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Enhancing the detection sensitivity of trace analysis of pharmaceutical genotoxic impurities by chemical derivatization and coordination ion spray-mass spectrometry

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ABSTRACT

Many pharmaceutical genotoxic impurities are neutral molecules. Trace level analysis of these neutral analytes is hampered by their poor ionization efficiency in mass spectrometry (MS). Two analytical approaches including chemical derivatization and coordination ion spray-MS were developed to enhance neutral analyte detection sensitivity. The chemical derivatization approach converts analytes into highly ionizable or permanently charged derivatives, which become readily detectable by MS. The coordination ion spray-MS method, on the other hand, improves ionization by forming neutral-ion adducts with metal ions such as Na^+, K^+, or NH_4^+ which are introduced into the electrospray ionization source. Both approaches have been proven to be able to enhance the detection sensitivity of neutral pharmaceuticals dramatically. This article demonstrates the successful applications of the two approaches in the analysis of four pharmaceutical genotoxic impurities identified in a single drug development program, of which two are non-volatile alkyl chlorides and the other two are epoxides.

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1. Introduction

During the manufacturing of active pharmaceutical ingredients (API), some starting materials, intermediates, reagents, and reaction by-products inevitably end up in the final products as impurities. Organic impurities that have the potential to induce genetic mutations, chromosomal breaks, and/or chromosomal rearrangements are considered as potential genotoxic impurities (GTIs), which may cause cancer in humans. Recently, the European Medicine Agency (EMEA) and US FDA have published separate guidelines with respect to the limit of genotoxic impurities in new commercial drugs. Both agencies have set threshold of toxicological concern (TTC) of 1.5 μg/day for genotoxic impurities in new commercial drugs [1,2]. As such, pharmaceutical R&D has devoted a great deal of effort to the development of manufacturing processes that can effectively control GTI levels [3]. While the usage and generation of potential genotoxic chemicals in any new manufacturing processes should be minimized whenever possible, complete removal of GTIs from drug substances or drug products is often impractical. This necessitates that GTIs are tested in the final products in order to ensure product quality and patient safety. As such, robust and sensitive analytical methods are required to support drug development. Moreover, from the process understanding point of view, analytical methods that are capable of measuring trace GTIs are needed to monitor the fate of GTIs during chemical synthesis. This in turn allows for the establishment of effective GTI control strategies. In response to the FDA's recent quality-by-design (QbD) initiative, the pharmaceutical industry has strived to gain process understanding and implement process controls. This requires analytical chemists to deliver sensitive and robust methods to support process development, and thereby an effective control strategy can be crafted and implemented to ensure API quality in terms of GTI levels [3].

In the past few years, much progress has been made in developing highly sensitive methods to analyze various GTIs [4,5]. Although conventional HPLC/UV methods are sometimes viable options [6], hyphenated mass spectrometry has gained popularity in the field [7–11] due to superior sensitivity and specificity. Because of the advantage of mass selective detection, MS methods are generally less prone to interferences compared to the non-specific detectors such as UV. Therefore, efforts needed for method development for GTI analysis could be greatly reduced. With regard to the ionization techniques in LC/MS, atmospheric pressure ionization MS, which includes electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), is by far the most popular tool because

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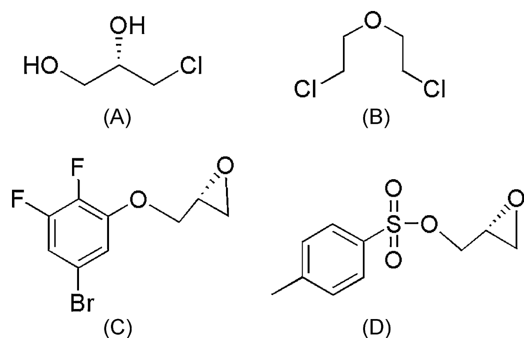

Fig. 1. Four potential GTIs identified in a single drug development program.

of simplicity and reliability. However, many GTIs are neutral molecules that lack proton affinity and thus are not amenable to the ESI. This necessitates the development of strategies to improve the analyte detectability. A solution can often be achieved in one of the following two ways: modify the analyte structure (chemical derivatization) or influence the ionization processes (coordination ion spray-MS). The chemical derivatization approach introduces an ionizable or even a permanently charged functional moiety to the neutral analyte to enhance the detectability. The coordination ion spray-mass spectrometry (CIS-MS) [12], on the other hand, converts neutral analytes into charged neutral-ion complexes in solution or gas phase. Both approaches have been previously shown to be able to dramatically improve the detection sensitivity of neutral analytes [13]. Since many genotoxins are reactive and unstable, chemical derivatization has been proven to be a useful strategy that simultaneously improves the analyte stability and MS detection [7].

In this article, we report the application of the above two approaches in the analysis of four alkylating agents (Fig. 1) of a single drug development program, including two alkyl halides (**A** and **B**) and two epoxides (**C** and **D**). Both GTIs **A**, and **D** are precursors of GTI **C** in two different synthetic routes; whereas GTI **B** is a suspected by-product in the manufacture process. Chemical derivatization approaches were developed for the analysis of **A**, **B**, and **C**, respectively, while coordination ion spray-mass spectrometry was applied to the analysis of **D**.

2. Experimental

2.1. Chemicals

(S)-(+)-3-Chloro-1,2-propanediol (**A**), bis(2-chloroethyl) ether (**B**), (2R)-(−)-glycidyl tosylate (**D**), dimethylamine (40% in water), ammonium formate, formic acid, potassium acetate, sodium acetate, lithium acetate, and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Purified materials of (R)-2-(5-bromo-2,3-difluoro-phenoxymethyl)-oxirane (**C**), and the API samples were manufactured by GlaxoSmithKline. Water was produced freshly by a Millipore water purification system (Billerica, MA, USA).

2.2. LC–MS methods

An Agilent 1100 LC-MSD system (Wilmington, DE, USA) with an electrospray ionization (ESI) source operated in the positive mode was used for all LC/MS analyses. The MS parameters were: nitrogen drying gas was set at 12 L/min with a temperature of 350 °C; nebulizer gas pressure was 30 psi; capillary voltage was in the range from 3.5 to 4.0 kV; fragmentor was set at 70; dwell time was set to 880 ms. The details of the HPLC conditions for individual analyte are described in Table 1.

2.3. Sample and standard preparation

Typical sample solutions are prepared at 5 mg/mL, either in 80% acetonitrile aqueous solution for GTIs **A** and **B**; or in 50% acetonitrile aqueous solution for GTIs **C** and **D**. A typical standard solution of 2 ppm is prepared by dissolving neat material and then diluted several times in the corresponding diluent to a concentration of 10 ng/mL. The spike solution was prepared typically by dissolving 5 mg sample in 1 mL of the 10 ng/mL standard solution to give a spike level of 2 ppm (this is in addition to the GTI level already present in the sample). The diluent solvent was chosen primarily based on the solubility of the sample and the compatibility with the chromatography and derivatization conditions. The concentrations of the standard solutions and samples were optimized to achieve a desired signal-to-noise ratio (S/N) and good peak shape, while maintaining the ratio of standard concentration to sample concentration at about 2 ppm (which is the targeted QL, see Section 3).

2.4. Derivatization reactions for GTIs *A*, *B* and *C*

The chemical derivatization was carried out by adding 20 μL of dimethylamine (DMA) solution (40%, w/w in water) into separate 2-mL vials containing 1 mL of the calibration standard, sample, or spike recovery solutions, respectively. A derivatization control blank was also prepared in the same fashion except that the analytes were omitted. All the vials were then placed in the thermostatic heating block on a Reacti-Therm III heating module (Pierce, Rockford, IL USA). The vials containing GTIs **A** and **B** were heated at 75 °C for 2 h and 1 h, respectively. For GTI **C**, the vials were heated at

Table 1
LC/MS methods.

Analyte	A	B	C	D
Column	Waters Atlantis HILIC silica 50 mm × 2.1 mm ID, 3 μm	Waters Atlantis HILIC silica 50 mm × 2.1 mm ID, 3 μm	Phenomenex, Luna C18(2) 50 mm × 2.0 mm ID, 3 μm	Phenomenex, Luna C18(2) 50 mm × 2.0 mm ID, 3 μm
Column temperature (°C)	35	35	40	40
Mobile phase	A: 0.1% formic acid plus 50 mM ammonium formate in water B: acetonitrile	A: 0.1% formic acid plus 50 mM ammonium formate in water B: acetonitrile	A: 0.1% formic acid in water B: acetonitrile	A: 0.1% formic acid plus 0.2 mM potassium acetate in water B: acetonitrile
Gradient	Flow rate: 0.3 mL/min %B: 83% for 8 min	Flow rate: 0.3 mL/min %B: 87% for 8 min	Flow rate: 0.5 mL/min %B: 10% for 4 min followed by column wash and equilibration	Flow rate: 0.5 mL/min %B: 30% for 10 min followed by column wash and equilibration
Injection vol. (μL)	5	5	5	2
SIM ion	m/z 120 $[M+H]^+$	m/z 116 $[M]^+$	m/z 310, or 312 $[M+H]^+$	m/z 267 $[M+K]^+$

55 °C for 20 min. The reaction rate of GTI **C** with DMA appeared to be the fastest, and **A** the slowest. After derivatization, all the vials were subjected to LC/MS analysis directly using the conditions described in Table 1. During the initial development of derivatization conditions, full scan MS experiments were useful for confirming the anticipated derivatization product.

3. Results and discussion

3.1. HPLC separation of analytes from the API peak

The proposed daily dose was 0.5 g for the clinical study, and the planned study duration was longer than 12 months. This led to an upper limit of 3 ppm of any GTI allowable in the API (1.5 μg/day/0.5 g/day = 3 ppm) according to the EMEA and FDA guidelines [1,2]. Therefore, a method with sensitivity of 3 ppm or lower was required. To achieve this, the LC method must provide adequate resolution between the main component (API) and the analyte(s) to avoid the ion suppression and interferences. The ion suppression from the extremely high level of API negatively impacted the MS signal response of the analytes. It is worth noting that sometimes minor impurities may also interfere however this was not the case here. Thus, the main focus of HPLC separation in this case was to resolve the target analyte from the API peak using an appropriate column and mobile phase combinations. The epoxide **D** eluted before the API on a reverse phase C18 column, and an isocratic elution using 30% acetonitrile yielded sufficient resolution between the API and the analyte peaks. The epoxide **C**, upon DMA derivatization, became much more polar than **D**. In order for the derivatization product to be retained on the column, the gradient elution started at 10% acetonitrile. After elution of the analyte peak, the gradient was then ramped to 90% acetonitrile to wash off the API from the column. In both cases, the flow rates were increased to 1 mL/min during column wash and equilibration. In cases of chlorides **A** and **B**, a HILIC phase column was employed to retain the polar derivative products (see Section 3.2). The relative hydrophobic API had little retention on the HILIC column and eluted near the void. In order to prevent contamination of the mass spectrometer by the large amount of API, the effluent was diverted to waste except for the retention regions of the analytes. As shown in Table 2, all the methods demonstrated S/N of greater than 10 at the concentration of 2 ppm or lower (considering the typical sample concentrations were 5 mg/mL).

3.2. Chemical derivatization approach for analyzing GTIs *A*–*C*

A chemical derivatization approach was developed for analyzing GTIs **A**, **B**, and **C**. The three analytes were derivatized by DMA, respectively and the derivatization products were then analyzed by LC/ESI/MS. The objective of the three derivatization reactions is to generate a strong basic center by introducing an amine functional group as shown in Fig. 2. A secondary amine instead of a more reactive primary amine was used to minimize the generation of reaction by-products, considering tertiary amines are not reactive toward the analytes. A large excess of DMA was used in all cases to overcome potential competing reactions. All three reactions afforded specific products through nucleophilic substitutions. The first derivatization reaction (Fig. 2a) between a less reactive alkyl chloride **A** and DMA produced a tertiary amine product. GTI **B** is more reactive toward the nucleophile due to the neighboring group effect of the oxygen atom. The initial substitution reaction product of **B** further reacts through intra-molecular nucleophilic substitution to produce a unique quaternary ammonium ion within a six member-ring (Fig. 2b). Having an epoxide functional group, GTI **C** is very reactive toward DMA. The substitution favors the less hindered position, and thus producing a dominant single reaction product (Fig. 2c). As anticipated, the positional isomer with the alkyl amine substitution on the hindered position was not observed.

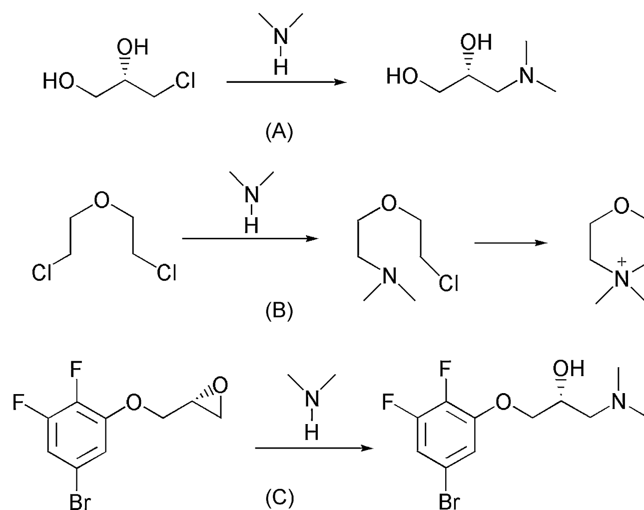

Fig. 2. Derivatization reactions of GTIs **A**–**C** with dimethylamine (DMA).

All three derivatization products are good candidates for ESI-MS due to the high proton affinity or the permanent charge. The derivatives of **A** and **B** were very polar and not well retained on C18 columns. However, this highly polar property makes them good candidates to be retained on a HILIC phase column (Table 1). The advantage of HILIC phase separation is that the API elutes near the column void due to high hydrophobicity; thus the API interference is easily separated from the very polar analytes. In addition, high organic content in the mobile phase allows for a larger sample injection volume, and helps desolvation in the ESI source to give a better signal. Figs. 3–5 show typical LC/MS chromatograms of the standards of GTIs **A**–**C**. The method validation data are summarized in Table 2.

It is critically important to demonstrate good spike recovery in trace analysis, which is also the accuracy measurement for the overall method. A low recovery may indicate the existence of competing side-reactions during derivatization and/or ion suppression during LC–MS analysis. It was noticed that the spike recovery for GTI **C** at 3 ppm level was relatively low (65%). This might be caused by the side reaction between the nucleophilic API (containing basic nitrogens) and the analyte. This was evidenced by the spike recovery from another less nucleophilic API, which yielded 75%. To account for the low recovery, the method S/N was corrected by multiplying

Table 2
Method validation summary.

Analyte	A	B	C	D
Sensitivity	S/N = 30 at 1 ng/mL	S/N = 36 at 1 ng/mL	S/N = 50 at 5 ng/mL	S/N = 260 at 7.5 ng/mL
Linearity	R^2 = 0.989 ranging from 1 to 80 ng/mL	R^2 = 0.9997 ranging from 1 to 100 ng/mL	R^2 = 1.0000 ranging from 7.5 to 100 ng/mL	R^2 = 0.9997 ranging from 7.5 to 100 ng/mL
Spike recovery (accuracy)	65% at 3 ppm	89% at 2 ppm	103% at 2 ppm	87% at 3 ppm
Injection precision %RSD (*N* = 6)	3.0% at 1.0 ng/mL;	0.4% at 10 ng/mL	1.5% at 10 ng/mL	1.3% at 15 ng/mL

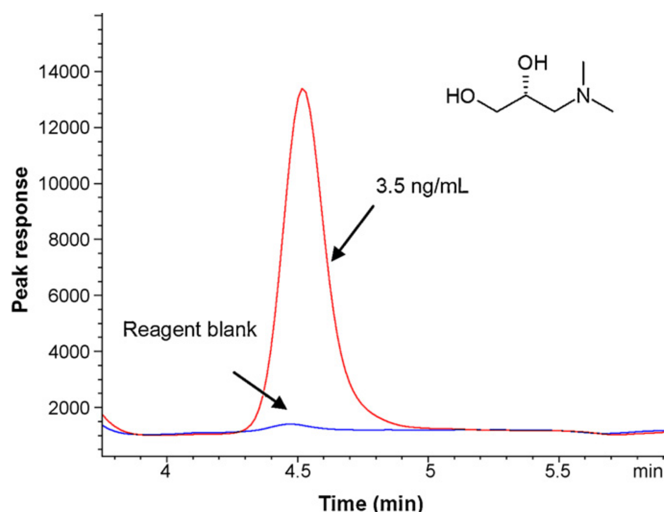

Fig. 3. Analyzing trace GTI **A**: overlay chromatograms of the standard at 3.5 ng/mL and the reagent blank.

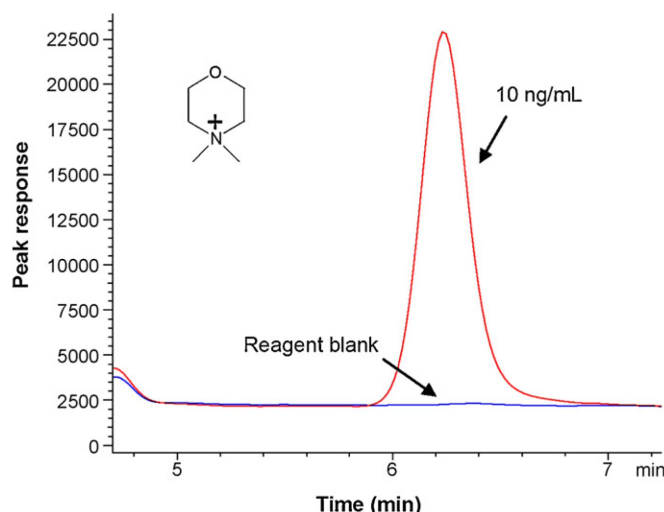

Fig. 4. Analyzing trace GTI **B**: overlay chromatograms of the standard at 10 ng/mL and the reagent blank.

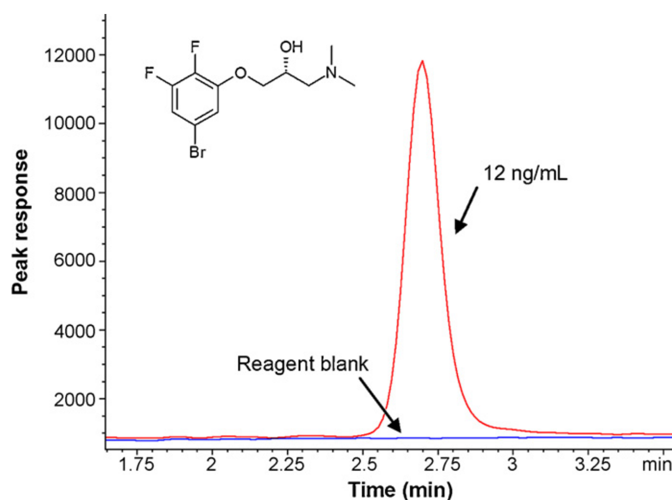

Fig. 5. Analyzing trace GTI **C**: overlay chromatograms of the standard at 12 ng/mL and the reagent blank.

0.65 to give the adjusted method sensitivity. In addition, the analytical results were also corrected by dividing the results with the spike recovery value from the concurrent experiment. Since the MS signal of the derivative peak was quite intense, the overall method sensitivity was not compromised by the slight signal loss due to the relatively low recovery.

Many publications reported the analysis of GTIs **A** [14–20] and **B** [21,22]. The former was found in several food products and classified as a carcinogen by the European Scientific Committee on Food [23]. They were typically analyzed by either GC or GC/MS methods. Most of the methods involved sample enrichment steps such as extraction, or some types of injection techniques such as SPME (solid phase microextraction). In addition, the hydroxyl groups were derivatized to improve volatility for GC analysis. However, these methods were not well suited for the current project for the following reasons. Sample extraction and other sample preconcentration methods to enrich analytes are primarily based on the difference in hydrophobicity or ionic interactions between the analytes and sample matrix. The insignificant difference between GTI **A** and the API is insufficient for the extraction methods to work effectively. Secondly, the API and/or minor impurities in the sample could potentially react with the reactive GTI analytes during extraction. We attempted direct analysis of **A** using GC/MS and a poor spike recovery (less than 20%) was experienced. This could be attributed to the reaction of the GTI analyte in the GC port (possibly with nucleophiles), resulting loss of the analyte during analysis as suggested in the literature [24]. Thirdly, methods using direct injection GC are often not rugged because the non-volatile API dissolved in organic solvent tends to precipitate out in the GC injection port and cause blockage or interference. In contrast to the previously reported methods, the derivatization approach presented in this paper converts the analytes into ionizable compounds, which facilitates the HILIC separation and the ESI detection simultaneously. Volatile DMA was used in excess over the API and other reactive species so it could convert GTIs into the desired derivatives quickly with minimum loss of the analytes due to side-reactions. The resulting derivatization products are very stable for LC/MS analysis.

3.3. Coordination ion spray-MS strategy for analyzing the epoxide ***D***

Even though GTI **D** is structurally similar to **C**, it could not be analyzed using the same derivatization approach. GTI **D** contains two electrophilic sites, thus, the derivatization reaction may produce multiple reaction products. To develop an approach that selectively produces one over the other, it may take significant effort to optimize the derivatization reaction conditions. Nevertheless, the GTI **D** structure that consists of several proximal oxygen atoms suggests the propensity to form an alkali metal ion adduct [25]. Indeed, it was found that **D** formed adduct ions with positively charged metal ions easily, presumably via charge–dipole interactions. Although metal ions such as sodium may be present as residual impurities in solvents, leachable from glassware, and contamination in the ESI source, the amount is insufficient for quantitative analysis. Considering the very low concentration, metal ions can be doped into mobile phases instead of post-column addition to provide a stable source of these cations. In order to screen for a suitable coordinating metal ion, a mobile phase containing 0.1 mM each of potassium, sodium, lithium, and ammonium acetates, plus 0.1% (v/v) of formic acid were employed and tested by monitoring the $[M+K]^+$, $[M+Na]^+$, $[M+Li]^+$, and $[M+NH_4]^+$ adduct ions, respectively. The signal response from each metal ion adduct was compared as shown in Fig. 6. Potassium appeared to give the highest response so was selected. The concentration of each cation can be optimized to produce the best signal response should it be desired. As a result, a stable and intense peak of potassium adduct was obtained

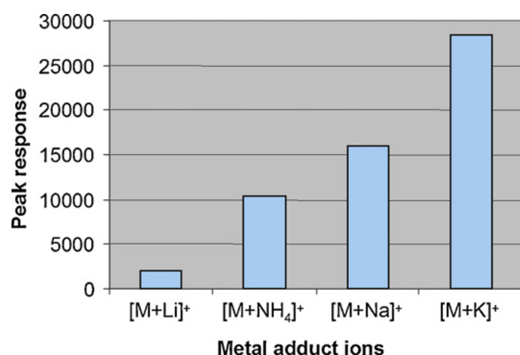

Fig. 6. Comparison of the peak responses from four metal adduct ions of GTI **D**.

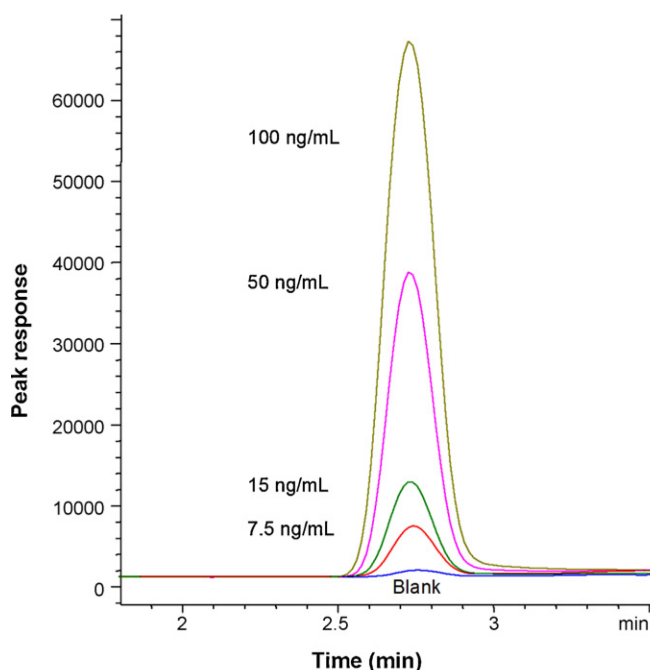

Fig. 7. Overlay chromatograms of the blank and the standard solutions of GTI **D** ranging from 7.5 to 100 ng/mL. The peak responses of potassium adduct ion was monitored at $[M+K]^+$, or $m/z = 267$.

when 0.2 mM potassium was doped into the mobile phase. Representative chromatograms of standard solutions at concentrations ranging from 7.5 to 100 ng/mL are shown in Fig. 7. The method was validated and the validation results are given in Table 2. The method afforded excellent sensitivity, linearity and accuracy.

It is worth noting that GTIs **A** and **C** also formed ion complex with the metal ions. For GTI **C**, a QL of 30 ppm was obtained using the CIS-MS method; however, the sensitivity was 10-fold lower than that of the derivatization approach. For GTI **A**, on the other hand, the potential of using CIS method was hampered due to chromatography issues. The diol analyte was poorly retained on the tested columns and difficult to resolve from the API peak. As such, the derivatization approach appeared to be a better choice for GTIs **A** and **C**. In general, the simpler CIS-MS method is the preferred choice in the authors' laboratory, while the derivatization method is investigated if the former does not provide adequate sensitivity.

4. Conclusion

Developing highly sensitive analytical methods for accurate determination of GTIs at low-ppm levels is non-routine for analytical scientists in pharmaceutical R&D in light of the emerging field. This is especially true for GTIs that lack structural features for sensitive detection. With routine use of LC/MS in combination with the right analytical approaches such as chemical derivatization and metal ion coordination, it is possible to establish highly sensitive analytical methodologies rather quickly. The effectiveness of the two approaches has been demonstrated in the analysis of GTIs **A**–**D** based on the understanding of their chemical structures and molecular properties. Four highly sensitive methods have been developed, validated, and applied successfully to the release of APIs for clinical uses. The approaches are considered to be generally applicable to the analysis of many other pharmaceutical genotoxic impurities.

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