Impurity analysis of phosphoramidites for producing oligo therapeutics

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Abstract
Solid-phase chemical synthesis based on phosphoramidite chemistry is one of the most widely employed approaches to synthesize therapeutic oligonucleotides. Many known groups of impurities in phosphoramidite synthesis are generated from self-generated nucleotide building blocks and control for them in the manufacturing process [1]. These complicated syntheses present the opportunity for many sources of potential impurities. This work goes on to characterize a typical 2’-fluoro-modified phosphoramidite that is used in production of oligonucleotides. 5’-dimethyltrityl-2’-fluoro-N-benzoyl-adenosine cyanethyl phosphoramidite (5’-DMT-2’-F-A(bz)-CEP). Impurity analysis of this phosphoramidite purchased from different vendors will be presented. Identification and structure elucidation of several dozen detected impurities was done using LC-MS and will also be presented in detail.

Introduction
With the growing interest in oligonucleotide therapeutics, analysis of impurities found in phosphoramidites (amidites) has taken on increased importance. Oligonucleotide therapeutics are produced from a variety of amidites and other starting materials. Common types of amidites and other materials used in producing phosphoramidites are shown in Figure 1.

Producing amidites of all types typically involve a multi-step reaction process [2]. These complicated syntheses present the opportunity for generation of impurities that can ultimately end up in an amidite being used as a starting material to manufacture an oligonucleotide therapeutic. Some typical impurities can be seen in Figure 2.

Results
The resulting UV and MS ion chromatograms for the analysis of the CEP phosphoramidite are shown in Figure 3, displaying the detection of several dozen impurities. LC-MS detects a number of impurities that are not readily detectable by UV.

Regulatory and industry guidance typically requires analytical methods to be able to detect and characterize impurities at levels down to or below 0.1% relative to the authentic raw material [3]. To establish the sensitivity of the LC-MS method for detection of impurities at or below the level required, spiked-in experiments were conducted using both the 5’-DMT-2’-F-A(bz)-CEP standard and dilution from this stock solution resulting from loss of the cyanethyl phosphoramidite group. Since this impurity was not detected at any level, it was instead spiked into the 5’-DMT-2’-F-A(bz)-CEP at relative concentrations ranging from 0.001% to 0.1% to model the case of an impurity that was not present in the sampled sample.

The resulting UV traces and positive-mode electrospray ionization extracted ion chromatograms (ESI(+) XICs) from the different injections of 5’-DMT-2’-F-A(bz), a potential impurity of 5’-DMT-2’-F-A(bz)-CEP. a) UV chromatogram and b) ESI(+) XIC of the impurity. Figure 3. Representative LC-MS chromatograms of 5’-DMT-2’-F-A(bz)-CEP. b) UV chromatogram and c) ESI(+) XIC of the impurity.

Figure 3. Representative LC-MS chromatograms of 5’-DMT-2’-F-A(bz)-CEP from vendor A at 1 mg/mL concentration (black trace), overlaid with solvent blank injection (red).

Materials and methods
Sample preparation
2’-modified RNA phosphoramidites were obtained from four different vendors (vendor A through D), with specified purity of 90% or higher. These amidites were then spiked into the final product, and they were used directly in the process [2]. The amidite analyzed in this work is 5’-DMT-2’-F-A(bz)-CEP.

Sample analysis by LC-MS
Impurity analyses were performed by LC-MS using a Thermo Scientific Vanquish™ Horizon UPLC system equipped with a diode array detector and coupled with the Thermo Scientific™ Orbitrap™ X300 mass spectrometer. The Thermo Scientific™ Chromatarn™ 7.2.10 Chromatography Data System (CDS) and Thermo Scientific™ Compound Discoverer™ 3.3 SP1 software were utilized for data analysis.

The data were first processed using the qualitative software in the Chromatarn 7.2.10 CDS to automatically detect all peaks in the total UV signal present at or above 0.01% relative intensity. Then, both expected and unexpected (i.e., “untargeted”) peak detection of the MS data were carried out using Compound Discoverer software, allowing detected UV peaks to be manually correlated to compounds detected in the MS data.

Expected compounds were generated by the software based on common transformations from the parent compound (including deamination, dehydration, reduction, methylolation, and combinations thereof), allowing for targeted compound extraction. Meanwhile, unexpected compound detection allowed for the unbiased detection of additional compounds, particularly in cases of substitutions or additions to the parent structure based on their relative abundances compared to a blank sample.

Based on the predicted composition and calculated transformation of the parent candidate, candidate structures could be proposed for the impurity. Using the acquired MS information generated with this software, it was possible to narrow down the possibilities and determine the site of incorporation to allow confident identification of the impurities as shown in Figure 4.

Conclusions
With the increased interest in therapeutic phosphoramidites, having a thorough analysis and characterization of phosphoramidite building blocks has gained importance. Here we highlight the need for highly sensitive, high-quality MS data to facilitate confident identification of phosphoramidite impurities and their profiling across different suppliers.

The analytical method’s suitability for sensitive detection of trace impurities at levels of 0.01%, and even lower, was demonstrated using an in-spike-in experiment.

Differences in the impurity profiles of 5’-DMT-2’-F-A(bz)-CEP from different vendors were readily determined, and while all investigated samples showed high purity exceeding the respective vendor specifications, different levels of impurities that are important to control for in the manufacturing of therapeutic phosphoramidites could be observed.

References
3. ICH: Q3A(R) Impurities in new drug substances (February 2003) and others.

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