Application Note

Biopharma/Pharma

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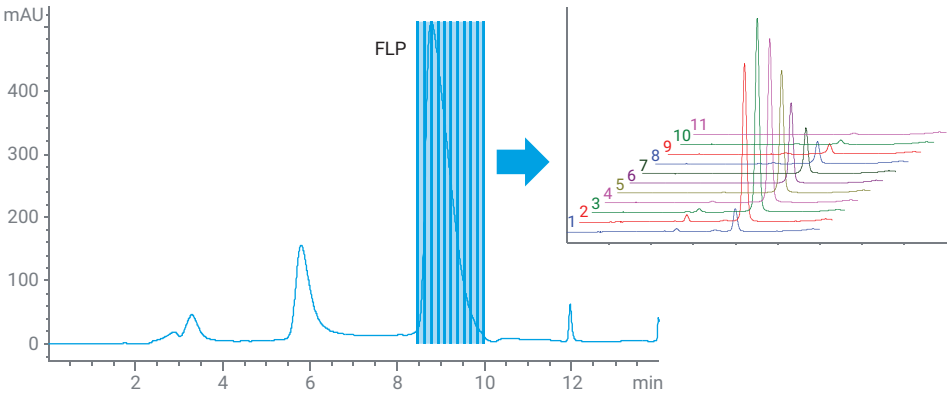
Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography



Author

Florian Rieck

Agilent Technologies, Inc.

Abstract

A crucial step in any pharmaceutical production is the purification of the product. The purification of oligonucleotides (ONs) can be particularly challenging because of the high similarity of target and impurities. Therefore, high-performance liquid chromatography (HPLC) is the method of choice if high-purity products are desired. This application note demonstrates the purification workflow of a single-stranded RNA ON by ion-pair reversed-phase HPLC. A method is developed at analytical scale and then transferred to preparative conditions. Using Agilent PLRP-S columns in analytical and preparative dimensions facilitates scale-up and increases reproducibility of the method. Purification is done on an Agilent 1290 Infinity II Preparative LC, which includes a preparative binary pump with titanium heads. These features ensure compatibility with a wide range of solvents and high reproducibility of shallow gradients typically employed for ON separation. Pooling the fractions after purification resulted in a >99% pure product at a yield >56%.

Introduction

Synthetic ONs are a class of compounds that have gained increasing interest over the last few years because of their use in biochemical research and as pharmaceuticals.[1] The process of synthesizing ONs has become much more efficient since its implementation in the 1980s. However, even with 99% coupling efficiency, a 25mer ON synthesis will yield less than 80% of the desired product. By-products are, for example, truncations of the target ON that have a sequence length of N–1, N–2, etc. Separating these impurities from the target molecule becomes more challenging with increasing sequence length.

An established separation method for ONs is reversed-phase HPLC. This technique employs separation columns with particles of 10 µm diameter or less, enabling high resolution of the analytes. By adding ion-pair reagents to the eluent, retention of the polar ONs on the nonpolar stationary phase is enabled. The ion-pair reagents interact with the charged phosphate backbone of the ONs and render them less polar with increasing sequence length. Elution and separation still require shallow solvent gradients of 1% slope or less, which can pose a challenge to HPLC instruments.

This application note demonstrates the separation and purification of a single-stranded RNA ON by preparative HPLC. The 1290 Infinity II Preparative LC System features a binary pump that is capable of precise gradient mixing even at high flow rates, which improves reproducibility and confidence in the results. Titanium pump heads are compatible with a wide pH range and salt concentration, stretching the range of applications that can be run on the instrument.

Method development is done at an analytical scale, then transferred to preparative conditions with added fraction collection. All experiments are conducted using PLRP-S columns, which are available in both analytical and preparative dimensions and thus enable easy scale-up and high reproducibility from analytical to preparative purification conditions.

Experimental

Instrumentation

The Agilent 1290 Infinity II Preparative LC System consisted of the following modules:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

Analytical method development and fraction reanalyses were conducted on an analytical system comprising the following modules:

- Agilent 1260 Infinity II Binary Pump (G7112A)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A)

Columns

- Analytical column: Agilent PLRP-S 100 Å, 4.6 × 150 mm, 8 µm (part number PL1512-3800)
- Preparative column: Agilent PLRP-S 100 Å, 25 × 150 mm, 8 µm (part number PL1212-3800)

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 [239] or later versions

Chemicals

Hexylamine >99% for synthesis, acetic acid >99% ReagentPlus, and analytical-grade urea were purchased from Sigma-Aldrich (Taufkirchen, Germany). LC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Mobile phase A was prepared freshly according to the following protocol: Prepare 450 mL of water, add 2.86 mL of acetic acid and 6.57 mL of hexylamine. Adjust pH to 7. Transfer solution to a 500 mL volumetric flask and fill to volume with water. For denaturing conditions, 10% (w/v) of urea was added to the buffer[2] to enhance the separation on preparative scale.

Sample

The sample to be purified was a crude synthetic, all-2'-O-methylated 22-mer oligonucleotide synthesized by Agilent NASD (Boulder, Colorado, USA) with the following sequence: 5'-aaaagcaccgacucggugccac-3'.

The sample was desalted and lyophilized before shipment. A fresh solution of 5 mg/mL in mobile phase A was prepared immediately before analysis.

Results and discussion

Analytical separation

The separation method was optimized on an analytical system using a sample concentration of 2 mg/mL. The optimized method was then scaled to preparative conditions using an HPLC method transfer calculator.[3] As the mobile phase and column under preparative conditions were not amenable to temperature control, urea was added to the mobile phase to act as a chaotropic, or denaturing, agent.

Figure 1 shows a chromatogram of the separation under preparative conditions. A total sample amount of 20 mg on column was well separated. Fraction collection was set to peak-based, collecting time slices of nine seconds width. This fraction mode enabled targeted collection of the full-length product (FLP) peak, based on signal threshold and slope. The combination of peak-based collection with time slices divided the FLP peak into 11 slices. These can be analyzed with respect to purity and product content to decide which fractions need to be pooled to meet purity and yield demands of the purification process.

Method settings

Table 1. Chromatographic parameters for purification.

Parameter	Value
Mobile Phase	A) 0.1 M Hexylammonium acetate in water, pH = 7.0, + 10% urea B) Acetonitrile
Flow Rate	30 mL/min
Gradient	Time (min) %B 0 32 0.26 32 10.10 42 11.09 100 12.07 100 13.06 32 Stop time: 14 min Post time: 1.5 min
Injection Volume	4,000 µL
Temperature	Ambient
UV Detection	260 nm No reference 5 Hz data rate
Fraction Collection	Peak-based from 7.0 to 12.0 min, collecting 9 s time slices UV threshold: 5 mAU UV upslope: 2 mAU/s UV downslope: 1 mAU/s

Table 2. Chromatographic parameters for fraction reanalysis.

Parameter	Value
Mobile Phase	A) 0.1 M Hexylammonium acetate in water, pH = 7.0 B) Acetonitrile
Flow Rate	1 mL/min
Gradient	Time (min) %B 0 28 8 36 9 100 10 100 11 28 Stop time: 11 min Post time: 6 min
Injection Volume	10 µL
Temperature	80 °C
UV Detection	260/4 nm Reference 360/100 nm 5 Hz data rate

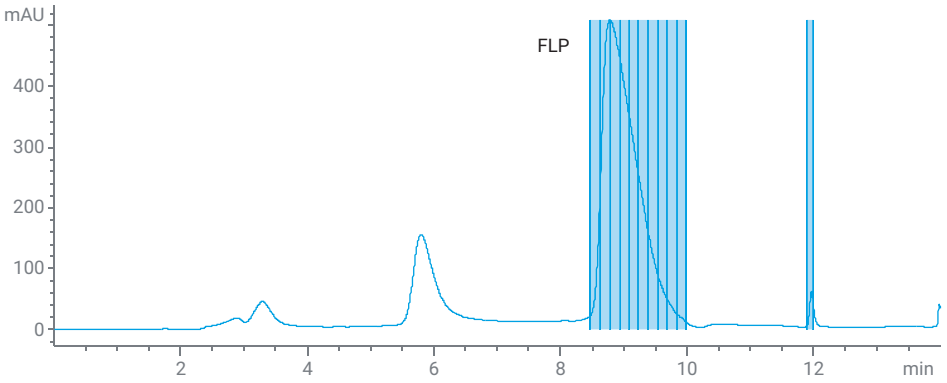


Figure 1. Chromatogram (UV 260 nm) of a 4 mL (20 mg on column) injection on the preparative column. Blue bars represent fraction collection of 9-second time slices.

Fraction reanalysis was carried out on an analytical HPLC system using the same stationary phase. To increase resolution, separate possible aggregates, and accelerate the analysis, the column and solvent were thermostatted to 80 °C and the gradient adjusted accordingly (see Table 2). All fractions collected over the elution of the FLP were reanalyzed and evaluated with respect to purity, measured by peak area percentage. Figure 2 shows a chromatogram overlay of all fraction reanalyses.

By injecting the crude sample and comparing peak areas at different dilutions with the fraction reanalysis, the FLP content in each fraction was calculated. With purity and content of the FLP determined, a pooling diagram and table can be created, which helps in judging which fractions to pool when a given purity or yield of the FLP needs to be achieved. Figure 3 shows the pooling diagram and table for the 11 fractions of the FLP. As expected, purity is highest in the center of the peak, whereas FLP mass is decreasing towards the tail. The pooling table quickly shows which fractions can be pooled if a minimum purity must be achieved and the yield is to be maximized. If, for example, the workflow requires minimum 99% purity, fractions 3 to 7 can be pooled and a yield of 56.3% be achieved.

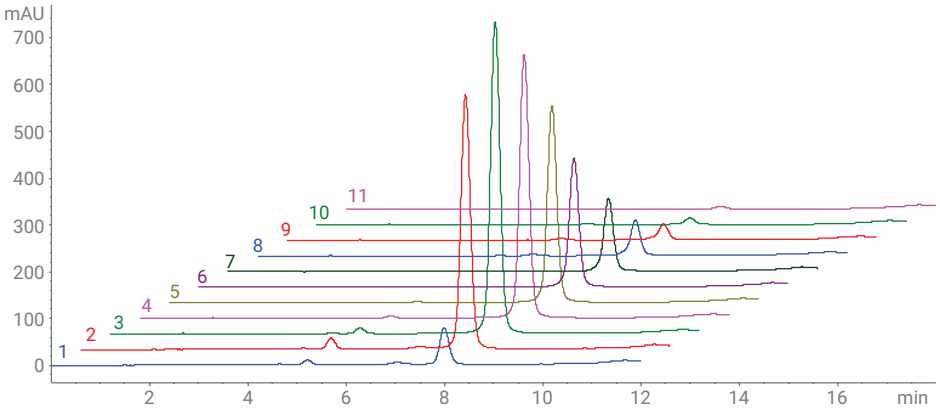
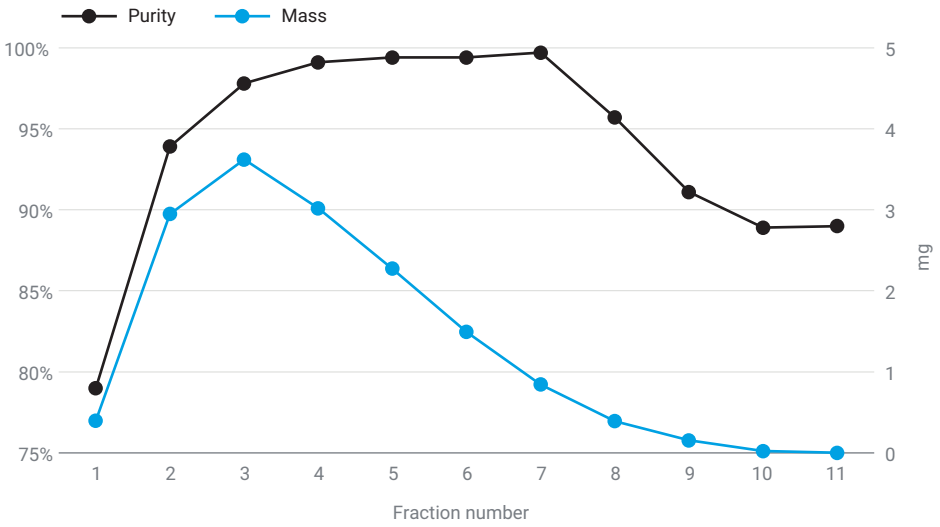


Figure 2. Chromatogram overlay (UV 260 nm) of the reanalysis of 11 fractions collected over the FLP.



Fractions	Pool Purity	Pool Yield
7	99.7%	4.2%
6 to 7	99.6%	11.7%
5 to 7	99.5%	23.1%
4 to 7	99.4%	33.9%
3 to 7	99.1%	56.3%
3 to 8	98.5%	58.2%
2 to 8	97.9%	72.9%
2 to 9	97.0%	73.7%
2 to 10	96.1%	73.8%
2 to 11	95.4%	73.8%
1 to 11	93.9%	75.8%

Figure 3. Pooling diagram and table for the 11 fractions of the FLP. Sorting fractions by purity, the pool to achieve highest yield at a given purity can easily be determined.

Conclusion

This application note demonstrates the purification of a short single-stranded RNA oligonucleotide by means of ion-pair reversed-phase chromatography. Separation conditions were scaled up from analytical to preparative scale, using a 25 mm id column that enables high sample load. The Agilent 1290 Infinity II Preparative LC, comprising a combined autosampler/fraction collector, proved to be the ideal tool for large-scale injections and flexible fraction collection into tubes of various sizes. Collecting the target peak in time slices enabled specific pooling of the fractions by purity to meet workflow demands. Using this technique, a >99% pure fraction pool could be created with a yield of >56%.

References

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