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Ian Neidigh
Andrews University, neidigh@andrews.edu

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J.N Andrews Honors Program

Andrews University

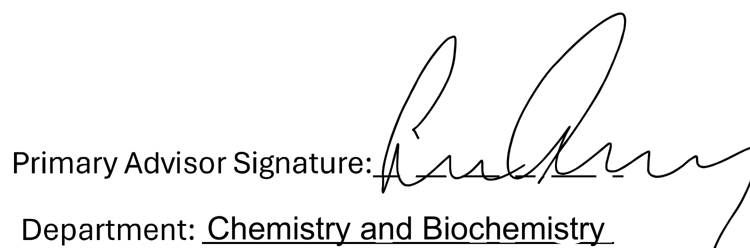
HONS 497

Honors Thesis

Understanding the Mechanism Behind HFIP's Effect on Diastereomeric Separation of Oligonucleotides

12 April 2024

Ian Neidigh

Primary Advisor Signature: 

Department: Chemistry and Biochemistry

Understanding the Mechanism Behind HFIP's effect on Diastereomeric Oligonucleotide Separation

Abstract

The goal of this research was to determine the mechanism behind 1,1,1,3,3,3-hexafluoroisopropanol's (HFIP) ability to suppress the separation of oligonucleotide diastereomers. This was accomplished by selecting different molecules that were like HFIP but differed in specific ways such as polarity, number and type of halogen(s), alcohol group location, carbon chain length, hydrophobicity, and chao/kosmotropicity. These molecules replaced HFIP in an IPRP chromatography method to determine if splitting of the diastereomers was observed and then they would be compared to HFIP's effect. Viscosity tests were run to acquire quantitative data to determine chao/kosmotropicity. No decisive trend among a single factor was observed meaning that more than one factor effects diastereomer selectivity.

Note that this research is qualitative in nature and has no statistical analysis.

Introduction

Therapeutic oligonucleotides, short RNA and DNA molecules that alter protein expression to treat diseases, can be degraded by enzymes in biological tissues before they reach their cellular target. For therapeutic efficacy, the naturally occurring phosphodiester bond is replaced with a phosphorothioate bond that is resistant to enzymatic degradation. However, the phosphorothioate group is chiral resulting in diastereomers which can coelute with impurities found in chromatographic methods that are required by the FDA to determine the purity of drugs. Inclusion of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) in the ion-pair reverse phase (IPRP) purity method suppresses the separation of diastereomers thereby improving the separation of chemical impurities. This research investigates the mechanism behind HFIP suppression of diastereomer separation which is currently unknown. Since the most common method for separating oligonucleotides is IPRP chromatography this research will be using this method.

Methods

IPRP chromatography was the primary method used for analysis. A mobile phase composition of 7%-17% of mobile phase B (MPB) (50:50 methanol:acetonitrile) with a mobile phase gradient of 1% increase in MPB per minute was utilized. Mobile phase A contained 25 mM of HFIP or another halogenated alcohol, otherwise known as the test analyte or molecule, alongside 14 mM of TEA (triethylamine) as the ion-pairing agent. The column used was an ACQUITY PREMIER Oligonucleotide BEH C18, 130A,1.7 uM, 2.1 x 100mm Column, 1/pk. The manufacturer was Waters and

had a part number 186009485 and lot number of 04543316518630.

The viscosity method used a viscometer; the MicroVISC manufactured by Rheosense, with serial number H1810-0039-S. An AO5 0-100 cP Chip was used (Part number: R23HA05100042). Solutions were inserted into RheoSense MicroVISC disposable pipettes and then the viscosity of each solution is measured by continuous dispensing from the MicroVISC. The units for viscosity are centi-poise.

Experiment and Results

Multiple oligonucleotides were evaluated for study similarly to Kadelcova (9) but 5'-T*TT GCA TTT TAC GTT T-3' alongside its total PS (5'-T*T*T G*C*A T*T*T T*A*C G*T*T T-3') and PO (5'-TTT GCA TTT TAC GTT T-3') oligonucleotides were chosen for this research and remained standard throughout. Note that the * denotes a phosphorothioate functional group.

pH and pKa of HFIP and NFtB

Initially pH was tested to determine how it affected the separation method. Each molecule tested could have different pKa's and thus depending on the pH of the mobile phase would be in their alkoxide and negatively charged form or in their alcohol form with no charge. An IPRP column separates based on charge thus an optimal pH is important to determine for running conditions since pH can vary molecules between a charged or neutral state. Since both HFIP and oligonucleotides can hydrogen bond determining which form, the alkoxide and negative or alcohol and positive, of HFIP achieves the desired result is crucial for determining testing conditions.

Figure 1 shows that as the pH of the mobile phase decreased then less separation of the peaks was observed.

Figure 1: Test of HFIP's separation at 25 mM varying pH from 9 to 6.

Figure 2 shows a similiar trend being observed over a smaller range with more gradations. HFIP's pKa is 9.3 thus when the pH of the solution is below this 9.3 then majority of HFIP is in its alcohol form. This means that below the pKa of HFIP poor separation is observed as intended. All future tests were run for each test molecule at least 2 pH units below the pKa of the test molecule to ensure standard operating conditions.

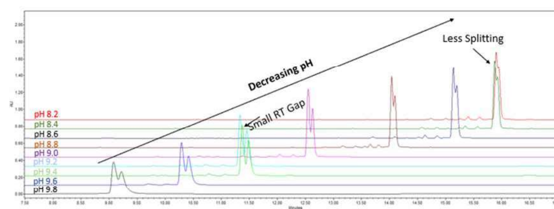


Figure 2: Test of HFIP's separation at 25 mM varying pH from 9.8 to 8.2.

The same test was run for NFtB as seen in **Figure 3**. Similarly to HFIP, NFtB shows that as the pH decreased less separation was observed. Since NFtB's pKa is 5.4 thus all tests with NFtB were run 1 pH unit below its pKa. Note that unlike HFIP, NFtB was run 1 pH unit lower than its pKa to avoid any acid base reactions with the oligonucleotide itself.

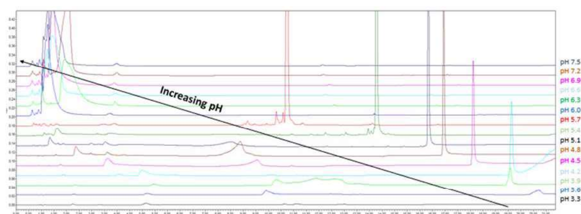


Figure 3: Test of NFtB's separation at 25 mM varying pH from 3.3 to 7.5.

When titrating mobile phases counter ions are added into solution. These counter ions have charge and could impact the ion interactions in the column. To test this an HFIP mobile phase was run at its initial preparation pH of 9.68 and then HCl and NaOH were added to get the solution's pH down to 2.34 and then back up to 9.44. This pH adjustment and its adjacent salt addition to the mobile phase was more extreme than this research would utilize since at pH of 2 or lower the oligonucleotide gets affected. **Figure 4** notes the result. The peaks have identical shapes with an RT difference of 0.335 minutes. Note that the figure does not show this 0.335 minute difference since both peaks were overlayed for visual inspection.

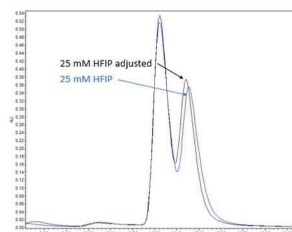


Figure 4: Test of HFIP at 25 mM adjusted from 9.68 to 2.34 and then back to 9.44.

The same test was performed for NFtB as seen in **Figure 5**.

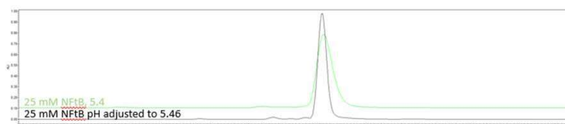


Figure 5: Test of NFtB at 25 mM adjusted from 5.34 to 3.2 then to 9.2 finally to 5.46.

To further test the hypothesis that salts would affect the column chemistry a test was performed by addition of 100 mM of salt to see if any difference could be observed. This test's results can be seen in **Figure 6**.

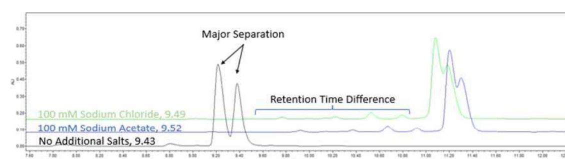


Figure 6: Test of HFIP at 25 mM alongside 100 mM of additional salts all at approximately 9.43 pH units.

Optimization Conditions

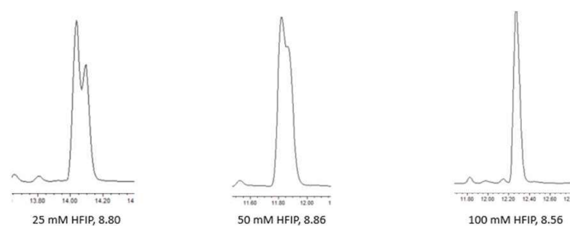


Figure 7: Test of HFIP at a pH of 8.80 at varying concentrations.

Figure 7 indicates that at a constant pH different concentration of HFIP changed the amount of peak splitting that is observed. This is expected, a higher concentration of HFIP indicates less peak splitting. All solutions after this test were run with their test analyte having a concentration of 25 mM since NFtB cannot exceed that concentration due to solubility problems.

Different halogenated molecules and their peaks

Multiple fluoroalcohols were chosen, many from Basiri (2), this research required acronyms for easier identification as seen in **Table 1**.

Table 1: Acronyms for each fluorinated alcohol tested.

Acronym	IUPAC Name
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
NFtB	1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-ol
TFE	2,2,2-Trifluoroethan-1-ol
TFP	3,3,3-Trifluoro-1-propanol
HFMIP	1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol
TeFP	2,2,3,3-Tetrafluoro-1-propanol
PFP	2,2,3,3,3-Pentafluoro-1-propanol
HFPIP	1,1,1,3,3,3-hexafluoro-2-phenyl-2-propanol
TFMP	1,1,1-trifluoro-2-methyl-2-propanol
TFIP	1,1,1-trifluoro-2-propanol
TFA	1,1,1-trifluoroacetone

Figure 8 shows the results of TFE, TFP, TeFP, PFP, HFMIP, HFIP, and NFtB. TFE and TFP both show major peak splitting while TeFP and PFP show less splitting. HFIP, HFMIP, and NFtB all show no splitting. Other molecules were tested as seen in **Figure 9**, **Figure 10**, and **Figure 11**.

Figure 9 shows fluoroalcohols that vary by hydrophobicity due to extra hydrophobic groups attached to the central carbon in the isopropanol structure. HFPIP, the addition of a phenyl group, shows a narrower peak indicating a stronger effect while HFMIP, HFIP with the addition of a methyl group, shows a broader peak indicating the opposite of HFPIP.

Figure 10 Shows data for TFMP and TFIP. Both molecules only have three fluorine atoms yet achieve the desired effect better than PFP and TeFP which have more fluorines.

Figure 11 shows that the alcohol group is not needed. TFA, a ketone, inhibits diastereomer splitting similarly to PFP.

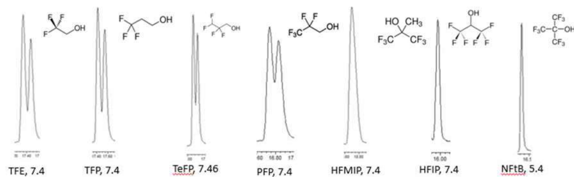


Figure 8: Multiple fluoroalcohols at 25 mM at pH of 7.4 (5.4 for NFtB). Structures are top right of each peak.

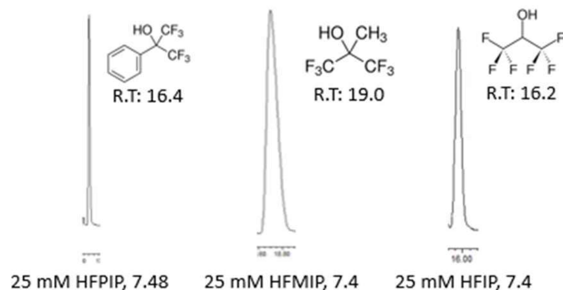


Figure 9: Multiple fluoroalcohols at 25 mM at pH of 7.4 varied by hydrophobicity group location. Structures are top right of each peak.

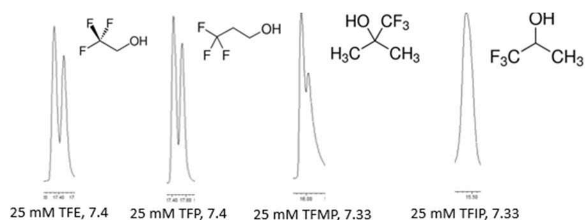


Figure 10: Multiple fluoroalcohols at 25 mM at pH of 7.4 varied by alcohol group location. Structures are top right of each peak.

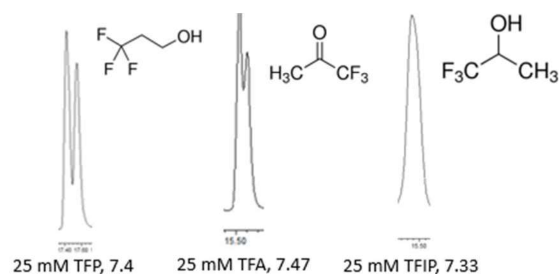


Figure 11: Multiple fluoroalcohols at 25 mM at pH of 7.4 varied by alcohol group. Structures are top right of each peak.

Different Metrics for comparison of fluorinated alcohols

A possible explanation for the inhibition of diastereomeric separation is that HFIP acts as a chaotropic or kosmotropic agent in solution. A chaotropic agent is a molecule that disrupts the hydrogen bonding network in aqueous solutions while a kosmotropic agent aids and strengthens the hydrogen bonding network. A UV-Vis method as described in Cray (3) but we were not able to replicate their results. Viscosity is related to the hydrogen bonding of a solution, the stronger the hydrogen bonding the more viscus the solution; thus the viscosity of each solution was measured as seen in **Figure 12**. This figure shows the difference for each fluoroalcohol's viscosity compared to water's viscosity.

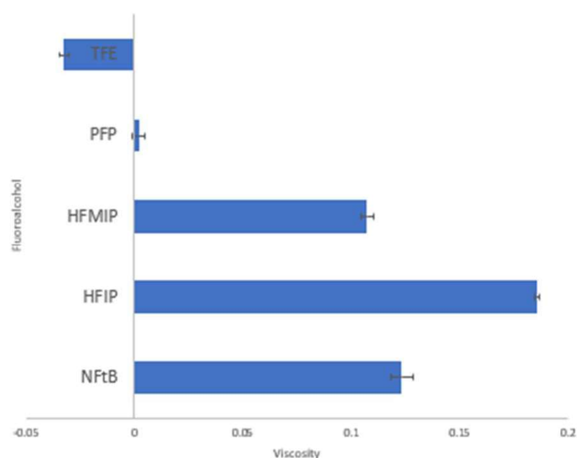


Figure 12: A bar graph of the viscosity (cP) difference from water for each fluoroalcohols.

Analysis and Discussion

To determine the mechanism behind HFIP's effect on diastereomeric oligonucleotide separation trends were identified and evaluated to see if they offer a comprehensive explanation for HFIP's effect.

A trend can be observed with the number of fluorines attached to the isopropanol (IPA) and inhibition of diastereomer separation. The more fluorine atoms attached to the IPA molecule the less the diastereomer peaks are resolved as seen in **Figure 8**. This trend is violated by the molecule TFIP seen in **Figure 9**. TFIP only has three fluorines yet has a single peak like HFIP, NFtB, and HFMIP. It seems that the number of halogens is an incomplete explanation for HFIP's observed effect.

While determining a trend for the number of fluorine atoms attached to an alcohol is useful, it's not comprehensive since the real effect lies in what those fluorine atoms do to the alcohol. Fluorine is the most electronegative element and thus one theory as to why the effect of HFIP is observed is because of the fluorines pulling electron density away from the alcohol group making the group a better hydrogen bond donor and a weaker hydrogen bond acceptor. TFA violates this assumption since it demonstrates similar inhibition of peak splitting yet has no hydrogen and cannot be a hydrogen bond donor as seen in **Figure 11**.

Another hypothesis would be that the hydrophobicity of the fluorinated compounds could change the results but as **Figure 9** shows no major trend can be observed. HFMIP, a more hydrophobic form of HFIP, demonstrates a wider peak showing better separation than HFIP. In contrast, HFPIP has a thinner peak showing that its peak inhibition is superior to HFIP while being even more hydrophobic than HFMIP. Two compounds that were both more hydrophobic than HFIP demonstrate opposite effects thus showing that no conclusive trend can be observed.

Chao/kosmotropicy is also a possible explanation for HFIP's effect on diastereomeric separation. If HFIP acts like a chaotropic agent weakening the hydrogen bonding network in water then it could possibly weaken the oligonucleotide's retention to the column

which is partially due to hydrogen bonds. The results in **Figure 12** indicate that fluorinated alcohols operate as kosmotropic agents. This may support a theory that as waters hydrogen bond network becomes stronger then the oligonucleotide gets pushed out and onto the column but that would indicate better separation opposite of the HFIP trend. A unique result from this trend is that NFtB doesn't conform to this trend but this could be due to NFtB being tested at a different pH as the other molecules since it's pKa is so different from the other molecules. This data set is small and requires more testing before a confirmed trend can be observed.

A unique result can be observed in **Figure 10** that shows branched alcohols achieve poorer resolution for diastereomers when compared to their straight chain counter points. TFP and TFIP are identical molecules except that TFIP has the alcohol group on the 2 positions yet achieves a desired effect. It is unknown why this trend is observed nor its implications, but more testing must be done to understand this observation.

The major explanation within the literature for HFIP's effect and effectiveness is that the molecule is the limit of solubility for fluorinated alcohols with more fluorinated molecules ceasing to be soluble. This isn't an explanation for what the fluorine atoms do to achieve this separation result thus it is incomplete. This research has not found the mechanism behind HFIP's effect on diastereomeric oligonucleotide separation but has eliminated some theories and has illuminated areas for future research.

In summation multiple factors may affect PS diastereomer selectivity!

Future Testing

This research while having achieved substantial progress is not conclusive thus below are future research opportunities.

This papers scope was limited to fluorinated compounds, addition of other halogenated compounds was not evaluated but may provide valuable insight into the mechanism.

Examining other ketone molecules with more fluorine atoms may demonstrate the same trend as with alcohols (more fluorine atoms greater effect) or may violate the trend; either way the result could illuminate what HFIP is doing in the column and how it is interacting.

A needed test is to determine viscosity of other fluoroalcohols and see if kosmotropicity is truly positively correlated while also reevaluating NFtB at a different pH.

Importantly, testing osmolality on each molecule with oligonucleotides would determine which ions are pairing up in the column and help generate new theories to test and determine legitimacy.

Bibliography

(1) Ball, P.; Hallsworth, J. E. Water Structure and Chaotropicity: Their Uses, Abuses and Biological Implications. *Physical Chemistry Chemical Physics* **2015**, *17* (13), 8297–8305. https://doi.org/10.1039/C4CP04564E.

(2) Basiri, B.; van Hattum, H.; van Dongen, W. D.; Murph, M. M.; Bartlett, M. G. The Role of Fluorinated Alcohols as Mobile Phase Modifiers for LC-MS Analysis of Oligonucleotides. *Journal of the American Society for Mass Spectrometry* **2016**, *28* (1), 190–199. https://doi.org/10.1007/s13361-016-1500-3.

(3) Cray, J. A.; Russell, J. T.; Timson, D. J.; Singhal, R. S.; Hallsworth, J. E. A Universal Measure of Chaotropicity and Kosmotropicity. *Environmental Microbiology* **2012**, *15* (1), 287–296. https://doi.org/10.1111/1462-2920.12018.

(4) Donegan, M.; Nguyen, J. M.; Gilar, M. Effect of Ion-Pairing Reagent Hydrophobicity on Liquid Chromatography and Mass Spectrometry Analysis of Oligonucleotides. *Journal of Chromatography A* **2022**, *1666*, 462860. https://doi.org/10.1016/j.chroma.2022.462860.

(5) Dong Pyo Hong; Hoshino, M.; Ryoichi Kuboi; Goto, Y. Clustering of Fluorine-Substituted Alcohols as a Factor Responsible for Their Marked Effects on Proteins and Peptides. *Journal of the American Chemical Society* **1999**, *121* (37), 8427–8433. https://doi.org/10.1021/ja990833t.

(6) Du, H.; Ozdemir, O.; Wang, X.; Cheng, F.; Celik, M. S.; Miller, J. D. Flotation Chemistry of Soluble Salt Minerals: From Ion Hydration to Colloid Adsorption. *Mining, Metallurgy & Exploration* **2014**, *31* (1), 1–20. https://doi.org/10.1007/bf03402344.

(7) Enmark, M.; Rova, M.; Samuelsson, J.; Örnskov, E.; Schweikart, F.; Fornstedt, T. Investigation of Factors Influencing the Separation of Diastereomers of Phosphorothioated Oligonucleotides. *Analytical and Bioanalytical Chemistry* **2019**, *411* (15), 3383–3394. https://doi.org/10.1007/s00216-019-01813-2.

(8) Hallsworth, J.; Heim, S.; Timmis, K. Chaotropic Solutes Cause Water Stress in Pseudomonas Putida. *Environmental Microbiology* **2003**.

https://doi.org/10.1046/j.1462-2920.2003.00478.x.
(9) Kadlecová, Z.; Kalíková, K.; Tesařová, E.; Gilar, M. Phosphorothioate Oligonucleotides Separation in Ion-Pairing Reversed-Phase Liquid Chromatography: Effect of Temperature. *Journal of Chromatography A* **2022**, *1681*, 463473. https://doi.org/10.1016/j.chroma.2022.463473.
(10) Ohgi, H.; Imamura, H.; Yonenaga, K.; Morita, T.; Nishikawa, K.; Westh, P.; Koga, Y. The Effect of 2,2,2-Trifluoroethanol on Water Studied by Using Third Derivatives of Gibbs Energy, G. *Journal of Molecular Liquids* **2016**, *224*, 401–407. https://doi.org/10.1016/j.molliq.2016.09.101.
(11) Roenfanz, H. F.; Paniak, T. J.; Berlin, C. B.; Van Tan Tran; Francisco, K. R.; Pierrik Lassalas; Devas, A.; Landes, O.; Rosenberger, A.; Rotella, M.; Ballatore, C.; Kozlowski, M. C. Hydrogen Bonding Parameters by Rapid Colorimetric Assessment: Evaluation of Structural Components Found in Biological Ligands and Organocatalysts. *Chemistry: A European Journal* **2023**, *29* (40). https://doi.org/10.1002/chem.202300696.
(12) Smith, S.; Poulson, S. *AGAR GELATION SPECTROPHOTOMETRIC ASSAY of CHAO-and KOSMO-TROPICITY of INORGANIC SALTS, and IMPLICATIONS for LIFE in TERRESTRIAL and MARTIAN BRINES*. https://www.hou.usra.edu/meetings/modernbrines2021/pdf/6017.pdf (accessed 2023-08-21).
(13) Vuluga, D.; Legros, J.; Crousse, B.; Slawin, A. M. Z.; Laurence, C.; Nicolet, P.; Bonnet-Delpon, D. Influence of the Structure of Polyfluorinated Alcohols on Brønsted Acidity/Hydrogen-Bond Donor Ability and Consequences on the Promoter Effect. *The Journal of Organic Chemistry* **2011**, *76* (4), 1126–1133. https://doi.org/10.1021/jo1023816.
(14) Coskun, O. (2016). "Separation techniques: chromatography." Northern clinics of Istanbul **3**(2): 156.

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