. Anal. 45 (2007) 472–479.
[14] J. An, M. Sun, L. Bai, T. Chen, D.Q. Liu, A. Kord, A practical derivatization LC/MS approach for determination of trace level alkyl sulfonates and dialkyl sulfates genotoxic impurities in drug substances, J. Pharm. Biomed. Anal. 48 (2008) 1006–1010.
[15] G.E. Taylor, M. Gosling, A. Pearce, Low level determination of p-toluenesulfonate and benzenesulfonate esters in drug substance by high performance liquid chromatography/mass spectrometry, J. Chromatogr. A 1119 (2006) 231–237.
[16] R.W. Beaver, Degradation of aflatoxins in common HPLC solvents, J. High Resolut. Chromatogr. 13 (1990) 833–835.
[17] I.K. Cigic, M. Strlic, A. Schreiber, M. Kocjancic, B. Pihlar, Ochratoxin A in wine: its determination and photostability, Anal. Lett. 39 (2006) 1475–1488.
[18] L. Di, E.H. Kerns, H. Chen, S.L. Petusky, Development and application of an automated solution stability assay for drug discovery, J. Biomol. Screening 11 (2006) 40–47.
[19] M. El Aatmani, S. Poujol, C. Astre, F. Malosse, F. Pinguet, Stability of dacarbazine in amber glass vials and polyvinyl chloride bags, Am. J. Health Syst. Pharm. 59 (2002) 1351–1356.
[20] R.B. Woodward, Recent advances in the chemistry of β-lactam antibiotics, Spec. Publ. Chem. Soc. 28 (1977) 167–180.
[21] E. Bayer, P. Gfrorer, C. Rentel, Coordination-ionspray-MS (CIS-MS), a universal detection and characterization method for direct coupling with separation techniques, Angew. Chem. Int. Ed. 38 (1999) 992–995.
[22] H.C. Chua, H.S. Lee, M.T. Sng, Screening of nitrogen mustards and their degradation products in water and decontamination solution by liquid chromatography–mass spectrometry, J. Chromatogr. A 1102 (2006) 214–223.
[23] J. Yin, J.W. Chu, M.S. Ricci, D.N. Brems, D.I.C. Wang, B.L. Trout, Effects of antioxidants on the hydrogen peroxide-mediated oxidation of methionine residues in granulocyte colony-stimulating factor and human parathyroid hormone fragment 13–34, Pharm. Res. 21 (2004) 2377–2383.
[24] M. Sun, S.C. Finnemann, M. Febbraio, L. Shan, S.P. Annangudi, E.A. Podrez, G. Hoppe, R. Darrow, D.T. Organisciak, R.G. Salomon, R.L. Silverstein, S.L. Hazen, Light-induced oxidation of photoreceptor outer segment phospholipids generates ligands for CD36-mediated phagocytosis by retinal pigment epithelium: a potential mechanism for modulating outer segment phagocytosis under oxidant stress conditions, J. Biol. Chem. 281 (2006) 4222–4230.
[25] M. Sun, L. Bai, D.Q. Liu, A generic approach for the determination of trace hydrazine in drug substances using in situ derivatization-headspace GC–MS, J. Pharm. Biomed. Anal. 49 (2009) 529–533.
[26] A. Ringbom, Masking and promoting of reactions in quantitative analysis, Pure Appl. Chem. 7 (1963) 473–488.