Organic Process Research & Development **2000**, *4*, 205−213

Process Development for Purification of Therapeutic Antisense Oligonucleotides by Anion-Exchange Chromatography

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Abstract:

This article describes the development of an anion-exchange chromatography method for purification of phosphorothioate antisense oligonucleotides. A variety of operating conditions were studied and optimized at small scale. These optimized parameters were used to generate data at multigram scale. The selected method can give a high purity product at a very good yield of the full-length desired product. This method can be suitably scaled up for antisense oligonucleotide purification at large scales.

Introduction

Synthetic oligonucleotides are now being routinely synthesized under cGMP conditions for clinical studies and commercial drug supplies.[1] Currently, there are over 12 antisense oligonucleotides undergoing human clinical trials internationally.[2] Vitravene is the first commercial drug in this class of compounds approved by the U.S. FDA in 1998. Both the discovery and development of Vitravene were accomplished here at Isis Pharmaceuticals. Isis conducts the GMP chemical synthesis and purification of the active pharmaceutical ingredient (API) required to produce Vitravene.

In the manufacture of these commercial and clinical grade oligonucleotides purification is a critical step that ensures the purity and quality of oligonucleotides. This article discusses the development of an anion-exchange (AX) chromatographic process that may be scaleable for the purification of such oligonucleotides. Key parameters involved in optimizing the purification process are reported, and our selection of critical process parameters based on scalability, feasibility, and performance are discussed.

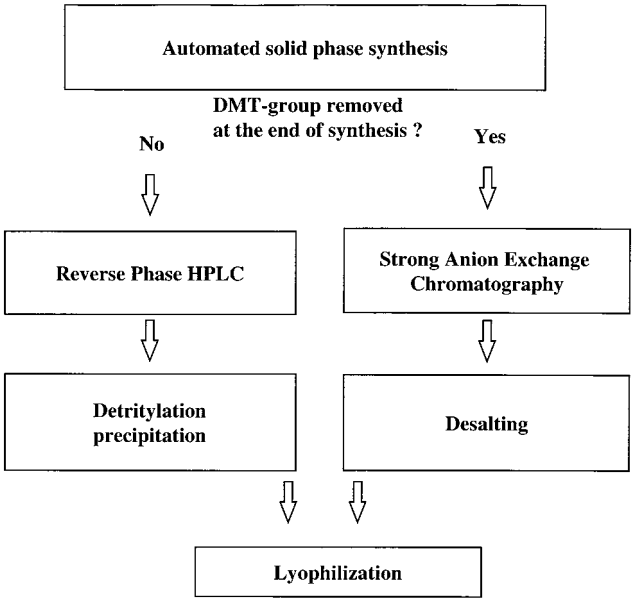

Figure 1. **Purification strategies for solid-phase synthesized oligonucleotides.**

Antisense oligonucleotides are typically synthesized on solid-phase supports using automated synthesizers. After cleavage of oligonucleotides from the support, two main strategies could be used to purify oligonucleotides at very large scale as indicated in Figure 1. In the first method, the 5′-DMT (4,4′-dimethoxytrityl) group is left intact on the oligonucleotide, acting as a hydrophobic handle for reverse phase (RP) purification. Effective separations of large quantities of oligonucleotides can be accomplished using this method. RP purification utilizing silica-based matrix is used for commercial manufacture of these drugs. The largest current manufacturing of clinical grade DNA is produced using the RP method in our facilities. This strategy yields oligonucleotides of high purity and high yields. A similar strategy using hydrophobic interaction media has been used elsewhere at relatively large scales.[3] However, there are few limitations with the RP strategy. The equipment for RP is typically versatile high-pressure equipment. The large volumes of organic waste generate significant solvent disposal costs and may require an explosion-proof facility at very large scales. For these reasons AX purification has been evaluated as an alternative to RP for large-scale and commercial-scale purifications.

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10.1021/op990091o CCC: $19.00
Published on Web 03/29/2000

There are several potential advantages in selecting an AX-based purification method. The purification can proceed without any protecting groups on the 5′-end of the oligonucleotide. This can decrease the number of process steps after purification. The equipment used for aqueous-only purification is low to medium pressure and represents a savings compared to high-pressure equipment required for RP purification. The saturation capacity of the AX resins is typically higher than RP materials for this application, and thus smaller-scale equipment may be used for the same batch size. However, the critical difference is that AX chromatography needs to be followed by an effective desalting step for the isolation of oligonucleotide. Also, the eluent volumes produced in AX are large compared to those in RP purification, and the product is relatively dilute. The equipment and scale-up of this unit operation is well understood for proteins and other biomolecules, however, some additional process development effort is required for effective large-scale purification of oligonucleotides. The yield per cycle for AX may be lower than that for RP, and recycling of side fractions may be required to obtain adequate yield. This may reduce the throughput.

Anion-exchange chromatography has been used extensively in purification of mononucleotides, oligonucleotides and natural nucleic acids. It effectively takes advantage of the charge on the phosphate linkage in such molecules. Separation of nucleotides and homopolymers of oligonucleotides has been described by Cohn.[4] Drager and Regnier[5] have studied the high performance chromatographic analysis of oligonucleotides by AX chromatography. Length based separation has been explored in detail by Baba[6] and Kasai.[7] The analytical aspects of AX chromatography, especially that of phosphorothioates, have been presented earlier by Srivatsa et al.[8] and Metelev et al.[9] Thayer et al.[10] have published protocols for phosphodiester and phosphorothioate analysis and micro-purification by AX. Other groups have studied large-scale separation of oligonucleotides including some phosphorothioates by AX chromatography.[11−15] Gerstner et al.[16] have explored displacement chromatography on anion-exchange resins. We recently reviewed the application of sample self-displacement for oligonucleotides.[17]

The solid-phase synthesis of antisense oligonucleotides and the major associated impurities have been described elsewhere.[2,18] The key impurities are deletion sequences $(n - 1)$, $(n - 2)$ etc., where n represents the desired full-length product. Phosphorothioates also contain partial phosphodiester components, $(P{=}O)_1$, $(P{=}O)_2$ etc. generated as a byproduct during the synthesis and workup steps. There are variety of other smaller impurities that are either discussed in the literature or are in the process of being characterized.[19] For simplicity all molecules strongly retained than the full-length product on either the analytical AX or capillary gel electrophoresis (CGE) are lumped together as $(n + x)$ for the purpose of this article.

On most commercial anion exchangers it is extremely difficult to achieve complete length-based resolution between $(n - 1)$ and *n*-mer phosphorothioates, especially oligonucleotides in the 20-mer range.[8] For example, a 19-mer all-phosphorothioate DNA deletion sequence is not completely resolved from the 20-mer phosphorothioate. If the backbone linkages are all phosphodiesters, there is increased selectivity between the 19-mer and 20-mer and they can be separated easily on most anion exchangers. The selectivity difference between a 20-mer all-phosphorothioate and a 20-mer monophosphodiester (where only one linkage is phosphodiester and the rest are phosphorothioates) is at between the two cases and we can get a good separation of these species. While the resolution between $(n - 1)$ and *n*-mer phosphorothioates is low, it is sufficient to get a good preparative purification of the product from crude material. Although methods exist for the large-scale AX chromatography of oligonucleotides, none describes the systematic process optimization of critical steps and their effects on the quality of the separation.

In this article we present a systematic evaluation of key variables that influence the purification of oligonucleotides by AX. Most of the data are for small-scale analytical columns, but the decisions and criteria used are applicable for process scale-up work. Preliminary preparative work is presented.

Experimental Section

Reagents and Chromatography Columns. ISIS 2302, a 20-mer all-phosphorothioate deoxyribonucleotide was used as the oligonucleotide sample in all separation studies. It is an antisense inhibitor of cell adhesion molecule ICAM-1 and is being developed to treat inflammatory diseases. Crude oligonucleotide was manufactured in-house via automated solid-phase synthesis, and the acid labile hydrophobic group 4,4′-dimethoxytrityl (DMT) was removed on the reactor bed itself.

Reagent grade NaCl and HCl were obtained from Sigma (St. Louis, MO), and NaOH from Mallinckrodt (Paris, KY). Deionized water used in experiments was obtained on-site through the MilliQ system (Millipore, Bedford, MA). Electrophoresis grade tris-borate/7 M urea buffer was obtained from Beckman Coulter (Fullerton, CA).

Most scouting experiments in this study were conducted on the Resource Q 1 mL pre-packed column from Amersham Pharmacia Biotech (Piscataway, NJ), packed with Source 15

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Table 1. **Summary of anion exchangers screened in this study**

		media		
tradename	manufacturer	ligand chemistry	bead chemistry	nominal particle size, μm
DEAE-Sepharose FF	Amersham Pharmacia Biotech (APB), Piscataway, NJ	DEAE[a]	Agarose	90−120
Express Ion Q	Whatman	Q[b]	regenerated cellulose	45
Q HyperD F	BioSepra, Inc. Marlborough, MA	Q	ceramic coated silica with gel inside pores	35
Poros HQ/50	PE Biosystems	Q	PSDVB[c]	50
Poros HQH	PE Biosystems	Q	PSDVB	10
Resource Q	APB	Q	PSDVB	15
Q Sepharose FF	APB	Q	agarose	90−120
Q Sepharose HP	APB	Q	agarose	35
TMAE-HiCap	Merck KGaA	TMAE[d]	poly methacrylate	40
TMAE	Merck KGaA	TMAE	poly methacrylate	40

[a] DEAE: diethylaminoethyl group. [b] Q: quaternary ammonium group. [c] PSDVB: polystyrene divinyl benzene. [d] TMAE: trimethylaminoethyl group.

(15 μm) media. The chromatography media has quaternary ammonium functional group on a polystyrene-based matrix. For larger columns Source Q 30 (30 μm) material was obtained from Amersham Pharmacia Biotech. A slightly larger 1.7 mL (4.6 mm i.d. × 100 mm length) column was packed in-house with the Source 15 and Source 30 media for some experiments. A 110 mL column (XK 26/20, 26 mm i.d. × 21 cm length) was packed with Source 30 media. Other media screened are listed in Table 1 and were obtained directly from the respective manufacturers.

Apparatus and Preparative Conditions. Most chromatographic experiments were performed on a BioCad 60 workstation (PE BioSystems, Framingham, MA) connected to a model SF-2120 fraction collector (Advantec Toyo Kaisha Ltd., Japan. Larger-scale runs (100 mL columns) were run on the Akta explorer 100 workstation (Amersham Pharmacia Biotech). Some preparative runs and all analytical runs were carried out on a Waters (Waters Corp., Milford, MA) chromatographic system, with 717 auto sampler, 600E system controller, 991 photodiode array detector and Millennium 2.10 operating software. Capillary gel electrophoresis (CGE) was performed on a P/ACE 5000 system (Beckman Coulter, Fullerton, CA).

In-process samples from preparative runs were stored in the refrigerator at 4 °C. The pH was not adjusted for analysis. Prior to further desalting, the pH of the product pool was adjusted to between pH 7 and 8 with dilute HCl.

Analytical Conditions. The partial phosphodiester content of fractions was determined by analytical AX chromatography, using a 1 mL (6.4 mm i.d. × 30 mm length) Resource Q pre-packed column (Amersham Pharmacia Biotech, Piscataway, NJ). The analytical separation was performed at 70 °C with the following buffer system: buffer A, 20 mM NaOH; buffer B, 2.5 M NaCl + 20 mM NaOH.

Length-based purity was determined by CGE as described earlier.[20] Fixed gel filled 47 cm (40 cm to detector) Beckman eCap ssDNA capillary (Beckman Coulter, Fullerton, CA) columns were used. The desalted sample was electrokinetically applied for 3−15 s at 7.5 kV and separated at 14.1 kV (300 V/cm) at 40 °C. The signal was monitored at 260 nm.

There was no sample prep in most cases for analytical AX. In some cases the fractions were diluted to 0.5 mg/mL with DI water prior to injection. For CGE, the samples were desalted using centrifugal membrane desalting devices, Centricon SR3 (Amicon div. Millipore, Bedford, MA). The samples were desalted thrice and then diluted to 0.1 mg/mL prior to injection in the CGE.

Results

Initial method exploration was carried out on 1 mL prepacked commercially available Resource Q columns. The solute for these experiments was ISIS 2302 DMT-off crude product solution. A variety of operational parameters were varied individually, and optimal parameters were chosen on the basis of qualitative separation of monophosphodiester impurity from phosphorothioate full-length material. Scalability and economics dictated some choices.

An unoptimized chromatogram is shown in Figure 2. The monophosphodiester (P═O) species is not well resolved in this example. To optimize the separation, some key parameters were explored. A detailed account follows.

Selection of the Chromatographic Media. For a commercially viable process the following criteria were considered, comprising the broad categories of performance, scalability, and regulatory issues. Media performance parameters included selectivity and dynamic binding capacity of the media. Scalability factors included size of the chromatographic beads, potential ease of packing in large industrial columns, and good flow characteristics. Scalability and regulatory concerns dictate that the media be available commercially at the scale desired, that the material be usable under cGMP conditions, that there should either be a comprehensive regulatory file provided by the vendor or

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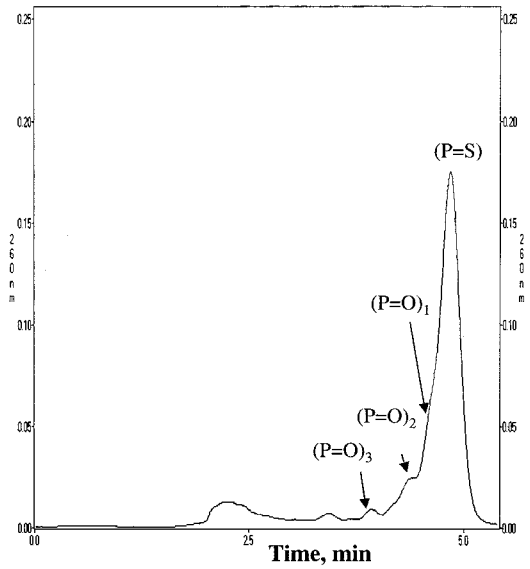

Figure 2. **Representative run without optimized conditions. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH/2.5 M NaCl, flow rate 5 mL/min, gradient 0−100% B in 20 CV, loading 50 μg (50 μl of 1 mg/mL ISIS 2302).**

there should be sufficient data in-house to indicate reproducibility between batches, and that adequate clean-in-place characteristics and low leachability be demonstrated. Cost is a consideration, but it needs to be evaluated on the basis of total process economics and not as an isolated unit operation.

We selected several AX media which satisfied most of the criteria listed above. These are listed in Table 1. For the purpose of this study we focus on Source 30 media. The base matrix of this media is polymeric. It is a rugged particle, with good chemical resistance in the pH range 2−14. Its rigidity and relatively large particle size makes it easy to pack. The selectivity of this media for oligonucleotides is among the highest we have observed. It has very good loading capacity. While the POROS HQ/H 10 μm media has similar selectivity, its loading is lower than the Source 15 μm media. Therefore, we selected the Source material for further studies. The surface characteristics of the Source 15 μm and 30 μm particles are identical; therefore, for most parameters a 1 mL screening column of 15 μm material was used. Resolution is higher on the 15 μm particles than on the 30 μm particles, as expected. Therefore, it is easier to judge qualitative changes using the 15 μm media. Some preliminary method development of DMT-on and DMT-off phosphorothioates purification on this media has been described earlier.[14,15] However, we present a more detailed analysis of the effect of critical parameters here and have scaled up the method to process larger quantitites of oligonucleotide. Most critical parameters were tested more than once on the larger particle size to ensure reproducible results.

Effect of Flow Rate. Higher separation flow rates can directly increase productivity. However, there may be equipment-related restrictions on high flow rate, and a higher flow rate can result in reduced resolution due to mass transfer

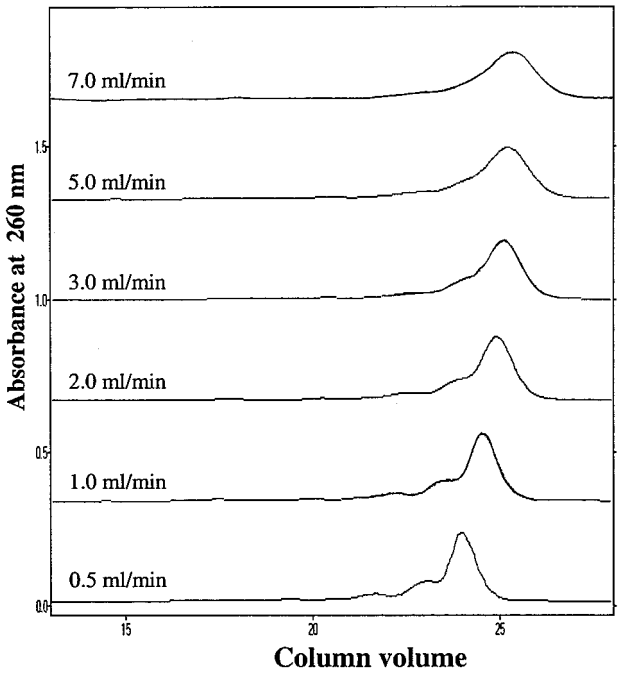

Figure 3. **Effect of flow rate on AX purification of oligonucleotides. The flow rate for the traces bottom to the top are (mL/min): (a) 0.5, (b) 1, (c) 2, (d) 5, and (e) 10. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH/2.5 M NaCl. Sample: 50 μg [50 μl of ISIS 2302 (1 mg/mL concentration)], Gradient: 0−100% B in 20 CV. Flow rates are indicated.**

limitations. Therefore, it is important to optimize flow rate at small scales for each media under study. A fixed injection volume and concentration and a simple gradient were used to study the effect of flow rate. Figure 3 shows the chromatographic profiles obtained when the volumetric flow rate was varied from 0.5 to 7 mL/min, equivalent to linear velocities of 90−300 cm/h. The lower flow rate shows superior resolution of the monophosphodiester and the phosphorothioate species. A compromise flow rate of 1 mL/min was considered optimal, equivalent to a linear velocity of 186 cm/h, which is quite practical for commercial chromatographic hardware. For faster screening, some experiments in this study were run at 5 mL/min rather than the optimal 1 mL/min for the 1 mL column.

Effect of Gradient Slope. Gradient slope can have a significant effect in maximizing resolution for closely eluting species at a particular load. Therefore, the slope of a linear gradient from 0 to 100% B, where B is the elution buffer, was evaluated. The number of column volumes (CV) was varied from 5 to 60 CV. As indicated in Figure 4, a shallower gradient of 60 CV increased resolution. While further slope reduction could potentially increase resolution, the zones would be additionally dispersed by diffusion. Therefore, 60 CV was considered optimal for this column scale.

Effect of Loading. When considering small-scale optimization experiments it is important that loading be within the linear range of adsorption isotherm. A series of experiments with increasing injection volume were run to determine the limit at which the elution profiles showed overload profiles. A 1 mg/mL stock solution was used, and the sample

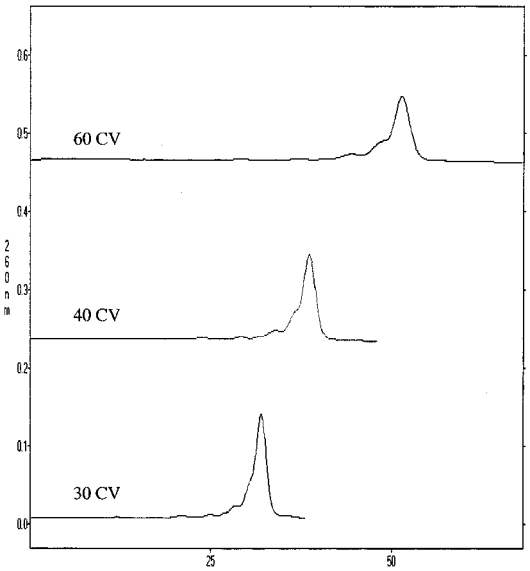

Figure 4. **Effect of gradient slope on AX purification of oligonucleotides. The gradient 0−100% B was varied with different eluent volumes expressed as column columns. The gradient column volumes for traces bottom to the top are (CV): (a) 30 ,(b) 40, and (c) 60. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH/2.5 M NaCl. Sample: 50 μg [50 μl of ISIS 2302 (1 mg/mL concentration)], flow rate 5 mL/min.**

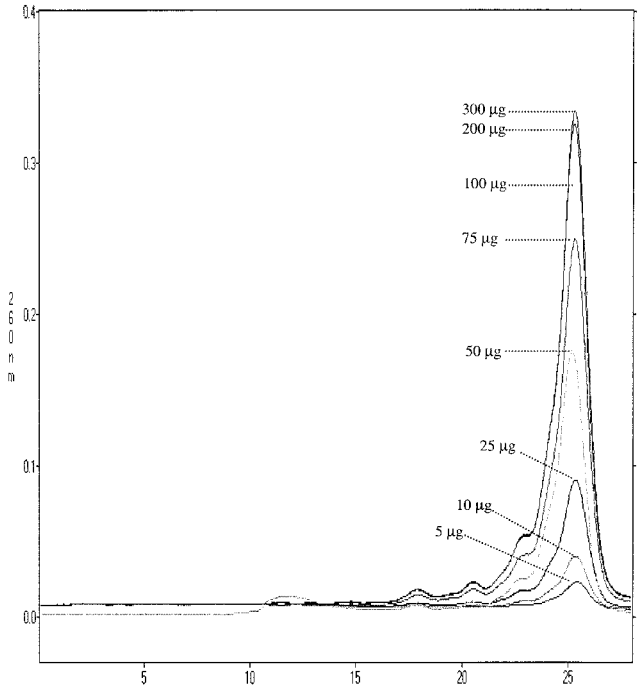

Figure 5. **Effect of sample injection (analytical loads). The sample injection was 5, 10, 25, 50, 75, 100, 200 and 300 μg in the traces indicated in the figure. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH/2.5 M NaCl. Gradient: 0−100% in 20 CV. Sample: stock [ISIS 2302 (1 mg/mL concentration)], flow rate 5 mL/min.**

injection varied from 5 to 300 μl (Figure 5). The response is predictable up to 200 μl injection and beyond that appears nonlinear. This is not a rigorous assessment, but for the purpose of optimization experiments, 50−200 μl injection was considered suitable. For preparative experiments, dynamic binding capacity of the media is more important as discussed below.

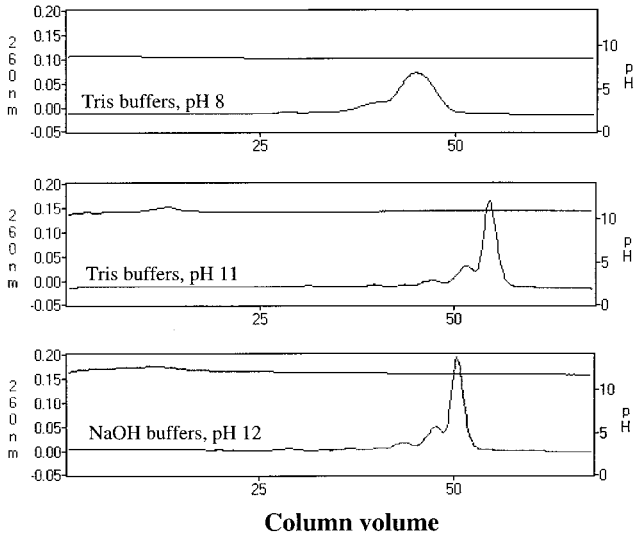

Figure 6. **Effect of buffer ions and pH. (Panel A) Buffer A: 100 mM Tris, pH 8.0. Buffer B: 100 mM Tris/2.5 M NaCl, pH 11.0. (Panel B) Buffer A: 100 mM Tris, pH 11.00. Buffer B: 100 mM Tris, 2.5 M NaCl, pH 11.0. (Panel C) Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH, 2.5 M NaCl. Gradient: 0−100% in 60 CV. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Sample: 50 μg [50 μl of ISIS 2302 (1 mg/mL concentration), flow rate 5 mL/min.**

Effect of pH and Buffer Ions. We compared resolution with different buffer ions at different pHs. The Tris/Cl buffer system at pH 8 and 11 was evaluated along with sodium phosphate and sodium hydroxide buffer systems at high pH. Figure 6 shows a comparison of using a 100 mM Tris/Cl buffered run at pH 8 and at pH 11, and their comparison with a separation with NaOH buffer system at pH 12.0. The higher pH shows dramatic improvement in resolution, but the experiment with 20 mM NaOH is superior to the two other buffer systems. Sodium phosphate (100 mM) buffer system at pH 12 was compared with the NaOH (20 mM) buffer system. A gradient from 0 to 3 M NaCl in 60 CV and 0−2.5 M NaCl in 60 CV was used for the phosphate and hydroxide experiments, respectively. The results are very close as seen in Figure 7, with the NaOH buffering showing a slight edge in performance. NaOH also is bacteriostatic and is also compatible with our post-purification processing. Therefore, all preparative experiments were based on a NaOH buffer system.

Molarity of NaOH. NaOH molarity needs to be selected with care. A low molarity may not give sufficient secondary structure denaturation or buffering capacity. A very high molarity could degrade the chromatographic column and aid deamination of dCyd to dUrd.[21,22] Therefore, experiments were conducted to study the effect of NaOH buffer molarity on AX separation. The following buffer system was used

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(22) Germann, M. W.; Pon, R. T.; van de Sande, J. H. *Anal. Biochem.* **1987**, *165*, 399−405.

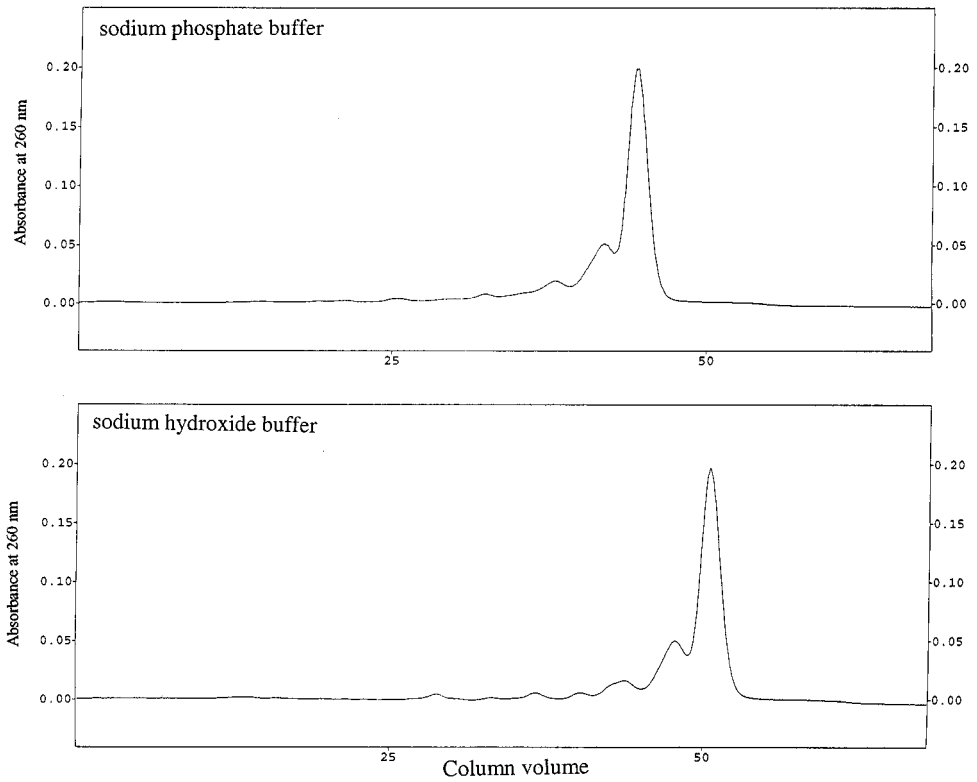

Figure 7. **Comparison of using sodium phosphate and sodium hydroxide as the buffers. (Panel A) Buffer A: 100 mM sodium phosphate, pH 12. Buffer B: 100 mM sodium phosphate + 3 M NaCl, pH 12.0. (Panel B) Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH + 2.5 M NaCl. Gradient: 0−100% B in 60 CV. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Sample: 200 μg [200 μl of ISIS 2302 (1 mg/mL concentration)], flow rate 1 mL/min.**

A: *X* mM NaOH, B: *X* mM NaOH + 2.5 M NaCl, where *X* was varied from 1 to 100 mM NaOH. The chromatograms from these runs are shown in Figure 8. Surprisingly, the elution pattern does not change markedly when NaOH molarity is varied from 1 to 100 mM. Binding is reduced above 50 mM but is relatively constant below that level. However, the pH profile does change during the run. The sample was at neutral pH for most of these runs and not adjusted to the running buffer pH. This causes injection-related pH disturbances as seen in Figure 9A. As expected, disturbances are reduced significantly when sample pH is adjusted to match the running buffer (Figure 9B). The change in pH during the run is minimal for 50 and 100 mM runs and much greater for lower molarity NaOH runs. Judged purely on chromatographic results, even 1 mM NaOH could be used; however, to ensure pH stability during the run, 10 or 20 mM is a better choice. We have elected to use 20 mM NaOH for all our experiments. One of the compounds we purified by using AX was an 8-mer with a very strong tetrad-forming tendency. In that case 50 mM NaOH was the minimal molarity that could be used to generate reproducible chromatographic performance.[16]

Effect of Organics in the Buffer. The heterocyclic bases contribute to the hydrophobicity of oligonucleotides. Likewise, all anion exchangers exhibit a degree of nonspecific solute binding, mostly hydrophobic in nature. Therefore, a small percentage of organics can be presumed useful to aid the separation. Some change was observed in the elution profile when methanol was added as an organic modifier to elution buffers; however it does not appear practically significant. Figure 10 compares traces with 0−50% methanol in the operating buffer. As expected, methanol reduced binding of oligonucleotides to the columns, and retention times were reduced. The buffers were miscible at the levels tested; however, at larger scales making such buffers is an issue. Results indicated that completely aqueous running buffers were the best approach for scale-up.

Effect of Column Length. A simple experiment was conducted to determine if increasing column length improved resolution. Two 3 cm long pre-packed Resource Q columns were carefully connected in series to simulate a 6 cm column and compared to a single 3 cm long column. The chromatograms are shown in Figure 11. The additional column length does not appear to make a difference to resolution. The injection in two experiments was identical and not scaled proportionally to the increased volume of the double column. This is consistent with experiments with other solutes on ion-exchange chromatography, where for the same particle size media the plate number does not affect resolution. For process scale purifications, the use of a longer column would be more economical than a wider column, provided that flow rates were sufficiently high.

Effect of Temperature. Figure 12 shows the comparison of a separation at room temperature (20 °C) and at 70 °C. The higher temperature demonstrates better resolution between $(P{=}O)_1$ and (P═S) peaks. High temperature also separates a peak between the $(P{=}O)_1$ and $(P{=}O)_2$ peaks. Large scale runs are feasible at high temperatures; however, the benefit of running at higher temperatures is not justified at higher scales due to the increased cost of the equipment.

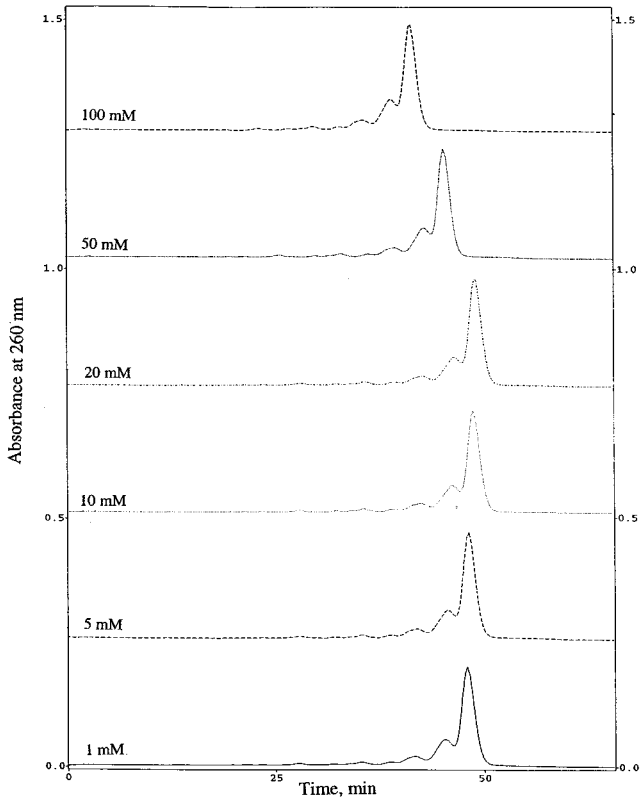

Figure 8. **Effect of NaOH molarity in the buffers on AX purification of oligonucleotides. Buffer system: A: *X* mM NaOH, B: *X* mM NaOH + 2.5 M NaCl. *X* is the NaOH molarity varied in the panel from bottom to the top traces as (1 mM): 5, 10, 20, 50, and 100 mM. Gradient: 0−100% B in 60 CV. Column: Resource Q 1 mL (6.5 mm i.d. × mm length). Sample: 200 μg [200 μl of ISIS 2302 (1 mg/mL concentration)], flow rate 1 mL/min.**

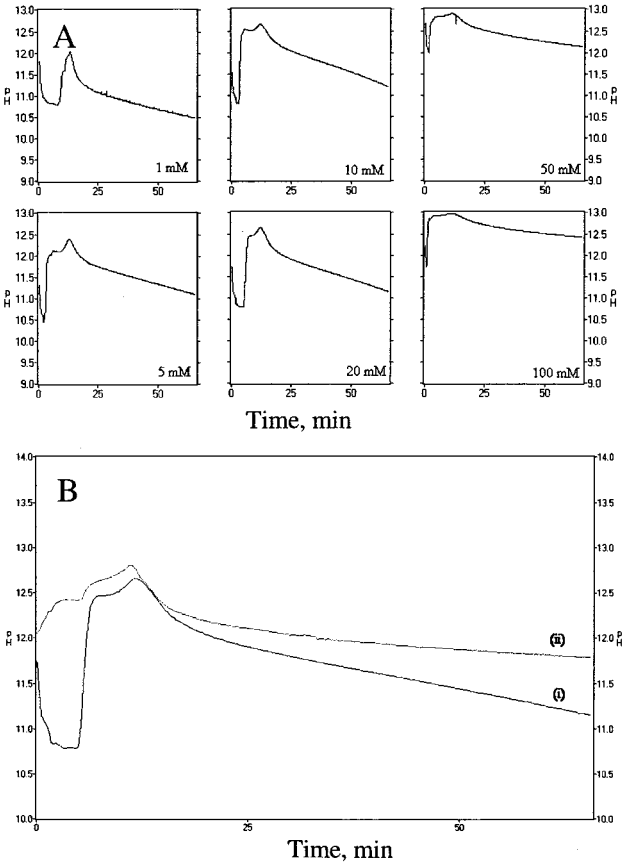

Figure 9. **(A) pH variation due to NaOH molarity change, Conditions as in Figure 6; (B) pH variation for 20 mM NaOH: (i) sample in water, (ii) sample pH adjusted to the equilibration buffer pH and molarity.**

Therefore, all further preparative runs experiments were performed at room temperature.

Dynamic Binding Capacity. Two larger 1.7 mL columns (4.6 mm i.d. × 100 mm length) were packed with Source 15 and Source 30 media, respectively. The sample was 0.92 mg/mL purified and desalted ISIS 2302. The column was set in the bypass mode and the sample solution allowed to flow through the UV flow cell at 1 mL/min. This allowed the sample to fill up most of the void volume and also enabled us to record the response of the UV detector at sample saturation. The column was then set in-line and sample allowed to flow continuously at 1 mL/min until the output response matched the initial bypass signal. The column was then flushed with strong eluent. The amount of oligonucleotide bound to the column at 5% breakthrough was determined. The dynamic capacity calculated for Source 15 was 24.4 mg/mL CV, and for Source 30 it was very close at 23.6 mg/mL CV.

Optimized Conditions for and Gram Scale and Process Scale Experiments

The optimized conditions were then utilized for separation of crude ISIS 2302. The chromatogram is shown in Figure 13. In contrast to the initial chromatogram shown in Figure 1, the resolution between $(P{=}O)_1$ and (P═S) peaks is now nearly 1. Experiments were conducted with loading higher

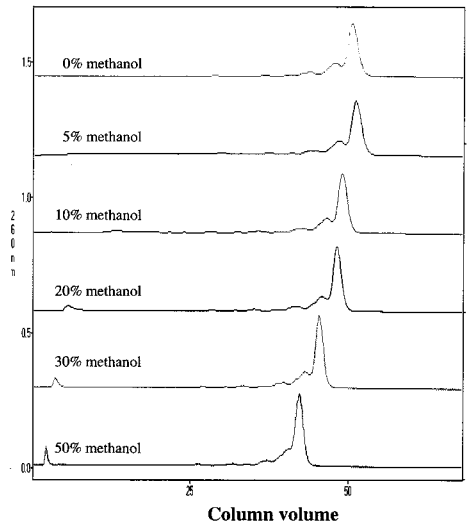

Figure 10. **Effect of organic modifiers in the operating buffers. Traces from top to bottom indicate elution profiles with (a) 0, (b) 5, (c) 10, (d) 20, (e) 30, and (f) 50% methanol in the buffers. Buffer A: 20 mM NaOH with above percentages of methanol in water. Buffer B: 20 mM NaOH, 2.5 M NaCl with above percentages of methanol and water. Gradient: 0−100% in 60 CV. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Sample: 50 μg [50 μl of ISIS 2302 (1 mg/mL concentration)], flow rate 5 mL/min.**

than that discussed in section 3.d. Higher loading experiments were conducted, and the gradient was shortened in the range

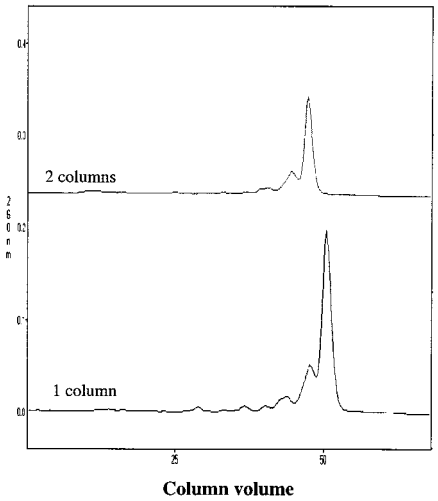

Figure 11. **Effect of column length on AX purification of oligonucleotides. Top profile shows the separation on two columns connected in series (6 cm effective length), and the bottom panel indicates the separation on a single column (3 cm effective length). The gradient 0−100% B in 60 CV. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH/2.5 M NaCl. Sample: 200 μg [50 μl of ISIS 2302 (1 mg/mL concentration)], flow rate 5 mL/min.**

of interest while maintaining the gradient slope. Loading of 1 mg on the small 1 mL cartridge is shown in Figure 14. Although the loading is higher than the previous sections, the essential elements of the chromatogram are still the same as in the optimized separation Figure 13.

The basic operating conditions were fixed at this stage by keeping the buffer ions and their molarity and the media unchanged. Several strong AX media (Table 1) were further tested at this stage (data not shown), as the effects of column loading and column capacity are more obvious at larger scales. Process conditions such as backpressure, packing effects, etc. also impact at this scale.

Extension of the basic method to 100 mL column sizes is shown in Figure 15. This column was packed with the Source 30 chromatographic media. The loading was in the low range (0.5 mg/mL CV) similar to that in experiments discussed in the earlier sections. The chromatogram at 260 nm is quite similar to the analytical trace obtained in Figure 13 and the preparative loading on small 1 mL column in Figure 14. Purity of the main peak by analytical SAX and analytical capillary electrophoresis (CE) were 95 and 92% respectively at a yield of full-length product of 85%. Additional fractionation can increase the purity at the expense of some yield loss. Similar experiments with higher sample loads are in progress, using a larger column packed with Source 30 media. The successful scale-up from 1 to 100 mL columns at the bench scale suggests that the data generated at lower analytical columns can be systematically utilized for further developmental work.

A method based on these results is being scaled to process crude quantities relevant to current production scales. This work is in progress and will be reported later.

Conclusions

These small-scale experiments have allowed optimization of key process parameters involved in the purification of ISIS 2302 by AX chromatography. In this study we have preferred to use single-parameter variation experiments rather than factorial design. At this stage, the use of an automated HPLC workstation simplified running single-parameter experiments. For larger sample loads, a factorial design could reduce the number of experiments.

For scale-up, the following criteria were found to be important from the parametric experiments. The flow rate studies indicate that a linear velocity of 190 cm/h was a good compromise between speed and resolution. A shallower gradient slope improves the separation at small scales. At

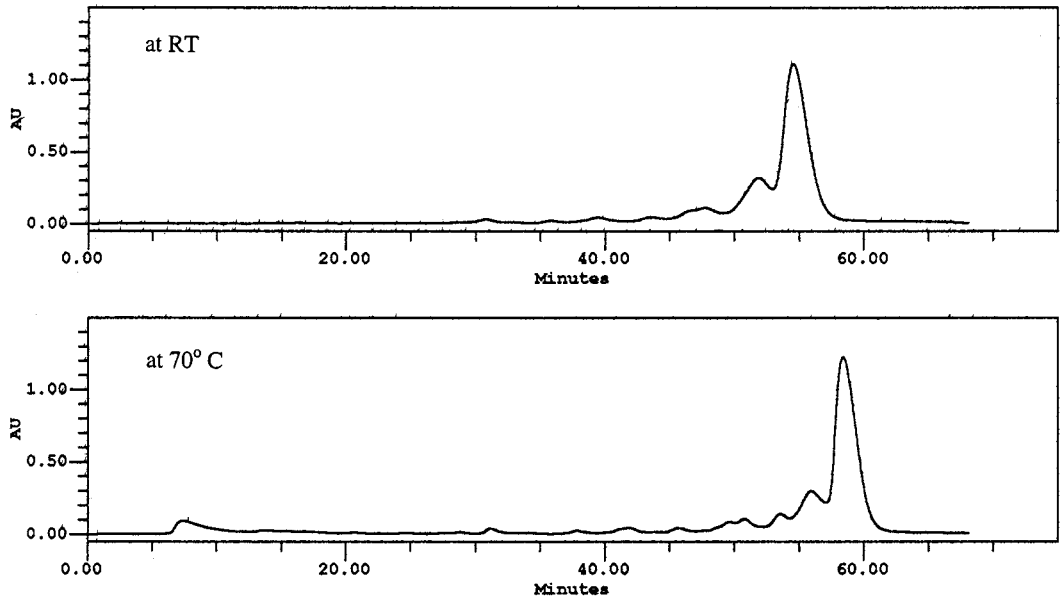

Figure 12. **Effect of temperature on AX purification of oligonucleotides. Top trace at room temperature and bottom trace at 70 °C. Separation was performed on an analytical Waters system with a heating block. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH/2.5 M NaCl. Sample: 200 μg [50 μl of ISIS 2302 (1 mg/mL concentration)], Gradient: 0−100% B in 60 CV. Flow rate 1 mL/min.**

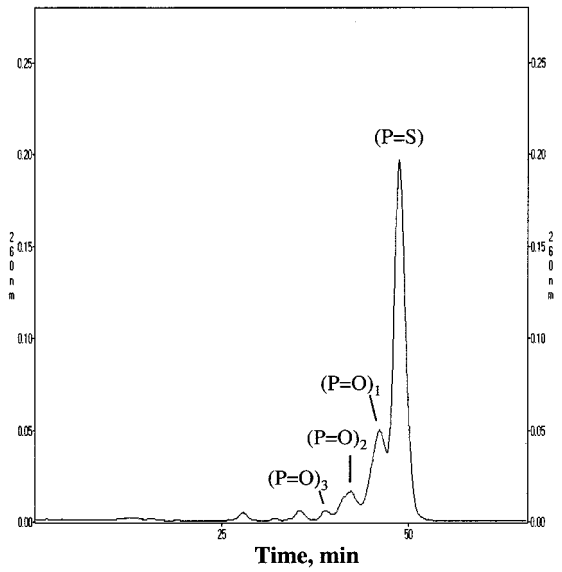

Figure 13. **Optimized parameters. Column: Resource Q 1 mL (6.5 mm i.d. × 30 mm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH, 2.5 M NaCl, flow rate 1 mL/min, gradient 0−100% B in 60 CV, loading 200 μg (200 μl of 1 mg/mL ISIS 2302).**

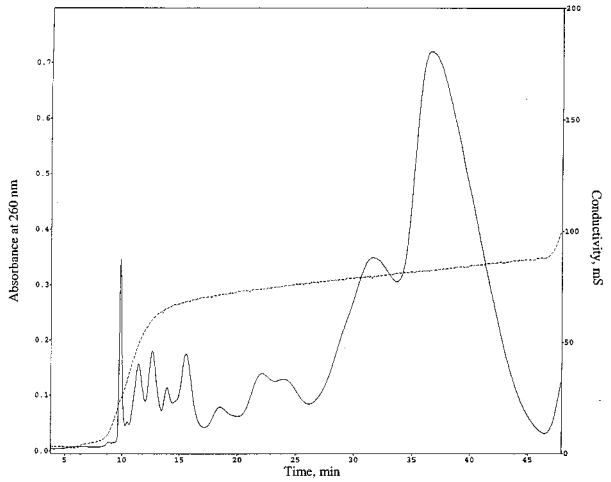

Figure 14. **Preliminary preparative experiments. Column: Resource Q 1 mL (6.5 mm i.d. × 3 cm length). Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH, 2.5 M NaCl, flow rate 1.4 mL/min, Elution sequence: equilibration 2 CV, 0% B, load 1.4 mL (0.75 mg/mL), wash 0% B for 2 CV, gradient 0−40% B in 5 CV, 40−60% B in 55 CV, clean 100% B in 2 CV, loading 1 mg (1.4 mL of 0.75 mg/mL ISIS 2302).**

larger scale, gradient optimization is key to successful separation. Regarding sample load, overloading has been shown to be an important parameter that can be optimized for oligonucleotide purification.[17] Therefore, experiments at large scale were needed to optimize this parameter. The pH

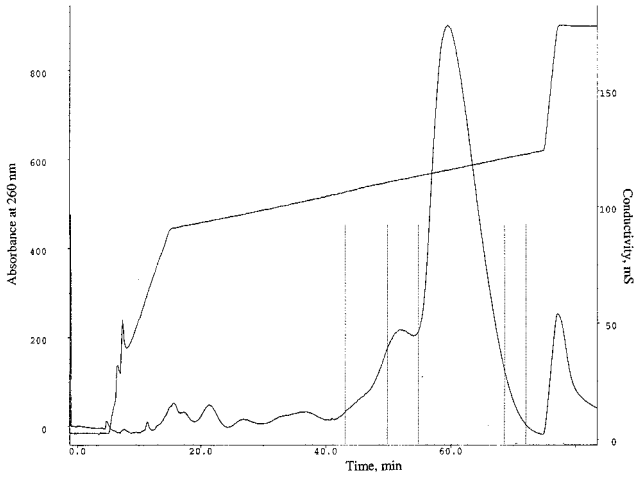

Figure 15. **Preparative experiment on 100 mL column. Column: XK 26/20 column, 110 mL (26 mm i.d. × 21 cm length), packed with Source 30 media. Buffer A: 20 mM NaOH. Buffer B: 20 mM NaOH, 2.5 M NaCl, flow rate 22 mL/min, Elution sequence: equilibration 2 CV, 0% B, load 2.5 mL, wash 0% B for 2 CV, gradient 0−40% B in 2 CV, 40−60% B in 12 CV, clean 100% B in 1 CV, loading 57 mg (2.5 mL of 23 mg/mL ISIS 2302).**

experiments have shown conclusively that higher pH is superior for this purification, with NaOH offering best selectivity. The molarity of NaOH appears to be less critical at this scale; we selected 20 mM to provide adequate buffering at higher sample loads. Cost or scalability concerns may alter the value selected for some variables at higher scales. For example, adding an organic modifier such as methanol does increase resolution marginally, but the increase is not, in our opinion, significantly relative to the reduced robustness of the separation, lower column capacity, and more complex buffer preparation. Resolution is not affected significantly by column length, and thus a longer column that allows desired flow at a suitable back-pressure rating can be chosen. This is generally a lower-cost alternative to a wider column with the same column volume. For cost reasons, operating the purification at ambient temperature was selected rather than operating it at higher temperatures. The optimized conditions scale linearly in the 1 to 100 mL range and can be similarly extended to larger columns. This analysis of critical variables narrows considerably the parameters that need to be tested and optimized at higher scales. This allows optimizing the purification close to the desired large scale with minimal experimentation.

Received for review October 18, 1999.

OP990091O