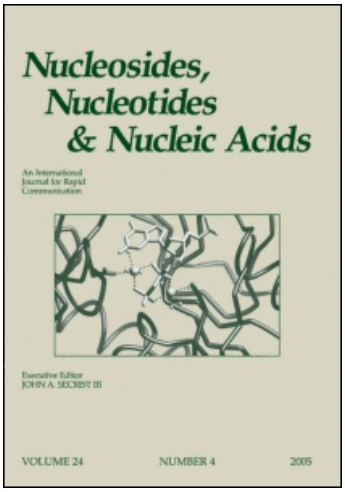

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A CASE STUDY: OLIGONUCLEOTIDE PURIFICATION FROM GRAM TO HUNDRED GRAM SCALE

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ABSTRACT

This article describes the purification and scale-up of ISIS 2302, a 20-mer phosphorothioate oligonucleotide by anion-exchange (AX) chromatography. The key operating parameters were optimized at gram scale and further scaled up to hundred gram. SOURCE 30Q, a high efficiency polymeric chromatographic media was used for both the small and large-scale work. High length-based purity and yield were maintained at scale-up. This purification is one of the largest demonstrations of AX purification of phosphorothioate oligonucleotide.

INTRODUCTION

Synthetic oligonucleotides are a relatively new class of drug molecules. With the successful launch of the first commercial antisense drug, Vitravene in 1998, there is anticipation of more oligonucleotides based drug therapies (1). Scaleable and cost effective production methods are thus needed to meet this potential need for large-scale therapeutic oligonucleotides (2). Most of the antisense oligonucleotide

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molecules in clinical trials are phosphorothioates, a first generation of backbone modified DNA molecules in which the non-bridging oxygen atom is replaced with a sulfur molecule (3).

Currently all oligonucleotides are manufactured on an automated solid-phase synthesizer. The basic starting blocks are the four phosphoramidites corresponding to the four nucleosides A, C, T, and G. The largest synthesis currently is accomplished using the OligoProcess^{TM} synthesizer. The coupling efficiency per addition of nucleotide is greater than 99% and upto 180 mmole of 20-mer phosphorothioate can be synthesized with typical purity of $>70\%$.

The key impurities in the crude oligonucleotide are length based. Deletions result at random locations along the oligonucleotide chain and these are termed N-x impurities, where N is the full-length oligonucleotide and x are number of deletions. Thus there are N-1, N-2 etc. deletions. There are also some longmers resulting from the synthesis process and post-synthesis processes, these are called N+x (4). Additionally, impurities related to phosphodiester linkages are generated during synthesis or in subsequent workup related steps. In these molecules the phosphorothioate linkage is oxidized into phosphodiester linkage. These are termed as $(P{=}O)_x$ impurities, x denoting the number of linkages where non-bridging sulfur is replaced by an oxygen atom. In this class, $(P{=}O)_1$ is the most abundant impurity generally between 15–20%.

As there is potential for commercial large-scale manufacturing, lower cost or more scaleable alternative purification methods are needed. To meet this need the anion exchange method presented here was developed for purification of ISIS 2302. The small-scale methods development along the critical parameter has been recently published (5). This article extends the work from small scale to pilot scale.

RESULTS

Methods Development at Small-Scale

Initial method was developed at small scale using 100–200 ml columns. The critical experiments were carried out on a 110 ml column and the sample load varied between 0.5 and 3 g. Elution was performed with a linear gradient from buffer A to buffer B in 20 column volumes (CV). Fractions were collected manually. Each fraction was then analyzed by UV, CGE and analytical AX chromatography. Fractions meeting certain purity criteria were pooled and the pools were analyzed again.

Media Scouting

While details of the chromatographic media selection are not presented herein, some key results are summarized. Purification at the gram scale was used to screen

the following media: SOURCE 30Q, SOURCE 15Q, Q Sepharose HP and Q Sepharose FF. Although SOURCE 15Q gives good results, pressure drop considerations at large scale influence choosing the medium sized particle SOURCE 30Q to give the added flexibility. Out of the four media screened, SOURCE 30Q was chosen for the majority of the experiments in this study. The SOURCE 30Q media is a strong anion exchanger, with a good chemical stability for routine use between pH 2–12 and cleaning at pH 1–14. It is suitable for packing in large columns and provides high resolution, productivity and scalability.

Bed Height

For SOURCE 30Q columns with bed heights of 10 and 20 cm were compared. The purity and yield were slightly higher on columns with a bed height of 20 cm. The pressure drop was also within comfortable operating regime for the two longer columns. Therefore, a 20 cm bed height was chosen for all experiments, including the pilot scale experiments.

Flow Rate

Initial experiments were done with a flow rate of 110 cm/hour. To increase the productivity flow rates upto 300 cm/hour were evaluated. Various experiments run at different flow rates shows that the results are independent of the flow rate (e.g. residence time) in this flow rate range, 110 to 300 cm/hour.

Temperature

It is well known from analytical separations of oligonucleotides on anion exchange columns that an increased temperature improves the resolution. A comparison of yield and purity on SOURCE 30Q run at 50°C and ambient temperatures while keeping all other conditions constant indicated that temperature has a decisive effect on both parameters. A conservative estimate on the difference between ambient temperature and 50°C is that at the same yield the purity of full-length oligo would be one per cent unit higher at 50°C compared to ambient temperature. The experiments run on the pilot scale was run at ambient temperature due to availability of equipment, but thermostated equipment/heat exchangers are available for large-scale.

Organic Solvent

Small-scale experiments had indicated that addition of organic solvent in the buffer improves the resolution. In two experiments 30% methanol was included in

both the A and B buffers. Surprisingly, the benefit of adding methanol was marginal. When methanol, or acetonitrile, is used the separation "train" becomes compressed compared to the appearance of chromatogram obtained without organic solvents. However, there are significant negative factors by including organic solvents in the buffers. The composition of the buffers is more critical, solubility of salts could be a problem, and disposing and explosion proofing may be needed if high percentage of organic is used.

Sample Loading

Sample self-displacement and displacement chromatography has been shown to play an important role in the purification of oligonucleotides. Therefore, sample load is an important parameter in optimizing the purification. At lab scale sample loads from 8 to 40 mg oligo/ml media were used to establish an optimal loading for the scale up (pilot scale) experiment. The yield and purity were consistent up to 24 mg/ml. However, above this loading the results started to deteiorate. At 24 mg/ml the purity of the product pool was 93.9% with a yield of 84%, whereas a loading of 30 mg/ml gave a higher purity (94.2%) but lower yield (60%). Therefore, for the pilot scale experiments, 24 mg/ml column loading was selected.

Optimization of Elution Conditions

After establishing the appropriate bed height (20 cm), sample loading (24 mg oligo/ml media) and flow rates (100–300 cm/hour), the elution conditions were then optimized. Until this point all screening experiments had been performed with a 20 CV linear gradient elution. Several experiments were run to optimize the various components of gradient elution program. This includes combinations of steps and linear gradient segments for elution (data not shown).

Recycling of the Side Fractions

In order to maximize the yield, recovery of oligonucleotide from side fractions was considered. In one experiement, side fractions were pooled from several campaigns and run on the SOURCE 30Q column again. One advantage of reprocessing on the anion exchange is that a simple dilution is generally sufficient to prepare the sample pool to be fed into the next cycle as feed. The purity of the resulting product pool was similar compared to the initial purification.

Sanitation

When reusing a column one must have guarantees that it is clean and sterile. Oligonucleotides are sensitive to hydrolysis under acidic conditions, so a cleaning-in-place (CIP) protocol based on a weak solution of phosphoric acid has been

developed. A solution of 4 mM H_3PO_4 was pumped through the column at a low flow rate for 30 minutes. This was immediately followed by a 30 minutes wash with 20 mM NaOH + 2.5 M NaCl. The impurities were effectively removed by the high salt solution. After cleaning the column is equilibrated in buffer A. The performance of the column does not change upon CIP. The elution profiles before and after the CIP procedure were virtually identical (data not shown).

PILOT SCALE EXPERIMENTS

Prior to performing the large-scale purification, the large column was evaluated with low loadings. The flow rate used throughout the experiments at pilot scale was 250 cm/hour, corresponding to 78.5 liter/hour. As a test, a small analytical load of the sample was injected on the large column to visually compare the elution profile of the crude to that obtained at small scale. Two experiments with low sample load of 0.5 mg oligo/ml media were performed. First with linear gradient elution, and then with a step gradient elution. These two experiments showed that the performance of the column was satisfactory. While the main product eluted at the same ionic strength, the programmed gradient (% buffer B values) had to be slightly changed to generate the same elution gradient profile. Several experiments were conducted to make minor elution condition optimizations at the large scale. In these experiments the entire column eluate was re-used for a subsequent experiment. This allowed judicious use of a costly feedstock to conduct as many experiments as possible. We had demonstrated at small scale that such complete recycling does not substantially change the elution profile, except the loading time. In such cases the sample conductivity was adjusted to 54 mS/cm and feed adjusted effective concentration of NaOH (20 mM). The column load was 24 mg/ml of the packed bed.

The final optimized large-scale process chromatogram is shown in Figure 1. The overall appearance of the chromatogram was similar to the chromatogram at small scale. The purity in single pass was >95% full length by CGE analysis. Side fractions from two preparative runs were combined and reprocessed on the same

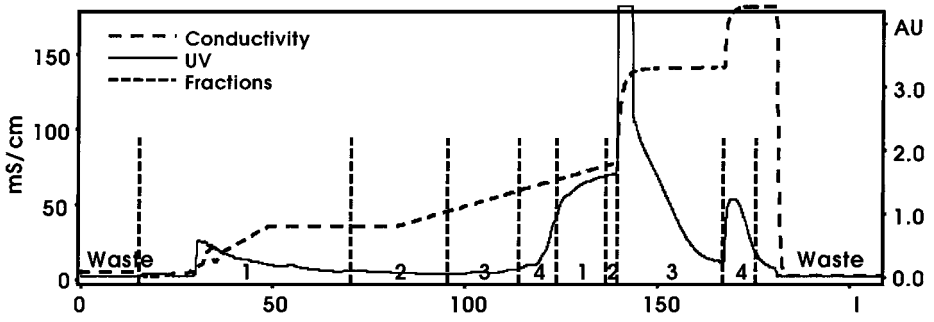

Figure 1. Optimized preparative run with 150 g of crude oligonucleotide load.

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Table 1. Summary of Analytical Data from Plot Scale Experiment

	Capillary Gel Electrophoresis Area % Purity			
	Total (N-x)	N-1	N-mer	N+x
Feed	22.5	5.3	71.7	0.5
Product	2.2	2.7	95.0	0.1
	Analytical anion exchange chromatography area % purity			
	Total (P=O)	(P=O)1	(P=S)	
Feed	21.7	11.4	66.9	
Product	0.2	1.8	98.0	

column. The analysis of the fractions shows good purification and the quality of the purified pool was as good as the first pass runs. Cumulative yield after mixing the first pass and reprocessed run was 72%. The complete analysis of the product pool is shown in Table 1 and Table 2 shows a comparison of results from the small and large-scale purifications.

POST PURIFICATION PROCESS

There are many processes to remove the high elution salts introduced during the anion exchange purification. Two widely used techniques, cross-flow membrane filtration and gel permeation chromatography (GPC) can both be used at production scales and give acceptable process for desalting oligonucleotides. In this article, we present data on the GPC process.

Desalting

Since oligonucleotides contribute to high viscosity at increased concentrations, viscous fingering is a problem in developing a suitable gel permeation method. This results in a second oligonucleotide peak that co-elutes with the salt elution

Table 2. Data Comparison During Small and Large-Scale Purification Experiments

Parameters and Variables	Small Scale	Large Scale
Column volume (liters)	0.1	6.8
Load of crude oligonucleotide (grams)	2.4	150
Purified oligonucleotide product (grams)	1.2	75
Product purity by CGE (% full length)	95	95
Total N-1 mer in purified product	2.9	2.5
Total P=S content in purified product by AX	98	98
Full length product yield (%)	75	81
Total time for purification (hours)	2	3

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zone. It can severly reduce the yield and reduce the maximum concentration of the oligonucleotide feed solution that can be used. Experiments were conducted to determine simplest variations in operating parameters that would minimize viscous fingering. Different grades of Sephadex G-25 corresponding to various particle sizes from fine to coarse were evaluated on a XK 26/20 column. The coarse grade material with an average wet particle diameter of 320 μm was found to give the best profiles. Flow rate was varied between 50–200 cm/hour and 100 cm/hour was found to be optimal and was selected for further experiments. Next, the sample load was optimized varying the volumetric sample load between 5–20% CV. The 15% CV was determined to be an optimal compromise between productivity and resolution. Individual fractions were analyzed for NaCl content using an indirect CE assay. Appropriate fractions were found to contain <0.1% w/w of NaCl. The yield was >85% and the dilution of the applied sample plug were between 1.8 and 2 fold. This was scaled up to a 6 L column volume. A 900 ml sample from the eluate of the optimized large-scale AX run was used as feed. Excellent separation of the salt and oligonucleotide peak was observed with a dilution factor of 1.8 (data not shown). The initial results indicate that the method is worthy of pusuing further.

DISCUSSION AND CONCLUSIONS

The purity and yield data at lab scale and at pilot scale clearly shows that purification scheme developed at lab scale is scalable and that the performance of the media SOURCE 30Q, also is similar on both scales. The results shown in Figure 1 can be considered to be close to being optimal concerning what can be achieved with ion exchange chromatography on SOURCE 30Q for purification of ISIS 2302. It has to be noted that the pooling of collected fractions in experiment shown in Figure 1 was conservative. With a less conservative cut the yield would be higher at the first pass itself.

While the purity and yield were slightly higher with the elevated temperature experiments, we decided agaist using high temperature for large-scale. It is technically feasible but more expensive and limiting to use high-temperature chormatography for production scale. A conservative estimate would be one per cent unit. The time of one cycle will be approximately 4 hours. This time include equilibration, purification, CIP and reequilibration. The actual run itself takes well under 3 hours. The separation mechanism seems to indicate a strong influence of sample-self displacement effect. There is a fine line between elution conditions and sample-self displacement, which may be hard to define. However, as per our prior work (6) and that of others, this phenomenon has been shown to be important in oligonucleotide purification. The impurity distributions presented in this article also indicate that sample-self displacement may be playing an important role.

AX chromatography has shown to be effective in purification of phosphorothioate oligonucleotides. Pilot scale experiments show that a linear scale-up is

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possible to 100–150 g crude oligonucleotide loading per run. Reprocessing of side-fractions has been shown to scale-up which results in product pool of as good quality as first run and allows higher overall yield. The basic units of this process are simple and could be scaled up to higher loads. Desalting by gel permeation chromatography has also been shown to be a viable method to complement AX chromatography of oligonucleotides.

EXPERIMENTAL

Chromatography Equipment, Columns, and Resins

Most small-scale developmental work was conducted on the Akta^{TM} Explorer 100 chromatography system (APB, Piscataway, NJ), and the BioCad 60 workstation (PE BioSystems, Framingham, MA). Large scale work was conducted on the Bio-process chromatographic skid (APB, Uppsala, Sweden). A standard high-pressure 6-mm chromatography skid (APB) equipped with two screw pumps and controlled via Unicorn software was used for the scale-up experiments. Detection was made at 280 nm with a path length of 2 mm. The flow rate used throughout the experiments at pilot scale was 250 cm/hour, corresponding to 78.5 liter/hour. This flow rate was in the middle of the flow rate range of the pump system. All experiments were run at ambient temperature (approximately 26–30°C).

Initial screening work was performed on an analytical 1 ml ReSOURCE Q (6.4 mm I.D. × 30 mm length) was purchased from Amersham Pharmacia Biotech (APB), Piscataway, NJ. For large-scale work SOURCE 30Q was used. Media was packed in XK columns (APB, Piscataway, NJ) of appropriate dimensions according to manufacturers instructions. A FineLine 20L column (200 mm I.D., packed to a bed height of 21.3 cm) was used for the large-scale work.

Chemicals, Buffers, and Oligonucleotides

For the small scale experiments sodium chloride was obtained for Sigma Chemicals (St. Louis, MO) and sodium hydroxide from Mallinckrodt, Inc. (St. Louis, MO). Deionized (DI) water from MilliQ water purification system (Millipore, Milford, MA) was used. For large scale work sodium chloride and sodium hydroxide were purchased from Merck kG (Darmstadt, Germany), and water obtained by an in-situ double distillation water purification plant. Buffers used for the AX chromatography were buffer A: 20 mM NaOH and buffer B: 20 mM NaOH + 2.5 M NaCl. No organic solvent additive was used unless specified.

Crude ISIS 2302 [sequence: 5′-GCC CAA GCT GGC ATC CGT CA-3′] was used for all experiments. This is a 20-mer phosphorothioate designed to bind to the 3′ UTR region of the ICAM-1 mRNA (7). It has been designed as a possible treatment for the following disease indications: Chrohn's disease, psoriasis, rheumatoid arthritis, ulcerative colitis, and renal allograft. It was synthesized at

80 mM scale using OligoProcess automated synthesizer. The crude oligonucleotide was detritylated on the synthesizer, thus it was 'DMT-off' crude. It was used at a concentration of about 45 g/L. The crude was diluted 1:1 with buffer A prior to injection.

ANALYSIS OF OLIGONUCLEOTIDES

The amount of oligonucleotide was measured by UV spectroscopy. The absorbance at 266 nm was with appropriate dilution of the solution was used to determine the OD units. This was divided by a multiplier (25.7 OD = 1 mg) to obtain the amount of oligonucleotide in the solution. The length-based purity was determined by capillary gel electrophoresis (CGE). A custom gel filled capillary was obtained from J&W Scientific and run on Beckman 5100 P/ACE capillary electrophoresis system. Samples were desalted by microdialysis on Millipore membranes. Capillary separation length was 40 cm, and 300 V/cm were applied.

The phosphodiester content was determined by analytical AX chromatography. This also served as a first estimate of the length based purity. ReSOURCE Q 1 ml column was run with same buffers as in preparative experiments (Buffer A: 20 mM NaOH, buffer B, 20 mM NaOH + 2.5 M NaCl). Column was placed in a column heater and operated at 70°C. In most cases the analytical method was run on a Waters HPLC system, Milford, MA. In some cases the method was adapted and run on Akta Explorer 10 (APB, Piscataway, NJ).

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