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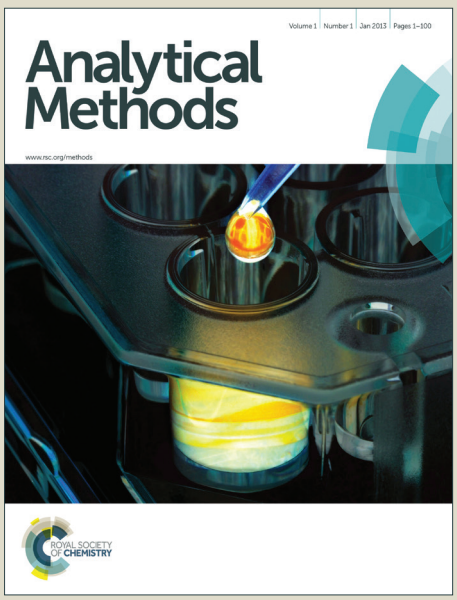

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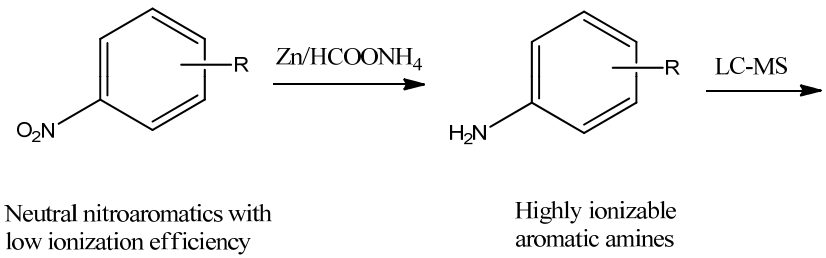
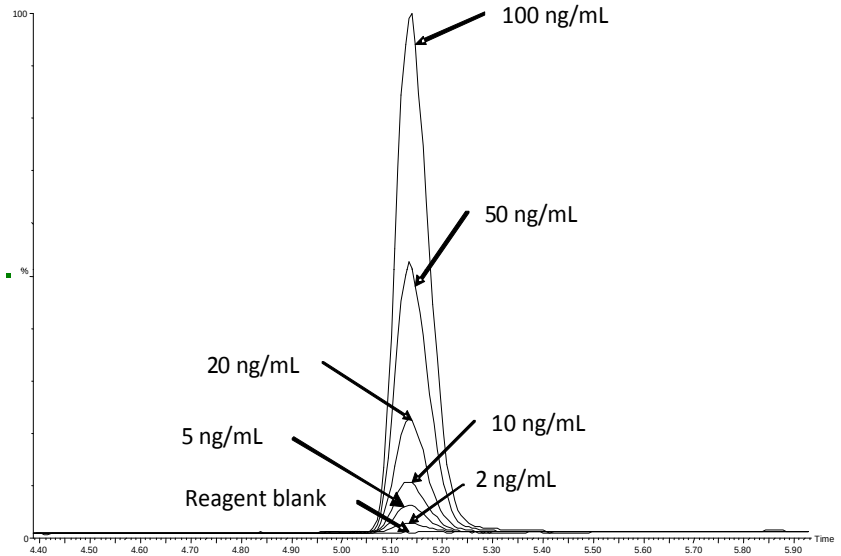


Neutral nitroaromatics with low ionization efficiency

Highly ionizable aromatic amines



Derivatization of Genotoxic Nitroaromatic Impurities for Trace Analysis by LC-MS

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Abstract:

Trace level analysis of genotoxic impurities requires highly sensitive analytical methodologies and poses tremendous challenges on the pharmaceutical industry. Nitroaromatic compounds, among the genotoxic structural alerts, are widely used as starting materials or intermediates for the synthesis of active pharmaceutical ingredients (APIs). If their genotoxicities are confirmed, the nitroaromatic impurities need to be controlled under the Threshold of Toxicological Concern (TTC) level. In general, the HPLC-UV methodology is not suitable due to the inadequate sensitivity of the UV detection. Many nitroaromatic compounds used in the pharmaceutical industry are non-volatile, and thus the application of GC is limited. If used directly, the LC-MS methodology is less useful to the neutral nitroaromatic compounds that lack ionization efficiency. In this article, the authors would like to report a new methodology to detect and quantify trace nitroaromatic compounds by LC-MS after chemical derivatization, where the nitroaromatic compounds are reduced to the corresponding ionizable aromatic amines. It is the authors' hope to provide a general methodology for detecting and quantifying nitroaromatics at

trace levels. The reported method was validated for sensitivity, linearity, precision and accuracy. This methodology has been successfully applied to one of Celgene's projects and the detailed case study is provided in this article.

Key Words

Genotoxic Impurity (GTI); Derivatization; Nitroaromatics; Quantitation Limit (QL); Detection Limit (DL); Electron Spray Ionization (ESI).

1. Introduction

Impurities that are reactive with DNA and have the potential of causing DNA damage and/or chromosomal breakage/rearrangement are considered as genotoxic impurities (GTIs). Genotoxic impurities in a drug product may come from chemical reagents, starting materials, intermediates, by-products of chemical reactions, and degradants from the active pharmaceutical ingredient (API) or other related impurities. From the quality-by-design (QbD) perspective, use of genotoxic chemicals shall be avoided or minimized in any manufacturing process. However, complete avoidance of GTIs from the process sometimes is not always practical. In that case, to ensure patient safety and product quality, a robust and sensitive analytical method should be developed and used to detect and quantify any GTIs in the drug substance and product. Many analytical strategies for GTI determination have been reported and reviewed recently.[1-6]

Nitroaromatic compounds are commonly used in the synthesis of APIs as starting materials or intermediates. *In silico*-based data systems suggest that the aromatic nitro functionality is one of the genotoxic "structural alerts".[7] If the genotoxicity of a nitroaromatic compound is confirmed by the Ames test that assesses the mutagenic potential of chemicals by exposing sample bacteria to the tested chemicals, it must be controlled to the level below the Threshold of Toxicological Concern (TTC).[8] Although a staged TTC approach, allowing greater daily intake, can be applied during the clinical development phases, an exposure of no more than 1.5 µg/day for each genotoxic impurity is generally accepted by the International Conference on Harmonisation (ICH), the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).[9, 10] For a given dose of 1.5 g/day, for example, it will require an analytical method capable of quantifying 1 ppm level of each GTI in the product. In comparison to the typical 200-500 ppm (0.02-0.05%) level of interest in the context of routine pharmaceutical

analysis, quantitation of a GTI at such a low level poses tremendous challenges on analytical method sensitivity. It becomes more challenging if the safety factors, depending on the age of patients, are considered or a total TTC may be applied to structurally similar GTIs with a shared genotoxic mechanism.[11] In either of these scenarios, an even lower quantitation limit may be required.

It has been reported that very low levels of nitroaromatic compounds can be analyzed by HPLC-UV or UPLC-UV due to their strong UV absorption.[12-14] In general, in order to detect and quantify the ppm or sub-ppm presence of GTIs in the API, high sample concentration is required. However, poor solubility of the API in diluent is common and could become a limiting factor when high sample concentration is required. Furthermore, at such high sample concentrations, some impurities below the detection limit at the typical analytical working concentrations are observed and interfere with the GTI peak. In recent years, LC-MS has been popular to analyze trace levels of GTIs due to the superior sensitivity and selectivity of the technique.

The reported analysis of nitroaromatics is largely from the forensic science as many nitroaromatics are explosives, like DNT, TNT, etc. Nitroaromatic compounds at high concentrations can be detected by either APCI or ESI with negative mode because of the strong electron-deficient nature of the nitro functionality.[15, 16] To achieve the desirable sensitivity for GTI analysis, other strategies are needed as many nitroaromatic compounds are neutral and lack of sufficient ionization efficiency. Two approaches are often used to enhance the MS detection sensitivity: one is chemical derivatization by introducing an ionizable or a permanently charged functional moiety to the molecule and the other is to use the coordination ion spray-mass spectrometry by converting a neutral analyte to a charged neutral-ion complex in solution.[17-19] In this article, the strategy of converting nitroaromatics to highly ionizable arylamines through

chemical derivatization was explored, taking advantage of the fact that many LC-MS methods for trace level arylamine analysis have been extensively reported.[14, 20, 21] In addition, many small molecule arylamines are volatile and GC-MS with a much cleaner chromatographic background may also be applicable if nitroaromatics are converted to arylamines.

In order to provide a general applicability of the methodology, the following criteria were considered: 1) reaction selectivity, where the reaction conditions shall have no or minor impacts on the API or related impurities; 2) simplicity, where the derivatization procedure shall be simple enough for most of the analytical laboratories to perform; and 3) mild reaction conditions, where the derivatization ideally occurs without extra sample manipulations, such as heating, cooling, extraction, pre-column concentration, etc. The ammonium formate/zinc system was reported to provide rapid, selective and mild reduction of nitros to amines, and was compatible with many other functionalities, including aryl/alkyl halides, ketones, aldehydes, esters, carboxylic acids, amides, carbamates, cyanides, etc.[22] Thus, this chemical derivatization methodology followed by LC-MS was evaluated for determining trace levels of aromatics and reported in this article. The general operation procedure includes: 1) dissolving a sample in a volumetric flask with the sample diluent, the mixture of organic solvent (e.g., MeOH, ACN) and 0.5 mM ammonium formate; 2) adding zinc dust and shaking or sonicating the solution for 20 minutes; 3) diluting to volume with the sample diluent and making serial dilutions to the desired concentrations; and 4) filtering or centrifuging the sample solution prior to LC-MS analysis. It is worth noting that if the API would react with the ammonium formate/zinc system, the amount of zinc and ammonium formate should be in excess to ensure complete reduction of the nitroaromatics.

In this article, we report a case study using the above stated approach to quantify a trace level of nitroaromatic GTI, methyl 2-(bromomethyl)-6-nitrobenzoate, in an API. It is the authors' hope to provide a simple, but useful methodology that can quantify trace levels of nitroaromatic GTIs with a typical sample preparation procedure that can be used in the majority of the analytical laboratories.

Table 1. LC-MS conditions

Analyte	**The model nitroaromatics:** Nitrobenzene, Nitrotoluenes, 4-Nitroanisole			**Methyl 2-(bromomethyl)-6-nitrobenzoate**		
HPLC parameters						
Column	Kinetex C8, 1.7 µm, 50 x 2.1 mm			Kinetex C8, 1.7 µm, 50 x 2.1 mm		
Col. temp. (°C)	25			40		
Mobile phase	A: 0.1% formic acid B: Methanol			A: 0.1% formic acid B: Methanol		
Gradient	Time (min)	%A	%B	Time (min)	%A	%B
	1	98	2	2	95	5
	2.5	60	40	10	0	100
	2.6	0	100	11	0	100
	3.6	0	100	11.1	95	5
	3.7	98	2	15	95	5
	7	98	2			
Flow rate	0.25 mL/min			0.4 mL/min		
Inj. vol. (µL)	7			7		
MS parameters						
Capillary voltage (kV)	3			2		
Cone Voltage (V)	30			18		
Source temp. (°C)	135			125		
Desolvation temp (°C)	400			350		
Cone gas flow (L/Hr)	50			50		
Desolvation gas flow (L/Hr)	900			750		
Dwell time (s)	0.1			0.1		
SIM ion	m/z 94 [M+H]+ for Aniline m/z 108 [M+H]+ for Toluidines m/z 124 [M+H]+ for 4-Anisidine			m/z 166 $[M+H]^+$		

2. Materials and Methods

2.1. Chemicals and Reagents

Model nitroaromatic compounds and the corresponding amines used as external standards were all purchased from Sigma-Aldrich (St. Louis, MO, USA) with ACS grade or analytical

grade. HPLC-grade acetonitrile, HPLC-grade ammonium formate, MS-grade formic acid and ACS-grade zinc dust were purchased from Sigma-Aldrich. Water was purified with a Milli-Q A10 system (Millipore, Bedford, MA, USA). The 0.5 M ammonium formate solution was prepared by dissolving 31.5 g of ammonium formate in 1000 mL of purified water.

2.2. UHPLC separation

Chromatography was carried out on an Acquity UPLC system from Waters (Milford, MA, USA) with a Kinetex C8 column (1.7 μm, 50 x 2.1 mm) from Phenomenex (Torrance, CA, USA). The LC-MS conditions were optimized to achieve acceptable sensitivity and resolution. The detailed parameters are shown in Table 1.

2.3. *Mass Spectrometry Detection*

A single quadrupole MS detector (SQD) from Waters (Milford, MA) with an electrospray probe in the positive mode (ESI^+) was used. Acquisitions were carried out in a selected ion recording (SIR) mode. A tuning solution was prepared by mixing individual amines at the concentration of 10 μg/mL. The MS was tuned by the combined perfusion of the tuning solution into the MS detector at 10 μL/min with the mobile phase at the flow rate of 0.25 mL/min. The tune parameters were optimized to provide an overall sufficient sensitivity for all tested arylamines. Thus, a universal setting was applied instead of optimizing the sensitivity for each individual arylamine with the different settings. The parameters are summarized in Table 1 above.

2.4. General Derivatization Procedure and Sample Preparation for Model Nitroaromatic Compounds

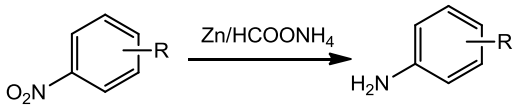
The sample diluent was prepared by mixing 500 mL of 0.5 M ammonium formate solution with 500 mL of MeOH. A nitroaromatic compound (~10 mg) was weighed into a 100 mL volumetric flask and dissolved in 70 mL of the sample diluent prepared above. Zinc dust (~6.5mg) was added and the resulting solution was shaken on an orbital shaker from VWR International (Radnor, PA, USA) for 20 minutes. The solution was then diluted to volume with the sample diluent. Serial dilutions were made to produce solutions of 0.2-10 ng/mL, respectively. The resulting solutions were centrifuged prior to LC-MS analysis. A reagent control blank was also prepared by following the same procedure except the absence of nitroaromatic compounds. External standard solutions were prepared from the commercially available arylamines.

2.5. Sample preparation and derivatization for Genotoxic impurity Methyl 2-(bromomethyl)-6-nitrobenzoate

An API sample stock solution was prepared at the concentration of 10 mg/mL in acetonitrile. Methyl 2-(bromomethyl)-6-nitrobenzoate sample stock solution (**A**) was prepared at the concentration of 100 ng/mL in acetonitrile. A typical spike solution of 0.4 ppm methyl 2-(bromomethyl)-6-nitrobenzoate in API was prepared by pipetting 200 µL of stock solution **A** into 5 mL of API stock solution in a 10-mL volumetric flask and then adding 4 mL of 0.5 M ammonium formate. The chemical derivatization was carried out by adding ~6.5mg of zinc dust into the spiked solution. The resulting solution was shaken for 20 minutes and then diluted to volume with acetonitrile. The solution was centrifuged, and the supernatant was injected for LC-MS analysis. Due to the unavailability of the amine **2**, the external standards with the

concentrations of 2, 5 and 10 ng/mL were prepared accordingly by derivatization of methyl 2-(bromomethyl)-6-nitrobenzoate following the similar procedure except the absence of the API. Solutions with different spiked levels of methyl 2-(bromomethyl)-6-nitrobenzoate were prepared by following the same procedure and varying the volume of stock solution **A**. A reagent blank was prepared in a similar fashion without the presence of methyl 2-(bromomethyl)-6-nitrobenzoate and the API. An API control blank was also prepared similarly except methyl 2-(bromomethyl)-6-nitrobenzoate was absent.

Scheme 1. Chemical derivatization of model nitroaromatic compounds



Nitrobenzene, R = H
2-Nitrotoluene, R = *o*-Methyl
3-Nitrotoluene, R = *m*-Methyl
4-Nitrotoluene, R = *p*-Methyl
4-Nitroanisole, R = *p*-Methoxyl

Aniline, R = H
2-Toluidine, R = *o*-Methyl
3-Toluidine, R = *m*-Methyl
4-Toluidine, R = *p*-Methyl
4-Anisidine, R = *p*-Methoxyl

3. Results and Discussion

3.1.Chemical derivatization for trace analysis of nitroaromatic compounds

As shown in Scheme 1, a chemical derivatization procedure followed by LC-MS analysis was first evaluated, using five model nitroaromatic compounds at trace levels. The objective of the chemical derivatization was to introduce an ionizable functionality to the molecule by reducing the nitro group chemically to an amine group. Five model nitroaromatic compounds, shown in Scheme 1, were deliberately chosen as they do not carry other ionizable functional groups and thus are not directly amenable to the ESI detection. In addition to the final product arylamines, the possible reduction intermediates, the corresponding hydroxyamines, were also

monitored by full-scan ESI to demonstrate the completion of derivatization reaction. Ammonium formate solution was used as a MS compatible diluent and a source of hydrogen donor for the proposed reduction reaction. The excess amount of zinc and ammonium formate as reducing agents ensured rapid and complete reduction of the nitroaromatic compounds. The leftover zinc powder was easily removed via centrifugation or filtration.

The reactions were complete within 20 minutes for all model compounds and produced the corresponding arylamines as the only final products. A UPLC method (Table 1) was developed to separate the mixture of aniline, 4-toluidine, and 4-anisidine in a single run. 2-Toluidine and 3-toluidine were analyzed separately as they were co-eluted with 4-toluidine. The MS detector was not tuned specifically to achieve the highest sensitivity for each individual arylamine. Instead, the MS parameters were optimized to provide general satisfaction for all five analytes with one universal setting. Commercially available arylamines were purchased and used as the external standards. The sensitivities were measured using 0.2 ng/mL of external standards, and the linearities were evaluated in a range of 0.5 to 10 ng/mL. The conversion was assessed at 0.5 ng/mL and 10 ng/mL, respectively. The calculation of the conversion was based upon the measured amount of arylamine against the theoretical amount of arylamine. The theoretical amount of arylamine was calculated using the following equation.

$$\text{Theoretical amount of arylamine} = \frac{\text{Wt}_{Nitroaromatic} \times \text{MW}_{Arylamine}}{\text{MW}_{Nitroaromatic}}$$

Where, $\text{Wt}_{Nitroaromatic}$ = Weight of the nitroaromatic compound

$\text{MW}_{Arylamine}$ = Molecular weight of the corresponding arylamine

$\text{MW}_{Nitroaromatic}$ = Molecular weight of the nitroaromatic compound

Table 2. Validation results for model nitroaromatic compounds

Analyte	Signal to noise (Mean, n=3) (0.2 ng/mL)	Linearity range (0.5 - 10 ng/mL)		Precision %RSD (n=6) 0.5 ng/mL	Precision %RSD (n=6) 10 ng/mL	Conversion (n=3) 0.5 ng/mL	Conversion (n=3) 10 ng/mL
Nitrobenzene	12	y=4807x-368	R^2= 0.9993	4.30%	3.60%	120%	121%
2-Nitrotoluene	25	y=4958x+628	R^2= 0.9968	5.40%	3.40%	106%	107%
3-Nitrotoluene	27	y=4061x-78	R^2= 0.9999	3.40%	4.50%	107%	103%
4-Nitrotoluene	27	y=5520x+88	R^2= 0.9996	2.80%	4.60%	117%	119%
4-Nitroanisole	13	y=5261x+50	R^2= 0.9989	3.30%	3.50%	107%	108%

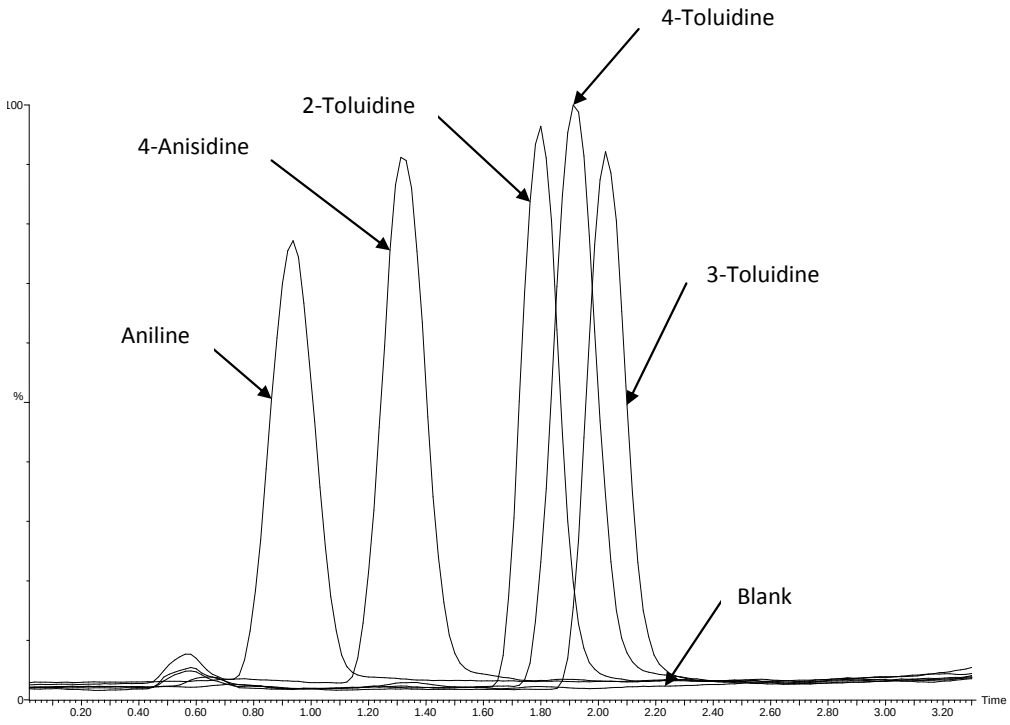
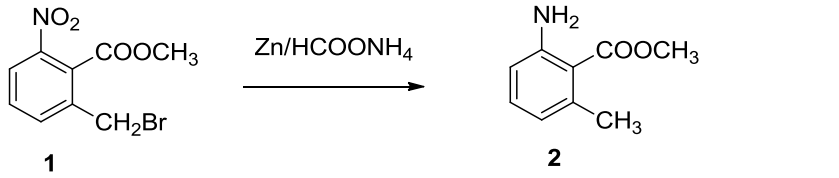


Figure 1. Overlaid LC-MS chromatograms of the arylamines at 10 ng/mL and the reagent blank

The method was evaluated for sensitivity, linearity, and precision. The validation data are summarized in Table 2. The overlaid chromatograms of five arylamines and a reagent blank are presented in Figure 1. The method is capable of detecting the model arylamines as low as 0.2

ng/mL with the signal to noise ratios above 10. At the concentration of 0.5 ng/mL, the method showed excellent RSDs ranging from 2.8% to 5.4% with six consecutive injections. The conversion ranged from 107% to 120% at 0.5 ng/mL and from 103% to 121% at 10 ng/mL. The slightly higher conversion than 100% could be due to the matrix effect by zinc ion as the external standards were prepared by using the sample diluent without the presence of znic. The linear range is between 0.5 and 10 ng/mL for all measured nitroaromatic compounds with the R^2 ranges from 0.9968 to 0.9999. The results showed that the method had good sensitivity, linearity and precision for the intended purposes.

Scheme 2. Chemical derivatization of the GTI 1



1 Methyl 2-(bromomethyl)-6-nitrobenzoate

2 Methyl 2-amino-6-methylbenzoate

*3.2. A Case Study of Detecting Methyl 2-(bromomethyl)-6-nitrobenzoate (**1**) in API at sub-ppm Level*

Methyl 2-(bromomethyl)-6-nitrobenzoate, shown in Scheme 2, is a genotoxic impurity that has been confirmed by the Ames test. Based on the currently proposed daily dose and clinical study duration, not-higher-than 4 ppm of this compound is allowed in the product. Considering a possible dosing increase in the future clinical studies, the objective was to develop a method with sub-ppm level sensitivity. It was initially attempted to develop an HPLC-UV method that can be easily correlated to the regular impurity method for the product . For the HPLC-UV strategy to be successful, very high concentration of API was needed due to the required sensitivity. However, two difficulties were encountered during the method development: 1) Although methyl

2-(bromomethyl)-6-nitrobenzoate was reasonably separated from the API peak, at a concentration of more than 500 times higher than the nominal analytical concentration, a previously undetected impurity peak was found to interfere with the methyl 2-(bromomethyl)-6-nitrobenzoate peak, and base-line separation was not achieved; 2) Another difficulty encountered was the solution instability due to the presence of benzylic bromide functionality in methyl 2-(bromomethyl)-6-nitrobenzoate. It was observed that methyl 2-(bromomethyl)-6-nitrobenzoate sample solution had a half-life of less than 2 hours in the solution. In addition, methyl 2-(bromomethyl)-6-nitrobenzoate itself lacks ionization functionality, applying LC-MS directly for detection was not successful. Therefore, derivatizing methyl 2-(bromomethyl)-6-nitrobenzoate became the strategy of choice. As shown in Scheme 2, the preliminary derivatization of methyl 2-(bromomethyl)-6-nitrobenzoate with ammonium formate and zinc dust showed that not only the nitro group was reduced to an amino group but also the bromomethyl group was converted to a methyl group, suggesting the involvement of hydrogenolysis at benzylic position. The investigated API is a small molecule clinic drug candidate, which carries a lactam and a sulfone functional groups. The API remained intact after exposed to the described derivatization conditions.

Table 3. Validation results for GTI 1

Analyte	Signal to noise (Mean, n=3)		Linearity range (2 - 100 ng/mL)		Precision RSD (n=6)			Recovery (n=3)		
	1 ng/mL	2 ng/mL			2 ng/mL	5 ng/mL	10 ng/mL	2 ng/mL	5 ng/mL	10 ng/mL
GTI **1**	23	37	y=3666x-217	R^2= 0.9999	3.60%	2.70%	2.10%	85%	80%	82%

The analytical method was then developed and validated for sensitivity, linearity, precision and accuracy. The validation results are summarized in Table 3. The sensitivity was measured at 1 ng/mL (DL=0.2 ppm) and 2 ng/mL (QL=0.4 ppm). The signal-to-noise is above 20 (n=3) at the DL level and above 30 (n=3) at the QL level. As the presence of methyl 2-(bromomethyl)-6-

nitrobenzoate above 20 ppm in the API was not expected, the linearity was evaluated in the range of 2 to 100 ng/mL corresponding to 0.4 to 20 ppm level. The overlaid chromatograms of linearity solutions and the reagent blank are shown in Figure 2. The good linearity was achieved with the R^2 of 0.9999 and Y% of 3.0% at the QL level. To avoid the potential interference from the matrix, an API control blank was prepared by the similar procedure in the absence of GTI **1**. As shown in Figure 3, there was free of interference at the retention time of methyl 2-amino-6-methylbenzoate from the API control blank, suggesting that the investigated API did not contain the GTI **1**. To minimize the potential contamination of the mass spectrometer by the large amount of the API injected, the unwanted API LC effluent was diverted to the waste after the elution of the arylamine **2**. The recovery was measured by spiking 0.4, 1, and 2 ppm of methyl 2-(bromomethyl)-6-nitrobenzoate into the API, respectively. The recovery was calculated by the peak area of the arylamine from the spiked sample against that from the external standard at the same concentration level. Recoveries at three different levels are all above 80% as shown in Table 3. Representative chromatograms of spike recovery at 0.4, 1 and 2 ppm are provided in Figure 3. The system precision was evaluated with six injections of derivatized methyl 2-(bromomethyl)-6-nitrobenzoate external standards at 2 (0.4 ppm), 5 (10 ppm) and 10 ng/mL (20 ppm), respectively. The RSDs at these three different concentrations were all less than 4%. The method was validated to be capable of detecting 0.2 ppm and quantifying 0.4 ppm of methyl 2-(bromomethyl)-6-nitrobenzoate in the API. The sample solution stability at 1 ppm spike level was evaluated by comparison of the recovery of each time point with that of the initial. The external standard at 1 ppm level was prepared freshly prior to anlaysis. The chromatograms shown in Figure 4 are from the stability solutions at QL and 1 ppm spike levels. The solution stability study was not continued after 2 days although the resulting arylamine **2** was stable with no significant degradation when stored under ambient conditions.

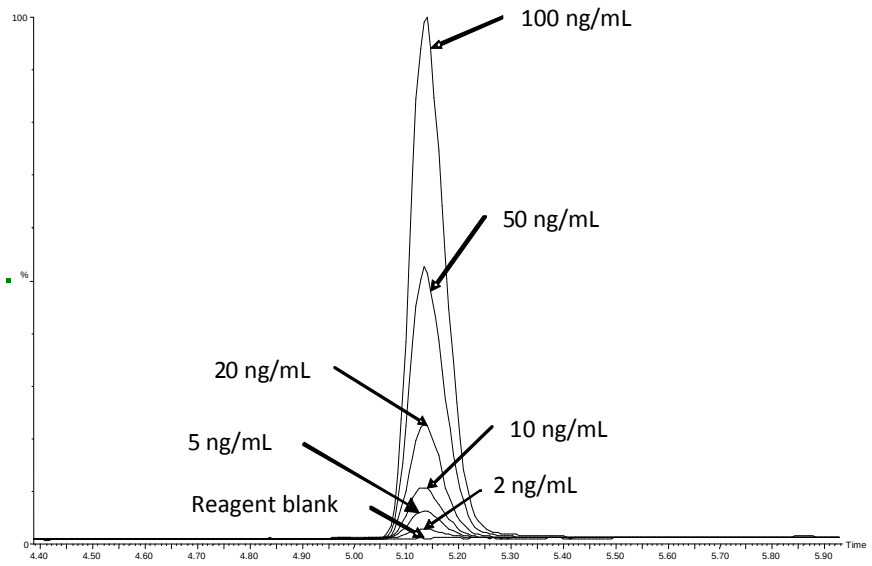


Figure 2. Overlaid LCMS chromatograms of the reagent blank and the amine 2 solutions ranging from 2 to 100 ng/mL.

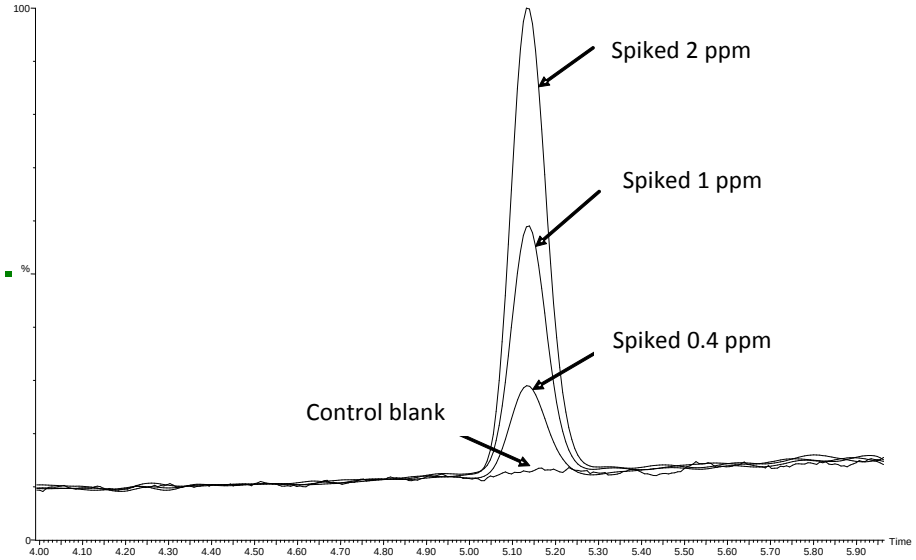


Figure 3. Overlaid LCMS chromatograms of the API control blank and the spiked solutions at 0.4, 1 and 2 ppm.

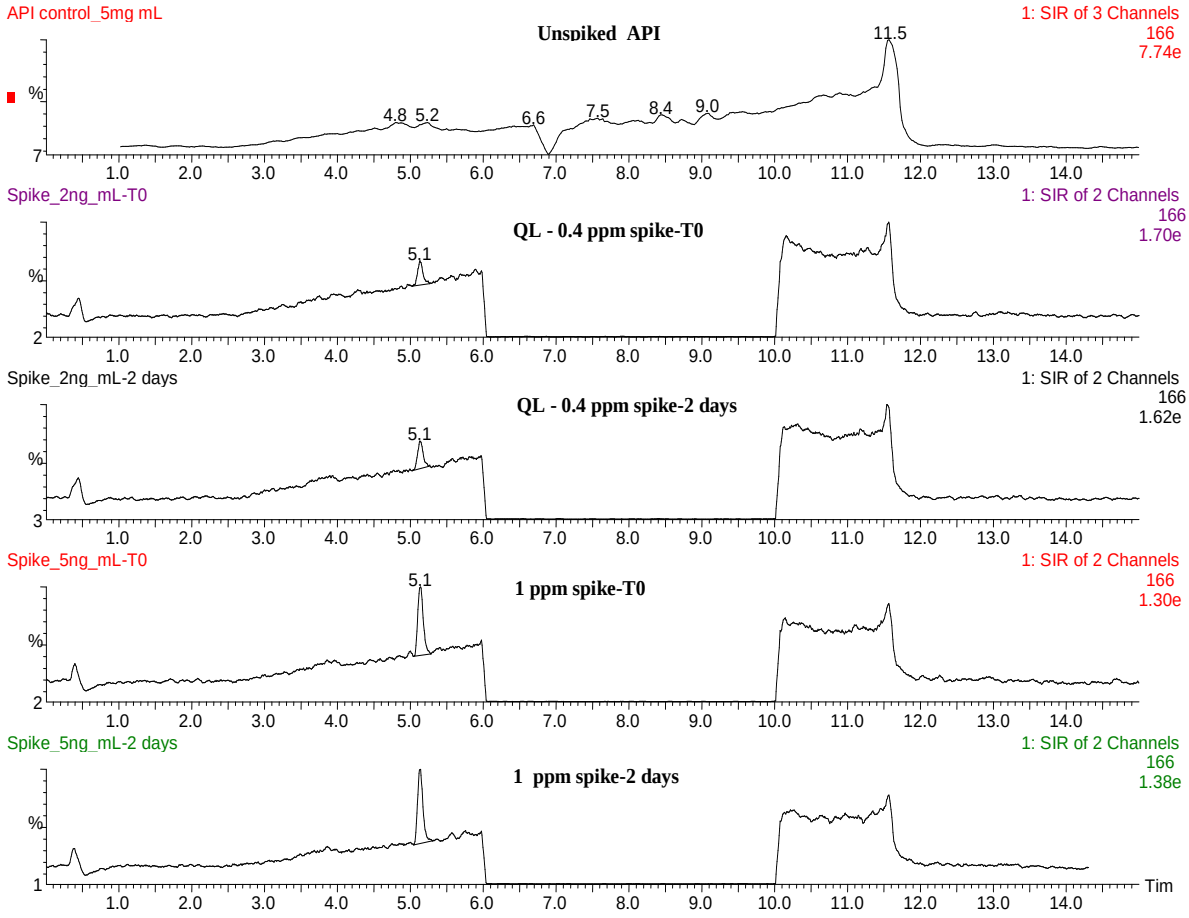


Figure 4. LCMS chromatograms of the API control blank and the stability solutions at QL and 1 ppm spike levels .

4. Conclusions

Even though the nitroaromatic compounds are categorized as GTI "structure alerts", they are commonly used in the synthesis of APIs. The quantitation of nitroaromatic compounds at trace levels is challenging pharmaceutical analysis although many methodologies have been explored and discussed recently. In the provided case study, the particular challenge of poor MS ionization and solution stability was resolved simultaneously by chemical derivatization, where the nitro group was converted to a highly ionizable amino group for LC-MS analysis. It was found that the benzyl bromide functionality was liable to hydrogenolysis while the lactam and sulfone functionalities were compatible to the described derivatization conditions.

The method has been successfully validated for sub-ppm quantitation. LC-MS was applied after derivatization, taking advantage of the high sensitivity and superior selectivity of MS. The described strategy, chemical derivatization coupled with LC-MS, which has been explored with both model compounds and real case studies, is simple to use and has the capability of quantifying trace level genotoxic neutral nitroaromatics in pharmaceuticals.

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