Streamlining Analytical Method Development with a Focus on Genotoxic Impurities

Developing analytical methods for a range of genotoxic impurities

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INTRODUCTION

The development of chromatographic methods to determine potential genotoxic impurities (GTIs) can be time consuming and laborious. While there are several approaches to method development, having a generic method development strategy can help streamline the process. This article provides an overview of method development strategies to help analysts, chromatographers, and researchers develop suitable, reliable, robust, and potentially validatable methods to determine GTIs. It includes several examples of successfully developed and validated methods for a range of GTIs, including nitrosamines, by liquid chromatography (LC) and gas chromatography (GC).

GTI METHOD DEVELOPMENT

GTIs are intermediates, reactants, or related substances from the synthetic pathway of a drug substance that must be monitored at very low levels, typically ppm or even ppb levels, 1,000 times lower than limits for classical impurities. Both the EMEA and ICH provide regulatory guidance to assess and characterize GTIs. Analytical methods used for their analysis include GC or LC, often combined with mass spectrometry (MS) detection.

GTI analysis methods require specificity to detect the compound of interest interference-free to distinguish between different chemical entities (i.e., resolution), and they must be fit for purpose. That is, perform what it is intended for the method to do. The method must also be capable of being validated, where parameters such as accuracy, precision,

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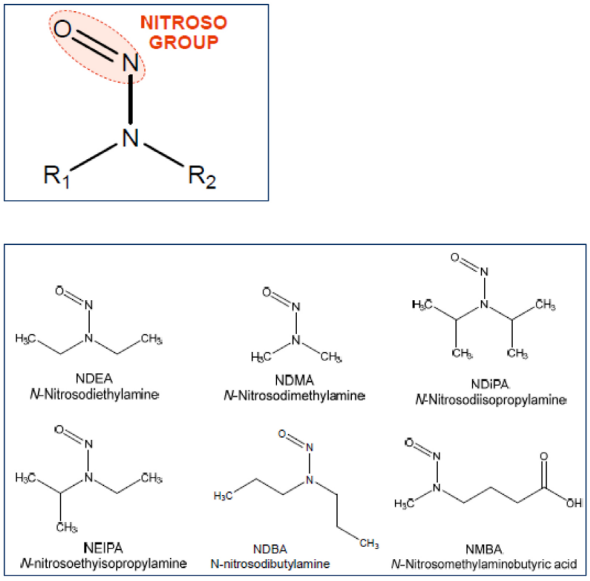
linearity, specificity, stability, and robustness are assessed for method suitability.

Several compound-specific details, including the compound pKa, the functional groups present, ionizable functionality, and molecular weight, should be considered when choosing the analysis mode. If LC is the technique of choice, the detection technique is governed by the presence or absence of a chromophore. If a chromophore is present, ultraviolet (UV) or photodiode array detection (PDA) can be used. If a chromophore is not present, then charged aerosol detection (CAD), evaporative light scattering detection (ELSD), refractive index (RI), or MS could be used. Other considerations include the solubility of the molecule and whether the molecule is polar or non-polar. It is also helpful to consider how any related substances, solvents, or reagents might affect the chromatography or identification of the target compound.

A rule of thumb for GTI method development is to not "reinvent the wheel." It might be possible that a similar method has already been developed. Compendia, like the U*nited States Pharmacopeia* (*USP*) or the *European Pharmacopeia* (Ph. Eur.), column manufacturer's or column supplier's application notes or technical services, the scientific literature, or even internal company documents can often provide the starting point for the method development process. If none of these sources yield results, other options are still available. One is a Quality by Design (QbD) approach, which defines the analytical target profile. QbB is based on factorial experimental design and can be automated using suitable software. Another option is a heuristic approach, which relies on the analyst's knowledge and an iterative experimental design, along with some trial and error to identify a suitable starting point.

Once a method has been identified, chromatography conditions must be optimized. A forced degradation study can be performed to generate degraded samples that can be used to determine and test method selectivity and whether further optimization is necessary. Process-related or raw material impurities or intermediates formed during the synthetic manufacturing process could also interfere and should be considered during method optimization. Once a suitable sample (or samples) is identified, optimization is carried out by manipulating parameters like mobile phase composition, pH, flow rate, and column temperature to adjust the resolution. Different column stationary phases can also impart significant changes in selectivity. Other method parameters that should be evaluated include detector settings, sample concentration, injection volume, sample diluent, and sample preparation.

Figure 1: Examples of nitrosamine impurities.



For rapid method development, often a generic starting method is used. A short C18 column and a rapid water/acetonitrile gradient are good starting points. The goal is to keep *k*' between two and ten. An acid modifier is used for ionizable compounds, keeping in mind detector (e.g., MS) compatibility. Following rapid screening runs, conditions can be further optimized to provide the desired resolution. However, if the initial runs do not provide the level of resolution required, alternative stationary phases or chromatographic modes should be considered. Alternative phases might include amino, diol, or pentafluorophenyl; alternative modes might include normal phase, hydrophilic interaction liquid chromatography (HILIC), GC, also including different column phases, or relying on MS to provide the selectivity.

DETERMINATION OF THE NITROSAMINES NDMA AND NDEA IN A SMALL MOLECULE API

The presence of nitrosamines in pharmaceutical products emerged as a public health concern in 2018 after reports that harmful levels of a nitrosamine impurity, N-nitroso-dimethylamine (NDMA), were observed in angiotensin II receptor blockers (ARBs, sartan products). The formation of these nitrosamines occurs by the reaction of secondary or tertiary amines and sodium nitrite. Following these reports, several public health alerts and guidance documents were issued by various health agencies, including WHO, FDA, and EDQM. Subsequently, based on animal studies, nitrosamines were classified as probable human carcinogens.

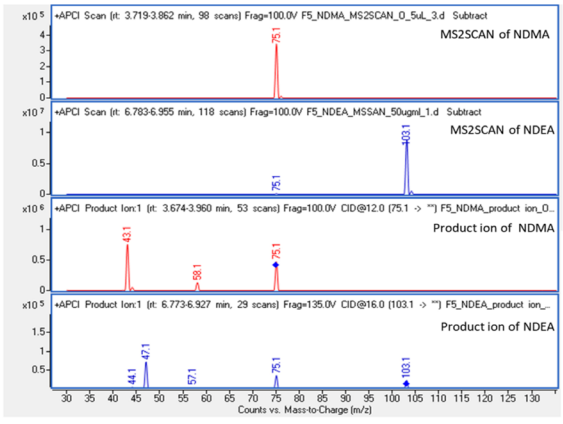
FIGURE 1 illustrates several different nitrosamine structures, all having the nitroso group in common. For a maximum daily API dose of 400 mg/day, the calculated acceptable intake levels are 0.24 ppm for NDMA and 0.07 ppm for N-nitrosodiethylamine (NDEA), thus sensitive methods are required. While various health agencies have published methods for analyzing these two GTIs using LC-MS/MS, headspace GC-MS, direct-inject GC-MS, and high performance liquid chromatography (HPLC)-UV techniques, LC-MS/MS was found to provide the best results for this example. The polar nature of the API and low vapor pressure ruled out GC.

To analyze these two GTIs in the API, several method objectives were established. A *k*' of

For rapid method development, often a generic starting method is used.

Figure 2: Analytical method development (LC-MS/MS).

- **Key Requirements for the Method Development**
  - To achieve the calculated LOQ level (for the acceptable intake of NDMA: 0.24 ppm, NDEA: 0.07 ppm)
  - To confirm any matrix effect from the sample (API): Specificity
- **MS/MS Information for NDMA and NDEA**



- **Column Screening**

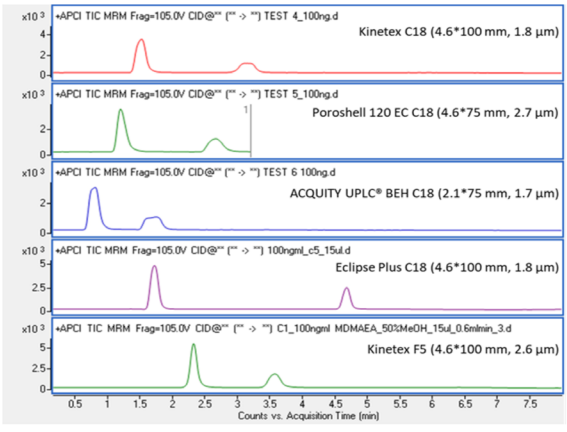
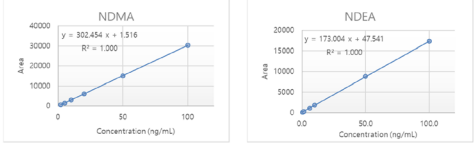
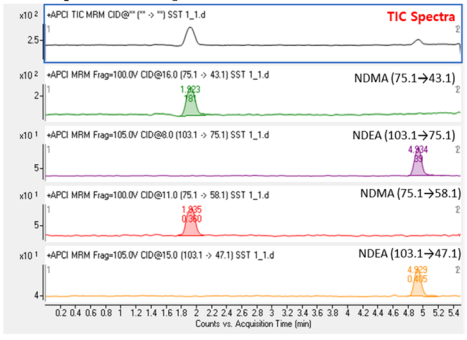


Figure 3: Analytical method validation.

- **Linearity: Pass**



- **Specificity: Pass**



- **Sensitivity (LOD and LOQ): Pass**

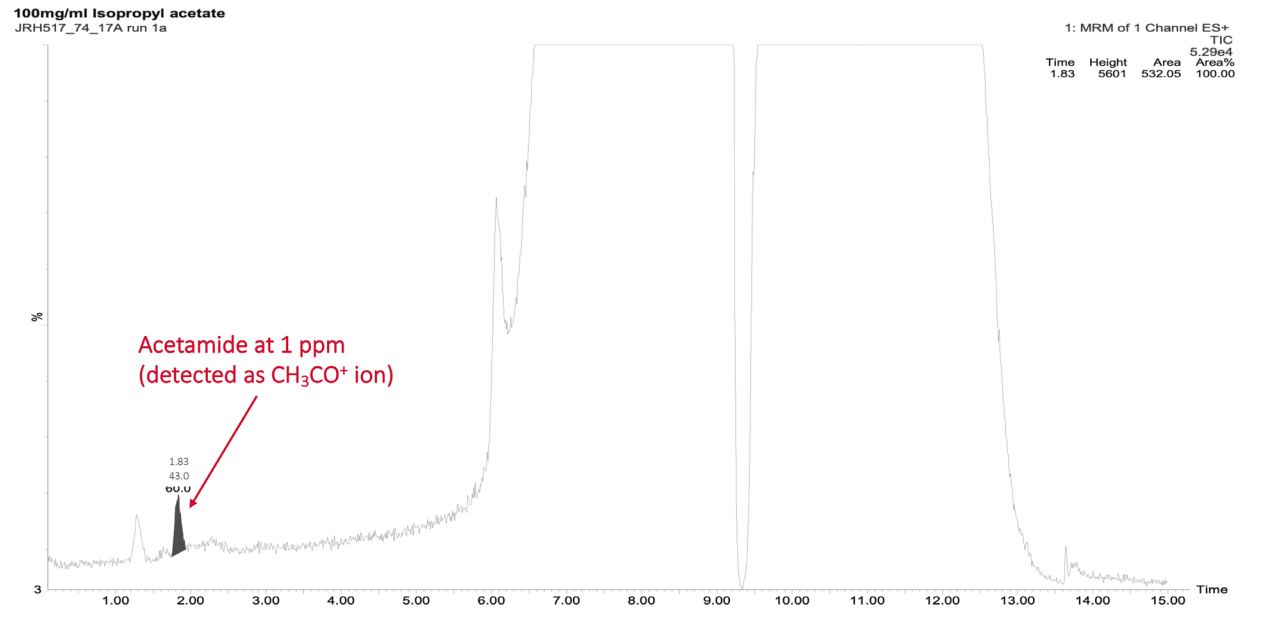
Solution	Compound	Absolute concentration (ng/mL)	Relative level in API (ppm)
LOQ	NDMA	2.0	0.25
	NDEA	0.5	0.06
LOD	NDMA	0.60	0.0075
	NDEA	0.15	0.0019

- **Accuracy and Precision: Pass**

Solution	Test	Level	%Recovery
Accuracy	%Recovery 70% - 130%	Spiked 0.025 ppm (NDMA); 0.006 ppm (NDEA)	NDMA: 89.1 % – 98.5 % NDEA: 91.4 % – 112.7 %
		Spiked 0.250 ppm (NDMA); 0.075 ppm (NDEA)	NDMA: 83.6 % – 88.1 % NDEA: 99.0 % – 102.5 %
		Spiked 0.500 ppm (NDMA); 0.250 ppm (NDEA)	NDMA: 81.2 % – 86.5 % NDEA: 95.8 % – 102.3 %
Precision	Spiked API sample	%RSD of the contents of NDMA and NDEA ≤ 30.0 %	NDMA: 1.7 % NDEA: 4.7 %
		%RSD of the contents of NDMA and NDEA ≤ 30.0 %	NDMA: 2.0 % NDEA: 2.9 %

- **Robustness: Pass**
  - **Solution stability (24 hours)**
  - **LC conditions: mobile phase, column temperature, flow rate**

Figure 4: Determination of acetamide.



at least two (providing adequate retention), resolution of all peaks of interest, symmetrical peak shapes to maximize sensitivity, and the ability to achieve the desired LOQs. Several reversed-phase HPLC columns were screened during method development, as shown in FIGURE 2. On the right-hand side of FIGURE 2, the fourth chromatogram down, using the Eclipse Plus column, yielded the best combination of peak shape and retention. On the left, the MS spectra for NDMA and NDEA, and their corresponding product ions are shown. In addition to the chromatography, it was also necessary to optimize the MS conditions, including the drying gas temperature, drying gas flow, nebulizer pressure, vaporizer temperature, APCI needle voltage, and capillary voltage. For NDMA, quantifier and qualifier product ions of 43.1 *m/z* and 58.1 *m/z* were selected, and for NDEA, 75.1 *m/z* and 47.1 *m/z,* respectively.

To ensure that the method was suitable for its intended use, validation was conducted by evaluating linearity, specificity, sensitivity, accuracy, precision, and robustness. FIGURE 3 summarizes the validation results. As can be seen, excellent results were obtained, and most importantly, the LOQs of 0.25 ppm and 0.06 ppm for NDMA and for NDEA, respectively, were easily achieved.

DETERMINATION OF PROCESS IMPURITIES

Acetamide is a process impurity that has a very poor UV and MS response. Consequently, quantitation is best performed by LC-MS/MS multiple reaction monitoring, as shown in FIGURE 4. In this example, an overloaded API sample concentration of 100 mg/mL was used to satisfy the 1 ppm detection limit required. A volatile ion pair reagent was added to the mobile phase to retain acetamide on the C18 hybrid column. The acetamide GTI was

Figure 4: Determination of acetamide.

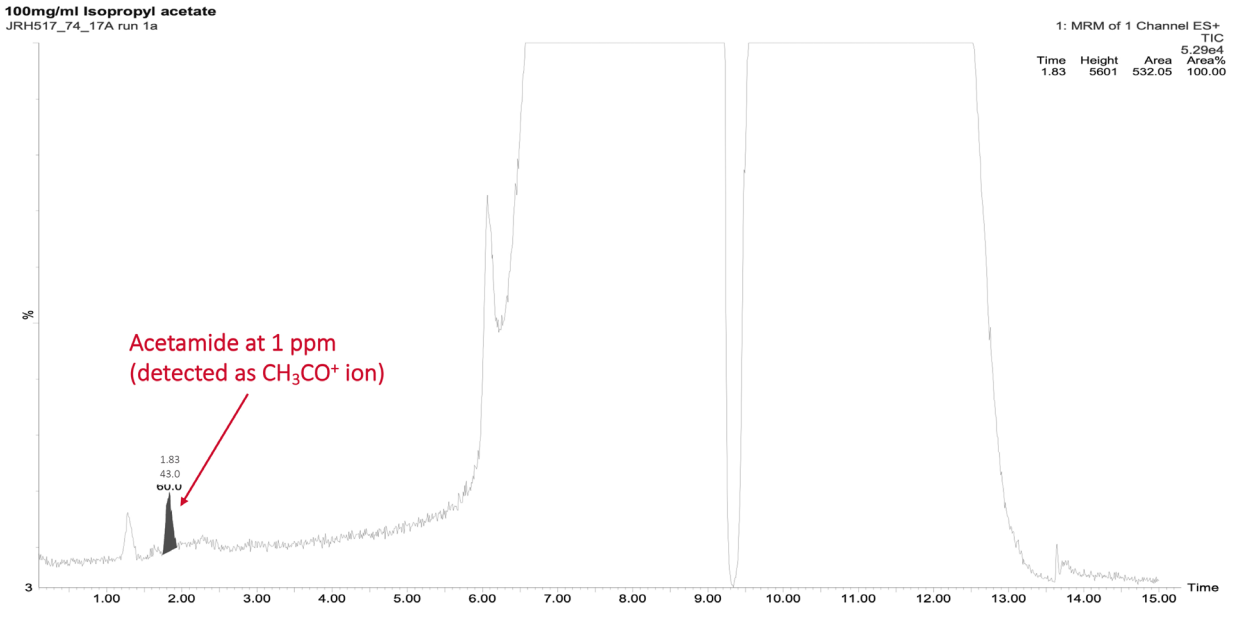


Figure 5: Separation by HILIC with CAD detection.

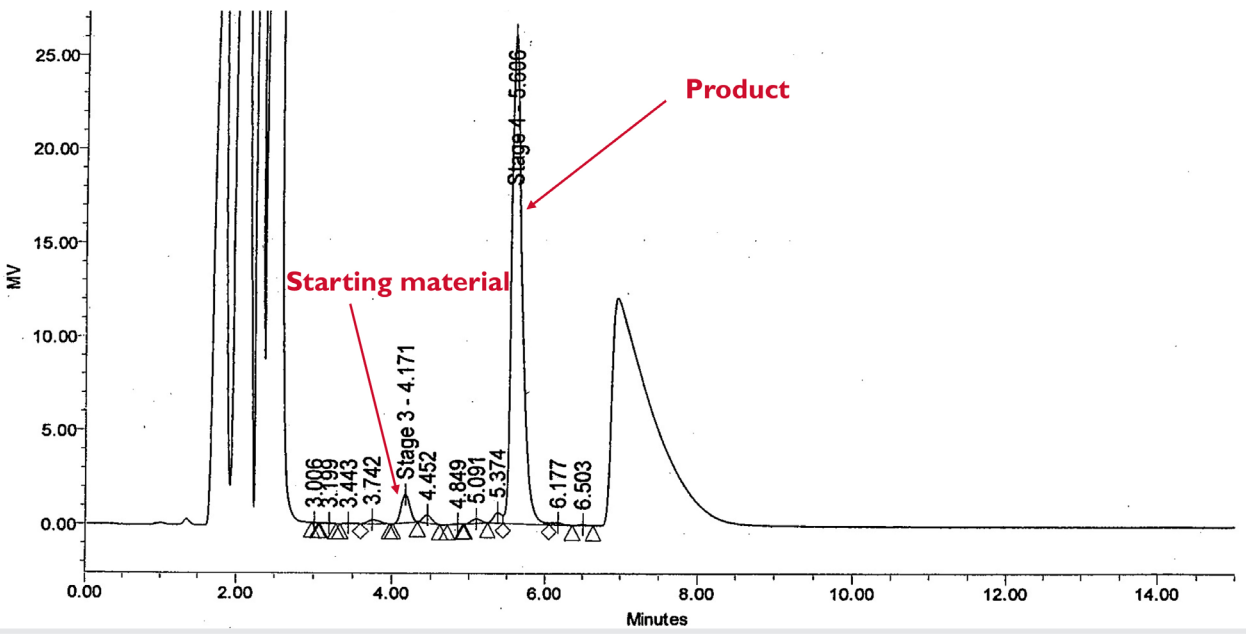
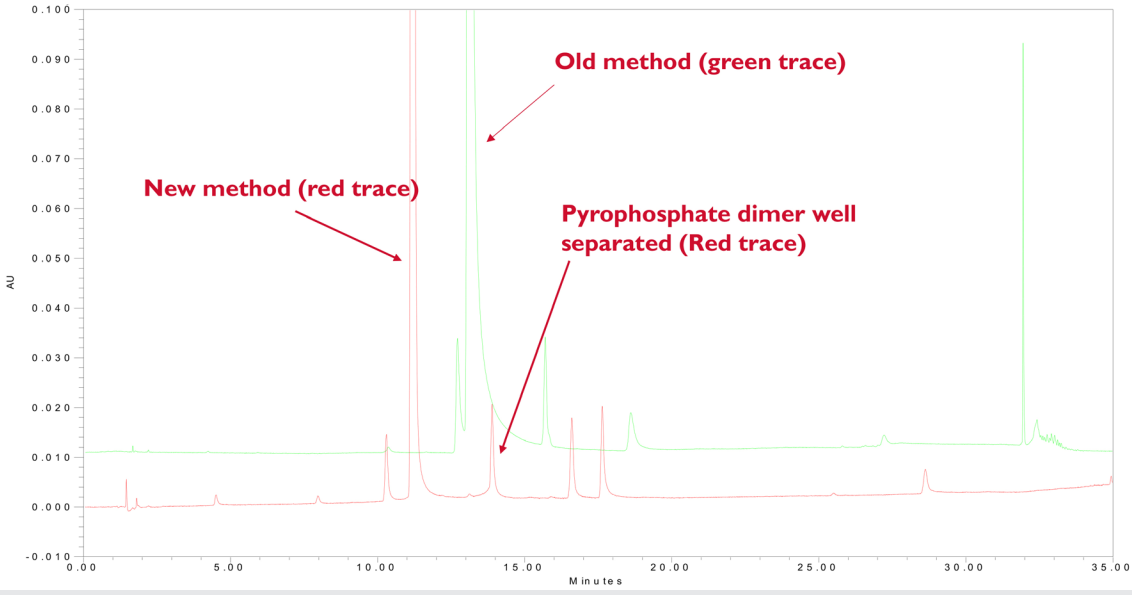


Figure 6: Redevelopment of an established method.



detected as its daughter ion, CH_3CO^+ at *m/z* of 43.

ANALYSIS OF HIGHLY POLAR COMPOUNDS BY HILIC

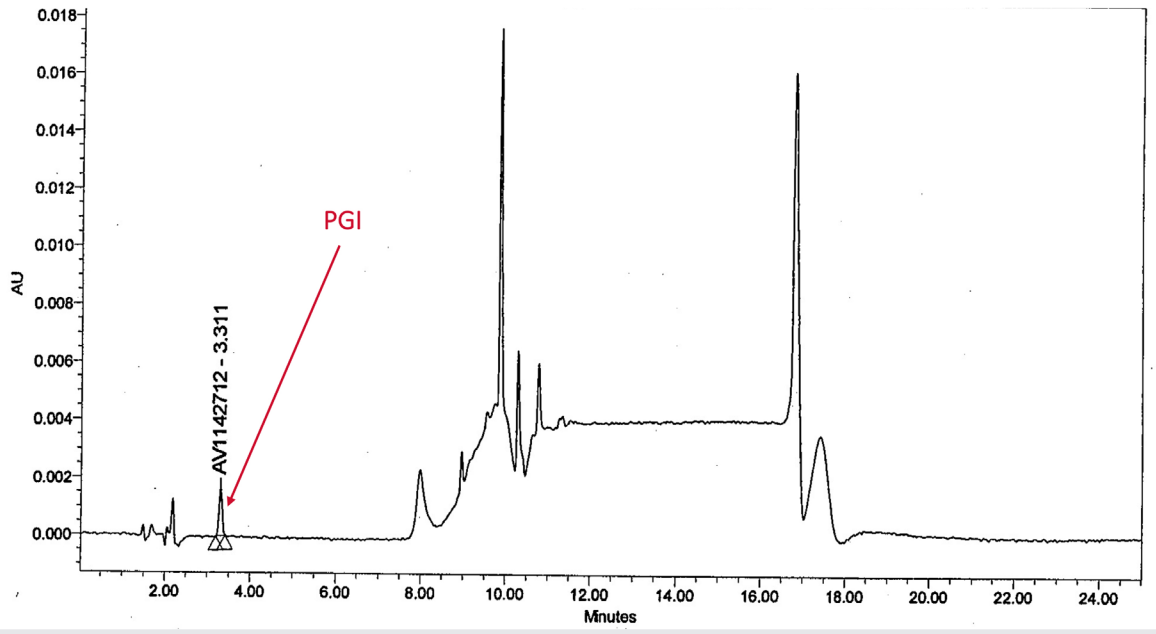
It is not easy to retain highly polar compounds using reversed-phase chromatography, and HILIC is frequently used for these applications. FIGURE 5 depicts a HILIC separation using a 50:50 ammonium acetate/acetonitrile mobile phase for a 5 mg/mL sample of a highly-polar starting material and product, a primary amine with two primary alcohols. To overcome the lack of a chromophore, a CAD was used. As shown in FIGURE 5, good retention was obtained with excellent resolution between the starting material and product, suitable for use as an in-process control and the release testing of the material.

REDEVELOPMENT OF ESTABLISHED METHODS

FIGURE 6 shows a redeveloped method (in red) of an older method (in green) that was delivering inconsistent results. The older method appeared to be modified over time, with the peak shape of the related substance deteriorating over time. The related substance was initially thought to have been removed during process optimization, but as it turned out, it was co-eluting with the main peak to the point where it could no longer be detected. Using a more modern hybrid C18 column and an MS-compatible ammonium formate mobile phase, the pyrophosphate dimer related substance was readily separated from the main peak and was much more reproducible across different column lots.

FIGURE 7 illustrates another example of developing a new hybrid C18 column method for the analysis of a 200 MW compound containing two amino groups and an amide, confirmed to be a GTI by a DEREK® test. HPLC was used for the analysis as the compound possessed a good

Figure 7: PGI at limit of 15 PPM.



UV chromophore, and sub-15 ppm detection limits were required. The method also required rapid elution of the GTI to avoid degradation on the column. It was validated as a limit test at the 15 ppm limit of quantitation, despite the overloaded API response.

CONCLUSION

Method development for the analysis of GTIs can be challenging due to GTI compound properties and the low detection limits required. Proper method development involves using all available resources, including literature references and vendor literature and services. While it is sometimes possible to adapt an existing method, starting with a generic or screening method and optimizing it according to method requirements and compound properties may be necessary, as the examples presented here illustrate.