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Technical Notes

Purification of DMT-On Oligonucleotide by Simulated Moving-Bed (SMB) Chromatography

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Abstract:

Recent clinical advances in the development of oligonucleotides as therapeutics have triggered a significant demand for these molecules in large quantities. To facilitate their development, purification of full-length oligonucleotide from various synthesis related byproducts is an important endeavour. Herein, we report for the first time an application of simulated moving-bed (SMB) chromatography as a purification tool for DMT-protected oligonucleotides. An efficient separation has been accomplished with polystyrene/divinyl benzene copolymer as the stationary phase and 0.05 M NH_4HCO_3 buffer (pH 8)/MeOH (6:4, v/v) as the mobile phase. A DMT-protected product purity of >99% with 77% yield for a 20-mer phosphorothioate oligonucleotide is reported.

Introduction

Interest in manufacturing large quantities of synthetic oligonucleotides has increased as a result of rapid evolution of their applications in antisense, aptamers, ribozymes, immunostimulatory CpG molecules, and miRNA and RNAi related therapeutics.[1] Currently, there are over 40 oligonucleotides that are under various stages of clinical trials with two products on the market. All of these products have been synthesized on an automated synthesizer using solid-phase amidite chemistry. The typical purity of the crude product obtained after automated synthesis is >70% full-length oligonucleotide. The desired product must be purified to a much higher full-length purity prior to use as a therapeutic agent. Oligonucleotide purification research has thus been critical to the successful development of this class of compounds.[2]

Frequently, postsynthesis oligonucleotides are cleaved from the solid-support with the 4,4′-dimethoxytrityl (DMT) protecting group intact at the 5′-hydroxyl end of the molecule (Figure 1). The hydrophobic DMT group serves as an excellent handle for the separation of DMT-protected (DMT-on) full-length product from the unprotected failure sequences (DMT-off) on reversed-phase high-performance liquid chromatography (RP-HPLC). This method has been used for the purification of a wide range of oligonucleotides at various scales where DMT-on purification is relatively easy and quick with good product purity and yields.[3] Although RP batch-HPLC is a well-established method for gram-scale oligonucleotide purification, it is not practical for commercial-scale production, due to its high costs dominated by long cycle times and high eluent consumption. To expand the scope of DMT-on reversed-phase chromatography, we explored the use of simulated moving-bed chromatography (SMB) as a scalable alternative. It is noteworthy that SMB is now considered a real production tool for multiton-scale separation of optical isomers, fine chemicals, and pharmaceuticals.[4] Herein, we describe for the first time application of SMB for the purification of synthetic DMT-on oligonucleotide.

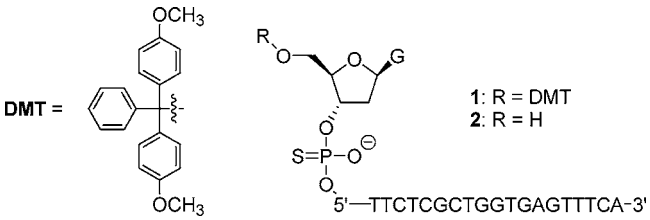

Figure 1. **Structural representation for DMT-on (1) and DMT-off (2) oligonucleotides.**

SMB chromatography is widely used today in the pharmaceutical industry mainly for the separation of racemates into their single enantiomers. Numerous examples are

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described in the literature where the use of continuous countercurrent chromatography results in both higher productivity and reduced solvent consumption, so that the overall process economy can be substantially improved.[5] The separation challenges in the field of active pharmaceutical ingredients (API) manufacturing is a daunting task with a greater emphasis on proteins and natural-products-based therapeutics.[6] The SMB technology has also been used to perform the purification of ionic molecules such as lysine and betaine.[7] However, to the best of our knowledge, there are no reports in the literature describing the purification of oligonucleotides using SMB.

Results and Discussion

The separation of DMT-off and DMT-on oligonucleotides is a classical two-compound hydrophobic separation. This separation should be well suited for SMB chromatography if an appropriate combination of mobile and stationary phase can be found. The challenge for the SMB-separation between DMT-off and DMT-on oligomers is given by the fact that isocratic elution conditions have to be optimised within a reasonable retention window for both compounds. As the retention of the oligo on a reversed-phase sorbent is governed by the hydrophobic DMT-group, substantial elution differences can be realized between DMT-on and -off products. Screening of various silica-based sorbents that were suitable for a separation under isocratic conditions resulted in unsatisfactory separation. If the elution strength of the mobile phase was set to high values by increasing the amount of organic modifier, the retention of the DMT-off products was reduced and if the strength of the eluting buffer was decreased, a broad DMT-on peak giving a very high elution volume was observed. When experimental porous polystyrene/divinyl benzene sorbent with an additional hydrophilic cross linker was used, it was possible to elute both DMT-on and DMT-off products of the crude oligonucleotide under isocratic conditions (Figure 2).[8] The principal benefit of the polymeric sorbent compared to traditionally used silica sorbents is the greater chemical stability at a high pH. The polymeric sorbent can be operated up to pH 14, while most silica sorbent are substantially degraded at pH values above 8. Operation of the chromatographic separation at a higher pH is preferred for three reasons. First, the cleavage of the hydrophobic DMT handle from the crude oligonucleotide is completely avoided during the separation process. Second, under high pH conditions, the oligonucleotides are denatured by abolishing the Watson−Crick hydrogen bonding, which may form secondary structures increasing the complexity of the separation. Third, the ammonium hydroxide solution, obtained after the cleavage of the protecting groups from the oligonucleotide, can be loaded directly onto the polymer-based sorbents. The polymeric sorbent was successfully packed into stainless steel columns of 25 mm inner diameter by applying a vacuum packing method. The polymer was axially compressed to 25 bar to ensure a stable chromatographic bed. A total of eight columns were packed for this study. These columns showed an equivalent behaviour when operated under linearly scaled-up conditions (Figure 3). The starting conditions for the SMB system have been determined by the peak maximum method described in detail by Nicoud et al.[9] With this methodology a first set of pump flow rates and a corresponding switching time were developed (see

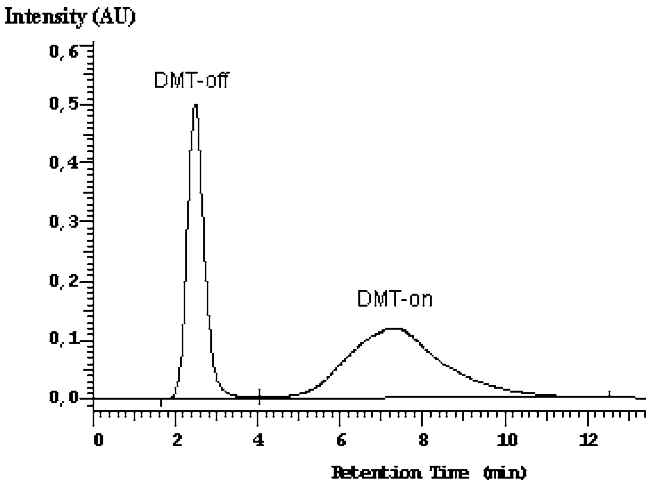

Figure 2. **Analytical chromatogram of crude 20-mer phosphorothioate oligonucleotide on experimental polystyrene sorbent using isocratic method.**

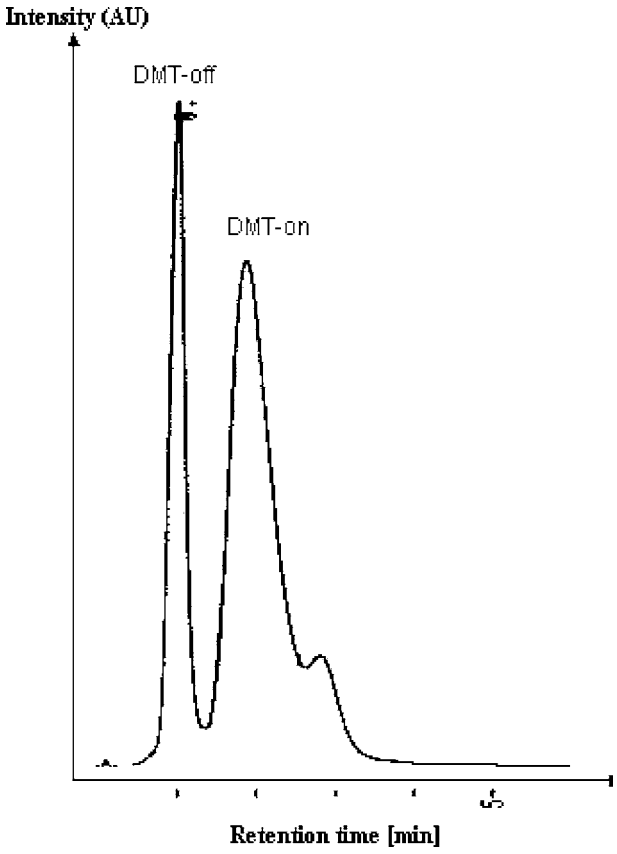

Figure 3. **Separation on one 25 mm column from the SMB system.**

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(8) Merck KGaA experimental media: Polystyrene/divinylbenzene copolymer, mean particle size 20 μm, mean pore size 30 nm.

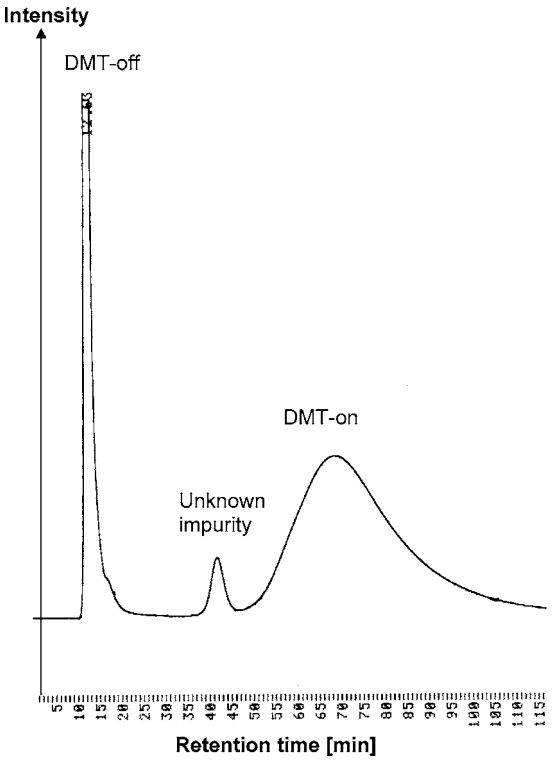

Figure 4. **Pulse injection of the eight SMB columns connected in series in an open loop.**

Table 1. **SMB system parameters**

parameter	calculated	profile 1	profile 2	profile 3
recycling flow [mL/min]	30.80	30.80	25.80	23.90
feed flow [mL/min]	4.59	4.59	4.59	4.59
feed conc [g/L]	8.38	8.38	8.50	5.70
raffinate flow [mL/min]	5.14	5.54	6.00	5.71
eluent flow [mL/min]	25.22	25.22	20.03	18.03
extract flow [mL/min]	24.67	24.27	18.62	16.91
period time [min]	5.94	6.10	6.10	6.10
purity raffinate [%DMT-off]	99.73	>99.9	64.46	80.88
purity extract [%DMT-on]	95.28	69.8	98.68	99.20
throughput feed [g/24 h]	55.39	44.83	58.06	38.93
product loss [%]	0	0	53.48	22.9

Table 1). After connecting all eight columns in series, a pulse injection on the SMB system in open loop mode was performed. The resulting chromatogram is shown in Figure 4. The SMB system was initiated, and after five cycles an internal concentration profile was determined by withdrawing samples at the half time point from the SMB system. In Figure 5 an overlay of three profiles, before and after optimisation of the purification steps, is shown.

The resulting profile 1 showed a good purity for the raffinate compound, which is the DMT-off oligonucleotide (Table 1). In zone 2, where the purification of the extract (DMT-on) compound was to take place, the separation was not efficient enough. The net flow rates in the single zones were adjusted by changing the pump inlet and outlet flow rates. In this case the eluent, extract, and recycling flow rate have been decreased, while the raffinate flow rate was

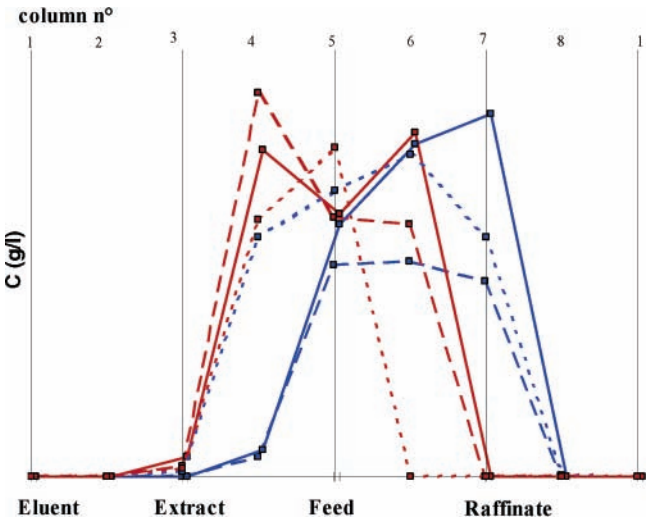

Figure 5. **Profile overlay before and after optimisation [parameter sets 1 = (···), 2 = (—), 3 = (− − −)].**

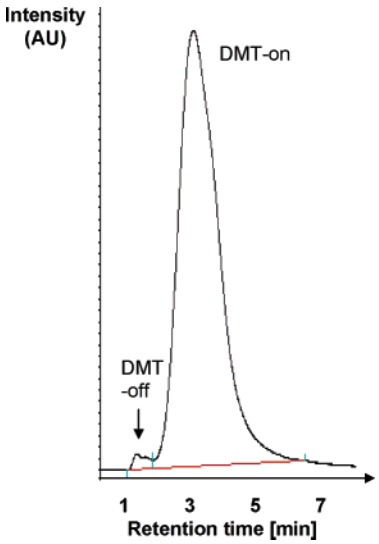

Figure 6. **DMT-on fraction purity (98.6%) determined on an analytical column according to method 2.**

slightly increased. This leads to a better separation of the two fractions in zone 2 and 3 of the SMB system. The influence on stationary and mobile phase velocities by pump flow rate adjustment is described in detail in the literature.[10] After restart and equilibration with the new conditions, a second profile was taken from the system (Figures 5 dotted lines [- - -]). With the new conditions (profile 2, Table 1) an extract purity of >98% was achieved, but the raffinate purity (DMT-off) was only moderate with 64%. This means that a substantial part (~54%) of the desired product DMT-on was eluted with the DMT-off byproducts. In a second optimisation step the parameters had been adjusted again. In this case the feed concentration as well as all flow rates had been decreased, except the feed flow rate. It can be seen from profile 3 that the concentration fronts have been sharpened. The analysis of the extract showed a purity of >99% (Figure 6), while the raffinate purity was 80% (Table 1).[11] Thus profile 3 appears to be the best condition for the separation

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of DMT-on from DMT-off among the three experimental conditions tried in this study.

Conclusion

The optimised system parameters fulfilled the demand for separation of DMT-on oligonucleotides from the crude postsynthesis products. With the lab-scale SMB system a total throughput of 39 g/day can be achieved. These parameters would give, on a typical pilot scale SMB system with 200 mm columns, a throughput of 2.5 kg/day. It is noteworthy that the given conditions have to be further optimised in two ways. First, the loss of DMT-on product into the DMT-off fraction is still high. This has to be optimised by adjusting the zone flow rates. Second, the total system pressure is still low at 35 bar, and a linear increase of all flow rates would therefore increase the total throughput. Based on the present study, we have demonstrated that SMB chromatography can be used for the purification of DMT-on oligonucleotides furnishing high purities as well as good throughput. We believe that this study paves the way for further development of SMB as an alternative to batch chromatography for the purification of therapeutic oligonucleotides.

Experimental Section

Chromatographic System: The feed mixture consisted of a crude DMT-on phosphorothioate oligonucleotide ISIS 3521 (sequence: GTTCTCGCTGGTGAGTTTCA) in buffer with an optical density of 625 ODUs, corresponding to a concentration of 25 mg/mL. The HPLC of the feed indicated ~68% full-length DMT-on ISIS 3521 in the mixture. The stationary phase was an experimental polystyrene/divinyl benzene copolymer with a particle size of 20 μm and a mean pore size of approximately 30 nm. The mobile phase consisted of 0.05 M NH_4HCO_3 buffer adjusted to pH 8 (60%) and methanol (40%). For the process determination study the sorbent was packed into 250 × 4 mm^2 stainless steel columns. The two compounds eluted at $k_1' = 0.65$ (DMT-off) and $k_2' = 3.89$ (DMT-on).

Determination of Adsorption Isotherms, SMB-Parameter Estimation: The starting parameters for the SMB separation (pump flow rates and period time) were determined by means of chromatograms obtained on the 250 mm × 4 mm column following the peak maximum method.[9] The data were processed using the software HELP from Novasep. In addition, the adsorption isotherms were determined by frontal analysis and modelled with a new proprietary software package of Bayer Technology Services GmbH.[12]

SMB System Preparative Columns: The SMB separation was performed on a Licosep Lab system from Novasep, Pompey, France. The system control was based on Siemens S7 PCS and control software developed at Merck Central Process department. The system was equipped with eight Merck Selfpacker columns with a diameter of 25 mm and a length of 100 mm. The columns were packed by the vacuum packing method with 2-propanol as the slurry liquid and afterwards axially compressed to a mechanical pressure of 25 bar. All single columns were checked for their retention time, pressure drop, column efficiency, and axial dispersion with a test mixture of uracil and diethylphthalate. The relative standard deviation of the k'-value of the test compound for all eight columns was determined to be 3.62%.The complete chromatographic bed was checked by a pulse injection over the eight columns connected in series (Figure 4).

Fraction Analysis: The fraction analysis was performed on an analytical column with a dimension of 125 mm × 4 mm filled with Purospher RP-18, 5 μm. The mobile phase was a linear gradient of methanol from 0 to 60% within the 38 column volumes. The aqueous phase was set to pH 7 by the addition of 10 mM triethylamine, and pH adjustment was done with formic acid. The flow rate was set to 1.0 mL/min. Under these conditions DMT-off eluted after 20.9 min, and DMT-on, after 24.3 min. A second method especially for quick in-process control of the SMB separation is using the experimental polystyrene sorbent packed into a 250 mm × 4 mm stainless steel column and operated under the mobile phase conditions of the SMB separation.

Extract + Raffinate, Purities, Throughput: At period half time fractions of 1.5 mL were withdrawn from the SMB system by means of a four-way Rheodyne valve and analysed by the method described above. From the fraction analysis, an internal concentration profile was constructed. Based on the profile shape, the pump flow rates and cycle time were adjusted.

Acknowledgment

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Note Added after ASAP Publication: In the version published on the Internet February 22, 2005, reference 7 was incomplete. In the final version published February 22, 2005, and in the print version, reference 7 is correct.

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(11) It is important to note that the DMT-on product pool may contain a small percentage of synthesis related impurities that coelutes with the DMT-on full-length product. For more details, see: Capaldi, D. C.; Gaus, H. J.; Carty, R. L.; Moore, M. N.; Turney, B. J.; Decottignies, S. D.; McArdle, J. V.; Scozzari, A. N.; Ravikumar, V. T.; Krotz, A. H. *Bioorg. Med. Chem. Lett*. **2004**, *14*, 4683−4690.

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