Analysis and Purification of RNAi and siRNA

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OVERVIEW

- Oligonucleotides are a rapidly emerging class of biopharmaceutical compounds which present unique analytical challenges.
- Sequences are generated via high yielding step-wise synthesis, however they often require analysis and purification prior to use.
- There is a desire for MS new compatible mobile phases for single stranded and duplex oligonucleotide separations.
- New mobile phases should provide sufficient resolution of single stranded and duplex oligos with adequate MS compatibility.
- A systematic comparison of resolving power and MS compatibility of mobile phase modifiers for oligonucleotide separations is presented.
- UPLC/MS analysis of siRNA and its corresponding impurities and intact siRNA UPLC/MS analysis methods using new mobile phases are presented.
- Semi-preparative methods for purification of both single stranded and siRNA which are fully scalable are presented.

HOMOMERIC OLIGONUCLEOTIDE SEPARATIONS

UV 260nm DETECTION

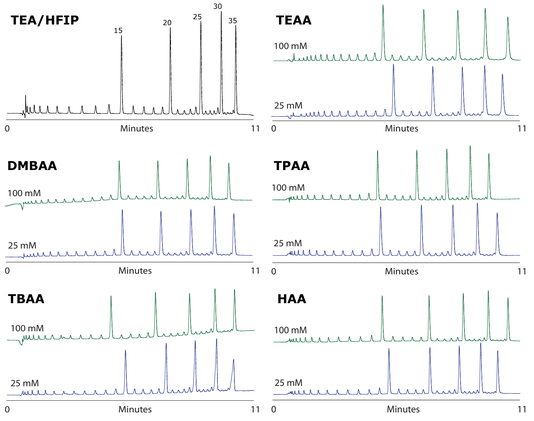

Figure 1: Separations were accomplished with a Waters ACQUITY UPLC® System using a Waters Oligonucleotide Separations Technology column (ACQUITY UPLC® OST C18, 1.7µm, 2.1x50) maintained at 60 °C. On column loading was 20 pmol/oligo of Waters MassPREP™ OST standard.

SINGLE QUADRUPOLE MS DETECTION

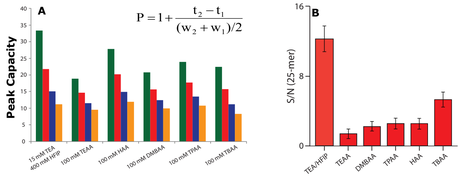

Figure 2: (A) Peak capacities for mobile phases at 100 mM for data shown in Figure 1. (B) Signal to Noise determined from three separate analysis of 25-mer oligo dT via single quadrupole MS.

- Gradient adjusted so 15-mer eluted at 5 minutes and 35-mer at 10 min.
- Generally, the more hydrophobic IP agents (longer alkyl chain) required higher ACN content for oligo elution.
- Mobile phases prepared by from equimolar ratios of acetic acid and appropriate base, pH adjusted to ≈ 7.0

RNA DUPLEX ANALYSIS

- LC analysis of siRNA is difficult due to melting and incomplete resolution from mis-matched duplexes and other impurities.
- OST column technology coupled with HPLC and UPLC, using duplex compatible mobile phases, solves these problems by giving predictable retention and non-denaturing separation conditions.
- Use of IP RP HPLC and UPLC analysis of duplexes can be interfaced with MS to provide MS data unlike PAGE and AX-HPLC

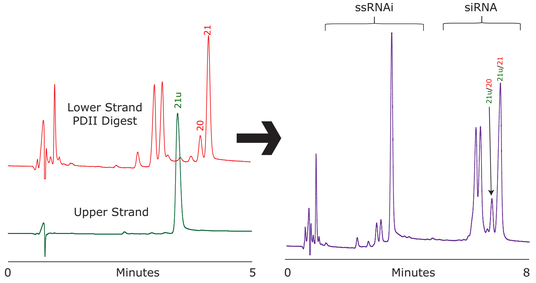

Figure 3. Separation of mis-matched duplexes via UPLC with HAA mobile phase. ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm column, 60 °C, UV 260 nm.

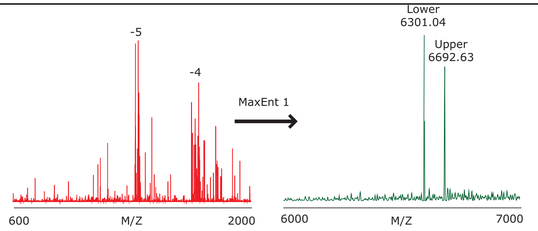

Figure 4: Raw and MaxEnt 1 deconvoluted spectra collected with single quadrupole detector from truncated siRNA separation shown in Figure 3. Data shown is for full length upper strand hybridized with N-1 lower strand (21u/20).

UPLC/MS ANALYSIS OF INTACT siRNA

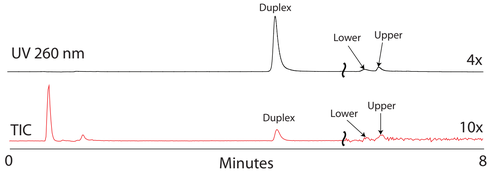

Figure 5. UPLC separation of siRNA. Mobile Phase A: 20 mM ammonium acetate, pH 6.5 B: Acetonitrile. ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm, 20 °C, UV 260 nm.

Polarity	ES-
Analyzer	V Mode
Capillary	2.7 kV
Sample Cone	31.0 V
Extraction Cone	3.0 V
Source	150 °C
Desolvation	350 °C
Cone Flow	0.0 l/h
Desolvation Flow	800 l/h

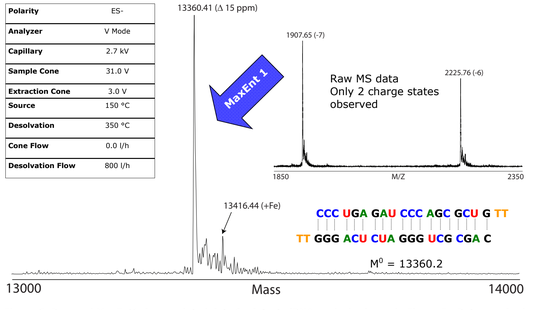

Figure 6. Raw and MaxEnt1 deconvoluted data for intact siRNA analysis shown in Figure 5. Data acquired using Synapt HDMS mass spectrometer.

RNA PURIFICATION

- Synthetic oligonucleotides are prepared by stepwise coupling
- Coupling efficiencies of 99.5% yield a 21 mer of approx. 90%
- Many applications require greater purity

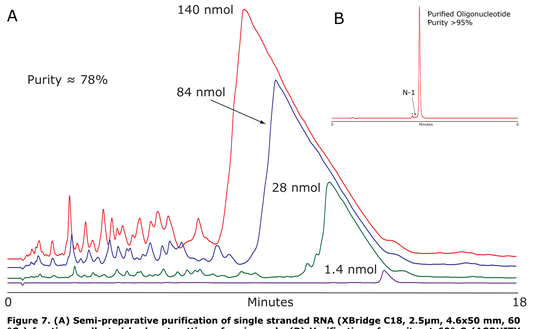

Figure 7. (A) Semi-preparative purification of single stranded RNA (XBridge C18, 2.5µm, 4.6x50 mm, 60 °C) fractions collected by heart-cutting of main peak. (B) Verification of purity at 60° C (ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm, 60 °C).

siRNA PURIFICATION

- Purification of siRNA is necessary to ensure specificity
- Purification accomplished by *on-column annealing* of siRNA
  - Inject first strand under initial gradient conditions immediately followed by injection of second complementary strand
  - After injection of second strand, gradient started to elute products
- Analysis of collected fraction confirms desired full length duplex composed of equimolar ratio of each complementary strand

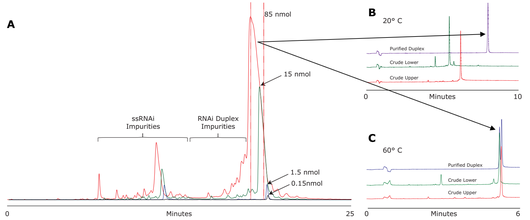

Figure 8. (A) Semi-preparative purification of siRNA via on column annealing of complementary single stranded RNA (XBridge C18, 2.5µm, 4.6x50 mm, 20 °C). (B) and (C) verification of duplex purity at 20° C and 60° C respectively (ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm).

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