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CHARACTERIZATION OF OLIGONUCLEOTIDE-BASED DRUGS BY CGE AND LC/MS

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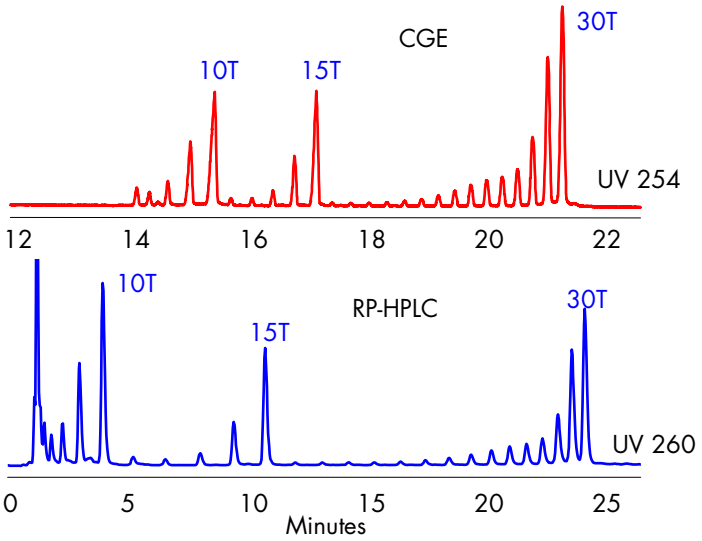
INTRODUCTION

Synthetic oligonucleotides are utilized for diagnostic and therapeutic purposes (antisense drugs). Synthesis of chemically modified oligonucleotides is challenging and often results in a product of limited purity. Electrophoresis is a traditional method for oligonucleotide separation and quality control (QC). However, it is difficult to interface with mass spectrometry (MS) and scale up for oligonucleotide purification purposes. We developed a high-performance liquid chromatography (HPLC) method that provided a resolution comparable to capillary gel electrophoresis (CGE). The method is scaleable for oligonucleotide purification without loss of resolution. The micro-HPLC column was coupled on-line with MS detection for the characterization of oligonucleotide-based drugs and diagnostic probes. The mobile phase was MS-friendly and optimized for maximum separation. The ion-pairing buffer based on triethylamine acetate (TEAA) was used for routine QC and purification, while a triethylamine-hexafluoroisopropanol (TEA-HFIP) buffer was employed for LC-MS, and for special applications such as separation of phosphorothioate and guanidine-rich oligonucleotides. Due to the chaotropic properties of TEA-HFIP buffers, the resolution of oligonucleotides with extremely strong secondary structure was possible. We also investigated the separation of diatereomers of phosphorothioate oligonucleotides in HPLC and CGE systems.

EXPERIMENTAL

HPLC system:	Waters Alliance® 2695 Separations Module.	
Capillary System:	Waters CapLC® System	
HPLC conditions:	See individual figure captions	
MS instrument:	Micromass® LCT™ ESI-TOF Mass Spectrometer	
MS conditions:	Capillary:	2000 V
	Cone	18 V
	Desolvation Temp:	120°C
	MCP:	2700 V
	Spectra were deconvoluted using MaxEnt1	



CGE:
Column: BioCAP™ 75µm, 27.5/34.5 cm
Temp: 30 °C
PEG sieving matrix, 15 kV run, 4s injection at 5kV

HPLC:
Column: XTerra® MS C18, 50 x 4.6 mm,2.5 µm (Part#186000602)
Temp: 60 °C
Flow: 0.5 ml/min
Mobile Phase
A: 10 % MeOH in 400mM HFIP, 16.3 mM TEA, pH 7.9,
B: 40 % MeOH in 400mM HFIP, 16.3 mM TEA, pH 7.9
Gradient: linear gradient from16.7 % to30 % B at 10 min, then from 30 % B to 47.15 % B at 30 min.

Figure 1: *RP-HPLC offers a similar performance for the separation of oligonucleotides as capillary gel electrophoresis.*

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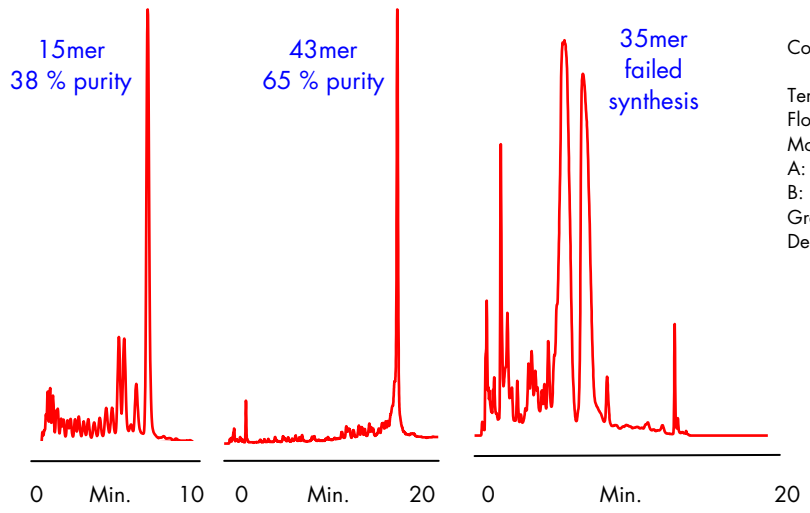
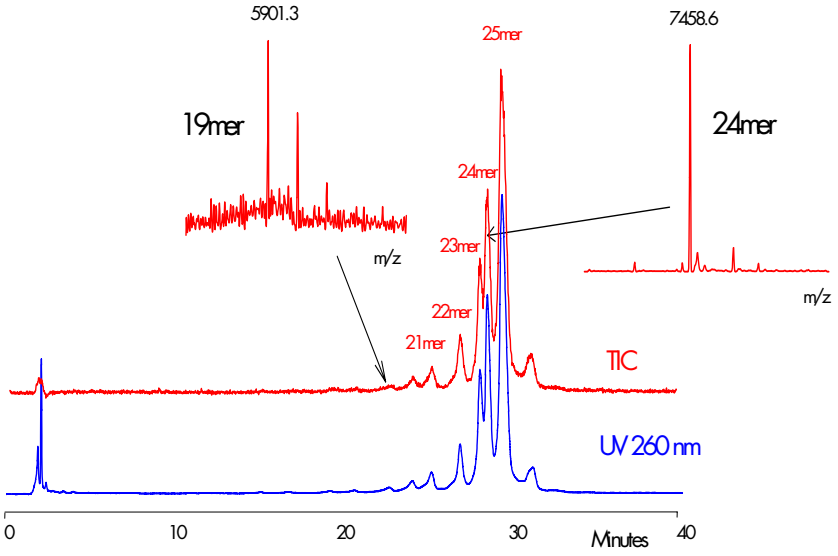


Column: XTerra® MS C18, 4.6x50 mm,2.5 µm (Part # 186000602)
Temp: 60 °C,
Flow: 1ml/min,
Mobile Phase
A: 5 % ACN in 0.1 M TEAA, pH 7
B: 20 % ACN in 0.1 M TEAA, pH 7
Gradient: 9.3 % B to 44 % B in 20.8 Min.
Detection: UV 260 nm

Figure 2: *Quality control of crude synthetic oligonucleotides. Clear separation of n-1 failure product is achieved up to ~ 30 mer. For longer oligonucleotides the n-1 partially co-elute with the target products. Separation with TEAA buffers partially depends on the sequence (Gilar et al., J. Chromatogr. A, 958 (2002) 167-182).*



Column: XTerra® MS C18, 1.0x50 mm, 2.5 µm (Part # 186000979)
Temp: 60 °C,
Flow: 23.6µl/Min,
Mobile Phase
A: 5% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9
B: 60% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9
Gradient: 18.2% to 36.4% B in 40 minutes
Detection: UV 260 nm
Injection: 0.6 µl injection represents 0.8 µg of 25mer, which is 103 pmole; the 19mer was detected at the level of ~ 1.5 pmole.

Figure 3: *LC-UV-MS analysis of 25mer phosphorothioate drug digestion. Metabolites are separated from parent drug and identified by MS.*

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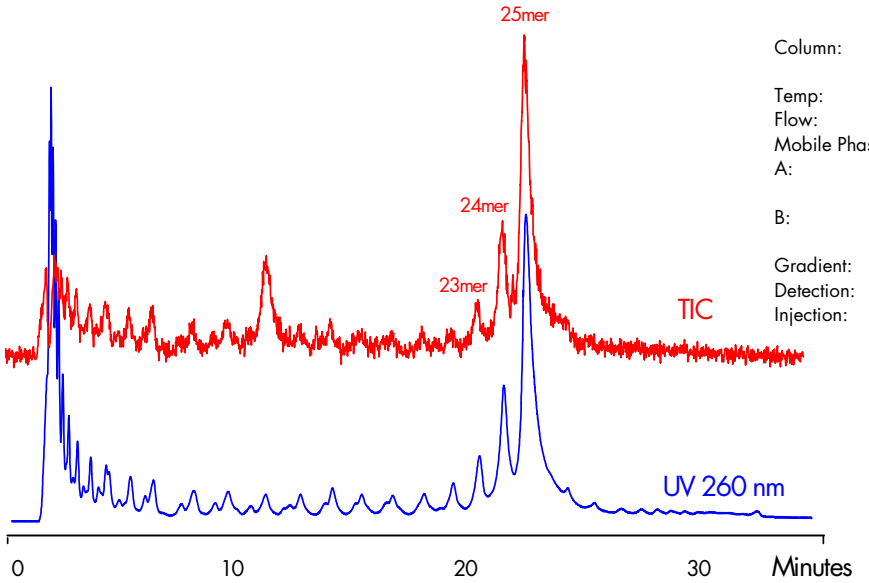
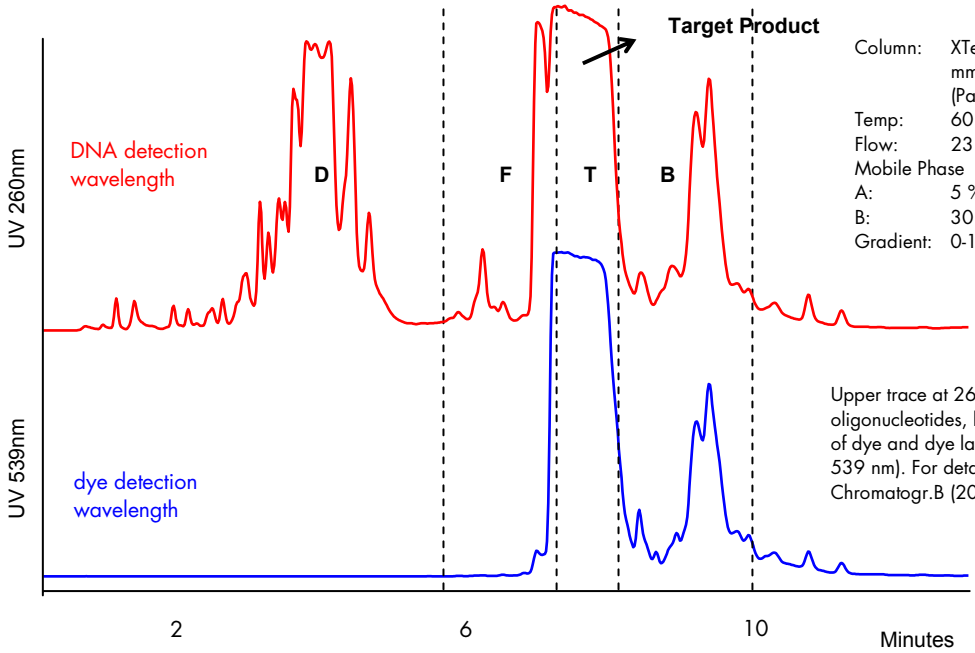


Column: XTerra® MS C18, 1.0X50mm,2.5 µm (Part #186000979)
Temp: 60 °C,
Flow: 23.6 µll/min,
Mobile Phase
A: 5% MeOH in 400mM HFIP16.3mM TEA, pH 7.9
B: 60% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9
Gradient: 14.5% to 69.1% B in 120 minutes
Detection: UV 260 nm
Injection: 2.5 µl injection; 2.6 µg (318 pmole) total mass load.

Figure 4: *LC-UV-MS analysis of crude synthetic 25mer oligoG (oligodeoxyguanidine). Failure sequences are clearly separated and identified by MS.*



Column: XTerra® MS C18, 4.6x50 mm,2.5 µm (Part #186000602)
Temp: 60 °C,
Flow: 23.6 µll/min,
Mobile Phase
A: 5 % ACN in 0.1 M TEAA, pH 7
B: 30 % ACN in 0.1M TEAA, pH 7
Gradient: 0-100 % B in 15 minutes

Upper trace at 260 nm shows signal of all oligonucleotides, lower trace represents a signal of dye and dye labeled oligonucleotides (λ max 539 nm). For details see Fountain et al., J. Chromatogr.B (2002), vol. 783, 61.

Figure 5: *Purification of 25mer 5'HEX labeled oligonucleotide. D fraction contains non-labeled failure sequences, F and B labeled failure oligonucleotides, T fraction was collected. 0.1 µmole was injected on column. Crude purity was 77 %, after purification ~ 94 %. Target yield was 85 %.*

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For separation conditions see reference: Gilar M., Belenky A., Cohen A.S., Electrophoresis (2000), vol.21, 2999. PVP sieving matrix act as a pseudo-stationary phase, providing for some hydrophobic interaction useful for separation of phosphorothioate diastereomers. Only partial resolution was achieved with hydrophilic PEG matrix. A highly hydrophilic polyacrylamide matrix does provide for pseudo-stationary phase interaction.

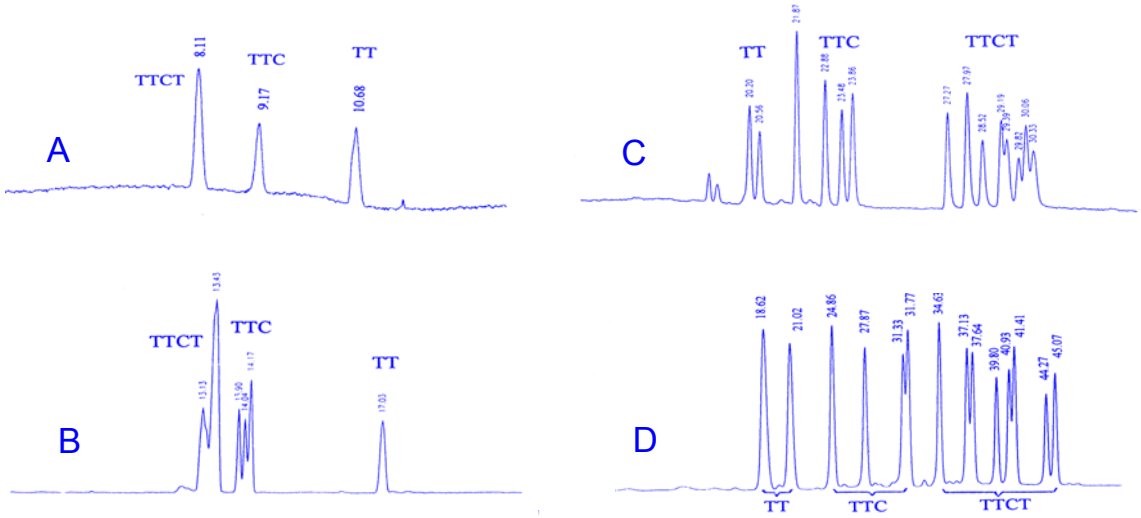

Figure 6: *Separation of diastereomers of 2mer TT (2 diastereomers), 3mer TTC (4 diastereomers), and 4mer TTCT (8 diastereomers). CGE with LPA matrix (A), PEG matrix (B), PVP matrix (C) and RP-HPLC (D) with C18 column.*

CONCLUSION

- Presented HPLC method provide a resolution comparable to CGE
- HPLC was applied for QC of oligonucleotides.
- HPLC with on-line MS method allows for characterization of therapeutic and diagnostic oligonucleotides
- HPLC method was used for oligonucleotide purification
- Separation of phosphorothioate diastereomers was achieved with both HPLC and CGE.
- We successfully analyzed
  - phosphorothioate oligonucleotides
  - G-rich oligonucleotide sequences
  - singly and dually labeled diagnostic oligonucleotides
  - locked nucleic acids

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