Nucleic acid therapeutics

Impurity profiling and analysis of TheraPure GMP nucleotides

Nucleoside triphosphates (NTPs) are key building blocks of many nucleic acid therapeutics and vaccines. These molecules are a vital component of revolutionary new medicines. Because of the importance of NTPs as a critical material used in the production of drug substances, it is crucial to understand and control the impurities that may be present in these building blocks.

Here we discuss the impurities found in Thermo Scientific™ TheraPure™ GMP* nucleotides. These nucleotides have the performance and quality attributes necessary for use in therapeutics, and they have been successfully incorporated into many. TheraPure GMP nucleotides are manufactured to relevant ICH Q7 GMP guidelines and are produced using validated manufacturing processes to provide a high level of analytical purity and product consistency.

The data presented below review the impurities that were identified and measured in three TheraPure GMP nucleotides—ATP, CTP, and GTP.

Impurities in the TheraPure GMP nucleotide products are typically closely related to the NTPs and come from these sources:

- Side products of NTP synthesis
- Degradation of NTPs

Representative samples of each NTP (ATP, CTP, and GTP) were thoroughly analyzed using HPLC-UV and HPLC-MS methods; in some cases, ^{1}H and ^{31}P NMR spectroscopy was applied to profile all the impurities present in each NTP product.

ATP impurity profile

Upon analysis of several lots of TheraPure GMP ATP, two types of impurities were found. One of them was identified as adenosine diphosphate (ADP), a typical degradation product of ATP. Nucleoside diphosphates (NDPs) are the most common impurities found in all types of nucleotide products, as they are generated by NTP degradation in water. This hydrolysis leads to the loss of a phosphate group from the NTP to generate the diphosphate impurity as follows:

$NTP + H_2O \rightarrow NDP + H_3PO_4$

We found that the level of the ADP impurity usually does not exceed 0.21% in fresh samples. However, the level of ADP may increase over time, since ribonucleoside triphosphates like ATP are energetically unfavorable, inherently unstable, and easily hydrolyzed. ADP identity was confirmed by high-resolution mass spectrometry (HRMS): a mass of 426.0242 m/z was determined (theoretical mass of the molecular ion is 426.0221 m/z), with an accuracy of 4.93 ppm.

* "TheraPure GMP" refers to the quality level of the raw, ancillary, or starting materials to be used for further manufacturing. TheraPure GMP products are manufactured in facilities with ISO 9001–certified quality management systems operating in accordance with relevant good manufacturing practice (GMP) principles as outlined in ICH Q7 or equivalent guidance documents or standards.

The second impurity found at very low levels in some of the ATP samples is a side product of the ATP synthesis process: bis(adenosine)-5′-triphosphate (A_2P_3). This impurity was identified by HRMS with a confirmed mass of 755.0782 m/z with a 4.64 ppm accuracy. A summary of the analysis of several lots of ATP for this impurity is presented in Table 1.

Both of these impurities are not critical for function, since they do not contain modified nucleotide bases. The presence of these impurities in TheraPure GMP ATP is routinely monitored and controlled by the standard operational procedure used to analyze and release this product (reversed-phase HPLC: RP-HPLC).

A representative HPLC chromatogram of a lot of TheraPure GMP ATP (lot 7200) with both impurities present is shown in Figure 1A. An analysis of multiple lots of TheraPure GMP ATP is shown in Figure 1B.

CTP impurity profile

Two types of impurities similar to those seen for ATP were found in several lots of Therapure GMP CTP (Table 2). The first impurity is cytidine diphosphate (CDP). This is a hydrolytic degradation product of CTP. The identity of this impurity was confirmed by HRMS. The molecular ion of CDP was identified at 402.0124 m/z with a mass accuracy of 3.73 ppm. Levels of CDP are low in fresh samples but may increase with time, since CTP is energetically unstable, making degradation likely.

The second impurity identified in the TheraPure GMP CTP product was CTP-(γ-methyl). This particular impurity is a side product of CTP chemical synthesis. It is unique to CTP, as the manufacturing process for CTP is different from that of other nucleotides. The process of synthesizing CTP starts with cytidine undergoing a one-pot phosphorylation/diphosphorylation cascade. The chemical reaction is carried out in a solvent that can methylate phosphate groups. To confirm the identity of the CTP-(γ-methyl) impurity, HRMS analysis was conducted, followed by tandem mass spectrometry (MS/MS) of the extracted ion (Figure 2).

Table 1. ATP impurities.

ATP, 100 mM sodium salt		
Lot no.	**ADP %**	**A_2P_3 %**
7400	0.16	0
7300	0.21	0
7200	0.20	0.07
7100	0.11	0
7000	0.13	0.05
6900	0.12	0

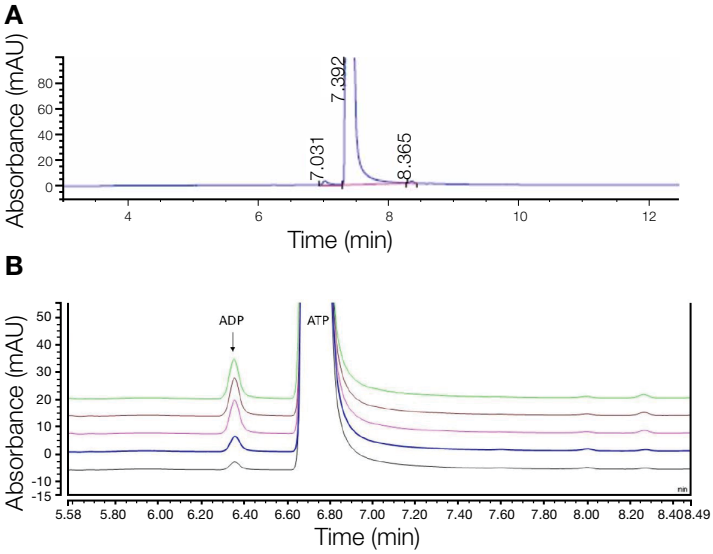

Figure 1. HPLC chromatograms. (A) ATP from lot 7200, and **(B)** ATP from multiple lots.

Table 2. CTP impurities.

CTP, 100 mM sodium salt		
Lot no.	**CDP %**	**CTP-(γ-methyl) %**
10400	0	0.12
10300	0.05	0
10200	0	0.15
10100	0	0.13
10000	0	0.11
9900	0.23	0.19

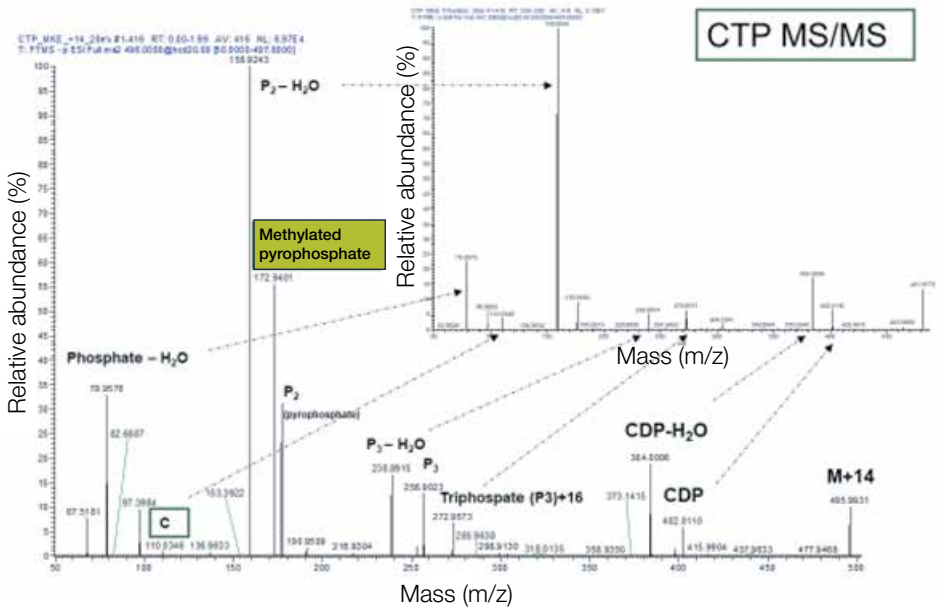

Figure 2. Compared MS/MS analyses of CTP-(γ-methyl) impurity and CTP.

The HRMS analysis identified the exact mass of the CTP-(γ-methyl) impurity as 495.9931 m/z, which corresponds to the theoretical mass of the methylated impurity. To evaluate the position of the methyl group, subsequent MS/MS analysis was performed. As shown in Figure 2, all degradation ions contain unmodified cytidine (CDP and cytidine). These and some other ions correspond to the expected CTP MS/MS spectra. The most important ion that was found is that with a mass of 172.9401 m/z, which corresponds to methylated pyrophosphate. This ion is missing in CTP mass spectra and is indicative of methylation of the triphosphate chain.

To further confirm the identity of the impurity, it was isolated by RP-HPLC and a more detailed structural analysis by NMR spectroscopy was performed.

The ^{1}H NMR spectrum of the isolated CTP-(γ-methyl) impurity is shown in Figure 3. There is a doublet of a methoxy group at 3.38 ppm with a coupling constant of ~11.5 Hz (due to vicinal coupling with ^{31}P) and signals of unmodified cytidine (doublets at 7.75 and 5.85 ppm) and ribose rings (signals at 5.62 and between 4.08 and 3.90 ppm).

The ^{31}P NMR spectrum of the isolated CTP-(γ-methyl) impurity is shown in Figure 4. There are signals corresponding to the triphosphate moiety: a triplet at –23.93 ppm and doublets at –12.20 and –10.50 ppm. It is important to note that the largest shift in comparison to the signals for natural CTP is for terminal phosphate nuclei (signal at –10.50 ppm). This means that the modification of the phosphate is on the terminal γ-phosphate.

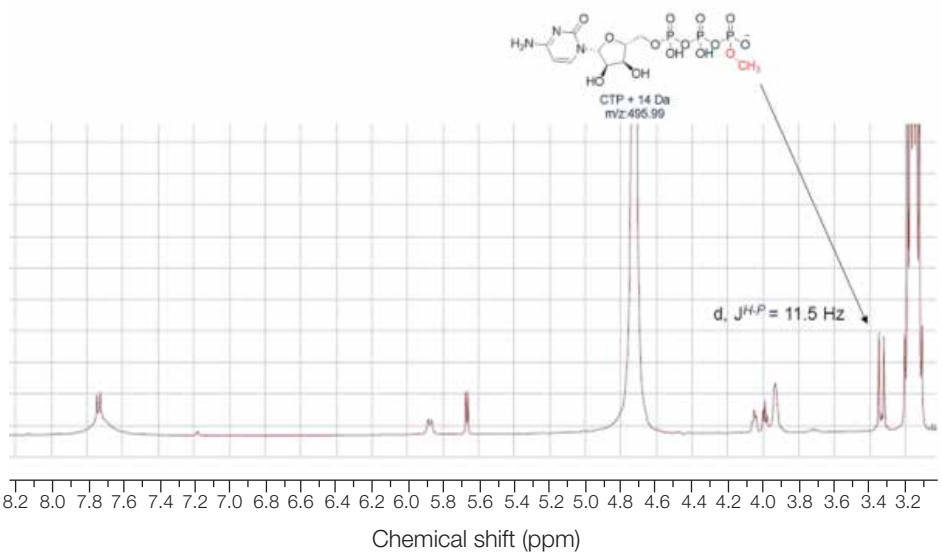

Figure 3. ^{1}H NMR spectrum of the CTP-(γ-methyl) impurity.

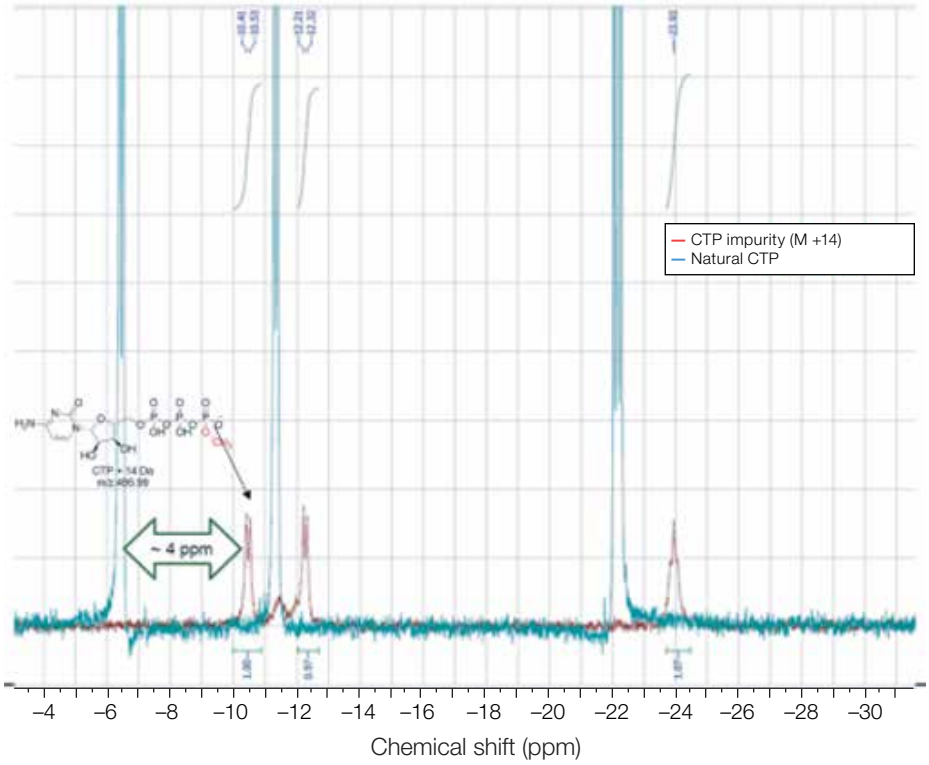

Figure 4. ^{31}P NMR spectra of CTP-(γ-methyl) impurity (red) and CTP (blue).

Regarding the CTP-(γ-methyl) impurity identified in the TheraPure GMP CTP product, it is important to note that this impurity is not critical for CTP's role in the formation of mRNA, as phosphates in both the terminal and middle positions are eliminated during the formation of phosphodiester bonds in an mRNA molecule:

N^1-P(α)-P(β)-P(γ) + N^2-P(α)-P(β)-P(γ) + … ➙ N^1-P(α)-N^2-P(α)-…

It also important to note that during the synthesis of CTP, modification of the α-phosphate is not possible; the α-phosphate remains electrophilic by nature, but methylation requires presenting of an HO-PO_3 (or nucleophilic) phosphate group. Thus, formation of a methylated impurity at this important phosphate position on the nucleotide is prevented.

Figure 5 shows a representative HPLC chromatogram as well as an analysis of multiple lots of CTP.

GTP impurity profile

When analyzing TheraPure GMP GTP, three types of impurities were identified in several lots of the product, as listed in Table 3.

More impurities were seen with GTP than with other NTP products. For example, in one of the lots of GTP that was analyzed, unreacted starting material guanosine monophosphate (GMP) was found at low levels (0.08%). GMP was seen only in one lot of GTP. The most common impurity identified is a product of hydrolytic degradation—guanosine diphosphate (GDP). This impurity is found at levels spanning the limit of detection (LOD) of the assays (0.05%) up to 0.6–0.7%.The third impurity identified is bis-(guanosine)-5′-triphosphate (G_2P_3). This impurity is a side product of the chemical reaction used to synthesize GTP and is formed from the interaction of activated GMP with GDP.

Identities of the GMP, GDP, and G_2P_3 impurities were confirmed by HRMS analysis and comparing them with standards. None of these impurities is considered critical, since they do not contain modified bases and cannot be incorporated into mRNA molecules.

Figure 6 shows a representative HPLC chromatogram as well as an analysis of multiple lots of GTP.

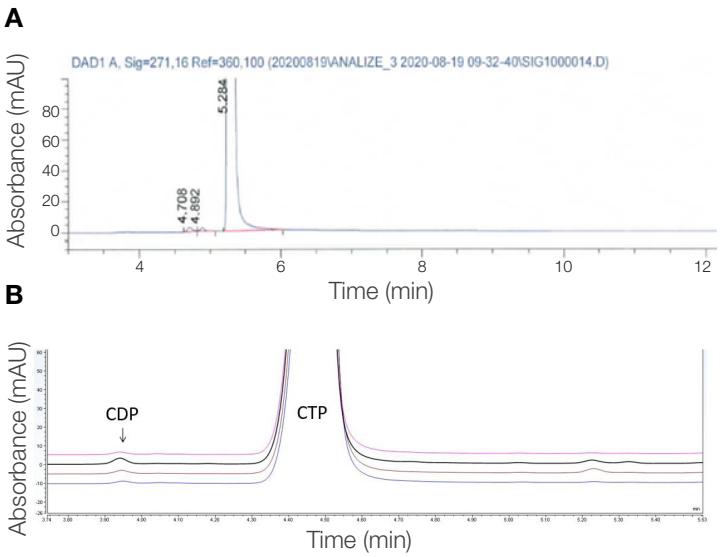

Figure 5. HPLC chromatograms. (A) CTP from lot 9900, and **(B)** CTP from multiple lots.

Table 3. GTP impurities.

GTP, 100 mM sodium salt			
Lot no.	**GMP %**	**GDP %**	**G_2P_3 %**
10700	0	0.09	0
10600	0.08	0.61	0
10500	0	0.15	0.05
10400	0	0.06	0
10300	0	0.20	0
10200	0	0.09	0.04

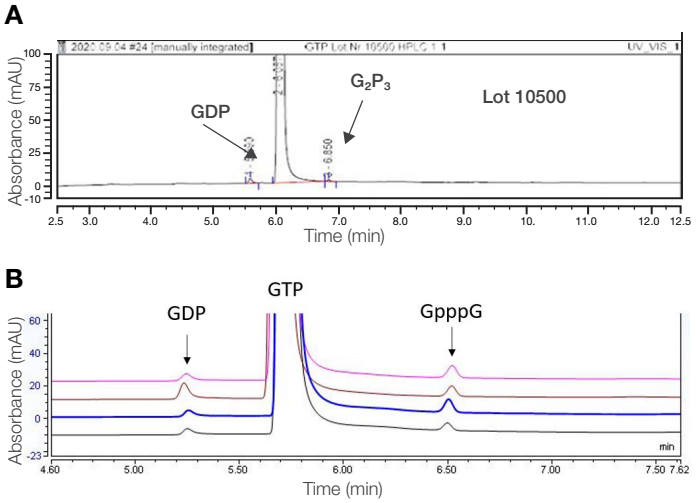

Figure 6. HPLC chromatograms. (A) GTP from lot 10500, and **(B)** GTP from multiple lots.

Conclusions

The quality of nucleoside triphosphates (ATP, CTP, GTP, and UTP) that are used for the production of mRNA therapeutics is extremely important. It is critical to identify any impurities that may exist in the crucial building blocks of a therapeutic. In this study, we examined the impurity profiles of several TheraPure GMP nucleotides (ATP, CTP, and GTP). In each product, a very limited number of impurities (2–3) was identified. These impurities were shown to be generated either by hydrolytic degradation of the energetically unfavorable NTP or as minor side products resulting from the chemical synthesis of the NTP.

In all TheraPure GMP NTP products analyzed, all significant impurities were identified and characterized. By analyzing several lots of each of these NTP products, it was ascertained that the impurities are present at very low levels. None of the identified impurities is sufficiently critical that it can interfere with the synthesis of mRNA when using these nucleotides, nor can any of these identified impurities be incorporated into mRNA synthesized from the NTP building blocks.

Finally, impurity profiling of several lots of TheraPure GMP NTP products shown here demonstrates the excellent consistency between lots in terms of their overall purity. The levels of impurities are consistent, and the types of impurities found in each NTP product are also consistent. This is most likely the result of using validated manufacturing processes that are tightly controlled to produce the same product in every lot and minimize lot-to-lot variability.

Ordering information

Description	Quantity	Cat. No.
TheraPure GMP ATP, 100 mM sodium solution	3.5 mL	R044SKB001
	17 mL	R044SKB002
	50 mL	R0441SKB003
	500 mL	R0441SKB009
TheraPure GMP CTP, 100 mM sodium solution	3.5 mL	R045SKB001
	17 mL	R045SKB002
	50 mL	R0451SKB003
	500 mL	R0451SKB009
TheraPure GMP GTP, 100 mM sodium solution	3.5 mL	R046SKB001
	20.5 mL	R046SKB002
	50 mL	R0461SKB003
	500 mL	R0461SKB009
TheraPure GMP UTP, 100 mM sodium solution	3.5 mL	R047SKB001
	20 mL	R0471SKB006
	100 mL	R0471SKB002
	500 mL	R0471SKB003

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