Development of separation method for analysis of oligonucleotides using LC-UV/MS

Ida Maria Björs
Abstract

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Introduction
Oligonucleotides are short nucleic acid chains, usually 19-27mer long. They bind to their corresponding chain, making a specific inhibition possible. In pharmaceuticals, this can be used to inhibit the expression of a gene or protein of interest. Oligonucleotides are usually analyzed based on separation using both hydrophobic and ion-exchange properties. In this project, the possibility to use a mixed-mode column to separate these oligonucleotides and their impurities were explored.

Method
Liquid chromatography is used as the separation method and the method of detection is both mass spectrometry and UV. Three different columns are evaluated; C18, DNAPac RP, and mixed-mode RP/WAX.

Results and discussion
Different compositions of mobile phases and gradients are evaluated based on a literature study. Triethylamine, triethylammonium acetate, ammonium formate, hexafluoroisopropanol is used along with both methanol and acetonitrile. Phosphate buffer is evaluated on LC-UV. The results from the C18 column displays a good separation of the oligonucleotides, whilst the DNAPac RP is not as sufficient using the same mobile phases. The mixed-mode column provides good separation and selectivity using phosphate buffer and UV detection.

Conclusion
Mixed-mode column has the potential to be used for separation of oligonucleotides and one future focus would be to make the mobile phase compatible with mass spectrometry. Phosphate buffer and UV detection seems to be the go-to mobile phase using mixed-mode column even though MS is a more powerful tool for the characterization and identification of oligonucleotides. This provides a hint about the challenge in making the mobile phase MS compatible.
1 Introduction

Antisense oligonucleotides are investigated for their use as potential drugs and have been under clinical advancement for about 30 years [1,2]. They are short nucleic acid polymers which bind specifically to a complementary nucleic acid (mRNA) by hydrogen bonding. After binding to the complementary nucleic acid it will enter the cell and activate an enzyme called RNase H. This will result in degeneration of the mRNA strand. In this way, the mRNA degenerates before it can start a translation step. Thanks to the fact that it is sequence-specific, it will not work unless perfectly synthesized, making the method very selective [3]. This makes it possible to use a sequence-specific oligonucleotide to inhibit the expression of a target gene or protein, this character is called the silencing effect [4]. The silencing effect has been seen to have the capability to be more effective than inhibiting the gene or protein itself [4,5].

Oligonucleotides as pharmaceutical candidates are often modified to increase the in vivo stability. This is done by replacing the non-bridging oxygen in the linkage with a sulfur atom. These oligonucleotides are called phosphorothioate oligonucleotides and have the same solubility and charge of the backbone as non-phosphorothioate oligonucleotides [6]. Oligonucleotides are often synthesized by solid-phase oligomerization [1] or phosphoramidite chemistry [7].

As early as 1978 Zamenick and Stephenson managed to inhibit the expression and growth of Rous sarcoma virus in cell culture by using the complementary antisense oligonucleotide to the virus RNA, which strengthens this theory [8,9]. Today, oligonucleotides are investigated as a part of the treatment of many diseases, such as Alzheimer’s disease and cancer to mention some [3]. Other applications where oligonucleotides are used today is genotyping, clinical diagnosis, polymerase chain reaction and DNA sequencing [4, 10]. To get a hint about the progress, six DNA pharmaceuticals were approved by the US Food and Drug Administration during 2017. These were the treatment for cytomegalovirus, age-related macular degeneration of the retina and homozygous familial hypercholesterolemia amongst others. The first antisense oligonucleotide pharmaceutical called fomivirsen got approved in 1998 [2,11].

The increasing interest for oligonucleotides in therapeutics also increases the demand for good separation methods for analysis, to be able to analyze important properties such as the purity of the sample [3,12,10]. This is important to make sure no unwanted gene silencing will occur [13]. The oligonucleotides are usually 19-27mer long and even though the synthesis and purification are well developed, impurities do occur [3,6]. Impurities can occur during the synthesis because of different parameters, such as impurities in the raw material or in the solvent. The longer the chain is, the larger is the risk of increasing impurities since the number of synthesis steps increase. A lot of different impurities can be analyzed, such as the formation of chloral adducts, acrylonitrile adducts, and isobutyryl adducts. The wrong length of the chain can also occur during the synthesis as a result of coupling inefficiency or inefficiency of cleavage [3,4,14]. Reactions as deamination, sulfur loss, and depurination can also produce potential impurities of the oligonucleotide. The most vigorous tool to identify the oligonucleotides and their possible impurities are considered to be liquid chromatography and detection using mass spectrometry. For oligonucleotides, separation using ion-pair reversed-phase and anion-exchange have given sufficient results [6].

Oligonucleotides are traditionally analyzed using both ion-exchange liquid chromatography and ion-pair reversed-phase liquid chromatography to get separation based on both charge and hydrophobicity [14]. In this study, a mixed-mode column will be used as a stationary phase, which combines both the separation based on charge and the separation based on hydrophobicity. Oligonucleotides are multiply negatively charged, hence
the anion-exchange liquid chromatography plays an important role in the separation.

Due to the negatively charged backbone of the oligonucleotide, it will also attract positively charged alkali metal ions as sodium and potassium. This attraction leads to the formation of metal adducts which have a negative impact on the mass spectroscopic analysis. The number of column volumes that flow through the system seems to have an impact on the formation of metal adducts. This is most likely due to the metal impurities in the mobile phase which contribute to the formation of metal adducts. Using a purer mobile phase will result in less spectral abundance loss over time [15].

When using ion-exchange chromatography the charge of the molecule is utilized. The charge results in different electrostatic affinities of the molecules for the stationary phase. In this case, where the oligonucleotides are negatively charged, the stationary phase should be positively charged (anion-exchange). Anions in the mobile phase will then be able to exchange the negative charges on the oligonucleotide. The mixed-mode, on the other hand, contains two or more sites of interaction, making the outcome of the separation much harder to anticipate [13].

Electron spray ionization (ESI) is a soft ionization method which doesn’t require any matrix. The sample is injected via the UPLC system into the instrument. ESI is good for oligonucleotides since they can be multiply charged and ESI can produce these higher numbers of charges [16]. Time of flight (TOF) mass spectrometry depends on the different masses of the molecules by operating with the equal kinetic energy of the molecules. In this way, when the molecules are projected into the detector the light ion will reach it earlier than the heavy ions. This makes mass separation possible [17].

The aim of this study is to develop a separation method for analysis of oligonucleotides using mixed-mode columns and MS-detection. The mixed-mode column used in this project is a reversed-phase/weak anion exchange based on silica packing material. The silica contains an alkyl chain which has an ionizable terminus. This combination produces both reversed-phase and weak anion exchange properties [18].

The method will be optimized to increase the selectivity and sensitivity and to decrease the formation of metal adducts. UPLC/ESI-qTOF together with MS/MS and MS$^E$ and ESI-MS has been examined and shown to be a good choice of instrumentation to get an effective analysis of oligonucleotide mixtures [19,20]. A C18 column and reversed-phase column are evaluated parallel to evaluation the mixed-mode column for comparison and as a compliment.

Figure 1: 16-mer long oligonucleotide, analyzed using both MS and UV-detection during this experiments. The sequence is GGG AAA mCmCmC TTT GAmCT.

2 Experimental

Information about the instruments, the chemicals and material are displayed under this section.

2.1 Chemicals

Chemicals used are compiled in this section. Triethylamine (TEA, $\geq 99\%$), triethylammonium acetate buffer (TEAA, 1.0 M in water, pH 7) and ammonium formate (AF) for mass spectrometry ($\geq 99\%$) were purchased from Sigma Aldrich. Acetonitrile (ACN, LC-MS grade), ortho-Phosphoric acid (reagent grade, 85%) and glacial acetic acid (analytical grade) were purchased from Fisher Scientific. Methanol (MeOH) was used both in mobile phases and to wash the glassware. In mobile phases, LC-MS grade purchased from Fisher Scientific was used. To wash, LC-UV grade MeOH from VWR chemicals were used. Hexafluoroisopropanol (HFIP, $\geq 99\%$) was
purchased from Sigma Aldrich and ammonium bicarbonate (LC-MS grade) were purchased from Fluka Analytical. Ammonium hydroxide solution (LC-MS, $\geq 25\%$ in H2O), also purchased from Fluka Analytical.

### 2.2 Materials

The oligonucleotide sample was provided by AstraZeneca containing synthesized 16-mer long oligonucleotides with a molecular weight of 4934.92 Da, the structure is displayed in Figure 1. The sequence of the oligonucleotide is GGG AAA mCmCmC TTT GAmCT.

The oligonucleotide absorbed light at 258 nm which was used to identify the peaks when analyzing with UV detection.

All glassware used, as mobile flasks, are washed with methanol before use. This is done to minimize the presence of metal adducts [15].

- **Reversed-phase column**: DNAPac™ RP, 4 $\mu$m, 2.1 x 100 mm, purchased from Thermo Scientific™. This is a column made of spherical wide-pore polymer resin.
- **Mixed-mode column**: Acclaim™ WAX-1, 3 $\mu$m, 3 x 50 mm, purchased from Thermo Scientific™. A column made of silica with a hydrophobic alkyl chain with an ionizable end [18].
- **C18 column**: ACQUITY UPLC BEH C18: 1.7 $\mu$m, 2.1 x 50 mm.

### 2.3 Instrumentation

UPLC-UV: Waters AQUITY FTN equipped with a PDA detector. Diode array, range: 190-300 nm.

UPLC-ESI-qTOF MS-detection Waters SYNAPT G2. The LC/MS system was managed by the software MassLynx™. MS method was operated in negative ion mode with a capillary voltage of 2.5 kV. The cone voltage was 40V, the desolvation gas flow rate was 800 L/h and the cone gas flow rate was 30 L/h. The desolvation temperature was 450°C and the source temperature was 150°C. The calibration range was 500-5500 Da due to the high mass of the oligonucleotides.

### 2.4 Method

#### 2.4.1 Sample preparation

The oligonucleotide sample was provided by AstraZeneca containing synthesized 16-mer long oligonucleotides with a molecular weight of 4937.32 Da. It was diluted into 0.672 mg/ml using pyrogen-free, RNase-free and DNase-free water (Biopak). Vinyl gloves and a mouth guard was used to minimize the risk of RNase contamination and degradation of the sample. The sample was divided into vials (600 µl in each). The vials were stored in -80°C freezer prior to use. Whilst in use, the sample was stored in 8°C. The injection volume was 5 µl for every analysis.

#### 2.4.2 Mobile phase preparation

A variety of mobile phases was evaluated in order to get the best separation and selectivity of the oligonucleotide and its impurities. A number of gradients were examined for the mobile phases, as well as different pH and temperature. The components and amounts of the mobile phases were chosen based on a literature study [14,20,21]. The different mobile phases are compiled and displayed in Table 1 and Table 2.

A stock solution of HFIP was prepared by diluting 52.5 mL HFIP to 500 mL using MilliQ-water (0.22 $\mu$m Millipak Express 40), this produces a solution of 1 M. Also, another stock solution is made by diluting 20 mL HFIP to 200 mL with MilliQ-water. This procedure produces a stock solution of circa 1 M (0.95 M). Another stock solution of 100 ml was made by adding 10 mL HFIP to 100 mL MilliQ-water (0.95 M).

A stock solution of TEA was prepared by diluting 7 mL TEA to 500 mL MilliQ-water, producing a solution of circa 100 mM TEA (100.44 mM).

A stock solution of AF was produced by weighing 3.1593 g AF and dissolve in 500 mL MilliQ-water. This procedure produces a stock solution of circa 100 mM AF (100.2 mM).

A stock solution of TEA was prepared by diluting 7 mL TEA to 500 mL MilliQ-water, producing a solution of circa 100 mM TEA (100.44 mM).

A stock solution of AF was produced by weighing 3.1593 g AF and dissolve in 500 mL MilliQ-water. This procedure produces a stock solution of circa 100 mM AF (100.2 mM). Phosphate buffer is prepared by adding 3.4 mL phosphoric acid to circa 125 mL MilliQ-
water. The volume is adjusted to 500 mL by adding MilliQ-water. This produces a solution of circa 0.1 M phosphoric acid. To make the solution of 0.2 M phosphoric acid 6.8 mL phosphoric acid is added to 125 mL water, following the same procedure. TEA or ammonium hydroxide is added to make triethylamine phosphate or ammonium phosphate until wanted pH is reached.

To make 0.1 M ammonium bicarbonate solution 3.9561 g ammonium bicarbonate is dissolved in 500 mL MilliQ-water. To make 0.2 M ammonium bicarbonate solution 7.90471 g ammonium bicarbonate is dissolved in 500 mL water. Glacial acetic acid is then added to the solutions to reach the wanted pH, 7 and 8 respectively.

3 Result and discussion

The aim was to develop a separation method for the analysis of oligonucleotides for an RP/W AX mixed-mode column and MS detection. Based on earlier research, triethylammonium acetate (TEAA), ammonium formate and triethylamine (TEA) were used as buffer components since good separation on C18 column has been reported [14,20,21]. When using mixed-mode column it is common to use different phosphate buffers and

<table>
<thead>
<tr>
<th>Method</th>
<th>Concentration in water</th>
<th>HFIP in water</th>
<th>OM</th>
<th>pH</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A,B: 20 mM TEA</td>
<td>A,B: 200 mM</td>
<td>A: 0% MeOH; B: 50% MeOH</td>
<td>A: 6.90, B: 6.95</td>
<td>n.e. on mixed-mode</td>
</tr>
<tr>
<td>2</td>
<td>A,B: 20 mM TEA</td>
<td>A: 100 mM; B: 400 mM</td>
<td>A,B: 30% MeOH</td>
<td>A: 6.68; B: 6.94</td>
<td>n.e. on mixed-mode</td>
</tr>
<tr>
<td>3</td>
<td>A: 5 mM TEA; B: 30 mM TEA</td>
<td>A,B: 200 mM</td>
<td>A,B: 30% MeOH</td>
<td>A: 6.90; B: 6.95</td>
<td>n.e. on mixed-mode</td>
</tr>
<tr>
<td>4</td>
<td>A,B: 15 mM TEA</td>
<td>A,B: 400 mM</td>
<td>A: 0% MeOH; B: 50% MeOH</td>
<td>7.68, 6.80</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>A,B: 15 mM TEA</td>
<td>A,B: 0 mM</td>
<td>A: 0% MeOH; B: 50% MeOH</td>
<td>7.55</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A,B: 6.67 mM TEA</td>
<td>A,B: 200 mM</td>
<td>A: 0% MeOH; B: 50% MeOH</td>
<td>6.64</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A,B: 6.67 mM TEA</td>
<td>A,B: 400 mM</td>
<td>A: 0% MeOH; B: 50% MeOH</td>
<td>7.54</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: The ammonium phosphate buffer (APB), triethylamine phosphate buffer (TPB) and ammonium bicarbonate buffer (ABB) concentrations are displayed as concentration in the water phase and not in the total volume. MP = mobile phase.

<table>
<thead>
<tr>
<th>Method</th>
<th>Buffer</th>
<th>ACN</th>
<th>pH</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>A: 0,1 M APB</td>
<td>A,B: 20%</td>
<td>A: 6,99</td>
<td>Elution on mixed mode</td>
</tr>
<tr>
<td></td>
<td>B: 0,2 M APB</td>
<td></td>
<td>B: 7,98</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>A: 0,1 M TPB</td>
<td>A,B: 20%</td>
<td>A: 7,03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B: 0,2 M TPB</td>
<td></td>
<td>B: 8,05</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>A: 0,1 M ABB</td>
<td>A,B: 20%</td>
<td>A: 7,01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B: 0,2 M ABB</td>
<td></td>
<td>B: 8,01</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>A,B: 0,1 M ABB</td>
<td>A: 0%</td>
<td>7,01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B: 50%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.1 ACQUITY UPLC BEH C18: 1.7 µm, 2.1 x 50 mm

The C18 column was evaluated using both UV and MS detection and it acted as a reference method for the mixed-mode column. A C18 column was chosen because of earlier studies producing good results when separating oligonucleotides on C18 columns. The results are compiled based on the type of detection.

3.1.1 UV detection

Evaluating both the usage of TEA in the mobile phase and the impact of HFIP was done by analysis of method 4 and 4.1, where method 4 contains HFIP and method 4.1 does not. Method 4 (A: 15 mM TEA, 400 mM HFIP, B: 15 mM TEA, 400 mM HFIP, 50% MeOH) with a gradient of 10-90% B over 25 minutes gave good separation of the peaks on a C18 column with UV-detection. The gradient used were decreasing both HFIP and TEA concentrations as the amount of methanol were increased over time.

When analyzing the separation without HFIP Method 4.1 is used (A: 15 mM TEA, B: 15 mM TEA, 50% MeOH). In Figure 2, this is displayed and the impact of HFIP is significant. The peaks seemed to change elution order and the separation was increased using 400 mM HFIP compared with 0 mM. An increased amount of HFIP increases the retention. Reducing the amount might result in the peaks eluting all at once while increasing the amount of HFIP could increase the separation of the peaks. Two replicates are analyzed after each other to examine the reproducibility of the method. This is made with a good result and the peaks shift <0,01 minutes.

Isocratic analysis is evaluated using method 4 (A: 15 mM TEA, 400 mM HFIP, B: 15 mM TEA, 400 mM HFIP, 50% MeOH) with both 60 % and 70 % mobile phase B, the analyte is eluted within the first minute, in the void. There is no separation of the peaks using either 60% or 70% of mobile phase B.

The amount of TEA was decreased to 6,67 mM as the starting concentration. This was made to make the method more compatible with mass spectrometry since TEA could be difficult to remove from the mass spectrometer. This gave a slight decrease in separation but a better shape of
the peaks, in terms of peak width. Using a more shallow gradient and a higher starting amount of mobile phase B resulted in improved separation and shorter analysis time.

Figure 2: Left: method 4.1 (0 mM HFIP), right: method 4 (400 mM HFIP). The time span shown is reduced to 6 min-12 min.

Evaluating a lower concentration of TEA was done using method 6 (A: 6.67 mM TEA, 400 mM HFIP, B: 6.67 TEA, 400 mM HFIP, 50% MeOH). Using a gradient of 10-90% B for 25 minutes with method 6 resulted in a separation of the peaks. The gradient was optimized using 35% to 45% B for 7 minutes, flow rate 0.3 ml/min. This provided sufficient separation of the peaks, starting after just 3 minutes. Integration of the peaks using these mobile phases indicated that the main peak constitutes 87.76% of the three largest peaks. This number could be slightly different when other small peaks were integrated as well. Decreasing the amount of HFIP from 400 mM to 200 mM decreases the separation of the peaks and the peak shape.

3.1.2 MS detection and peak identification

MS detection provides information on the masses of the different peaks. This makes it possible to identify which peak that belongs to the known structure of the oligonucleotide sample, and also to evaluate possible structures of the other impurity peaks based on their mass. The optimized mobile phase including a low concentration of TEA and 400 mM HFIP, method 6 (A: 6.67 mM TEA, 400 mM HFIP, B: 6.67 TEA, 400 mM HFIP, 50% MeOH), was analyzed using MS detection.

From the results of MS-detection, the exact mass of the main peak and the two smaller peaks could be calculated. The main peak has charged state up to z=-7 and the two smaller peaks have charged states up to z=-6. It can be seen from the spectrum that the analyte is present as both sodium adducts and potassium adducts together with other adducts which were harder to evaluate, see Figure 3.

The exact mass of the structure displayed in Figure 1 is 4934,92 Da calculated using ChemDraw. When manually calculating the molecular weight of the main peak using the mass spectrum it is seen to be 4934,917 Da as average which correlates very good with the exact mass of the oligonucleotide. This indicates that the main peak belongs to the oligonucleotide structure shown in Figure 1.

The first peak (of the three large peaks) has an average mass of 4276,799 Da and the second peak has an average mass of 4605,861 according to manual calculations from the mass spectrum. This means that one peak is 329,056 Da lighter and one is 658,118 Da lighter. This indicates that the heavier of the two smaller peaks miss one part of the chain, and the lighter of the peak miss two parts since these two lost masses correspond to 329,056*2=658,112. Based on the mass of the missing part it is likely that the peaks have lost one resp. two guanine parts of the chain, see Figure 4 and Figure 5. The difference in molecular weight of the two guanine parts (330,06-329,056=1,004 Da; 659,11-658,112=0,998 Da) matches the fact that the chain binds an extra hydrogen to form a hydroxy group at the end of the chain. This result further indicates that the step where the guanine is introduced to the chain during the synthesis of the oligonucleotide could be insufficient in some way.
Figure 3: Mass spectrum of the main peak, using Method 6 (A: 6.67 mM TEA, 400 mM HFIP, B: 6.67 TEA, 400 mM HFIP, 50% MeOH). C18 column, flow rate 0.300 ml/min. Gradient 35-45% B for 7 minutes. Column temperature 40 °C.

As expected, the peak belonging to the TEA is seen in the mass spectrometer after the analysis. To solve this, washing had to be done. After washing using both acid and basic wash the TEA peak was reduced. After repeated washing using water and methanol, the TEA peak disappeared. Time to wash the system has to be taken into account when using TEA in mass spectrometry.

3.2 DNAPac™ RP, 4 µm, 2.1 x 100 mm

The DNAPac™ RP column was recommended by a manufacturer, specifically for separation of oligonucleotides. Therefore, the column was evaluated as an alternative reference column for the C18.

The same mobile phases as evaluating the C18 column was evaluated on the DNAPac™ RP as well. Method 4 (A: 15 mM TEA, 400 mM HFIP, B: 15 mM TEA, 400 mM HFIP, 50% MeOH) did provide elution of the peaks at 7.76, 7.91 and 8.04 minutes using the gradient 10-90% B for 15 minutes, and then holding 100% B for 1 minute, with a flow rate of 0.2 ml/min. The flow rate was low to make sure the maximum pressure of 4000 psi was not exceeded. All the three earlier detected peaks were visible, but not baseline separated.

TEAA had been used in earlier studies on
a C18 column and were also found in an example mobile phase in the product manual of the DNAPac™ RP, therefore this was evaluated. Method 9 (A: 100 mM TEAA, B: 100 mM TEAA, 75% ACN) was used as mobile phase without success. The analyte was eluted within the first minute using both the initial gradient 14-39% B for 4 minutes and 5-15% B for 6 minutes, flow rate 0.4 ml/min. When an isocratic method with 0% B, flow rate 0.4 ml/min, is evaluated the analyte is eluted after 2 minutes as one large peak. When using method 4 (A: 15 mM TEA, 400 mM HFIP, B: 15 mM TEA, 400 mM HFIP, 50% MeOH) on the DNAPac™ RP column three peaks are eluted. The separation of the peaks is better than when using method 9 (A: 100 mM TEAA, B: 100 mM TEAA, 75% ACN), but with poorer selectivity than when using the same mobile phase on a C18. Method 4 gives the best separation on both the C18 and the DNAPac™ RP column, therefore the TEA is further analyzed.

The mobile phase containing TEA and HFIP, method 4 (A: 15 mM TEA, 400 mM HFIP, B: 15 mM TEA, 400 mM HFIP, 50% MeOH), provided the best result produced on the DNAPac™ RP column at a point where the time limit of the project pushes towards the decision to stop the method development of the DNAPac™ RP column. The focus was therefore shifted towards the C18 and mixed-mode column only.

### 3.3 Acclaim™ Mixed Mode WAX-1, 3 μm, 3 x 50 mm

The initial focus of the project was to explore the feasibility of developing an optimized separation method for oligonucleotides analysis on a mixed-mode column. The RP/WAX type of mixed-mode was evaluated since earlier studies have displayed good results when using it to separate oligonucleotides. However, this was conducted using phosphate buffers and UV detection only and this project initially focused on MS detection. This turned out to be more of a challenge than expected. The analyte seemed to bond very strongly to the stationary phase, making strong eluting mobile phase necessary.

The mobile phases evaluated on the C18 and the DNAPac™ RP was evaluated on the mixed-mode column as well. This was investigated even though no earlier studies using this was found. Due to the low solubility of HFIP and TEA in acetonitrile, methanol was used as organic eluent. Mobile phases more suitable for ion exchange was also evaluated.

It turned out that methanol in the range 5-50% was not enough in regards of elution strength to get the analyte to elute when used with HFIP (100-400 mM) and TEA (5-30 mM), see Table 1, method 1-3. In the first experiment, the amount of MeOH is increased from 0-50% over time. In the second experiment the amount of HFIP is increased from 100-400 mM, and in the third experiment, the amount of TEA is increased from 5-30 mM over time. Neither the method 1,2 or 3 resulted in elution of the analyte. The column was equilibrated for 30 minutes with the current mobile phase before the first injection. Mobile phase including TEAA (A: 100 mM TEAA, 20% ACN, B: 200 mM TEAA, 20% ACN) was evaluated with the gradient 50-100 % B for 25 minutes and 100 % until 52 minutes, see method 8. Flow 0.3 ml/min was used and a column temperature of 40°C. This gradient was evaluated based on earlier experiments. No elution of the analyte is detected. Due to lack of time and the difficulty to optimize a method when no elution is seen at all, TEAA is not used in further analyses on the RP/WAX mixed-mode column. The C18 column has shown to produce better results based on the analyses made in this project.

Phosphate buffer was found to be the recurrent mobile phase used in earlier studies separating oligonucleotides on a mixed-mode column. Both phosphate buffer including ammonium hydroxide and phosphate buffer including TEA was evaluated. Using ammonium phosphate buffer on the mixed-mode column provided a good separation of the peaks when using UV detection. The retention is heavily dependent on the temperature of the column, probably making the robustness of the method lower. This is illustrated in Figure 6. The retention increases with increasing column temperature between the temperatures 30°C and 50°C. The most significant change is seen between 40°C and 45°C, and the smallest is seen between 30°C and 35°C. Changing the temperature in the range of 30°C to 50°C resulted in the retention of the main peak shifting almost 10 minutes.
The gradient used was 20-80% B for 25 minutes, 80% B until 30 minutes, 20% B another 10 minutes. This significant influence of the temperature indicates that different interaction mechanisms are dominant under different temperatures, in this case, it would be the reversed-phase and the weak anion exchange properties that are affected. Different interactions being dominant at higher/lower temperatures could also explain the fact that the retention time seems to be least stable at temperatures between 40°C and 45°C.

Figure 6: RP/WAX, 0.3 ml/min, method 10 (ammonium phosphate buffer/ACN). Green: 30°C, orange: 35°C, grey: 40°C, yellow: 45°C, blue: 50°C. The time span displayed is reduced to 15 min-45.2 min. Gradient: 20-80% B for 25 min, 80% B until 30 min, 20% B 10 min.

Figure 7: Three replicates of method 10. Gradient: 50-100% B for 25 minutes, 100% until 52 minutes, 50% B for another 8 minutes. The time span displayed is reduced to 16 min-35 min to display the changes in the runs.

Three replicates are analyzed with the mobile phase A: 0.1 M APB, 20% ACN, mobile phase B: 0.2 M APB, 20% ACN, see Figure 7. The main peak shift +0.11 and +0.12 minutes between the runs.

Using triethylamine phosphate buffer in the same concentration (Method 11, Table 2) as ammonium phosphate buffer provided a similar result to the ammonium phosphate buffer (Method 10, Table 2) although the analyte were eluted much earlier (the first substantial peak eluting at circa 9 minutes compared to circa 21 minutes when using the ammonium phosphate buffer). The elution time was much less temperature sensitive when using triethylamine phosphate buffer, see Figure 8.

Mass spectrometry is important for the qualitative analysis of the oligonucleotide. Phosphate buffer is a nonvolatile liquid which will clog up and damage the ion source. Therefore, a different solution for the mobile phases was desired.
Ammonium bicarbonate was evaluated as a possible solution for the RP/WAX mixed-mode column thanks to its compatibility with mass spectrometry. Method 12 (A: 0.1 M ABB, 20% ACN, B: 0.2 M ABB, 20% ACN) was analyzed using a gradient of 50-100% B for 30 minutes, and then 100% for another 20 minutes. This was not sufficient to get any elution of the analyte. One suspicion was that this could be due to the earlier usage of phosphate buffer, and washing the column could be difficult. Therefore, ammonium bicarbonate was evaluated on a new RP/WAX mixed-mode column of the same kind. Method 13 (A: 0.1 M ABB, 0% ACN, B: 0.1 M ABB, 50% ACN) was evaluated on the new column, using a gradient of 50-100% B for 25 minutes, 100% until 52 minutes, 50% B for another 8 minutes. No elution of the analyte was detected. The concentration of ammonium bicarbonate or the pH might have been too low, but this could not be further evaluated due to lack of time.

The reason for not trying out the same method on the new RP/WAX mixed-mode column was because the recommended pH for the column was 2.5 - 7 pH units. When using method 12 the mobile phase reached 8 pH units which might damage the column or impair its original condition. It might be that pH >7 is needed for elution of the analyte. One other difference from the earlier analysis was the concentration of ammonium bicarbonate. In the earlier analysis, the concentration rises from 0.1 M to 0.2 M ammonium bicarbonate. In this analysis, only 0.1 M ammonium bicarbonate was used. This might also play a vital role in the elution of the analyte.

4 Conclusion

The project took a different turn than expected early on, due to the challenge of getting elution of the analyte on the mixed-mode column. Also, the initial plan was to use MS detection but UV detection turned out to be much more fruitful at this stage of the project. The structure and mass of the oligonucleotide examined in this project were known, but as long as a sufficient method and the time of elution wasn’t known, UV detection was more useful. Mass spectrometry was used when a working method was determined. Using mostly UV detection made it possible for me to be self-going during most of the time early on, but when using the mass spectrometer I had to be supervised. This made it possible to maximize the time doing analyzes.

The main challenge of the project was to develop a mobile phase for the mixed-mode column compatible with mass spectrometry. Since all the mobile phases evaluated during this project, except for the phosphate buffers, was insufficient. None of the mobile phases resulted in elution of the analyte. One thought to solve this problem was to connect the RP/WAX mixed-mode column to another column that would bind the phosphate before entering the mass spectrometer. Whether or not this could be a possible option was not further evaluated.

Examination of the impact of different pH values using the mixed-mode and further work using ammonium bicarbonate would be made if more time was provided. If ammonium bicarbonate would be sufficient to get elution on the mixed mode, then the problem with finding an MS compatible mobile phase would be cleared and further optimization could be made. Such as the impact of different pH values, temperatures and the usage of HFIP.

The impact on metal adducts have not been examined as a part of the project due to the lack of time, but this would be an interesting part to fo-
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cus on for the ongoing project. All the glassware used in this project were rinsed with methanol before use to minimize the presence of metal adduct, whether or not this actually gives a significant difference would be a useful result.

Evaluation of the impact of HFIP could easily be done, for example by analyzing amounts of 100 mM, 200 mM, and 300 mM HFIP as a complement to the more extreme values of 0 mM and 400 mM HFIP. The way the peaks shift would be interesting to investigate. Since the oligonucleotides are investigated as pharmaceuticals, examination of degradation products would be important work for the future of the ongoing project.

Using the mixed-mode column to separate oligonucleotide samples have good potential since the peaks are very well separated. The fact that the mixed-mode column separates the oligonucleotides based on both the hydrophobicity and the charge makes the separation much greater than using the C18 and the DNAPac™ RP column with respect to separation of structurally similar oligonucleotides, this can also be seen in the result of this project. When being able to separate using the mixed-mode column the main oligonucleotide and its structurally similar impurities are well separated using both ammonium phosphate and triethylamine phosphate buffer as mobile phase.

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6 Populärvetenskaplig sammanfattning


Detektionen av molekylerna i provet görs i detta projekt på två olika sätt, antingen med UV eller masspektrometri (MS). De som analyseras med MS laddas inne i instrumentet och styrs vidare till den del av instrumentet som kallas qTOF. Molekylerna laddas här med samma mängd kinetisk energi, men på grund av deras olika massor så kommer de att flyga olika långt och träffa detektorn vid olika tidpunkter. Detta ger upphov till separation av molekyler med olika massor, resultatet blir som visas i Figur 8.


Resultatet som sågs på mixed-mode-kolonnen var att vid användandet av en viss mobilfas var analysen väldigt temperatureberoende medan med en annan mobilfas var den inte det. Oligonukleotiden i mitt prov var väl separerad från de orenheter som var närvarande. Med hjälp av en vanlig C18-kolonn och MS kunde de två största orenheterna i provet undersökas. Baserat på massorna verkar de vara kortare kedjor än den huvudsakliga strukturen, dessa saknade en respektive två nukelinsyror i sekvensen, se Figur 4 och 5.

Mixed-mode-kolonnen har stor potential att separera sturkutrellt liknande oligonukleotider men den stora utmaningen är att lyckas hitta en mobilfas som klarar av att göra detta och samtidigt är kompatibel med MS.
7 References

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