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Analysis of Therapeutic and Diagnostic Oligonucleotides Using Reversed-Phase Chromatography with Mass Spectrometry Detection

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Introduction

Synthetic oligonucleotides are utilized for diagnostic as well as therapeutic purposes (antisense drugs). Synthesis of chemically modified oligonucleotides is challenging and often results in a product of limited purity [1,2]. An LC-MS method has been developed for the characterization of oligonucleotide-based drugs and diagnostic probes [3]. Ion-pair reversed-phase HPLC was utilized for analysis of phosphorothioate oligonucleotides, dually labeled probes and native oligonucleotides.

LC-MS is useful for analysis and characterization of nucleic acids [4,5]. We used an optimized LC-MS system for analysis of synthetic oligonucleotides and their failure products. We explored mass-directed target fraction collection. Phosphorothioate as well as guanidine-rich antisense oligonucleotide drugs are known to be particularly difficult to analyze [6]. Due to the chaotropic properties of triethylamine-hexafluoroisopropanol (TEA-HFIP) buffers, we were able to successfully separate <60mer antisense oligonucleotides [3] and identify the failure oligonucleotides in a drug form.

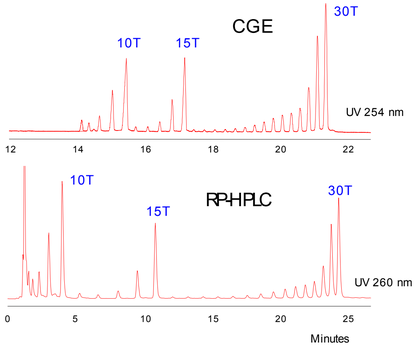
Guidelines for column selection and optimization of mobile phase composition are discussed. The ion-pair reversed-phase HPLC separation benefits from the use of 2.5 µm sorbent and elevated temperature. The separation performance was equivalent or better than ion-exchange HPLC and in some cases rivaled capillary gel electrophoresis separation. Using a 50 x 1 mm XTerra column, we were able to obtain molecular weight confirmation for ~ 1-10 pmole of oligonucleotides injected.

Experimental

HPLC system: CapLC®, Waters, equipped with a photodiode array detector
Column: 50 x 1 mm XTerra® MS C18, 2.5 µm
Column temperature: 50-60 °C
Mobile phase flow rate: 23.6 µl per minute.
HPLC conditions: see figure captions

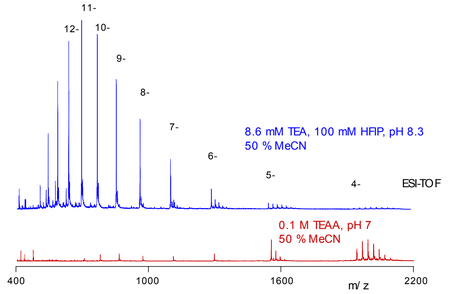
Ion pairing buffers: Triethylamine ion-pairing agent (8.6 mM) was buffered with 100 mM hexafluoroisopropanol (weak acid), TEA-HFIP buffer pH was 8.3. Alternatively, 16.3 mM – 400 mM TEA-HFIP buffer, pH 7.9 was used. Oligonucleotides were eluted from the column with a methanol gradient.
MS instrument: ESI-TOF mass spectrometer, LCT, Micromass
MS conditions: capillary 2000 V
cone 18 V
desolvation temperature 120 °C
MCP 2700 V
spectra deconvoluted using MaxEnt1

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



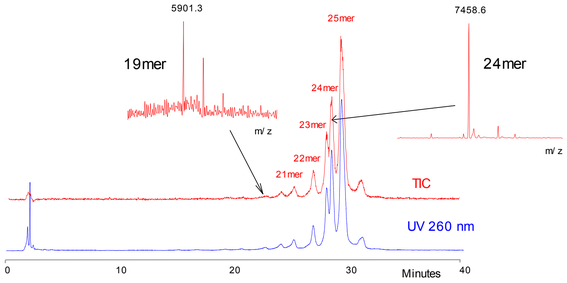
CGE: BioCAP™ 75 µm, 27.5/34.5 cm, 30°C, polyethylene glycol sieving matrix, 15 kV run, 4s injection at 5kV, 15 kV run
HPLC: XTerra® MS C18, 50 x 4.6 mm, 2.5 µm; 60°C, 0.5 ml/min, A: 10 % MeOH in 400 mM HFIP, 16.3 mM TEA, pH 7.92, B: 40 % MeOH in 400mM HFIP-16.3 TEA, pH 7.92, linear gradient from 16.7 % to 30 % B at 10 min, then from 30 % B to 47.15 % B at 30 min.

Figure 2: TEA-HFIP ion-pairing buffers allow for more sensitive oligonucleotide MS detection than triethylammoniumn acetate mobile phases.



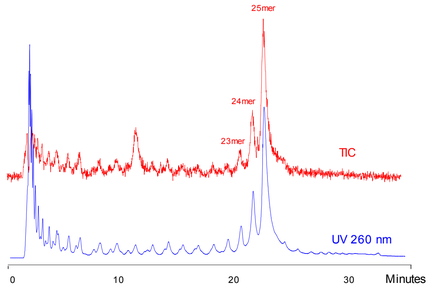
1 mg/ml solution of 25mer phosphorothioate oligonucleotide (7776.42 Da) was infused in suggested buffer in 50 % acetonitrile solution. The infusion flow rate was 10 µl/min.

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



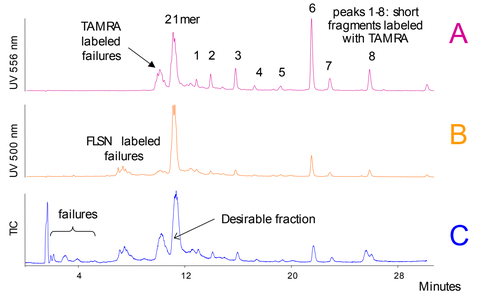
XTerra® C18, 50 x 1 mm, 2.5 µm; A: 5% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9; B: 60% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9; gradient from 18.2% to 36.4% B in 40 minutes; 23.6 µl/min.; 60°C; UV 260 nm;
0.6 µl injection represents 0.8 µg of 25mer, which is 103 pmole; the 19mer was detected at the level of ~ 1.5 pmole.

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



XTerra® C18, 50 x 1 mm, 2.5 µm; 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 from 14.5% to 69.1% B in 120 minutes; 23.6 µl/min.; 60 °C; UV 260 nm, 2.5 µl injection; 2.6 µg (318 pmole) total mass load.

Figure 5: LC-UV-MS analysis of 21mer dually-labeled diagnostic oligonucleotide 5' (FLSN)-CAC CTC CAG TGG AAA TCA AGT -(TAMRA) 3'. Oligonucleotide was synthesized in one step ("one pot" synthesis).



XTerra® MS C18, 50 x 1mm, 2.5 µm; A: 8.6 mM TEA—100 mM HFIP, pH 8.25; B: methanol gradient starts from 15 % to 45 % MeOH in 30 minutes, 23.6 µl/min, 50°C. Upper trace shows oligonucleotides labeled with TAMRA dye (λ max 556 nm), middle trace oligonucleotides labeled with fluorescein (λ max 500 nm), lower trace — total ion current. For more details see paper [3].

Conclusion

- Presented LC-MS method is simple, robust, and reproducible
- It allows for characterization of therapeutic and diagnostic oligonucleotides
- We successfully analyzed
  - phosphorothioate oligonucleotides
  - G-rich oligonucleotide sequences
  - singly and dually labeled diagnostic oligonucleotides
  - locked nucleic acids
- We achieved excellent LC separation with MS friendly mobile phases
- The LC-MS method is capable of detecting ~1-10 pmole of oligonucleotides

References

[1] W.J. Warren, G. Vella, Mol. Biotechnol. 4 (1995) 179.
[2] M. Gilar, E.S.P. Bouvier, J. Chromatogr. A 890 (2000) 167.
[3] M. Gilar, Anal. Biochem. 298 (2001) 196.
[4] C.G. Huber, A. Krajete, Anal. Chem. 71 (1999) 3730.
[5] A. Apffel, J.A. Chakel, S. Fischer, K. Lichtenwalter, W.S. Hancock, Anal.Chem. 69 (1997) 1320.
[6] P.J. Oefner, J Chromatogr. B 739 (2000) 345.