LC-MS Analysis of Therapeutic and Diagnostic Oligonucleotides

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Overview

- LC-MS method was developed for characterization of oligonucleotides.
- Method was applied for analysis of therapeutic and diagnostic oligonucleotides.
- Excellent LC separation was achieved
- Mobile phase is compatible with MS detection.

Introduction

Synthetic oligonucleotides are utilized for diagnostic as well 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]. The method was utilized for analysis of phosphorothioate oligonucleotides, dually labeled probes and native oligonucleotides.

LC-MS is useful for nucleic acids analysis [4,5]. We used an optimized LC-MS system (see Methods) for analysis of synthetic oligonucleotides and their failure products.

Phosphorothioate as well as guanidine-rich antisense oligonucleotide drugs are known to be particularly difficult to analyze [6]. Due to the chaotropic properties of triethlylamine-hexafluoroisopropanol (TEA-HFIP) buffers, we were able to successfully separate <60mer antisense oligonucleotides [3] and identify the failure oligonucleotides in a drug form.

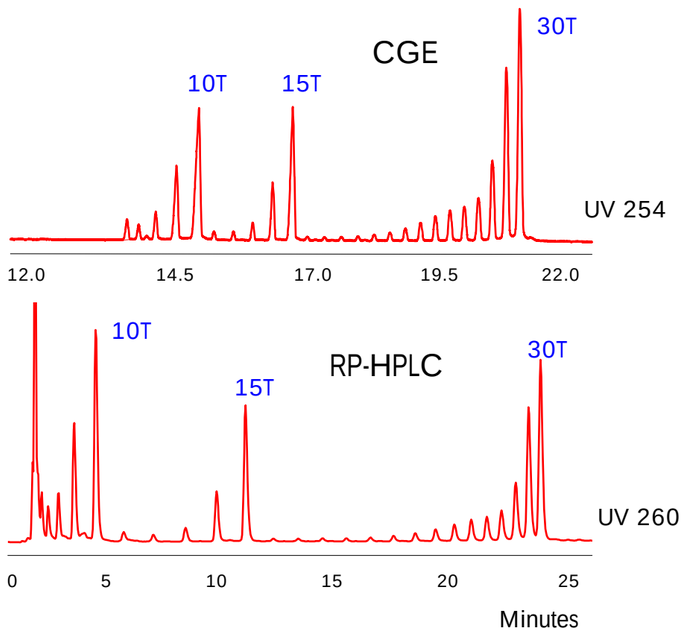
Guidelines for column selection and optimization of mobile phase composition are discussed. The ion-pair reversed-phase HPLC separation benefits from the use of 2.5 µm sorbent and elevated temperature. The separation performance was equivalent or better than ion-exchange HPLC and in some cases rivaled 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.

Methods

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 hexafluoroiso-propanol (weak acid), TEA-HFIP buffer pH was 8.3. Alternatively, 16.3mM – 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 desolv. temperature 120 °C MCP 2700 V spectra were deconvoluted with MaxEnt1 software

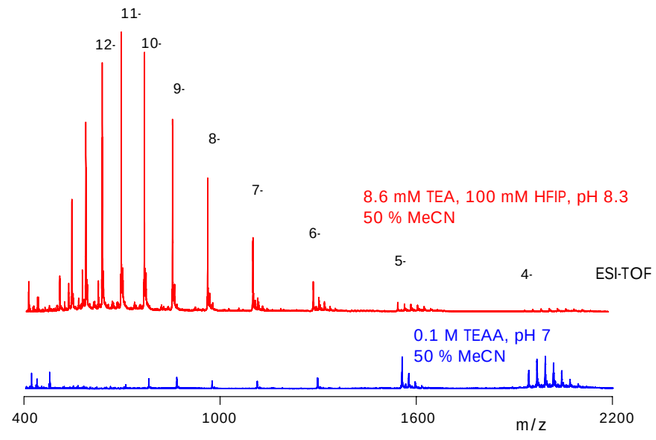
Results

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



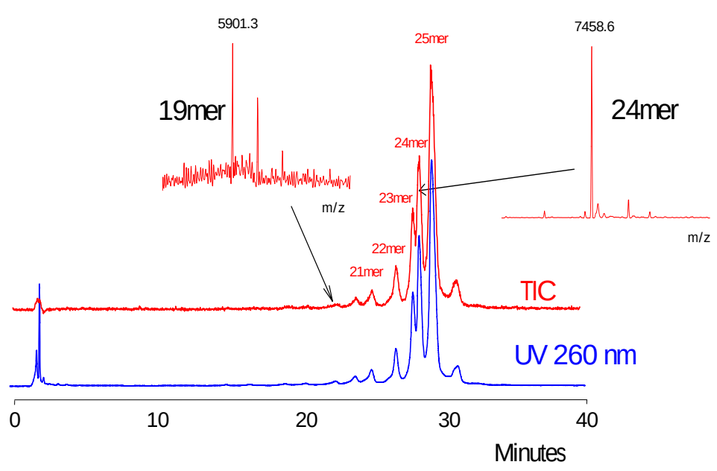
CGE: BioCAP™ 75 µm, 27.5/34.5 cm, 30°C, polyethylene glycol sieving matrix, 15 kV run, 4s injection at 5kV, 15 kV run
HPLC: XTerra® MS C18, 50 x 4.6 mm, 2.5 µm; 60°C, 0.5 ml/min, A: 10 % MeOH in 400 mM HFIP, 16.3 mM TEA, pH 7.92, B: 40 % MeOH in 400mM HFIP-16.3 TEA, pH 7.92, linear gradient from 16.7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



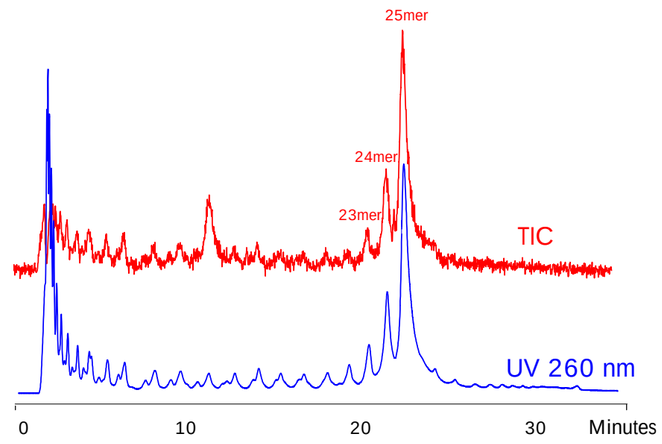
1 mg/ml solution of 25mer phosphorothioate oligonucleotide (7776.42 Da) was infused in suggested buffer in 50 % acetonitrile solution. The infusion flow rate was 10 µl/min.

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



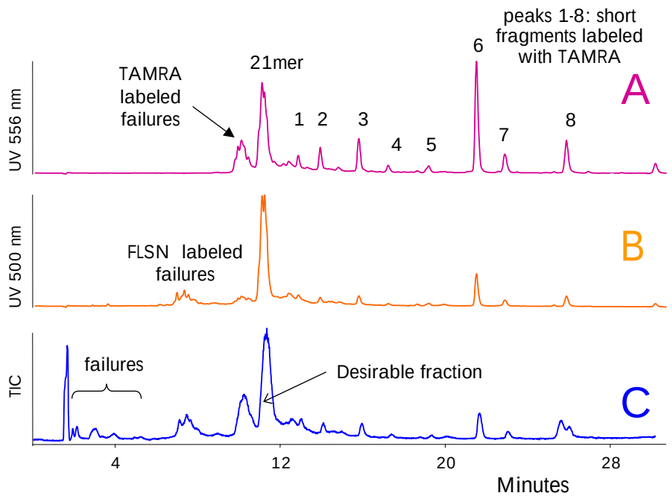
XTerra® C18, 50 x 1 mm, 2.5 µm; A: 5% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9; B: 60% MeOH in 400mM HFIP: 16.3mM TEA, pH 7.9; gradient from 18.2% to 36.4% B in 40 minutes; 23.6 µl/min.; 60°C; UV 260 nm;
0.6 µl injection represents 0.8 µg of 25mer, which is 103 pmole; the 19mer was detected at the level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

Conclusions

- 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.