Optimization of IPRP-UPLC Method to Prevent PS to PO Conversion on Column

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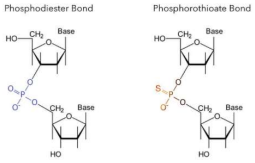
Abstract

Several chemical modifications have been developed in antisense therapeutic drugs to date. The phosphorothioate (PS) bond is one of the original and most widely-used backbone variants. PS bond oligo-modification alters the phosphodiester (PO) bond by replacing one of the non-bridging oxygens with a sulfur atom. This modification improves the metabolic stability and cellular uptake of oligonucleotide without affecting their affinity for target. During development of the analytical IPRP method for compound X, which contains PS backbone, we observed a phenomenon that PS bond was being oxidized to a PO bond on-column. Here, we present an approach to improve an IPRP method that prevents on column PS to PO oxidation by utilizing synergetic effects of temperature, addition of EDTA and DTT into the mobile phase.



Materials & Methods

Instruments:

- Waters ACQUITY UPLC H-Class
- Xevo G2-S ToF

Column:

- Waters ACQUITY Premier Oligonucleotide C18 Column, 130Å, 1.7 µm, 2.1 x 100 mm, Part # 186009485

Reagents:

- **Water**, LC/MS Grade, Fisher, W6-4
- **HFIP**, ≥ 99%, TCI, H0424
- **DIPEA**, 99.5%, Sigma, 496219
- **Acetonitrile**, LC/MS Grade, Fisher, A955-1
- **DTT**, 98%, Sigma, D9779
- **EDTA**, ≥99.5%, Fisher, #BP118-500

Compound X:

- Oligonucleotide with PS bond modification

Software:

- Empower 3
- MassLynx

Original Method Condition:

- **Column Temp:** 80 ºC
- **Mobile Phases:**
  A: 1% HFIP, 0.1% DIPEA in water
  B: 40% Acetonitrile in water
- **Gradient:**

Time	%A	%B	Curve
Initial	98.0	2.0	*
2.0	98.0	2.0	Linear
10.0	71.0	29.0	Linear
32.0	61.0	39.0	Linear
33.0	50.0	50.0	Linear
34.0	98.0	2.0	Linear
41.0	98.0	2.0	Linear

Results

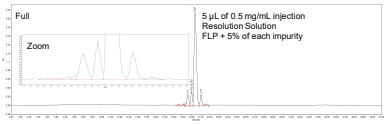


Fig. 1: LC chromatogram for the injection of resolution solution in August 2021 using original method. Good resolutions (USP) was achieved with the original developed method. The minimal resolution (USP) is 0.9 (between FLP peak and PS to PO peak).

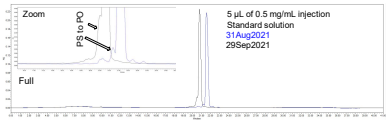


Fig. 2: Overlay LC chromatogram of two injections of the standard solution one month apart. The LC profiles were significantly different and the %Area for PO impurity increased from **2.7%** in Aug2021 to **8.2%** in Sep2021. Both samples were prepared freshly.

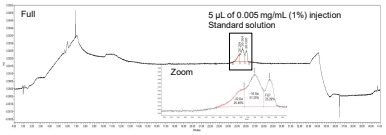


Fig. 3: LC Chromatogram of 1% level of standard solution injection. Three peaks were observed when the sample was injected at 1% level. Using MS, three peaks were identified as di-PS to PO impurity, mono-PS to PO impurity and FLP, respectively. This data indicates that PS is oxidated to PO on column.

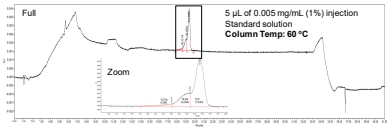


Fig. 4: LC Chromatogram of 1% level injection with column temperature at 60 ºC. Reducing column temperature partially prevented the PS oxidation to PO. FLP purity increased to ~72%. Mono-PS to PO impurity and di-PS to PO impurity decreased to ~24% and ~4%, respectively. This indicates that high column temperature partially drives PS to PO conversion.

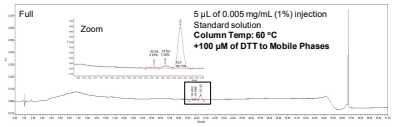


Fig. 5: LC Chromatogram of 1% level injection with additional 100 µM of DTT in mobile phases. Adding reducing agent DTT to mobile phases minimized the PS conversion to PO. FLP purity increased to ~90%. Mono-PS to PO impurity and di-PS to PO impurity decreased to ~7% and ~3%, respectively. This indicates that DTT inhibits the PS oxidation to PO.

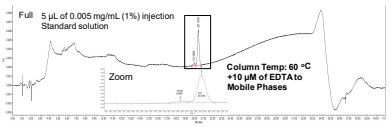


Fig. 6: LC Chromatogram of 1% level injection with additional 10 µM of EDTA in mobile phases. Adding chelating agent EDTA to mobile phases also inhibited the PS conversion to PO. FLP purity increased to ~93%. Mono-PS to PO impurity decreased to ~7% and no di-PS to PO impurity was detected. This appears to indicate that cation metal contributes to the PS oxidation as well.

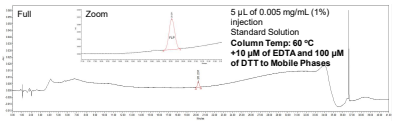


Fig. 7: LC Chromatogram of 1% level injection with additional 10 µM of EDTA and 100 µM of DTT in mobile phases. Adding both DTT and EDTA to mobile phases completely prevented PS conversion to PO. Only one FLP peak was observed and no PS to PO impurity was detected using this condition.

Final Method

- **Column Temp:** 60 ºC
- **Mobile Phases A:** 1% HFIP, 0.1% DIPEA, 10 µM of EDTA, 100 µM of DTT in water.
- **Mobile Phases B:** 40% Acetonitrile, 10 µM of EDTA, 100 µM of DTT in water.
- **LG Gradient:**

Time	%A	%B	Curve
Initial	98.0	2.0	*
2.0	98.0	2.0	Linear
10.0	69.0	31.0	Linear
32.0	59.0	41.0	Linear
33.0	40.0	60.0	Linear
34.0	40.0	60.0	Linear
35.0	98.0	2.0	Linear
42.0	98.0	2.0	Linear

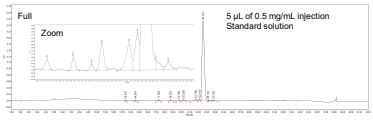


Fig. 8: LC chromatogram of standard solution injection using the final method. The FLP purity is ~92% and the %Area for PS to PO impurity is ~2%.

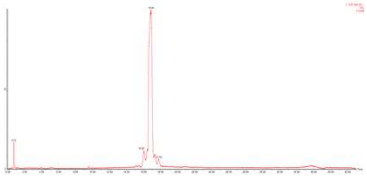


Fig. 9: Total Ion Count TOF mass spectrum of standard solution injection using the final method. Addition of DTT and EDTA in the mobile phases did not affect the MS responses. The MS signal for the FLP peak is as high as 5.53 X 10^6.

Conclusion

- The PS bond on compound X appears to favor being converted to PO using the originally developed method.
- The PS to PO oxidation is probably attributed by the high temperature and cation metals.
- Lower temperature, metal chelating agent (EDTA) and reducing agent (DTT) all contribute to prevent the PS to PO conversion.
- Addition of DTT and EDTA in the mobile phases do not affect the UV and MS responses.

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