Mass Spectral Accuracy Applied to the Quantitative Analysis of Deamination Impurities in Oligonucleotide Therapeutics

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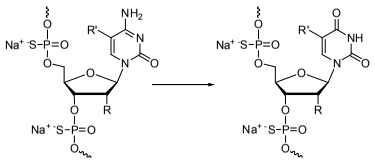
Introduction

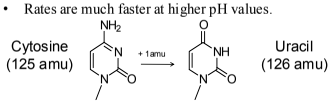
Deamination of cytosine and 5-methylcytosine residues of DNA and RNA, resulting in the formation of uracil and thymine, respectively, constitutes an important degradation pathway for oligonucleotides therapeutics. Unfortunately, deamination is difficult to detect and quantify because the deaminated components cannot easily be resolved chromatographically from the parent molecule. Furthermore, detection and quantification by mass spectrometry is hampered by the fact that the deaminated oligonucleotides have masses increased by only one mass unit from the non-deaminated species, which is present at much higher levels. For larger oligonucleotides, the overlapping isotopic distributions cannot be sufficiently resolved even with high resolution mass spectrometers, especially since electrospray ionization requires the analysis of higher charge states.

Hydrolytic Deamination of Cytosine to Uracil and 5-Methylcytosine to Thymidine

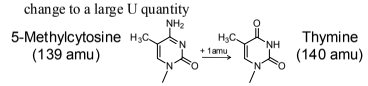


Some Facts about Deamination

- Deamination rates at 37°C and pH 7.4 for cytosine and 5-methylcytosine residues in ssDNA are 2e-10s-1 and 1e-9s-1, respectively [ref 1], or
- After one year at pH 7.4 and 37°C, a sequence with four 5-methylcytosine residues will contain about 11% of molecules with one deamination site.
- Rates are slower for ssRNA, dsDNA, dsRNA.
- Rates are much faster at higher pH values.



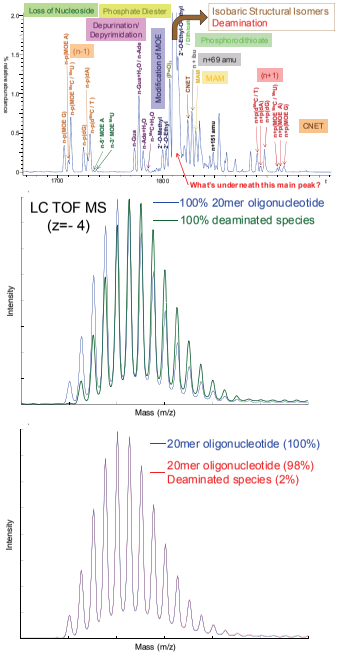
- For DNA, one may digest and measure amount of U as DNA does not contain Uracil bases.
- For RNA, most likely U bases are already present in non-deaminated sequence, requiring detection of a small change to a large U quantity



- For DNA, most likely T bases are already present in non-deaminated sequence, requiring the detection of a small change to a large T quantity via digestion.

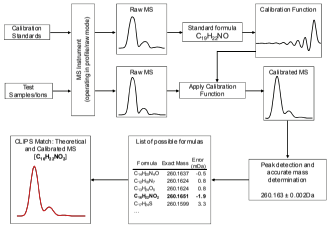
Method

Measuring deamination is difficult at the oligonucleotide level even with the most advanced MS instrumentation because the parent compound and the singly deaminated compound are only 1 mass unit apart (0.25 amu at the z=4 charge), which would be extremely difficult to detect from typical impurity profiling experiments.

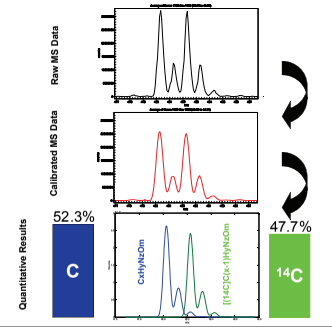


While difficult to detect the spectral differences corresponding to 1-2% of the deamination impurity in the presence of 98-99% of the non-deaminated species, there are consistent and systematic differences between the two mass spectra above and beyond the random noise in measurement. In order to discern such level of spectral differences, a novel calibration involving both m/z and MS peak shape needs to be performed to allow for exact isotope modeling based on the known elemental compositions of the species involved and for the accurate calculation of the relative concentrations of these species in a mixture [ref 2].

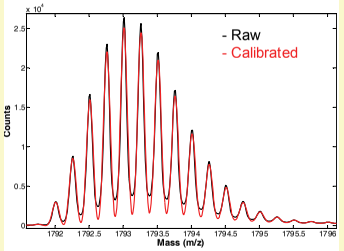
Novel MS Calibration and Spectral Accuracy



Spectral Accuracy for Mixture Analysis

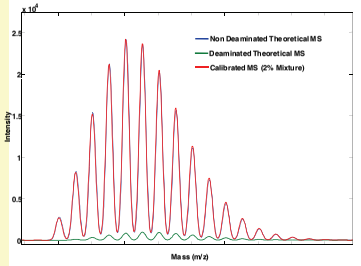


Results and Discussion

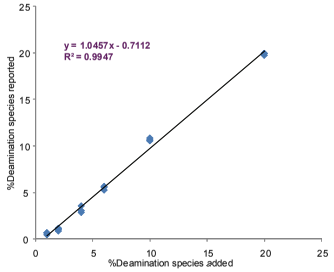


With one or multiple standards of known elemental compositions, the mass spectrum measured of a multiply charged oligo can be calibrated with both accurate mass and, more importantly, a known mathematical peak shape function, enabling exact modeling in the next step of mixture analysis for the quantitation of deamination impurities.

LC TOF Infusion Data

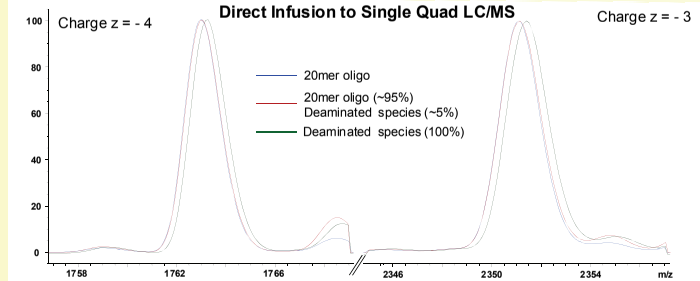


Calibration Curve: Reported vs Actual



When the reported percent concentration of the deaminated species is plotted against the known spiked %deamination, a good linear regression line is obtained from 20% all the way down to 1% through this triplicate experiments. Though both intercept and slope are significantly different from 0.0 and 1.0, which deserves further study, the detection of deamination at practically meaningful level of 1-5% is quite achievable.

Direct Infusion to Single Quad LC/MS



Initial experimentation with a single quad LC/MS has also demonstrated the presence of observable and consistent spectral differences between the oligo and its deaminated species, at least to 5% level, indicating the possibility of deamination analysis on a routine workhorse LC/MS instrument.

Conclusions

- Tiny mass spectral differences are of high importance in differentiating and quantitating closely related species such as deamination and its oligo counterpart.
- In order to exploit small mass spectral differences in a meaningful way, the mass spectral data have been calibrated both in terms of m/z and, more critically, in its peak shape.
- With TOF LC/MS, oligo deamination impurities can be determined down to the practically important level of 1.0%.
- A performance similar to or at least approaching that of a TOF LC/MS may be possible with just a single quad LC/MS, though further experimentation will be required.

References

1. Ehrlich, M. et al, *Biosci. Rep.* **1986**, *6*, 387-393.
2. Wang, Y.; Gu, M. *Anal. Chem.* **2010**, *82*, 7055-7062.