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Exploring Liquid Chromatography Method Development Strategies: Enhancing Analytical Performance in Pharmaceutical Applications

Master's Thesis in Biotechnology

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Abstract

From discovery to the final phase of drug development, Liquid Chromatography (LC) is considered one of the core separation techniques. Its broad capabilities in identifying and quantifying complex compound mixtures find application in quality control, formulation studies, pharmacokinetics, environmental monitoring, and purity determination [1]. This 60-credit thesis aims to develop and optimize LC methods through exploration of three individual studies, each detailed in separate chapters.

Chapter one is focused on evaluating separation performance under various conditions using a selected compound mixture as reference. This study examined factors such as buffer selection, mobile phase composition, and instrument compatibility based on the separation of these reference compounds. Despite high separation capability of trifluoroacetic acid (TFA) due to its strong ion pairing strength, its significant drawbacks include signal suppression in mass spectrometry (MS) and environmental impacts. The obtained results suggest that difluoroacetic acid (DFA) and ammonium acetate (AA) by showing acceptable performance, can be potential replacements for TFA.

In chapter two, the LC method development process for separating impurities of metoprolol was examined. Fusion QbD software was employed in this section to assist in the design of experiments (DoE), analysis of screening experiments, and optimization of the separation methods. Ultimately, the investigation revealed a method using a 10 mM potassium phosphate buffer with a pH of 8.1 and a gradient from 15 to 42% acetonitrile over 7 minutes at 50°C yielded the most optimal separation of the target compound from its impurities. Furthermore, the study compared the performance of an MS-friendly buffer, ammonium acetate, with potassium buffers based on the parameters of the optimized method.

In the final chapter, an optimized experimental procedure for assessing the stability of Inclisiran, a commercial siRNA used for reducing low-density lipoprotein cholesterol (LDL-C), was studied. This exploration focused on measuring the melting point (Tm) and involved testing various parameters such as the number of thermal cycles, the effectiveness of silicone oil, and the choice of temperature ramp. In the end, it was demonstrated that setting only one cycle, adding 10 µL of silicone oil, and using a temperature ramp of 0.5°C/min were the optimal parameters for obtaining reliable data. On the other hand, experiments assessing Inclisiran stability in the TEA/HFIP system revealed that TEA concentration did not significantly impact stability at 5 and 15 mM, but adverse effects were observed at 100 mM TEA, resulting in a lowering of the melting point by 11.6°C. LC separation experiments provided additional insights into Inclisiran's behaviour, showing complete denaturation at 65°C but non-denaturation conditions at lower temperatures.

Keywords: Liquid Chromatography, Analytical Method Development, Alternative LC Buffers, Fusion QbD, Metoprolol Impurity Profiling, Inclisiran Stability, siRNA Melting Temperature

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Finally, it should be noted that the third part of this project has been supported by the Swedish Knowledge Foundation via the project “Improved Methods for Process and Quality Controls using Digital Tools” (grant number 20210021).

[1]Dr. Jufang Wu Ludvigsson and Malin Wallberg, both supervised the first and second parts of this thesis project, while the third part was carried out under the supervision of Dr. Eivor Örnskov.

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List of Abbreviations

Term/Symbol	Description
LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
RPLC	Reverse Phase Liquid Chromatography
SST	System Suitability Test
PDA	Photo Diode Array
TFA	Trifluoroacetic Acid
DFA	Difluoroacetic Acid
AA	Ammonium Acetate
FA	Formic Acid
MS	Mass Spectrometry
BEH	Ethylene Bridged Hybrid
UV	Ultraviolet
Gly	Glyburide
Ace	Acetaminophenol
DHQ	$(DHQD)_2PHAL$
Amt	Amitriptyline
API	Active Pharmaceutical Ingredient
QbD	Quality by Design
DoE	Design of Experiment
RoI	Responses of Interest
PDA	Photo Diode Array
MeCN	Methyl cyanide (Acetonitrile)
MeOH	Methanol
CVD	Cardiovascular disease
ASCVD	Atherosclerotic Cardiovascular Disease
LMT	lipid modification therapy
TG	Triglycerides
ON	Oligonucleotides
RNA	Ribonucleic acid
siRNA	Small Interfering RNA
RISC	RNA-induced silencing complex
LDL-C	Low-density Lipoprotein Cholesterol
GalNAc	Triantennary N-acetylgalactosamine
AEX	Anion Exchange Chromatography
IPRP-LC	Ion-Pair Reversed-Phase Liquid Chromatography
TEA	Ttriethylamine
HFIP	1,1,3,3,3-hexafluoro-2-propanol
ESI-MS	Electrospray Ionization-Mass Spectrometry
Tm	Melting Temperature
$\Delta G°$	Gibbs free energy
Abs	Absorbance
$\frac{d(Abs)}{dT}$	Derivative of absorbance values over temperature
RSD	Relative Standard Deviation

Chapter 1

Investigation on Liquid Chromatography Separation Performance with a Selected Test Mixture

1.1 Introduction

In bioanalytical chemistry, liquid chromatography (LC) plays a crucial role in separating and analysing complex mixtures of compounds. However, achieving an optimal separation can be challenging due to various factors such as analyte characteristics, choice of stationary and mobile phases, column attributes, and operational conditions. Understanding the interplay between these factors is vital for developing efficient and reliable chromatographic methods [2].

In reverse phase liquid chromatography (RPLC), additives by regulating the pH of the mobile phase have significant influence on the separation of the ionizable analytes and are able to improve the chromatographic separation considerably [3]. There have been multiple studies to investigate and compare different mobile phase additives to enhance the separation capabilities, for instance in peptides and proteins separation [4], [5], or for bioactive compounds [6]. Additionally, research indicates that the configuration of analytical instruments can impact the performance and transferability of the methods [7].

The primary objective of this study is to address critical inquiries related to buffer selection, mobile phase composition, and instrument compatibility based on the separation of a standard mixture of compounds on LC. Ultimately, these insights will contribute to deepening our understanding and making more informed decisions in method development trials.

To evaluate separation performance and compare different factors, four reference compounds were selected: 3-Acetamidophenol, $(DHQD)_2PHAL$, Amitriptyline, and Glyburide. These compounds were previously investigated by Klarqvist et al. [8] and deemed apt for system suitability tests. Such tests are standard procedures conducted with well-characterized compounds to assess the accuracy and precision of instruments or methods. As outlined in that study, the rationale behind choosing these compounds was their relevance to the drug discovery phase, where numerous unknown compounds can complicate suitability tests. This selection of four compounds covers a broad chemical space and aligns with pharmaceutical research requirements, making it an appropriate analytical approach. Following the conclusions of their research, we incorporated the same compounds for this analysis.

Following the selection of test compounds, this study will address three primary research questions:

1) Which mobile phase additive can demonstrate superior performance in separating a standard system suitability mixture?
2) What are the critical differences between two LC instruments with different configurations, and how do these differences impact separation and reproducibility?
3) How does the presence or absence of additives in the organic mobile phase affect key chromatographic parameters, such as plate numbers, resolution, and peak symmetry?

By implementing a simple LC method and evaluating the separation of the test mixture, this study will illustrate how these three factors influence chromatographic outcomes. This preliminary investigation aims to provide insights for future studies focusing on analytical method developments.

1.2 Methods

1.2.1 Procedure

Test mixture

The System Suitability Test (SST) ensures the proper functioning of the system and instrument. Typically, it involves several injections of a batch of standard or previously known samples before conducting the actual analysis. The level of SST conducted depends on the complexity of the analysis and the associated risk. Simple and robust assays may not mandate extensive suitability testing, whereas complex assays with limited sample volumes may require more comprehensive testing to mitigate risks [9].

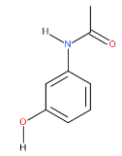
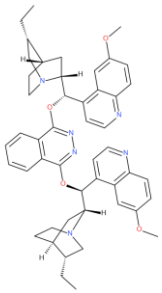
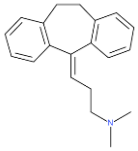
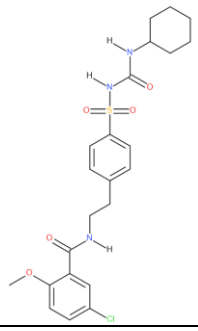
SSTs typically serve two primary objectives. Firstly, they aim to verify the qualification of the instrument, ensuring it meets the expected levels of accuracy and precision. Secondly, they monitor the performance of both the instrument and columns to identify any changes that may occur over time within the system. This guarantees that the data collected, especially in the initial runs of the analysis, remains consistent with subsequent injections and confirms the robustness of data acquisition method [9].

SST finds application across various domains, ranging from meeting regulatory requirements to instrument validation. Different regulatory standards, such as Good Manufacturing Practice (GMP) and General Laboratory Standard (GLS), impose varying levels of requirements for validating data collection methods. In addition to regulatory compliance, certain qualifications must be verified for the instrument. Initially, Installation Qualification (IQ) entails a broad inspection of the instrument. SST implemented at this stage is typically more general and are often conducted by the manufacturer. Subsequently, during Operational Qualification (OQ) and Performance Qualification (PQ), SST must be tailored to the specific environment in which the instrument will operate. The PQ phase is typically performed continuously by the analyst and may vary significantly for each analysis [9].

Selection of the compounds for SST

In a study conducted by Klarqvist et al. [8], they undertook an extensive virtual screening process to identify suitable compounds for establishing a standard SST mixture. Their approach involved mapping a diverse range of components onto a chemical space using principal component analysis (PCA) based on common physicochemical properties such as lipophilicity, hydrogen bond properties, and polar surface area. Afterwards, the most appropriate components were selected based on their availability and with the aim of covering as much of the generated chemical space as possible. These four components comprise 3-Acetamidophenol, $(DHQD)_2PHAL$, Amitriptyline, and Glyburide. Detailed information regarding the chemical structure and calculated properties of these compounds can be found in Table 1.

Table 1. Physicochemical properties of the SST compounds, calculated by ACD Lab software [10]. [1]Hydrogen Bond Doner. [2]Hydrogen Bond Acceptor.

Component	Structure	MW	pKa values	LogP	No. of HBD[1]	No. of HBA[2]
Acetamidophenol		151.16	9.5	0.77	2	3
$(DHQD)_2PHAL$		778.98	3.8; 4.4; 7.9; 8.5	6.66	0	10
Amitriptyline		277.40	9.2	4.67	0	1
Glyburide		494.00	5.2	3.81	3	8

Selection of buffers

In this study, four LC buffers were selected for comparative analysis. These buffers are Trifluoroacetic Acid (TFA), Difluoroacetic Acid (DFA), Formic Acid (FA), and Ammonium Acetate (AA). The reason behind choosing this set of buffers is twofold. Firstly, to assess the performance of each additive and ascertain its impact on separation efficiency. Secondly, to compare buffers with TFA to determine if they exhibit comparable separation efficiency, qualifying them as potential alternatives.

Trifluoroacetic Acid (TFA) stands out as one of the most commonly used mobile phase additives owing to its strong ion-pairing capability. It has earned recognition as the gold standard mobile phase additive for protein biopharmaceuticals [5]. However, when LC is coupled with mass spectrometry (MS), TFA suppresses MS signals and hampers analyte ionization [4][11]. Moreover, TFA is recognized for its negative environmental effects, particularly on aquatic ecosystems, supporting the motivation to search for alternatives [1].

Consequently, efforts have been made to identify alternatives to TFA that offer similar performance in LC, while being more compatible with MS [5]. Similarly, in this study by selecting other buffers, it is aimed to explore how these candidates could serve as suitable

alternatives to TFA. These results hold significance for future method development, particularly in the context of buffer selection.

Selection of instruments

Following the design of a platform to compare buffers, it was deemed advantageous to also compare two different instrumentations. To achieve this objective, two sets of identical experiments were conducted on state-of-the-art instruments from two well-known vendors: Waters Co. and Agilent Technologies. The Agilent 1290 Infinity II system featured a quaternary pump, a Photo Diode Array (PDA) detector, a 100 µL flow-through needle, and a jet weaver (380µL) mixer [12]. Additionally, the Waters ACQUITY H-Class utilized in our experiment was equipped with a quaternary pump, a PDA detector, a 15 µL flow-through needle, a 50 µL extension loop, and a 100 µL mixer [13].

The measured dwell volumes for the Agilent and Waters systems were 0.79 mL and 0.44 mL, respectively. It is important to highlight that, with the exception of the instrument comparison section, all chromatography data was obtained using Waters H-Class system, counted as a reference for our comparative analysis.

1.2.2 Materials

Chemicals and chromatography system

For the preparation of aqueous mobile phases and samples, water with a conductivity of 18 MΩ from an in-house Milli-Q water purification system was utilized. Acetonitrile (gradient grade for LC), trifluoroacetic acid (≥99.0%, suitable for HPLC), difluoroacetic acid (≥97.5%, GC), formic acid (98-100%, purity for analysis), and ammonium acetate (7.5 M solution for molecular biology) were all procured from Sigma-Aldrich. Additionally, all test compounds, including 3-acetamidophenol, $(DHQD)_2PHAL$, amitriptyline, and glyburide, were also purchased from Sigma-Aldrich at analytical grade or higher.

This study was conducted using reverse phase liquid chromatography mode. For column selection, an ACQUITY UPLC BEH (Ethylene Bridged Hybrid) C18 column from Waters Co. was chosen. This column has dimensions of 2.1 mm (inner diameter) x 100 mm (length), a pore size of 130 Å, and a particle size of 1.7 µm.

1.2.3 Preparation of samples and mobile phases

As detailed in Table 2, a total of six mobile phases were utilized for this study. All the buffers were measured volumetrically, and the mobile phases were prepared to a total volume of 1 L. It was decided to include two concentrations of both TFA and DFA to increase the number of observations for comparison purposes. The selected concentrations align with typical values for these additives in similar studies [5], [8]. Following preparation, the pH of the mobile phases was measured and confirmed to be in close approximation with the reference values reported in the literature [3].

Table 2. List of all the aqueous mobile phases utilized in this study and their measured pH.

Aqueous mobile phase	Measured pH
0.1% (v/v) TFA	2.0
0.03% (v/v) TFA	2.5
0.1% (v/v) DFA	2.0
0.03% (v/v) DFA	2.4
0.1% (v/v) FA	2.7
25mM AA	6.8

For sample preparation, the necessary amount of each compound was weighed and dissolved in 5 mL of the reference diluent (30:70 v/v acetonitrile/water) to achieve a concentration of 0.2 mg/mL. The prepared solution was then placed in an ultrasonic bath for 15 minutes. Subsequently, the primary 5 mL stock solution was stored in the freezer, while the required amount for analysis was transferred into amber glass vials and sealed with a pre-slit silicone septum.

Software

Empower 3 software (Waters, Milford, MA) was employed to control and monitor the LC instrument throughout the experiments. This software facilitated the handling of chromatography experiments, creation of sample sets, and final processing of chromatography data.

1.2.3 Definitions

There are different chromatography parameters one could study to determine the performance of the analysis. In this study our focus primarily revolves around three essential factors related to efficiency, peak shapes, and separation ability. These terms will be defined in the following.

Plate count

This terminology originates from the concept of efficiency in distillation towers. The term "plate" stems from the idea that at each stage of the distillation separation, an equilibrium between the liquid and gas phases is established. Essentially, a higher number of plates indicates more available separation stages and thus, more efficient separation. Similarly, in chromatography, this term is synonymous with the efficiency of the separation. In other words, a higher plate count signifies that a shorter column length is required for superficial equilibration in the chromatography column, resulting in higher efficiency. The number of theoretical plates (or plate counts) can be expressed by the term N in the following equation:

$$\mathrm{N} = 16\left(\frac{\mathrm{t_R}}{\mathrm{w}}\right)^2$$

Where t_R is the retention time of the analyte, and w is the peak width at the baseline [14].

Peak tailing

The peak tailing factor or asymmetry factor (As) is an indicator of how far the peak shape deviates from an ideal Gaussian shape. This parameter quantifies the degree of skewness in the peak shape. Various expressions of this equation exist, but here we adopt the definition provided by the European Pharmacopeia (EP):

$$A_s = \frac{w_{0.05}}{2d}$$

Where, $w_{0.05}$ is the width of the peak at one-twentieth of the peak height, and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height. For a perfect Gaussian peak shape, the A_s value is 1.0, but if $A_s > 1.0$, peak is tailing and if $A_s < 1.0$, the peak is fronting [15].

Resolution

Resolution is a measure of the column's capability to separate two consecutive peaks effectively. Resolution (R) can be defined by the following equation [16]:

$$R = \frac{2(t_{R2} - t_{R1})}{w_2 + w_1}$$

Where the index 2 refers to the later eluting peak.

1.3 Results

As outlined in the introduction sections, this study is structured around three focal points, and accordingly, the results are presented in three sections, each addressing specific aspects of the research questions.

1.3.1 Comparison between four buffers

In this stage, six distinct experiments were designed, as indicated in Table 3. This design features a singular varying parameter, which is the type of buffer utilized. Such a design offers a suitable platform to observe and analyse the effects only associated with changes in the buffer type.

Table 3. Designed experiments to compare the separation efficiency of four buffers.

	Mobile phase A (Aqueous phase)	Mobile phase B (Organic phase)
Experiment 1	0.1% TFA in water	0.1% TFA in acetonitrile
Experiment 2	0.03% TFA in water	0.03% TFA in acetonitrile
Experiment 3	0.1% DFA in water	0.1% DFA in water
Experiment 4	0.03% DFA in water	0.03% DFA in water
Experiment 5	0.1% FA in water	0.1% FA in acetonitrile
Experiment 6	25 mM AA in water	Acetonitrile

A noteworthy aspect of Experiment 6 is the variation in the organic phase for ammonium acetate. In this experiment, the buffer was only added to the aqueous mobile phase. This decision was motivated by the buffer's low solubility in acetonitrile [17]. Consequently, the injected analytes did not encounter a consistent concentration of buffers in the mobile phase throughout elution, which makes the comparison with other buffers slightly complicated. However, the next section will further investigate the elimination of buffers from the organic mobile phase, allowing us to determine the sensitivity of the separation of these SST compounds to this factor.

All experimental setups were initially defined on Empower software and subsequently run on a Waters H-Class system, specifications of which are explained in the methods section. The applied gradient for the chromatography was from 5 to 95% of mobile phase B over 7 minutes and the UV wavelength was set to 254 nm.

After completing the experiments, all the chromatography data was processed and parameters such as plate counts, and peak tailing factors were extracted. Figure 1 depicts an overlay of all the obtained chromatograms. Upon collection of the data, these values were also visually compared in a bar chart, as shown in Fig. 2 for peak tailing factors and Fig. 3 for plate counts.

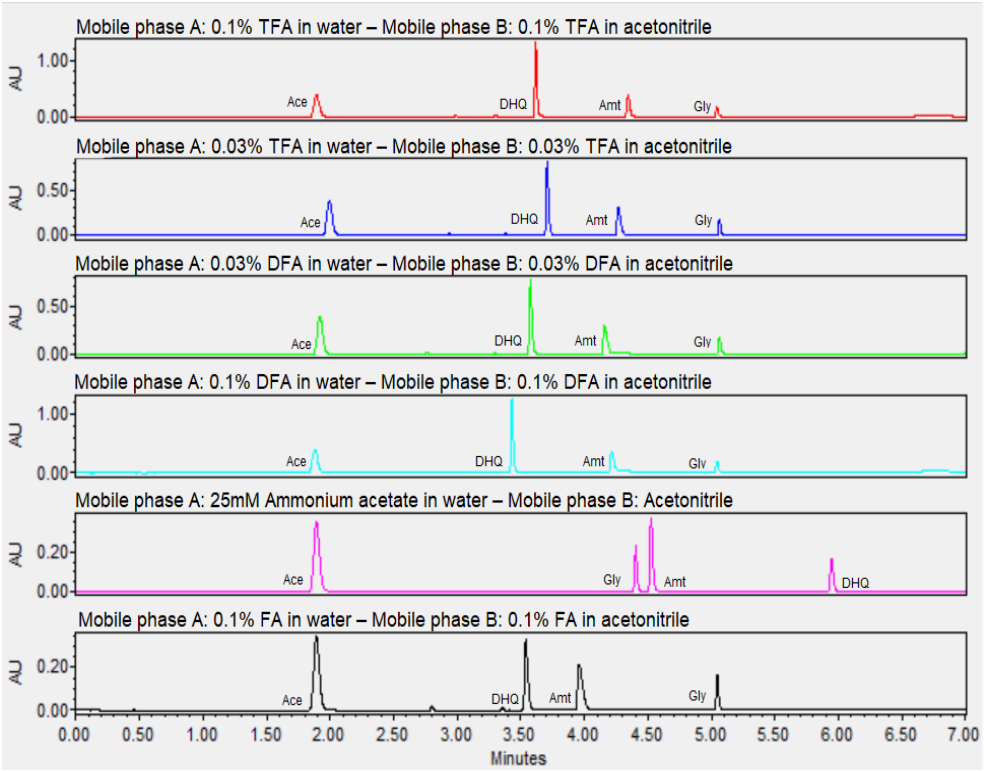


Figure 1. Chromatograms from six experiments utilizing different buffers. All components are denoted by the initial three letters of their names. "AU" is the abbreviation for "Absorbance Unit."

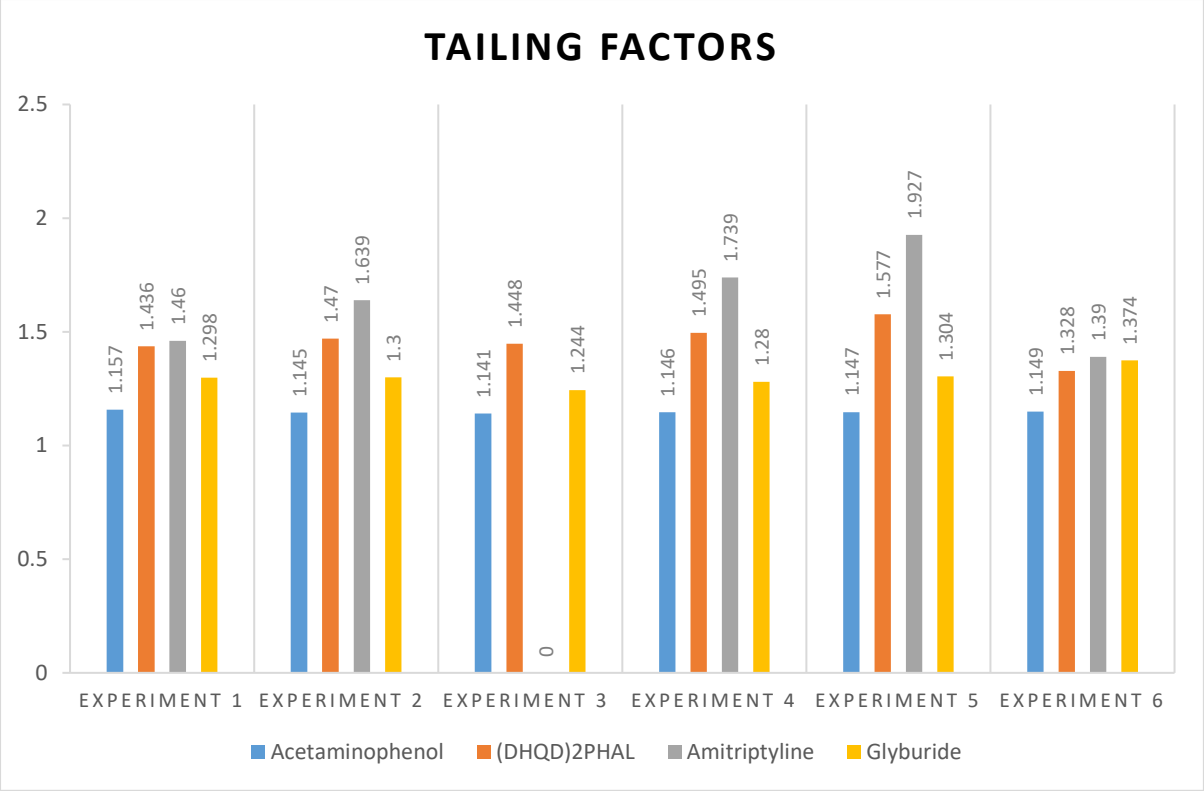


Figure 2. Peak tailing factors for SST compounds in six experiments.

Figure 3. Plate counts for SST compounds in six experiments.

As shown in Fig. 2, the tailing factors for amitriptyline in experiment 3 is reported as zero. This inability arises from the challenge of calculating the tailing factor for peaks with shoulders or unclear baselines. Fig. 4 depicts the exact shape of this peak, in which the two legs of the peak are not aligned at the same level. Therefore, accurate calculation of the tailing factor is not feasible in such cases.

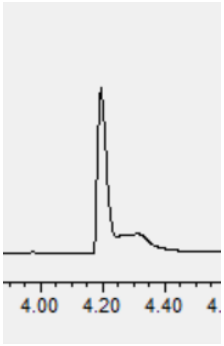


Figure 4. Peak shape of Amt in experiment 3 that has no reported value for tailing factor.

1.3.2 Removing buffers from the organic mobile phase

In the following section it was decided to run the exact same methods in the previous step, with the distinction of having the buffer present only in mobile phase A. When defining a gradient elution in this manner, it is crucial to note that besides the strength of the mobile phase in

eluting analytes from the column (gradually transitioning from polar to non-polar solvent), the concentration of buffers will also change along the gradient.

Thus, to observe the impact of varying buffer concentration throughout the gradient elution, compared to the initial setup (Table 3), buffers from mobile phase B were removed. For this comparison, only acidic buffers were selected, and ammonium acetate was excluded from this section. As a result, a new set of experimental setups was created, detailed in Table 4.

Table 4. New sets of experiments to address the effect of eliminating buffers from mobile phase B.

	Mobile phase A (Aqueous phase)	Mobile phase B (Organic phase)
Experiment 7	0.1% TFA in water	Acetonitrile
Experiment 8	0.03% TFA in water	Acetonitrile
Experiment 9	0.1% DFA in water	Acetonitrile
Experiment 10	0.03% DFA in water	Acetonitrile
Experiment 11	0.1% FA in water	Acetonitrile

Table 5 presents the final results of the experiments conducted in this section. This table also provides a comparative analysis of two critical factors extensively discussed in this study, plate counts and tailing factors, with their change compared to the reference values from the previous section.

Table 5. Results from running experiments 7-11, and comparison with the reference values. *These changes are calculated compared to the initial setup in Fig. 2 and 3.

	Compounds	Tailing factors	Change* (%)	Plate counts	Change* (%)
Experiment 7	Acetaminophenol	1.16	0.26	9.34E+03	-0.41
	$(DHQD)_2PHAL$	1.436	0.00	2.16E+05	-6.59
	Amitriptyline	1.527	4.59	1.29E+05	-20.34
	Glyburide	1.304	0.46	3.67E+05	0.13
Experiment 8	Acetaminophenol	1.156	0.96	9.28E+03	-5.99
	$(DHQD)_2PHAL$	1.502	2.18	1.40E+05	-18.45
	Amitriptyline	1.749	6.71	7.07E+04	-23.26
	Glyburide	1.294	-0.46	3.65E+05	-2.37
Experiment 9	Acetaminophenol	1.144	0.26	8.97E+03	-0.27
	$(DHQD)_2PHAL$	1.454	0.41	1.79E+05	-10.20
	Amitriptyline	-	-	9.52E+04	-8.85
	Glyburide	1.261	1.37	3.52E+05	-2.09
Experiment 10	Acetaminophenol	1.148	0.17	9.50E+03	0.14
	$(DHQD)_2PHAL$	1.52	1.67	1.27E+05	-13.36
	Amitriptyline	1.782	2.47	6.11E+04	-16.30
	Glyburide	1.258	-1.72	3.65E+05	-0.62
Experiment 11	Acetaminophenol	1.145	-0.17	8.45E+03	2.69
	$(DHQD)_2PHAL$	1.587	0.63	7.61E+04	-4.30
	Amitriptyline	1.955	1.45	3.83E+04	-4.34
	Glyburide	1.3	-0.31	3.41E+05	0.79

1.3.3 Instruments

In this final section, identical experiments to those in Table 3 were again conducted, this time using a different instrument. Hence, the only variable in this comparison is the type of LC

instrument utilized. Similar to that section, the obtained data, this time from the Agilent 1290 Infinity instrument, is presented in Figures 5 and 6.

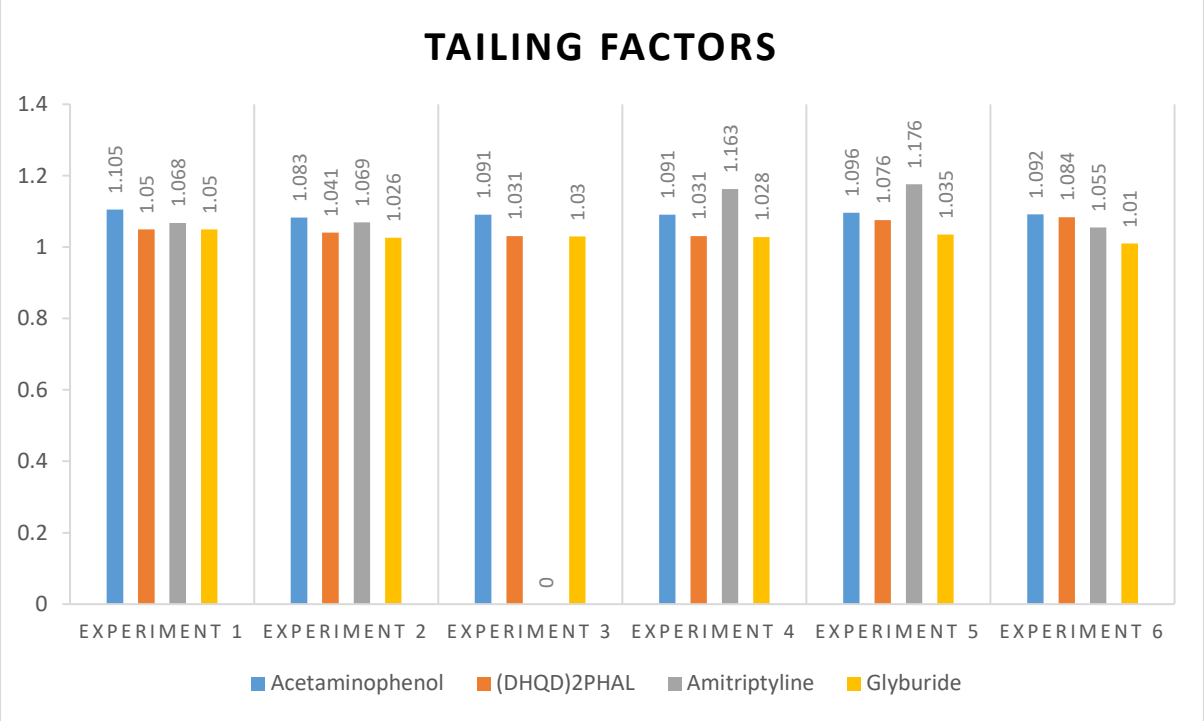


Figure 5. Tailing factors of the peaks from experiment 1-6, conducted on Agilent 1290 Infinity II system.

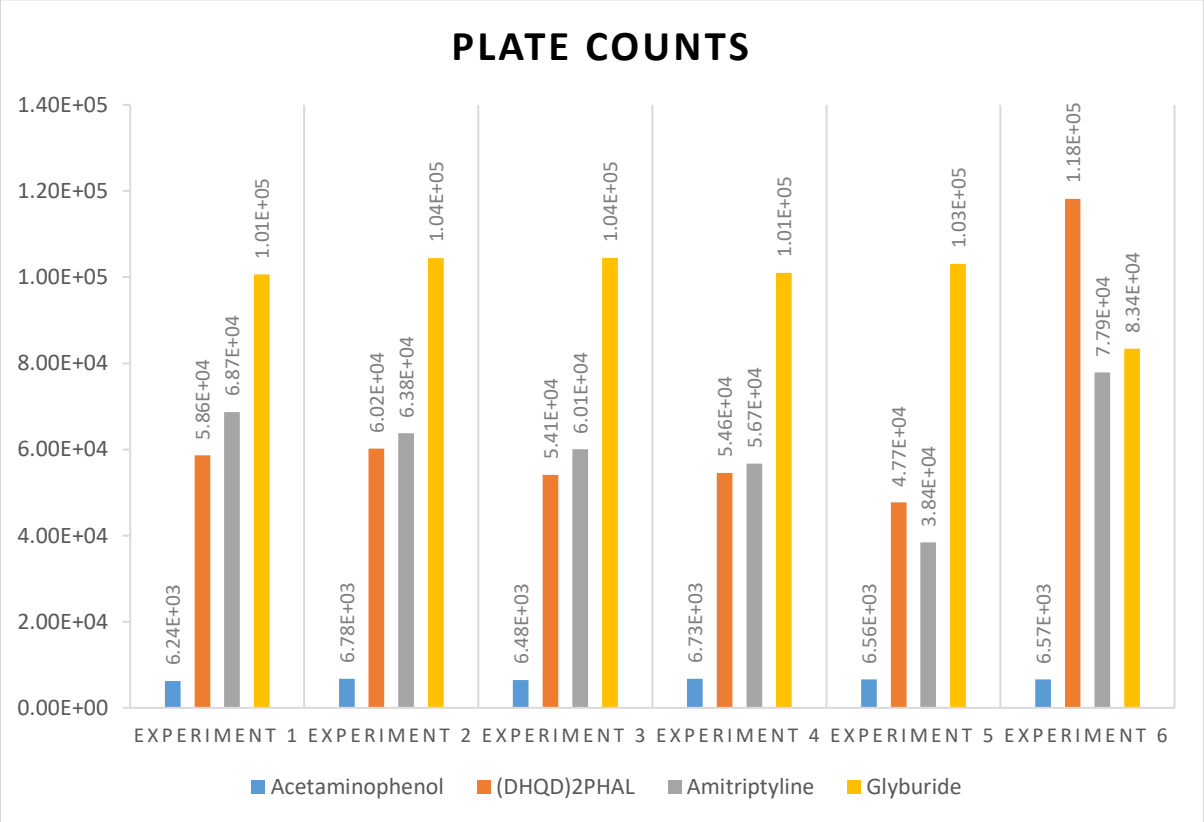


Figure 6. Plate counts from experiment 1-6, conducted on Agilent 1290 Infinity II system.

1.4 Discussion

Restating the primary objectives of this study, the discussion section will be divided into three main segments. Firstly, the performance of four buffers in separating a mixture of SST compounds will be compared. Within this part, results will be explored to determine whether any suitable alternatives to TFA exist. Moreover, the following discussion will be focused on the types of mobile phases, aiming to address the effectiveness of additives in the organic mobile phase. Lastly, a brief section will be dedicated to examining the differences between two LC instruments.

1.4.1 Buffer Comparison

As shown in Fig 1, the separation of the selected SST compounds under acidic conditions would result in the elution order from Acetaminophenol (Ace), $(DHQD)_2PHAL$ (DHQ), Amitriptyline (Amt), to Glyburide (Gly). Whereas, within the neutral pH range, and by utilizing ammonium acetate, the elution order changes to Ace, Gly, Amt, and DHQ, respectively. Overall, excellent separation between the components can be observed, with desirable resolutions among them. Moving forward, our attention turns to the critical parameters selected for evaluation: plate counts and peak tailing factors, in Fig 2 and 3.

As highlighted in the introduction section, TFA has been recognized as an ideal and robust acidic additive partly due to its ion-pairing capability. However, the challenge with ion suppression in RPLC-MS and its detrimental effects on environment persist as an issue [1]. While it was anticipated that TFA would exhibit superiority over other buffers, our objective was to evaluate whether other additives could achieve comparable performance to TFA, potentially indicating their suitability as alternatives.

As shown in in Fig. 3, it is noticeable that the results from TFA and DFA have close resemblance to each other. The data indicates that the average plate counts for DFA are approximately 10% lower than those for TFA. This finding could be significant, suggesting that by substituting TFA with DFA, the efficiency of the separation remains largely consistent. In terms of peak tailing factors, our comparison indicates that the utilization of DFA results in higher tailing of the peaks compared to TFA. This behaviour can be attributed to the higher ion-pairing strength of TFA, which reduces the ionic interaction between analyte and stationary phase, resulting in sharper peak shapes. With DFA, tailing factors for all four compounds do not exceed 1.8, which may not be ideal but could still be considered acceptable.

However, there is a notable phenomenon regarding Amt when using 0.1% DFA as the mobile phase additive (Experiment 3). As indicated in the results section, the peak tailing data for this condition is not provided by Empower, suggesting an undesirable peak shape from this condition. Further insights into the reason for this phenomenon can be found in a study by Kasagic et al. [18], where they presented a method development process for optimizing the separation of Amt from its impurities. This suggests that the choice of DFA for this specific compound in not optimal, complementing that in the plate count data this difference for Amt between having TFA and DFA is also noticeable and the biggest differences between these two buffers in plate counts stems from this compound.

Based on this information, the overall conclusion that can be drawn regarding DFA is that it holds promise as a potential alternative to TFA. As demonstrated by previous studies [5], DFA

does not exhibit the issue of ion suppression in MS. Additionally, according to the findings of this study, its LC performance closely resembles TFA, making it a viable option.

When examining the data for FA, it becomes evident that both parameters of plate counts and peak tailing are the least favourable for this additive, with the highest tailing factors and lowest plate counts observed. According to the literature, the usage of formic acid may indicate good MS detection properties, however, this suboptimal behaviour in LC can be associated with its weaker ion pairing strength [5].

Finally, AA can be discussed as the last buffer in this study. According to the data acquired, this buffer demonstrated the highest plate counts for two of the test compounds (Gly and Amt). Additionally, AA offered better peak shapes with increased symmetry. Thus, a promising performance in RPLC was demonstrated, suggesting improvements compared to TFA. Additionally, this buffer is more MS friendly and can serve as a potential candidate to replace TFA. However, one downside of this buffer is that in this separation, the resolution between two of the peaks is quite low. As shown in the chromatogram in Fig. 1, the resolution between Gly and Amt is 3.2.

In conclusion, this study suggests DFA and AA as potential candidates for TFA replacements when both acidic and neutral buffers work for the aimed separation. DFA has a similar pH range to TFA, although with a slight reduction in efficiency and peak shape. Conversely, AA shows improvements in separation performance but operates in a higher pH range. On the other hand, FA demonstrated the least favourable performance among the buffers tested in this experiment.

1.4.2 Comparison of Organic Mobile Phases

In the second phase of this study, the buffers were removed from the organic mobile phase to observe their effect on separation. According to Table 5, it can be seen that this new set of experiments has slightly decreased the performance in terms of both plate counts and tailing factors. The most significant reduction in plate counts, as a measure of separation efficiency, was observed for TFA, with an average reduction of 10% in plate counts. Additionally, peaks for almost all components exhibited slightly more tailing upon removing the buffers. However, an interesting observation pertains to the retention times of the analytes and their resolutions, which remained unchanged in this setup.

The conclusion drawn from this observation was that these test compounds are not significantly affected by changes in buffer concentration during the gradient elution. Even with the removal of buffers from the organic phase, the separation performance remained almost unchanged. This suggests that for future SST tests, reducing the consumption of these buffers could be a possible option, potentially leading to a more environmentally friendly SST protocol.

1.4.3 Instrument Comparison

In the final phase of this comparative study, the same experiments outlined in Table 3 were repeated, this time using an Agilent 1290 Infinity system instead of the Waters H-Class.

According to Fig. 5, concerning the asymmetry of peaks, it is apparent that tailing factors in the Agilent systems are much closer to 1, indicating highly symmetrical peaks were obtained in this new configuration. However, one of the significant findings is observed in the plate counts between these two instruments. As shown in Fig. 6, plate counts on the Agilent system

were reduced by an average factor of 50%. Moreover, this comparison reveals a 32% reduction in peak heights and a 46% increase in peak widths in the Agilent system.

While the Agilent instrument appears to maintain peaks more symmetrical, both the plate counts, and the broadness of the peaks seem to be adversely affected. This difference, however, can be attributed to the higher dwell volume of the Agilent instrument. As mentioned in previous sections, the Agilent system has almost twice the dwell volume of the Waters system. Although this study did not measure the extra-column volume of both systems, if it is assumed that the Agilent system also features a higher extra-column volume, this could be a possible explanation for this trend. Overall, our results suggest that while Agilent system provides more symmetrical peak shapes, for other operational parameters this system does not seem to be a better choice compared to Waters H-Class instrument.

1.5 Conclusion

In conclusion, this study undertook an investigation into the factors impacting liquid chromatography (LC) separation, aiming to provide insights for method development strategies. Through systematic analysis, the importance of various factors affecting chromatographic performance was investigated, which can help researchers to make informed decisions.

The comparison of four common buffers, namely trifluoroacetic acid, difluoroacetic acid, formic acid, and ammonium acetate, revealed some interesting results. Although TFA is recognized for its robust ion-pairing capability, its adverse impact on mass spectrometry signals is notable. Our study highlights DFA, operating within a similar pH range with slightly reduced performance, and AA, offering improved separation though at a different pH, as potential alternative candidates. Furthermore, our findings indicate that FA, among the selected buffers, exhibits the lowest suitability for LC separation of the SST compounds.

Further exploration into the influence of buffers on separation, particularly the removal of buffers from the organic mobile phase, revealed minimal impact on separation efficiency and peak shapes. This observation suggests the insensitivity of the SST compounds to the presence of buffers in the organic mobile phase, indicating potential reductions in their consumption and thereby enhancing environmental sustainability.

Moreover, the instrument comparison between Waters H-Class and Agilent 1290 Infinity systems revealed notable differences in chromatographic performance. While the Agilent system generated more symmetrical peaks, it also demonstrated decreased plate counts and wider peaks compared to the Waters system. This discrepancy could be attributed to the higher dwell volume of the Agilent instrument and extra post column volume, underscoring the importance of considering instrument characteristics in method development.

By addressing critical questions regarding buffer selection, mobile phase composition, and instrument compatibility, this research provided a foundation for future advancements in LC method development and optimization. As the next step, one can test and compare the performance of these buffers in MS as well, to determine whether the proposed candidates are still able to demonstrate promising performance compared to TFA or not.

Chapter 2

Analytical Method Development for Metoprolol and Its Related Impurities Using Fusion QbD Software

2.1 Introduction

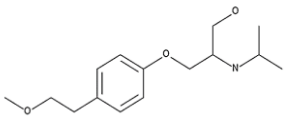
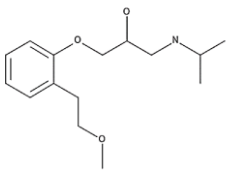
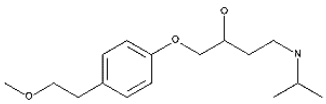
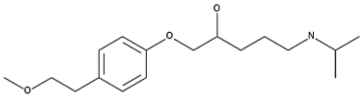
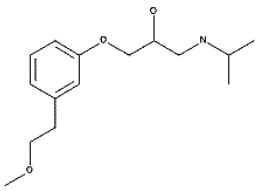
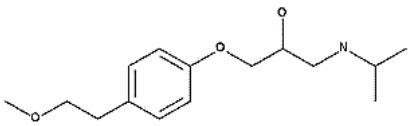
Metoprolol ($C_{15}H_{25}NO_3$) is characterized as an aromatic ether, a secondary alcohol, and a secondary amine compound [19]. Alongside other drugs such as betaxolol and bisoprolol, metoprolol falls under the category of cardio-selective beta-1-blockers, functioning by inhibiting receptor sites for epinephrine (adrenaline) and norepinephrine (noradrenaline), primarily present in heart [20]. This mechanism allows beta-1-blockers to efficiently control blood pressure and heart rate. Metoprolol is commonly prescribed for different cardiovascular conditions, such as hypertension, high blood pressure, vasodilation (relaxation of blood vessels), angina (chest pain), heart failure (inefficient blood pumping), and atrial fibrillation (irregular heartbeat). This Active Pharmaceutical Ingredient (API) is available in succinate and tartrate forms, providing short-term and long-term release, respectively, and its formulation is customised to meet specific treatment needs [19].

According to our literature review, metoprolol, as a model compound, has emerged as a common target in various separation studies. These studies can be generally categorized into three groups. Firstly, there are studies focusing on combination drugs, where formulations typically consist of multiple APIs, in addition to metoprolol, with the primary objective of developing a separation method for them [21], [22]. Secondly, some studies have focused on separating metoprolol from complex sample matrices like blood or urine, which contain numerous compounds that can hinder the separation [23],[24]. Thirdly, other studies have aimed to separate metoprolol from its impurities or related compounds [25].

Similar to the latter category, the objective of this study is to use an automated approach to develop an analytical method capable of separating metoprolol from its impurities. Various impurities and degradation products with structures and properties similar to metoprolol have been identified previously [26]–[28]. This underscores the complexity of developing an accurate separation method for the purification of metoprolol. In pharmaceutical sciences, the identification and quantification of impurities play a crucial role, especially during the product development phase, ensuring the quality of the final product. Therefore, the establishment of an automated and faster process of identifying these methods is a valuable aim that this study strives to achieve.

Method development refers to the process of adjusting experimental parameters to optimize the separation of a specific target compound. Analytical method development, therefore, can be described as the exploration for the most effective separation conditions within a defined analytical technique. According to the target of this method development, five structurally similar compounds (impurities) to metoprolol, labelled as H84/79, H106/59, H170/31, H170/40, and H177/19, have been chosen (Table 6).

Table 6. Physicochemical properties of metoprolol and its related substances, predicted by ACD lab software [10].

Components	Chemical structure	Chemical formula	Molecular weight	pKa
H84/79		$C_{15}H_{25}NO_3$	267.2	8.1
H106/59		$C_{15}H_{25}NO_3$	267.2	9.4
H170/31		$C_{16}H_{27}NO_3$	281.2	10.2
H170/40		$C_{17}H_{29}NO_3$	295.2	10.6
H177/19		$C_{15}H_{25}NO_3$	267.4	9.5
Metoprolol succinate		$C_{15}H_{25}NO_3$	267.2	9.4

The time-consuming nature of the method development is one of the frequently encountered obstacles in this process. This emphasizes the importance of utilizing computational tools in examining a wider operational range, thus accelerating the identification of potential solutions. These statistical tools not only boost efficiency but also contribute to greater confidence in determining the optimal method.

Fusion Quality by Design® software, developed by S-matrix, was utilized in this research as the primary computational tool. This software holds promise as a crucial step in automating LC method development, in line with Quality by Design (QbD) principles. Examples of integrating Fusion QbD into analytical method development process can be found in similar literature [29], [30]. By using the capabilities of Fusion QbD, this software was the main guide throughout the initial Design of Experiment (DoE), screening, and final optimization stages of method development.

During method development, the pH of the mobile phase deemed one of the most critical factors influencing the optimization of the separation. Additionally, the column type, temperature, and gradient slope were also selected as influential variables in this method development.

Ultimately, by following the screening through optimization steps, a method utilizing 10 mM potassium phosphate buffer with a pH of 8.1 and a gradient from 15 to 42% acetonitrile over 7 minutes was identified as the most optimal approach. As this method was developed using a phosphate buffer and considering the incompatibility of such buffers with mass spectrometry (MS), the final step involved exploring an alternative using ammonium acetate with the same pH as a more MS-friendly option.

This research serves as an example of utilizing state-of-the-art software for analytical method development in accordance with the QbD approach. The findings of this research can contribute to the advent of automated method development processes by minimizing manual interaction and offering the required statistical tools.

2.2 Theory

Analytical method development is indispensable in the pharmaceutical industry, being involved across various stages of drug development. Its applications range from quality control and drug discovery to impurity profiling, stability studies, and regulatory validation of products prior to launch [16]. Analytical chemists typically strive for separation methods that can precisely identify and quantify all drug substances while achieving efficient separation within practical time and cost limitations. Method development involves systematically exploring optimal conditions within an analytical technique or instrument to fulfil these objectives.

The necessity for analytical development generally arises from several factors: the absence of a suitable method for a particular sample matrix, the presence of error- or contamination-prone methods leading to poor precision in analysis, the existence of time-consuming or expensive methods, and the development of new instrumentation and techniques offering the potential for improved methods [31]. Therefore, any effort aimed at enhancing separation capability can fall under the category of method development.

To describe this searching process, one can divide the entire method development process into four distinct levels:

1. Defining variables and specifying their range
2. Design of Experiments (DoE)
3. Screening
4. Optimization

It is important to note that Design of Experiments (DoE) and screening steps are often interchangeable, indicating that data gathered during the screening experiment can feed back into the DoE step to improve the design space. In other words, there may be many iterations between steps 2 and 3, aimed at creating a refined space for the optimization phase [16].

Depending on the target for method development, the desired criteria can be translated into various parameters in LC. Defining an optimal range for parameter values is crucial, and it should be based on the properties of the target analyte. Moreover, with the same aim and in case there is limited knowledge about the analyte(s), the screening phase of method development can also function as an exploratory step to gain deeper insights into system behaviour and unknown interactions.

Once an acceptable set of variables and their ranges are defined, the next step involves the Design of Experiment (DoE). This process aims to create sets of experiments where an optimal method within the defined parameters is sought. DoE entails multivariate analysis with the goal of identifying significant associations among multiple factors. In DoE, determining the cause-and-effect relationship between experimental parameters and separation performance is crucial [32].

Various models and approaches exist to define the experimental space (see Fig 7), with key considerations including the number of required experiments and the precision in adjusting the correlation between variables. Different DoE algorithms such as Response Surface Mapping (RSM), serve as tools that provide a set of experiments containing a combination of response factors, enabling an effective exploration of the defined space. Following the execution of these

experiments, the collected data is analysed by fitting it to various mathematical models, which may include linear, quadratic, cubic, and quartic models [32].

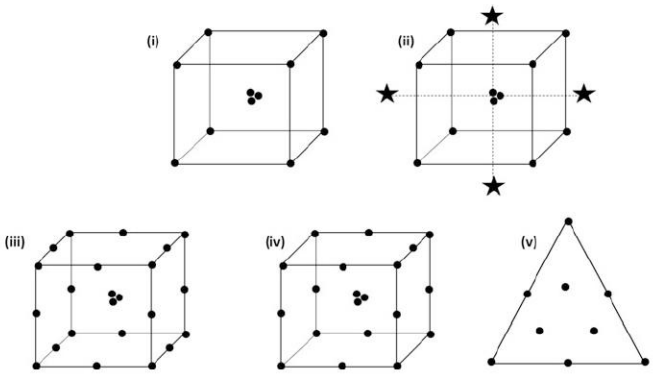


Figure 7. Examples of approaches used for factor screening study, (i) Full factorial design, (ii) Central composite design, (iii) Box-Behnken design, (iv) Optimal design, (v) Mixture design [32]. Adapted with permission, ©Springer Nature.

Furthermore, it is crucial to identify the parameters that exert the most significant influence on the desired response. In method development, numerous factors are typically involved, making it challenging to precisely determine the individual or collective impact of each on the final outcome. Consequently, conducting screening experiments is important to acquire essential information, eliminate undesired responses, and, most importantly, prioritize the most influential factors [32].

Finally, in the optimization phase, based on all the obtained chromatography results in previous steps, some parameters are defined as the Responses of Interest (RoI). These RoIs can be set to be maximized, minimized, or targeted to a specific value. Figure 8 illustrates an example of how this parameter definition is conducted using Fusion QbD, the software utilized in this study. Additionally, relative rankings can be assigned to indicate the priorities of these responses in the final optimized method. The RoIs may include resolution, peak shape, plate counts, tailing factor, peak capacity, elution time, detection limits, limit of quantitation, and overall accuracy in quantifying the specific analyte(s) [31].

Enabled	Response	Goal	Lower Bound	Target	Upper Bound	Relative Rank
☑	No. of Peaks	Target	4.0	6.0	6.1	1
☑	No. of Peaks >= 2.00 - USPResolution	Maximize	0.0		4.0	0.8
☑	No. of Peaks <= 2.00 - USPTailing	Maximize	0.0		6.0	0.8
☑	Metoprolol - USPTailing	Maximize	0.82		2.86	0.8
☑	Metoprolol - USPPlateCount	Maximize	2851.222		53806.811	0.8
☑	Metoprolol - USPResolution	Maximize	0.705		4.572	0.8

Figure 8. Defining RoIs within Fusion QbD software.

Following method development, method validation becomes necessary to evaluate the suitability of the developed method for commercial application. This entails thorough testing to ensure the method meets predefined criteria for accuracy, precision, specificity, linearity, and robustness. One validation approach involves method transfer, where the same method is performed, and results are compared in an interlaboratory manner. This process contains transferring the validated method from one laboratory to another, ensuring consistent and reliable results across different settings. Such tests aid in verifying the robustness and reproducibility of the methods [31].

However, it is important to note that this study will solely focus on the method development phase, excluding further testing and validation of the method, like those mentioned earlier. Thus, the primary emphasis will be on devising the most effective separation method for metoprolol impurity profiling on RPLC, along with the integration of an automated tool for analytical method development.

2.3 Methods

2.3.1 Procedure

Introducing the variables in the design

In our method development process, four variables that are considered to be the most influential factors were identified. The objective is to optimize a method by adjusting these four variables. This section will provide a more in-depth introduction to these four factors.

Firstly, the pH of the buffers was selected as a variable in this study. The pH of the mobile phase alters the state of the analytes, whether they are negatively charged, positively charged, or neutral, thereby affecting their affinity to bind and interact with the stationary phase. This state is determined by the pKa values of the analytes, making it crucial to set the pH of the mobile phase accordingly. For example, if the pH of the mobile phase is close to the pKa of the analytes, even a slight change in pH can significantly impact the chromatographic outcome [3].

Temperature represents another critical factor in LC and is frequently incorporated into method development. One of the direct impacts of temperature is on the viscosity of the mobile phase. Elevated temperatures result in decreased viscosity of the mobile phase, requiring lower backpressure to push the solvents into the column. Moreover, higher temperatures lead to an increased diffusion rate from the mobile phase into the stationary phase in chromatography. Nevertheless, the drawback of operating at high temperatures is the potential stability issues of the stationary phase. Most LC columns are recommended to operate at temperatures up to 60-70°C. Beyond this range, there is a risk of degradation of the silica backbone or its bonding, which can result in column bleeding or the loss of the stationary phase [33], [34].

The third factor considered in this design is the type of LC column. Here, a total of three columns were utilized. The first column selected is an ACQUITY UPLC BEH C18 column from Waters, which is among the most common choices. This column type is favoured for its robustness and durability against harsh pH and temperature conditions. The particles within this column are fully porous, offering a greater surface area for interaction with the mobile phase.

In addition, two core-shell columns were also chosen, featuring a superficially porous core. This solid core restricts analyte absorption to the surface of the particles, resulting in lower back pressure and minimizing the effect of diffusion on band broadening. This structure enables them to shorten the analysis time and be more resistant to higher flow rates. Wu Ludvigsson et al. conducted a comprehensive test on various core-shell columns to determine their performance [35]. Among the options available, it was decided to select two columns from different manufacturers, namely EVO Kinetex from Phenomenex [36] and HPH Poroshell from Agilent Technologies [37]. In the following, Fig. 9 compares these two particle structures.

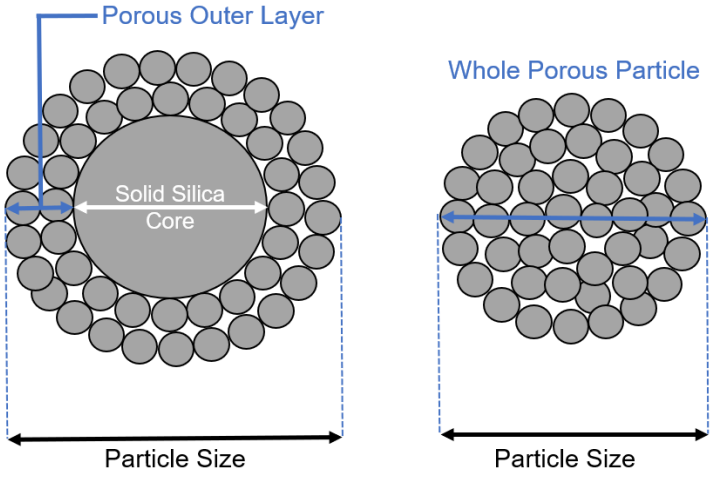


Figure 9. Comparison of two structures for particles used in LC columns. Left: Core-shell particles with solid core. Right: common fully porous particles [37].

The last variable in this study was the slope of the gradient in the elution. This is a factor mainly changing resolution of the peaks by compressing or stretching the chromatograms and peak shapes. If a gradient is too fast it might make all the peaks collide in a same region, and on the other hand it should not be too shallow that leads to band broadening of the peaks, also if the gradient is not strong enough, analytes will retain in the column.

Fusion workflow

In this project, Fusion Quality by Design® software developed by S-matrix Corp., Eureka, CA, was utilized for multivariate analysis. Fusion QbD is tailored for the development, validation, and transfer of LC, LC-MS, and Supercritical Fluid Chromatography (SFC) methods. Serving as an advanced statistical tool, Fusion QbD is instrumental in method development for this experiment. This computational tool integrates Design of Experiments, chromatography data software automation, and advanced data modelling, which provides an effective approach to screening and optimizing multiple systems [38].

Once the variables of the experiment are determined, the next step involves introducing the values into the software and creating a design. In Fusion QbD, it is necessary to specify the type of each variable, whether discrete or continuous. Additionally, the software allows for the definition of different stages of the gradient elution as a variable. For example, users can create a design consisting of various equilibration times to explore an optimized duration. Figure 10 illustrates the interface of the software for creating a new design.

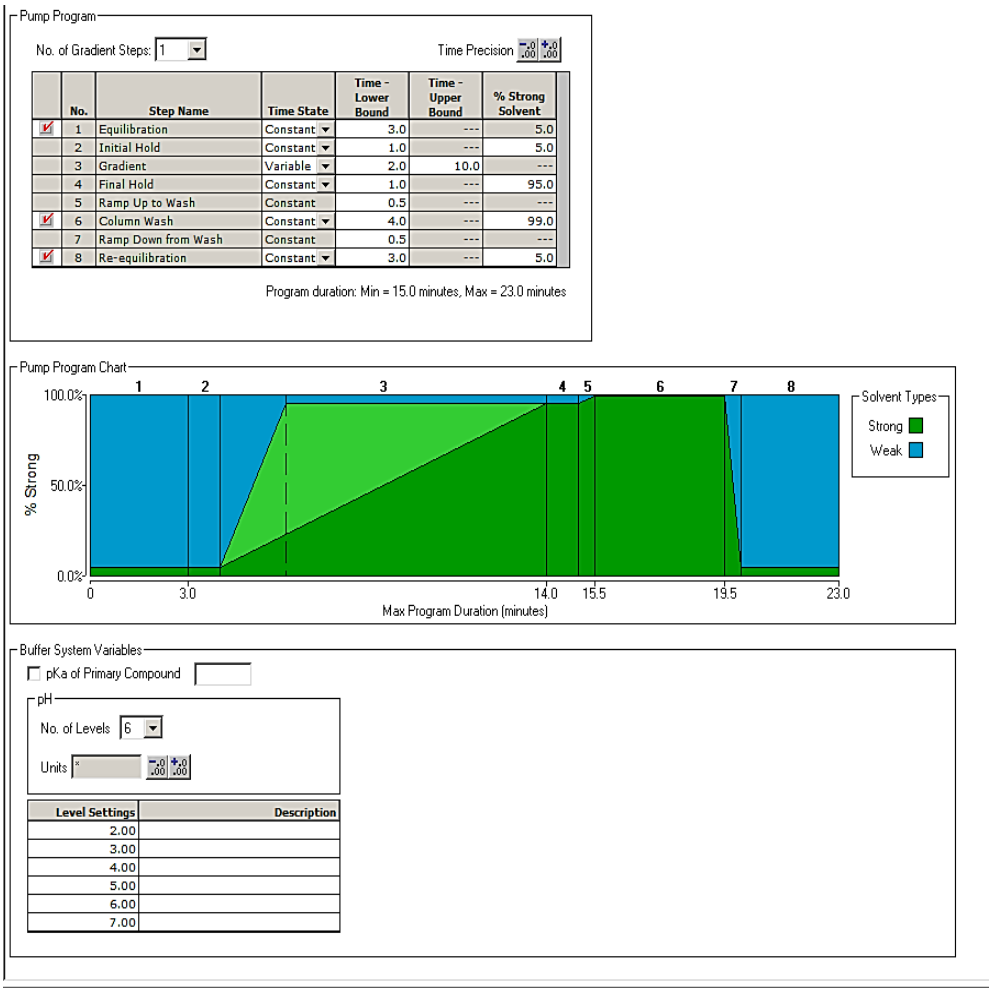


Figure 10. Optimizing different stages of gradient elution in Fusion QbD.

Once all the variables are defined and their desired ranges are set, Fusion QbD will generate an experimental design. This software has the capability to directly connect to Empower, allowing for convenient export of the experimental setup to the LC instrument. This feature, in fact, enables the immediate execution of the experiments. Upon completion of LC runs, the obtained data is imported to Fusion QbD for further analysis. Based on the defined RoIs (Fig. 8), the software can model the responses and predict the best possible answer. Furthermore, Fusion QbD can provide contour plots and various visualization tools to elucidate the relationships between parameters.

2.3.2 Materials

Chemicals

Water with a conductivity of 18 MΩ was obtained from a Milli-Q water purification system. Acetonitrile (Gradient grade for LC), trifluoroacetic acid (≥99.0%, suitable for HPLC), potassium phosphate dibasic (≥99.0%), potassium phosphate monobasic (≥99.5), ammonia solution 25%, and ammonium acetate (7.5 M solution for molecular biology) were all supplied from Sigma-Aldrich. The reference components for analysis, including metoprolol succinate and all impurities (listed in Table 6), were internally provided by AstraZeneca.

An ACQUITY UPLC BEH C18 from Waters, featuring dimensions of 2.1 mm (inner diameter) x 100 mm (length), a pore size of 130 Å, and a particle size of 1.7 µm was utilized. The two core-shell columns selected for this experiment were Kinetex EVO C18 (100 x 3 mm, 2.6 µm, 100 Å) from Phenomenex, Torrance, CA, and InfinityLab Poroshell HPH C18 with dimensions of 3.0 x 100 mm, 2.7 µm from Agilent Technologies, Santa Clara, CA.

Chromatography system

The method development process was conducted on a Waters H-Class instrument, featuring a Photo Diode Array (PDA) detector and a quaternary pump within the solvent manager section. This instrument was equipped with a 15 µL flow-through needle, a 50 µL extension loop, and a 100 µL syringe. The dwell volume of the system was independently measured and reported as 490 µL. The injection volume was set at 5 µL, and the UV detection wavelength was set to 220 nm.

Preparation of buffers

In this experiment, different buffers were chosen to match the desired pH of the aqueous mobile phase. Trifluoroacetic acid (TFA) buffer was selected for separations close to a pH of 2. For the screening phase of this study, a 0.03% v/v solution of TFA was prepared, resulting in a measured pH of 2.5. In the neutral pH range, potassium phosphate buffers with the ionic strength of 10 mM and pH of 6.3, 7.3 and 8.1 were prepared. Ammonium buffer was identified as another suitable choice for this experiment. Mobile phases for both neutral and basic pH ranges were prepared in the presence of ammonium cations. For neutral pH, an ammonium acetate solution (20 mM) was utilized, while for the high pH condition, ammonium hydroxide (25% solution) was diluted directly with water. Table 7 summarises the preparation procedures for all the selected aqueous mobile phases in this study.

Table 7. All the aqueous mobile phases used in this study with their preparation procedure. *PB: phosphate buffer. **AA: ammonium acetate

Aqueous mobile phase (1 Litre)	Preparation procedure
pH 2.5	300 µL of TFA
pH 6.3	6.1 mL of 1M KH_2PO_4 + 1.3 mL of 1M K_2HPO_4
pH 7.3	1.4 mL of 1M KH_2PO_4 + 2.9 mL of 1M K_2HPO_4
pH 8.1 (PB*)	0.24 mL of 1M KH_2PO_4 + 3.3 mL of 1M K_2HPO_4
pH 6.8	20 mM of ammonium acetate
pH 8.1 (AA**)	Adjusting pH of 20 mM ammonium acetate solution by adding droplets of ammonium hydroxide solution (25%)
pH 10.8	1.6 mL of 25% ammonium hydroxide solution

Sample preparation

At first, 5 mL of stock solutions were made by precisely measuring and dissolving the respective masses of each component in a diluent comprising 40% water and 60% acetonitrile, resulting in a concentration of 1 mg/mL for each component. These prepared stock solutions were then stored in a freezer for preservation.

The final working solution was prepared by mixing different volumes of each stock solution to achieve concentrations of 0.1, 0.05, 0.04, 0.03, 0.02, and 0.01 mg/mL for metoprolol, H177/19, H170/40, H84/79, H106/59, and H170/31, respectively. The order of these concentrations was

selected arbitrarily to aid in peak tracking and identification. Subsequently, aliquots of the resultant mixture were transferred to LC vials and stored in the freezer for further use.

2.4 Results and Discussion

This section of the report goes through the method development process, focusing on the results of screening and optimization experiments. The initial stages of this process, as outlined in the preceding section, involved firstly identifying the key parameters influencing LC separation: pH of the aqueous mobile phase, temperature, gradient, and column type. Then, these parameters were defined within the Fusion QbD software to generate the experimental design. Therefore, the results obtained from the third and fourth phases are presented in this section, with the first series of screening experiments detailed in Table 8.

Table 8. Screening experiments with their defined parameters.

	Screening 1 (Acidic)	Screening 2 (Basic)	Screening 3 (Basic)
Column type(s)	BEH	BEH	HPH, EVO
pH	2.5, 6.8	7.3, 10.8	7.3, 10.8
Temperature (°C)	30, 40, 50	30, 40, 50	30, 40, 50
Final %MeCN[1] in gradient	60 – 90 Initial: 5%	30 – 50 Initial: 10%	30 – 50 Initial: 10%
No. of injections	11	11	25
Flowrate (mL/min)	0.4, 0.5	0.4	0.8
Gradient time (min)	10	6	6

The first two experiments aimed to evaluate separation performance across high and low pH ranges. For these sets, a BEH C18 column was selected due to its versatility across a pH range of 1 to 11 [39]. However, based on the outcomes of screening experiments 1 and 2, it was decided to investigate two additional columns (HPH and EVO) only under basic conditions. Further details will be provided in following discussions.

The chosen responses of interest for analysing the results of screening experiments comprised the total peak count, the numbers of peaks with a resolution greater than 2, and the number of peaks with a tailing factor less than 2. It is common to observe inadequate separation and peak co-elution in screening experiments. Therefore, the primary objective here was to identify chromatograms where a higher number of peaks had been successfully separated. However, in the final optimization stage, the responses of interest will be adjusted to be more restrictive. In the following, results of each screening experiment will be presented along with accompanying discussions, guiding us in determining the most suitable operational range for the optimization step.

2.4.1 Screening 1

In Screening 1, the results of experiments conducted at two pH levels were compared: pH 6.8, containing 20 mM ammonium acetate, and pH 2.5, with 0.03% TFA. It was noted that the more acidic pH (2.5) did not produce satisfactory separation. Only up to four peaks were visible in the chromatogram at this pH, suggesting considerable coelution and compromised resolution. On the other hand, regardless of variations in temperature, gradient, and flow rate, all chromatograms at pH 6.8 displayed five peaks consistently. A visual comparison of these two conditions is illustrated in Figure 11, where A represents the method at pH 2.5 and B for pH 6.8.

[1] Methyl cyanide or acetonitrile

Moreover, the impact of temperature in conjunction with pH was also considered. On the BEH column at pH 2.5, running experiments at 50°C resulted in three peaks, whereas at 30°C, four peaks were detected, suggesting that lower temperature improves the separation in this case.

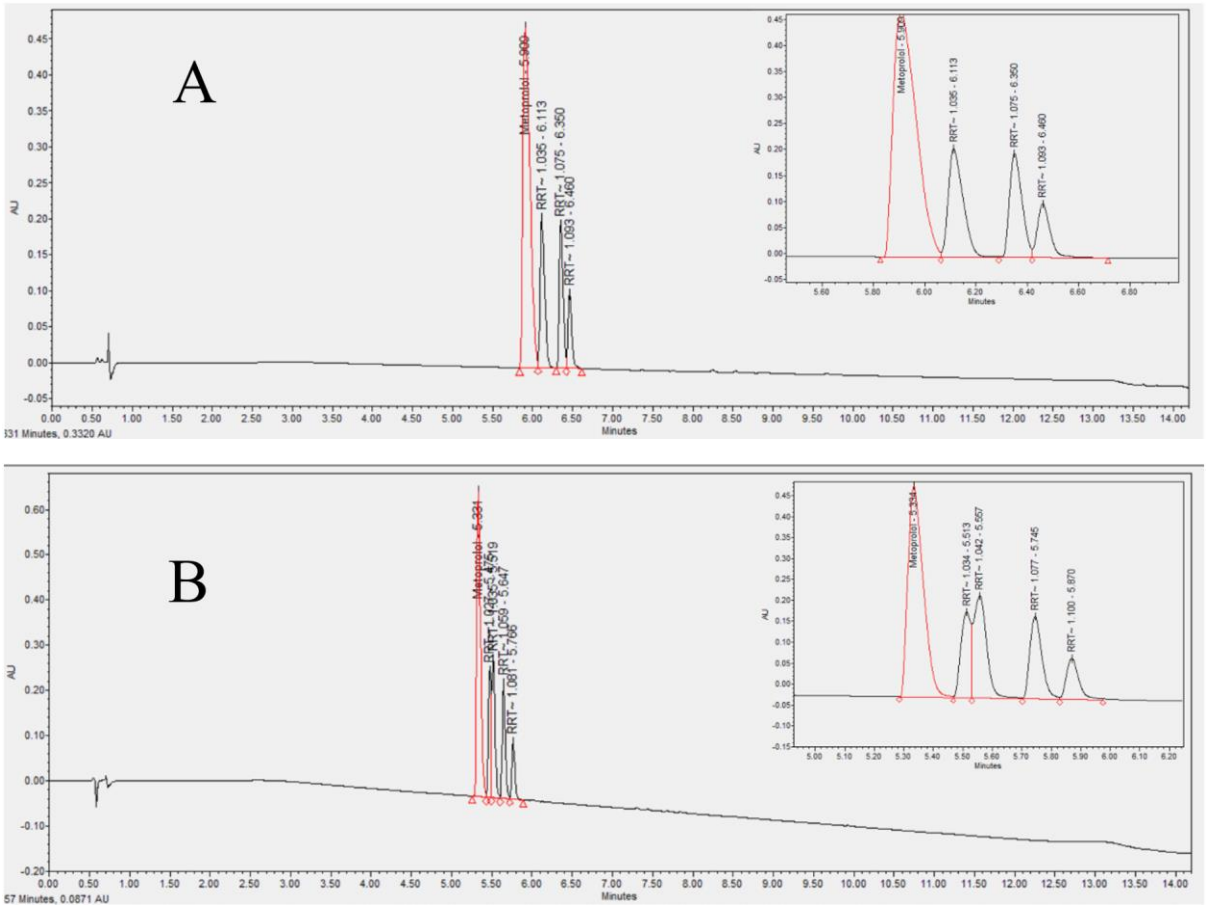


Figure 11. Results obtained from screening 1. A) Flowrate: 0.4 mL/min; 5-60% MeCN in 10 minutes; Oven Temperature: 30°C; pH: 2.5 B) Flowrate: 0.5 mL/min; 5-60% MeCN in 10 minutes; Oven Temperature: 30°C; pH: 6.8.

2.4.2 Screening 2

In the next screening phase conducted under basic pH, a potassium phosphate buffer at pH 7.3 and a diluted ammonium hydroxide solution at pH 10.8 were utilized. Initial observations indicated an improved separation efficiency within this pH range compared to Screening 1. Two representative chromatograms from these two experiments are illustrated in Fig. 12A and B.

Interestingly, both pH 7.3 and 10.8 conditions demonstrated the capability to detect all six peaks under various conditions, which contrasted with the findings of Screening 1. However, both conditions exhibited two peaks closely eluting immediately after the metoprolol peak (H177/19 and H170/40). It is noteworthy that the retention times for all components were prolonged at pH 10.8. Consequently, in two injections at this pH, at temperatures of 40°C and 50°C, both with a final gradient of 30% MeCN, no peaks were eluted before 9 minutes, even after the re-equilibration time. This underscores the necessity for stronger mobile phase in the end of the gradient for these conditions.

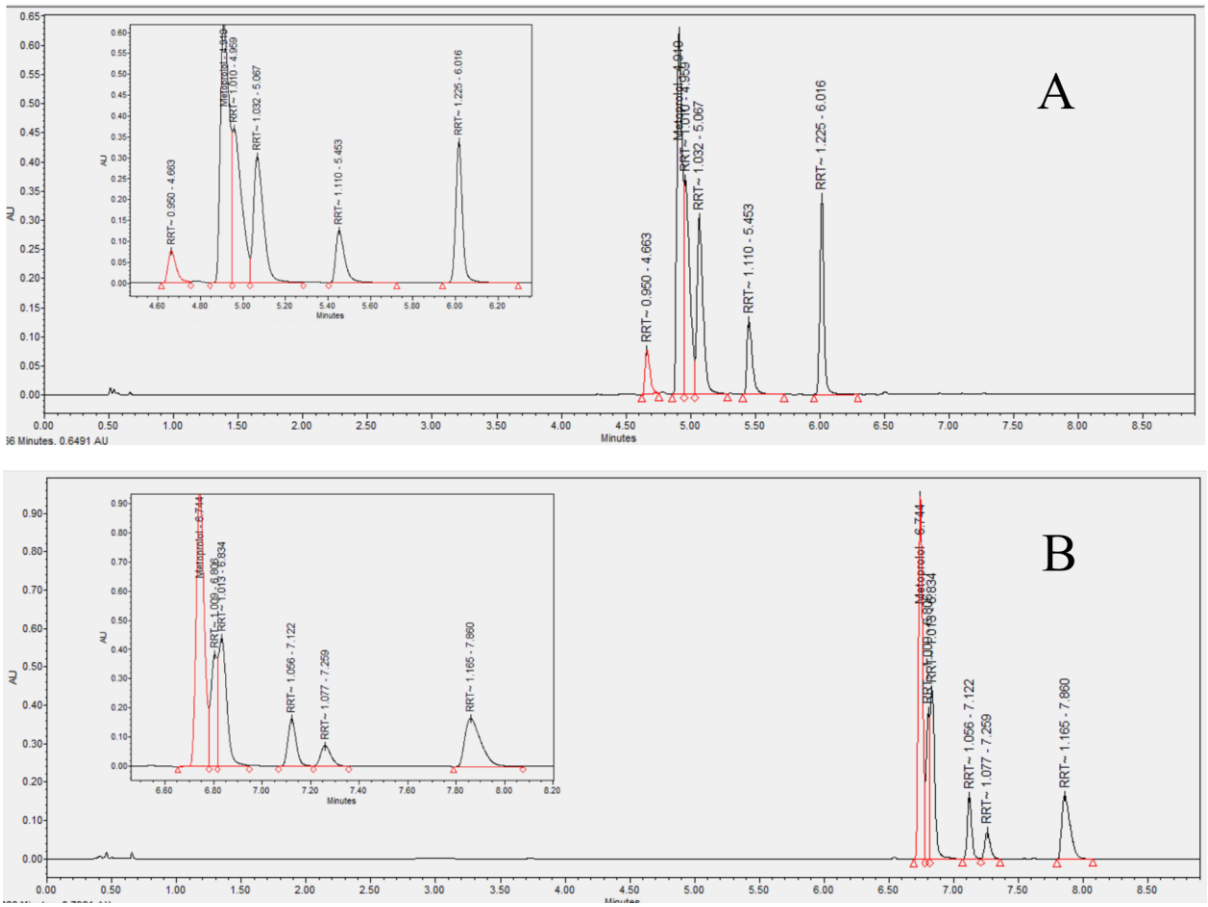


Figure 12. Results obtained from Screening 2. A) Flowrate: 0.4 mL/min; 10-50% MeCN in 6 minutes; Oven Temperature: 50°C; pH: 7.3 B) Flowrate: 0.4 mL/min; 10-50% MeCN in 6 minutes; Oven Temperature: 50°C; pH: 10.8.

2.4.3 Screening 3

Before delving into the results from this screening experiment, it should be noted that due to the superior performance of the basic pH in this method development, the decision was made to exclude the acidic condition run on the core-shell columns after comparing the results from screenings 1 and 2. Thus, screenings on two other columns focus only on studying the separation performance within the same pH range as Screening 2.

By analysing the outcomes of Screening 3, it was interesting to observe the contrasting compatibility of the columns with pH. While pH 10.8 yielded better separation for the EVO Kinetex column, the HPH Poroshell column exhibited superior performance at a pH of 7.4. Figures 13A and B illustrate the separation performance of the EVO Kinetex column at pH 10.8 and the HPH Poroshell column at pH 7.4, respectively. Additionally, an observation worth mentioning is the noticeable tailing factors, particularly evident in later eluting peaks for the EVO column as depicted in Fig. 13A.

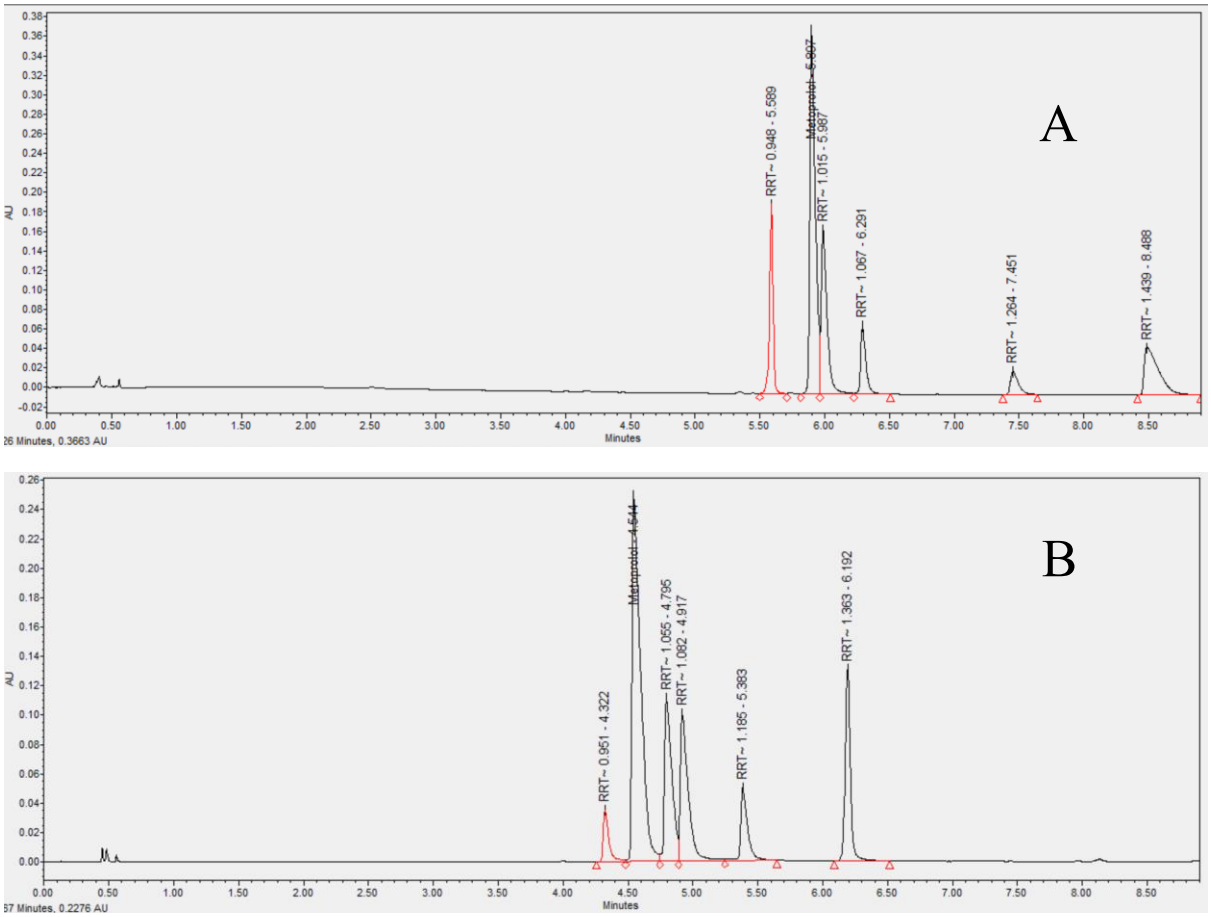


Figure 13. Results obtained from Screening 3. A) Column: EVO Kinetex; Flowrate: 0.8 mL/min; 10-50% MeCN in 6 minutes; Oven Temperature: 40°C; pH: 10.8. B) Column: HPH Poroshell; Flowrate: 0.8 mL/min; 10-30% MeCN in 6 minutes; Oven Temperature: 40°C; pH: 7.4.

Therefore, at this point, a decision was required regarding the pH range for the optimization experiments. Considering that developing a method at extremely high pH levels could potentially reduce the column lifetime, and notably, as per the results from data analysis conducted with Fusion QbD, a preference was given towards operating within a more neutral pH range. Consequently, it was decided to proceed in favour of the HPH Poroshell and BEH C18 columns, opting for a pH range of 6-8 for the subsequent phase of experimentation.

2.4.4 Optimization

Based on the outcomes derived from the screening runs, the primary aim at this stage is to fine-tune parameter ranges to increase confidence in approaching the optimal method. As indicated by Fusion QbD, two pH levels around 7, used in the screening experiments (i.e., pH 6.8 and 7.4) were found to deliver the most effective separation. Therefore, for further optimization, a new experimental space involving pH values of 6.3, 7.3, and 8.1 was delineated. It is worth noting that due to the well-established high performance of phosphate buffers, all mobile phases investigated in the optimization phase contained potassium phosphate buffers with a strength of 10 mM [3]. For a more comprehensive overview, detailed information on the two optimization sets is provided in Table 8. Similar to previous experiments, two separate conditions for optimization were established, differing only in their flow rates, to account for the potential capability of running the HPH column at higher flow rates.

Table 8. Optimization experiments with a narrower pH range.

	Optimization 1	Optimization 2
pH	6.3, 7.3, 8.1	6.3, 7.3, 8.1
Temperature (°C)	40, 50	40, 50
Final %MeCN in gradient	35 – 50 Initial: 15%	35 – 50 Initial: 15%
Flowrate (mL/min)	0.4	0.8
Gradient time (min)	7	7
Column	BEH C18	HPH Poroshell

Following the optimization experiments, three additional responses were incorporated into the data analysis alongside the initial three RoIs. These new factors included the tailing factor, plate counts, and resolution of the metoprolol peak. As a result, a total of six RoIs were established for optimization in the pursuit of the best possible answer. The results of this analysis will be discussed in the next section.

2.4.5 Finding the best possible answer

In the final phase of this method development, based on the results from optimization 1 and 2, multivariate analysis was conducted using Fusion QbD software to determine the most optimal separation method. Figures 14 and 15 illustrate the outcomes of this analysis from optimization 1 and 2, respectively.

Answer #1

Variable Settings

Name	Level Setting
Final % Strong Solvent	42.8
Oven Temperature	47.9
pH	8.00

Predicted Results

Response Name	Goal	Predicted Result	Desirability	-2 Sigma Conf. Limit	+2 Sigma Conf. Limit
No. of Peaks	Target	6.0	0.9996	5.8	6.2
No. of Peaks >= 2.00 - USPResolution	Maximize	3.6	0.9119	3.0	4.2
No. of Peaks <= 2.00 - USPTailing	Maximize	5.7	0.9647	4.6	6.8
Metoprolol - USPTailing	Maximize	1.40	0.3662	0.79	2.00
Metoprolol - USPPlateCount	Maximize	48,289.107	0.9124	47,344.732	49,233.483
Metoprolol - USPResolution	Maximize	4.589	1.0000	4.532	4.645

Cumulative Desirability Target = 1.0000
Cumulative Desirability Result = 0.8154

Figure 14. The recommended best possible answer for BEH C18 column from Fusion QbD

Answer #1

Variable Settings

Name	Level Setting
Final % Strong Solvent	41.9
Oven Temperature	48.2
pH	8.00
Column Type	Poroshell HPH

Predicted Results

Response Name	Goal	Predicted Result	Desirability	-2 Sigma Conf. Limit	+2 Sigma Conf. Limit
No. of Peaks	Target	6.0	0.9998	5.8	6.2
No. of Peaks >= 2.00 - USPResolution	Maximize	4.6	0.9296	4.3	4.8
No. of Peaks <= 2.00 - USPTailing	Maximize	3.8	0.9513	3.1	4.5
Metoprolol - USPTailing	Target	1.00	0.9996	0.75	1.25
Metoprolol - USPPlateCount	Maximize	47,967.616	0.8369	34,212.130	61,723.102
Metoprolol - USPResolution	Maximize	3.162	0.5925	2.634	3.867

Cumulative Desirability Target = 1.0000
Cumulative Desirability Result = 0.8715

Figure 15. The recommended best possible answer for HPH Poroshell column from Fusion QbD

As observed in these findings, a method recommended by the Fusion QbD includes approximate parameters for gradient elution of 15-40% MeCN over 7 minutes, a temperature of 50°C, and a pH of 8.1 for both BEH and HPH columns. As these recommended answers (Fig. 14 and 15) are just a prediction based on the provided data, exact values for each parameter in the method set may not align precisely with these answers. It is believed that these minor discrepancies do not significantly change the final outcome of the separation. The actual methods chosen, which closely match the characteristics in those figures, are detailed in Table 9.

Table 9. Specifications of the actual optimized methods on BEH C18 and HPH Poroshell columns. [1]10 mM Phosphate buffer

Column	Flowrate (mL/min)	Final %MeCN	Temperature	pH	Metoprolol tailing factor	Metoprolol plate count
BEH C18	0.4	35%	50℃	8.1[1]	0.82	4.4E+04
HPH Poroshell	0.8	42.5%	50℃	8.1[1]	0.90	5.9E+04

The chromatograms corresponding to these methods can be seen in Figure 16. In the first chromatogram (Fig. 16A), which belongs to the BEH C18 column, it is observed that all peaks exhibit a resolution ≥2, with tailing factors for all peaks being smaller than 2. However, in the optimized method for the HPH column (Fig. 16B), 5 peaks had a resolution greater than 2, while 4 peaks were seen with a tailing factor less than 2.

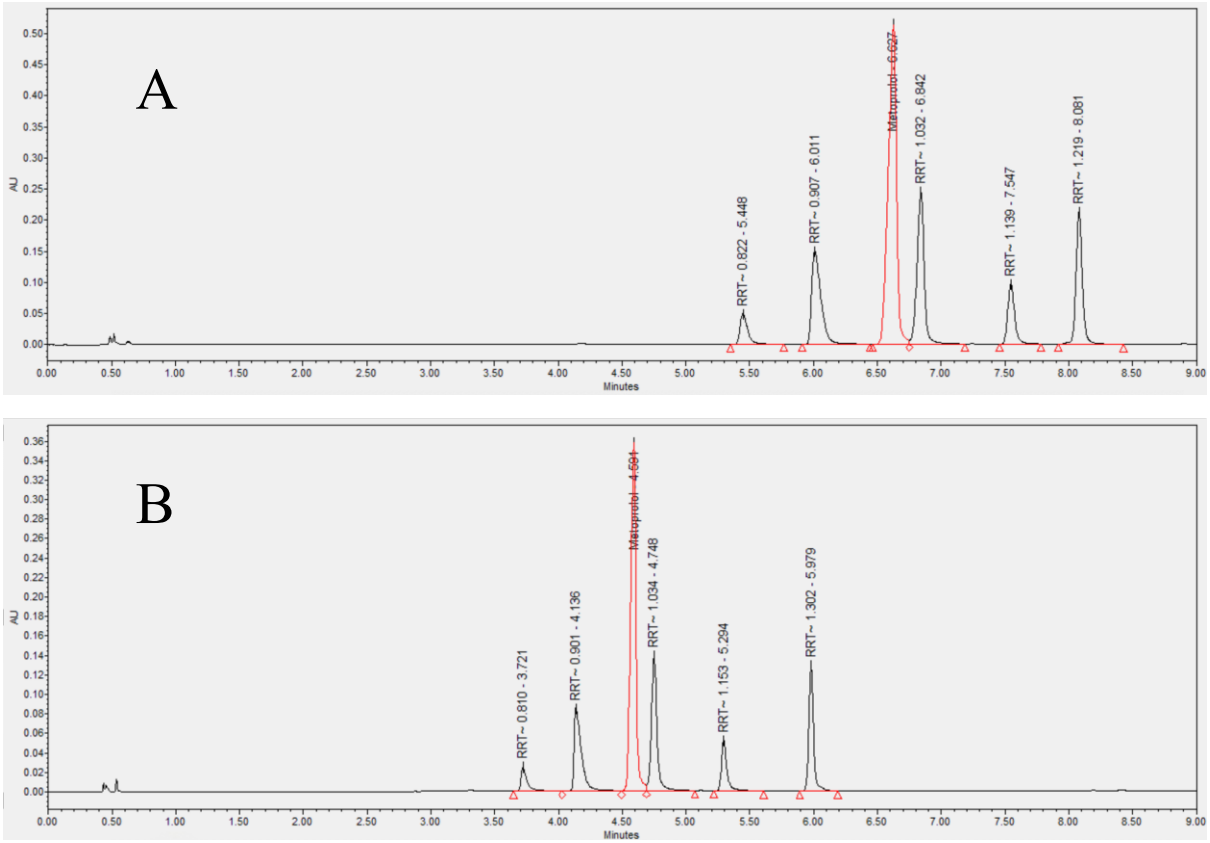
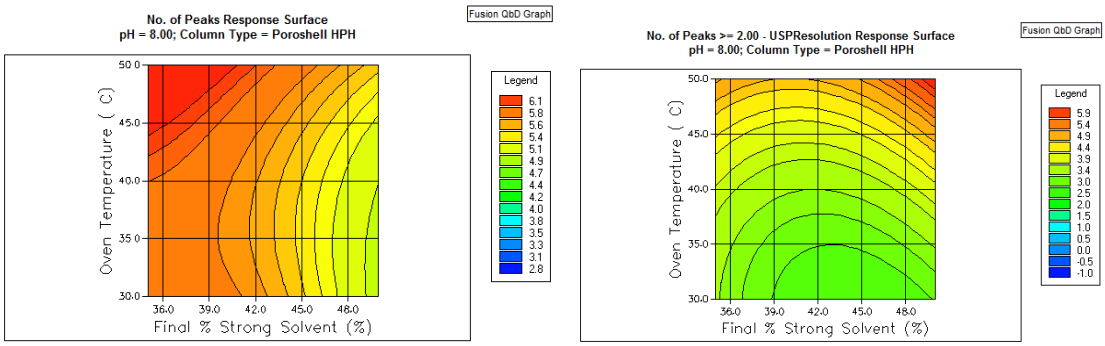


Figure 16. The performance of the optimized methods. A) BEH C18 column, flowrate: 0.4 mL/min, 15-35% MeCN in 7 minutes, Oven temperature: 50°C, pH 8.1. B) HPH Poroshell column, flowrate: 0.8 mL/min, 10-42.5% MeCN in 7 minutes, Oven temperature: 50°C, pH 8.1. Elution order for both conditions: H170/31, H170/40, metoprolol, H177/19, H106/59, and H84/79.

As mentioned earlier, Fusion QbD enables the visualization and examination of the entire experimental regions using chromatographic data. Figure 17 illustrates the contour plots and the dependency of each RoI on experimental variables such as temperature and gradient slope, based on the results obtained from the selected method on the HPH column.



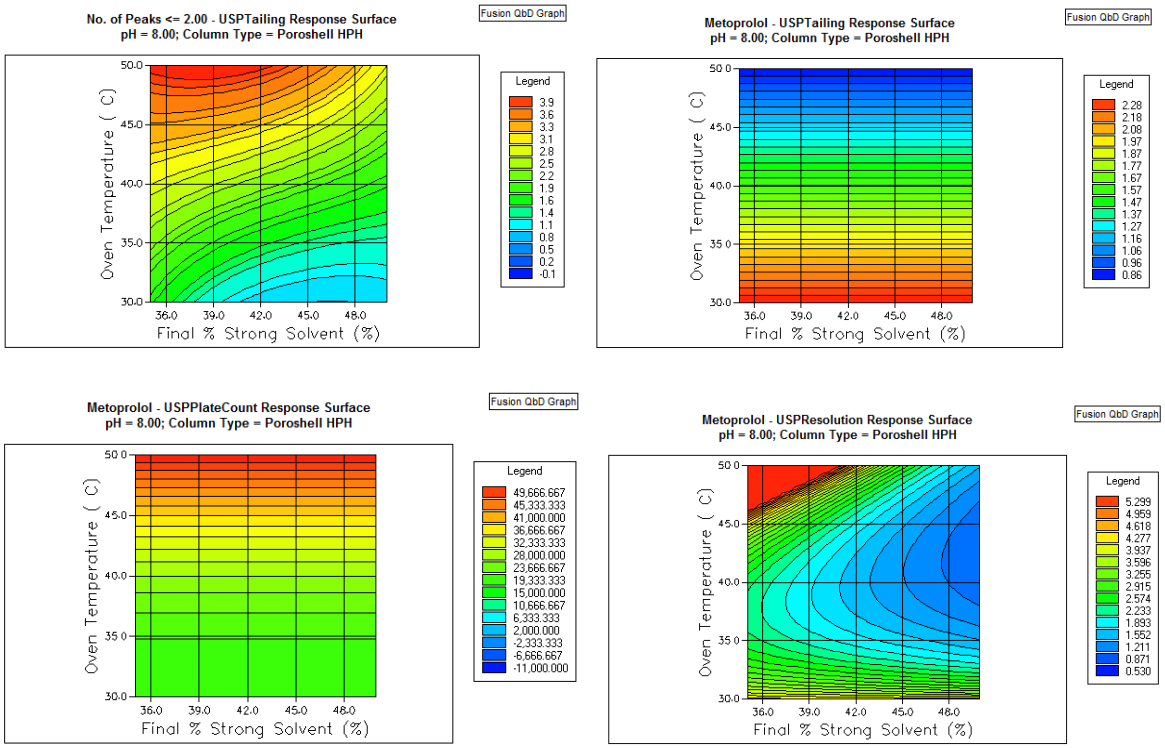


Figure 17. Contour plots of results from the optimization 2 on HPH Poroshell column.

Among the six selected RoIs, it appears that the plate counts, and tailing factor of metoprolol are not highly influenced by the percentage of MeCN during elution but rather by temperature. Additionally, two RoIs show contrasting trends, while a higher percentage of MeCN appears to decrease the number of peaks with a tailing factor less than 2, it increases the number of peaks with a resolution greater than 2. However, calculations from Fusion QbD suggest that a final percentage of MeCN at 40% is the most suitable value to finish the gradient elution.

2.4.6 Comparing the performance of ammonium acetate with phosphate buffer

As previously mentioned, only potassium phosphate buffers were utilized during the optimization phase. However, phosphate buffers offer incompatibility with mass spectrometry. Consequently, after identifying a suitable method, the performance of ammonium acetate as an alternative MS-friendly buffer at the same pH was also explored. Table 10 outlines the differences between the two buffers' performance in impurity profiling of metoprolol.

Table 10. Comparison of ammonium acetate and potassium phosphate buffers at the optimum pH (8.1) in separation of impurities.

Buffer/ Column	No. of peaks	No. of peaks with resolution≥ 2	No. of peaks with tailing factor ≤2	Metoprolol tailing factor	Metoprolol plate count	Metoprolol resolution
PB/ BEH	6	4	6	0.82	44839	4.78
PB / HPH	6	5	4	0.90	58966	5.13
AA / BEH	6	2	2	2.70	2661	1.23
AA / HPH	6	4	5	2.24	15391	1.68

As Table 5 indicates, using ammonium acetate with the HPH Poroshell column seems to offer superior separation compared to the BEH C18 column. This trend implies that phosphate buffer

still surpasses ammonium acetate across all separation criteria. Therefore, a potential trade-off arises when considering the combination of LC separation with MS and replacing phosphate buffer with ammonium acetate. However, this study suggests that if ammonium acetate were chosen, the HPH Poroshell column presents significant advantages over the BEH C18 column (Figure 18). This claim gains further support from the substantially higher plate count observed for the HPH column, nearly five times greater, along with its ability to provide superior tailing and resolution of peaks.

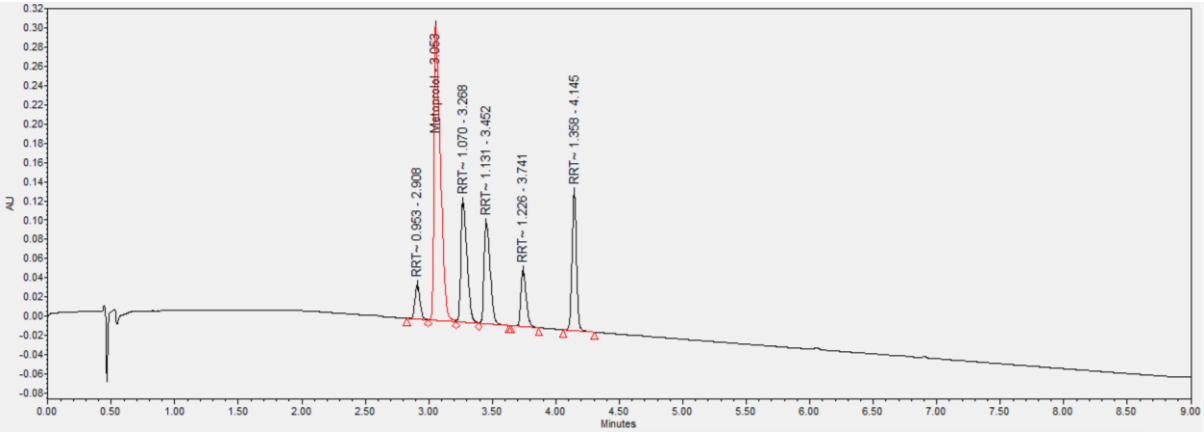


Figure 18. Performance of ammonium acetate buffer at pH 8.1 on HPH Poroshell column. Flowrate: 0.8 mL/min, 10-42.5% MeCN in 7 minutes, Oven temperature: 50°C. Elution order: H170/31, metoprolol, H177/19, H170/40, H106/59, H84/79.

2.5 Conclusion

In conclusion, the development of a reverse-phase liquid chromatography method for the impurity profiling of metoprolol has been successfully achieved in this study. By incorporating five selected impurities, the aim was to achieve a comprehensive separation of the target Active Pharmaceutical Ingredient (API) from its related substances. Throughout this method development process, Fusion Quality by Design® software played a pivotal role, facilitating the creation of a suitable design space and providing robust statistical analysis.

For this method development, four factors of pH, temperature, column type, and gradient slope were considered as variables to modify the separation method. Screening experiments conducted under different pH conditions highlighted the significance of pH in influencing separation performance. Notably, while acidic conditions proved inadequate, basic pH ranges exhibited promising separation capabilities. Optimization experiments further refined parameter ranges, leading to the determination of the most optimal method. Fusion QbD recommended a method with a gradient of 15-40% MeCN over 7 minutes, a temperature of 50°C, and a pH of 8.1 (10mM phosphate buffer) for both BEH C18 and HPH Poroshell columns, offering optimal separation according to defined responses of interest.

Furthermore, the performance of ammonium acetate and phosphate buffer was compared at the optimum pH of 8.1. While phosphate buffer exhibited superior separation criteria overall, ammonium acetate can also exhibit acceptable performance if LC is hyphenated with MS. Overall, the findings of this study contribute to the advancement of analytical method development, offering insights into automated strategies and the optimization of chromatographic conditions. Additionally, this study provides an optimized and rapid UPLC method for the complex separation of metoprolol along with its five structurally similar impurities.

For future work, it is recommended to incorporate Fusion QbD into more method development processes due to its desirable performance and features. While this study primarily focused on method development, further analysis could involve method validation to investigate terms like transferability and precision of this proposed method.

Chapter 3

Optimization of Experimental Procedures for Assessing Inclisiran Duplex Stability During Ion Pair Liquid Chromatography

3.1 Introduction

Cardiovascular disease (CVD), more specifically, atherosclerotic cardiovascular disease (ASCVD), stands as a leading cause of mortality, notably prevalent in western nations. As per the 2019 ESC/EAS Guidelines, CVD claims over 4 million lives annually in Europe alone, with approximately 2.2 million female and 1.8 million male fatalities [40]. Given the significant association between total cholesterol levels and CVD, considerable efforts have been dedicated to managing lipid levels in both primary and secondary prevention protocols, focusing particularly on reducing plasma concentrations of low-density lipoprotein cholesterol (LDL-C). In this regard, lipid modification therapies (LMTs) have emerged as transformative strategies in CVD prevention [41].

LDL is structured as a spherical molecule, comprising a lipid-rich core containing cholesteryl esters and triglycerides (TG), enveloped by a dual-layered coat consisting of phospholipids, free cholesterol, and apolipoprotein B [42].

In recent years there has been significant progress in therapeutic oligonucleotides (ON) as the next generation of drugs. One notable class within ON therapeutics is Small Interfering RNA (siRNA). siRNAs exist in double-stranded or hairpin forms, typically comprising 20-30 base pairs. In this structure, the antisense strand, integral to the formation of the RNA-induced silencing complex (RISC) within the cell, is linked to a complementary strand that aids in the intracellular transport of siRNA. The primary function of siRNAs lies in gene silencing or the downregulation of specific proteins, achieved through RNA degradation [43], [44].

A promising approach to managing LDL-C levels involves a novel therapeutic modality. Inclisiran, a siRNA marketed under this commercial name, has been engineered to silence the *PCSK9* gene, thereby regulating LDL receptors. The European Medicines Agency (EMA) granted approval for the medical use of this drug throughout Europe in December 2020. Studies have reported a significant reduction of approximately 50% in LDL-C levels in randomized controlled trials of Inclisiran [45], [46].

A visual representation of the Inclisiran structure is provided in a review paper conducted by Pietro et al. [46], illustrating the sequence of sense and antisense strands (Fig. 19). The antisense strand of Inclisiran consists of 23 nucleotide bases, while the sense strand comprises 21 nucleotides. Additionally, the 3′ end of the sense strand is conjugated with triantennary N-acetylgalactosamine (GalNAc), facilitating its uptake into hepatocytes through interaction with asialoglycoproteins [46].

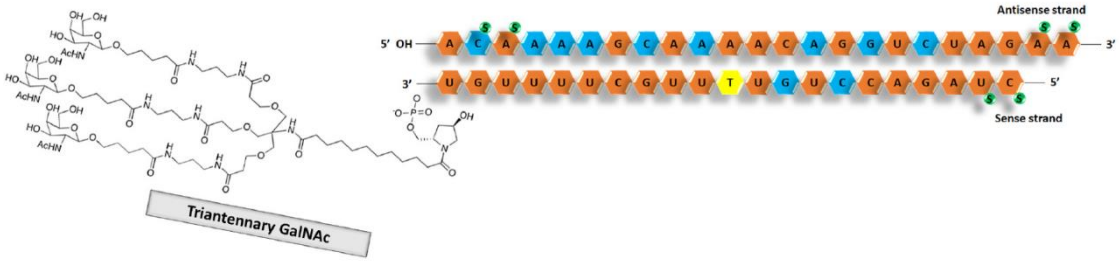


Figure 19. Structure of Inclisiran showing the sequences of the sense and antisense strands, along with the conjugated GalNAc [46].

Depending on the ON synthesis method employed, there is a potential for generating various impurities at different stages. These impurities can typically be categorized into shortmers (n-1, n-2), longmers (n+1), residual phosphodiester (PO) oligonucleotides (ONs) in phosphorothioate (PS) products, and basic ONs. Characterization of these impurities often relies on separation methods such as anion exchange chromatography (AEX) and ion-pairing reverse-phase (IP-RP) liquid chromatography, which are considered as the gold standards separation methods for this aim [44].

When it comes to impurity profiling of siRNAs, there are typically two approaches, depending on the purpose. One method involves denaturing the double-stranded structure of the siRNA duplex and quantifying each individual strand. The other approach entails maintaining the hybridized duplex intact and developing a non-denaturing method. Developing a method that meets the requirements of the latter approach is complicated and requires careful consideration [47].

The nearly identical physicochemical characteristics of optimal duplex and non-optimal duplex variants pose a challenge for chromatographic separation. The lack of adequate chromatographic resolution is presently the primary obstacle preventing the detection and quantification of individual duplex impurities using non-denaturing techniques [47].

One of the most recognized methods that offers a non-denaturing separation condition, is through IP-RPLC using triethylamine (TEA) with 1,1,3,3,3-hexafluoro-2-propanol (HFIP) buffer system. Utilizing HFIP in ion-pairing buffers holds significant interest due to its compatibility with Electrospray Ionization-Mass Spectrometry (ESI-MS) and its frequent delivery of desired separation performance [47]. However, a drawback of HFIP is its high cost compared to other typical chemicals used in this separation [48].

The key in developing non-denaturing method for siRNA separation is to understand factors affecting the stability of the siRNA duplex during the LC-separation. Several factors within LC can affect siRNA stability, potentially leading to duplex denaturation. These include temperature, stationary phase chemistry, type and concentration of the ion-pairing agent, and organic solvent concentration [49].

While numerous studies have focused on developing separation methods for siRNAs, there is limited knowledge regarding the individual contributions of each factor to siRNA duplex stability during the separation. Our study aims to establish an experimental setup capable of investigating these factors individually through melting point measurements, the temperature at which double-stranded siRNAs dissociate into two single-stranded species. This preliminary study precedes LC separation and offers insight into the stability of siRNAs that can be valuable in development of new separation methods.

In this chapter, initially, various factors in the melting temperature (Tm) measurement experiment will be explored to determine an optimal experimental approach. This first phase can also be categorized as method development since the goal is to identify the most suitable conditions. Following this, the optimized procedure will be applied to measure the Tm of the siRNAs under different conditions within the TEA/HFIP system. Once these findings are obtained, the final phase involves conducting several runs on LC to evaluate the separation performance at different temperatures.

3.2 Theory

In this section, two important aspects in this study will be further discussed. Firstly, the concept of the melting temperature of oligonucleotides will be explained, following by its implications, calculation methods, and the various factors that can influence it. In the second part, a brief overview of the necessity for using ion pair chromatography in the separation of siRNAs will be provided.

3.2.1 Melting Point of Oligonucleotides

The temperature referred to in this study as the melting temperature is not the same as the classic definition of the phase transition point from solid to liquid phase. In this context, the term "melting" denotes the dissociation of double-stranded oligonucleotides into their single-stranded forms. Therefore, in our study melting is synonymous with the dissociation or denaturation of ONs.

More accurately, the melting temperature of ONs is the temperature at which a 50/50 proportion of intact and denatured ONs is achieved. In other words, it is the temperature at which 50% of the total duplexes are denatured into their single-stranded forms in the solution. Thus, this temperature indicates the mid-transition point of this denaturation process [50].

The method by which this temperature is monitored and measured is based on the absorbance of the solution. According to the Beer-Lambert law [2], there is a linear relationship between the concentration and the absorbance of the species. Additionally, during this transition, as single strands are generated from previously intact double strands, the total concentration of the ONs in the solution increases. As a result, a technique is required that can simultaneously measure the absorbance of the solution while heating it up to the denaturation temperature [50], [51]. Figure 20 provides a clear illustration of this transition mechanism.

Moreover, by recording the melting profile of ONs, thermodynamic and kinetic values can be calculated. For instance, it is possible to extract $\Delta G°$ at 37°C in this experiment and obtain the association constant. Mathematically, this term can be expressed as the temperature at which the maximum of the derivative of absorbance over temperature is reached ($max(\frac{d(Abs)}{dT})$) [50].

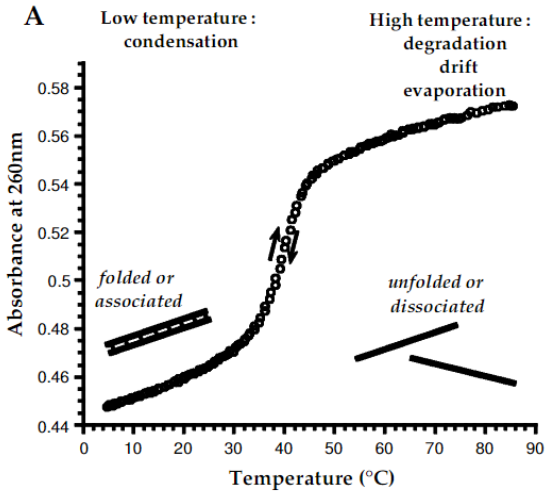


Figure 20. The melting profile of typical oligonucleotides and the various stages of their denaturation [50]. Adapted with permission, ©Mary Ann Liebert, Inc.

The purpose of using this experiment is to assess the stability of Inclisiran as the target siRNA. Implementing such a study requires the establishment of a suitable method, considering several factors to ensure the most reliable measurement. Since this experiment is temperature-dependent, it is important to investigate if the set temperature ramp is adequate to achieve thermal homogeneity in the samples. Additionally, the potential impact of evaporation on the results should be taken into account. As the recorded melting temperature is influenced by the oligo concentration, the evaporation can superficially show a higher absorbance value, creating an error in the data. Another practical consideration is the impact of oil, which is recommended by the instrument manual [52], on melting curves. All these factors can potentially impact the results and therefore must be carefully addressed in this method development.

3.2.2 Ion-Pair Reversed-Phase Chromatography (IPRP-LC)

Ion-pair chromatography represents a common method, facilitating the separation of hydrophilic analytes. In this method the same stationary and mobile phases as in RPLC are used, with the key distinction being the incorporation of an ion pair reagent into the mobile phase [53].

The necessity for IPRP-LC in this separation arises from the structure of the ONs. When analysing siRNAs on reverse phase liquid chromatography, the electron-rich phosphate backbone of siRNA leads to minimal retention of these analytes, causing them to elute rapidly from the column phase [48]. By adjusting the mobile phase concentration of the ion pair reagent, the retention factor for such analytes can be increased by a factor of 10 to 20 compared to when the absence of ion pair reagent [53].

A typical choice of ion pairs is n-alkylamines, which provide strong ion pairing capabilities while preserving the siRNAs intact. In chromatography, the retention time of the duplex peak may vary depending on the hydrophobicity of these ion pairs. TEA is regarded as a weak ion pairing reagent, resulting in rather rapid elution of the duplexes. As previously stated, this study will primarily investigate the TEA/HFIP system, which appears to offer adequate chromatographic performance [48].

3.3 Methods

3.3.1 Procedure

The primary focus of this study involves establishing a robust method for measuring the melting points of siRNAs. Three key factors are selected for in-depth investigation: the number of thermal cycles, the impact of silicone oil on absorbance curves, and varying temperature ramps in this experiment. Based on the results obtained from these three aspects, an optimal experimental setup will be devised. Subsequently, this method will be utilized to measure samples with varying concentrations of TEA/HFIP, enabling exploration of their effects on the stability of Inclisiran. Finally, our findings will be supplemented by conducting LC runs at three different temperatures.

3.3.2 Chemicals

For this study, water (18 MΩ) was obtained from a Milli-Q purification system. LC-MS grade methanol was purchased from Honeywell. Silicone oil was supplied from Alfa Aesar (ThermoFisher). Other chemicals, including HFIP (≥99%) and TEA (≥99.5%, GC), were sourced from Sigma-Aldrich.

3.2.3 Instruments

In this study, Cary 3500 UV-Vis Spectrophotometer, manufactured by Agilent Technologies, was selected for measuring the thermal stability of oligonucleotides. This instrument allows for the recording of sample absorbance while precisely controlling temperature, enabling the acquisition of absorbance data as a function of temperature. This instrument features four heating blocks, each adjustable to different temperatures. Additionally, each heating block accommodates two cuvettes, which allows for handling of up to eight samples per run. However, it is important to note that one of these spots is designated for the reference sample and for absorbance calibration. Consequently, in practice, seven spots are available for sample placement.

For these experiments, Micro Cell Rect quartz cuvettes manufactured by Agilent Technologies were selected due to their compatibility with the instrument. These cuvettes have a nominal volume of 100 µL and a path length of 1 mm. Fig. 21 displays images of the Cary 3500 instrument along with the cuvettes utilized in the experiments.

Figure 21. Cary 3500 UV-Vis Spectrophotometer and Micro Cell Rect quartz cuvettes, both manufactured by Agilent Technologies [52]. Adapted with permission, ©Agilent Technologies Inc.

The instrument calculates the melting temperature (Tm) by determining the derivative of the absorbance value over temperature. For example, if the data interval in the program was set to 0.2°C, it implies that the absorbance is measured for every 0.2°C change in temperature. Therefore, the dT value equals 0.2, representing the smallest ΔT measured in the experiment.

Subsequently, the difference between the absorbance values of these two points is divided by 0.2, yielding the derivative of the curve at that specific point.

In the Tm measurement experiment, a standard concentration of 15.3 µM of Inclisiran was selected as the reference. As previously mentioned, since the absorbance of the oligos depends on their concentration in the sample, all samples were prepared to contain the same concentration of siRNAs.

Following the recommendation in the instrument manual to prevent solution evaporation, it is advised to add mineral oil on top of the solutions in cuvettes. In this study, we aim to evaluate this suggestion and determine the efficacy of silicone oil. As such, a volume of 90 µL was allocated for all the solutions, allowing space for the addition of 10 µL of silicone oil.

For the chromatography experiments, an Agilent 1260 Infinity II system was utilized. The chosen column was an AdvanceBio Oligonucleotides C18 (120 Å, 2.7 µm, 2.1 x 50 mm). Furthermore, for the preparation of the mobile phases, all components were measured volumetrically and then stirred for 30 minutes before use. The detector wavelength was set to 260 nm, with a flow rate of 3 mL/min and an injection volume of 2 µL.

3.4 Results and Discussion

As outlined in the method section, initially, the establishment of an optimized method for measuring Tm will be explored. This will entail considering various factors, including determining the appropriate number of thermal cycles, assessing the influence of silicone oil, and selecting a suitable temperature ramp to ensure accurate measurement. Through this investigation, an optimized approach for measuring Tm will be identified. Afterwards, a series of experiments using the TEA/HFIP system will be conducted. Finally, these findings will be complemented by performing three LC runs to evaluate the separation performance.

3.4.1 Evaluating Experimental Parameters in Melting Point Measurement

Number of thermal cycles

Within the Cary 3500 instrument, the capability to create many cycles of heating and cooling allows for the collection of extensive data on the melting curves. However, with the addition of more thermal cycles in the experiment, there is an increased risk of evaporation. To assess the reliability of Tm measurement with different numbers of cycles, a sample of Inclisiran was utilized without the addition of any ion pairing reagent, and in the presence of only 1x PBS, in which the siRNA had been annealed and prepared previously.

As depicted in Fig. 22, the orange and red lines belong to the first cycle. Upon comparing these results with other absorbance curves, it becomes evident that the absorbance increases with subsequent heating and cooling cycles. This difference is approximately 0.2 absorbance units in the second cycle and around 0.3 in the third cycle. Such pronounced changes in absorbance indicate an increase in siRNA concentration, due to the evaporation of the diluents.

Another aspect to consider is that in the second and third cycles, the peak of the curve (maximum absorbance) gradually shifts to the left. While the peak of the curve is barely observable in the first cycle, a distinct pattern can be observed in the third cycle. As explained in the theory section and according to the method of Tm calculation, this shift introduces inaccuracies in the obtained data and the reported Tm value. As shown in Figure 22, only in cycle 1 are the recorded Tm values closely clustered and with higher cycle numbers, this discrepancy increases.

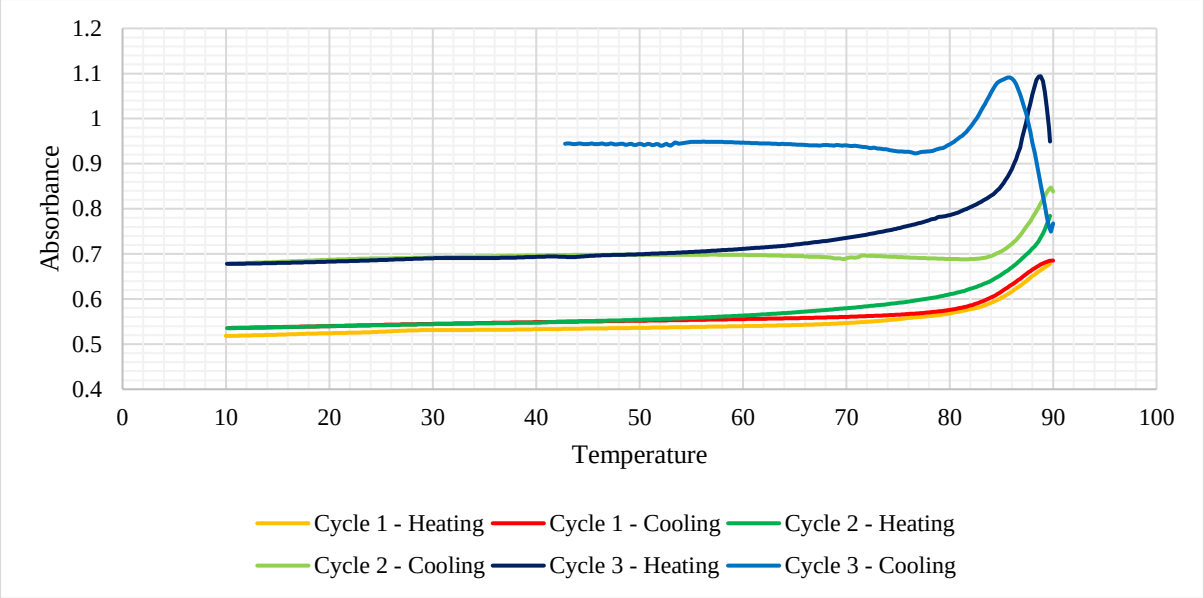


Figure 22. Melting curves for Inclisiran in 1x PBS solutions, with temperature ramp of 0.3°C/min. It should be noted that the cooling of the third cycle in this experiment was aborted before completion. However, even with the exclusion of the absorbance data from this cycle, spanning from 40 to 10°C, all the conclusions drawn still hold true.

Based on this data, it became evident that only the absorbance curves in the first cycle could be considered reliable, while substantial evaporation occurred in the second and third cycles. Although the intention was to gather more data on the Tm, these findings indicate that data from other cycles are inaccurate due to the effects of evaporation and changes in absorbance values.

Effect of silicone oil

The second factor to consider in this study is the effect of silicone oil on evaporation. To test this effect and evaluate how effective the presence of this oil is on the absorbance curve, a solution of 95/5 water/methanol with 50 mM HFIP as the diluent was selected. In one set, 3 thermal cycles were conducted on a sample without adding oil (Fig. 23), while in the next experiment, 10µL of the oil was added on top of the solutions inside the cuvettes (see Fig. 24).

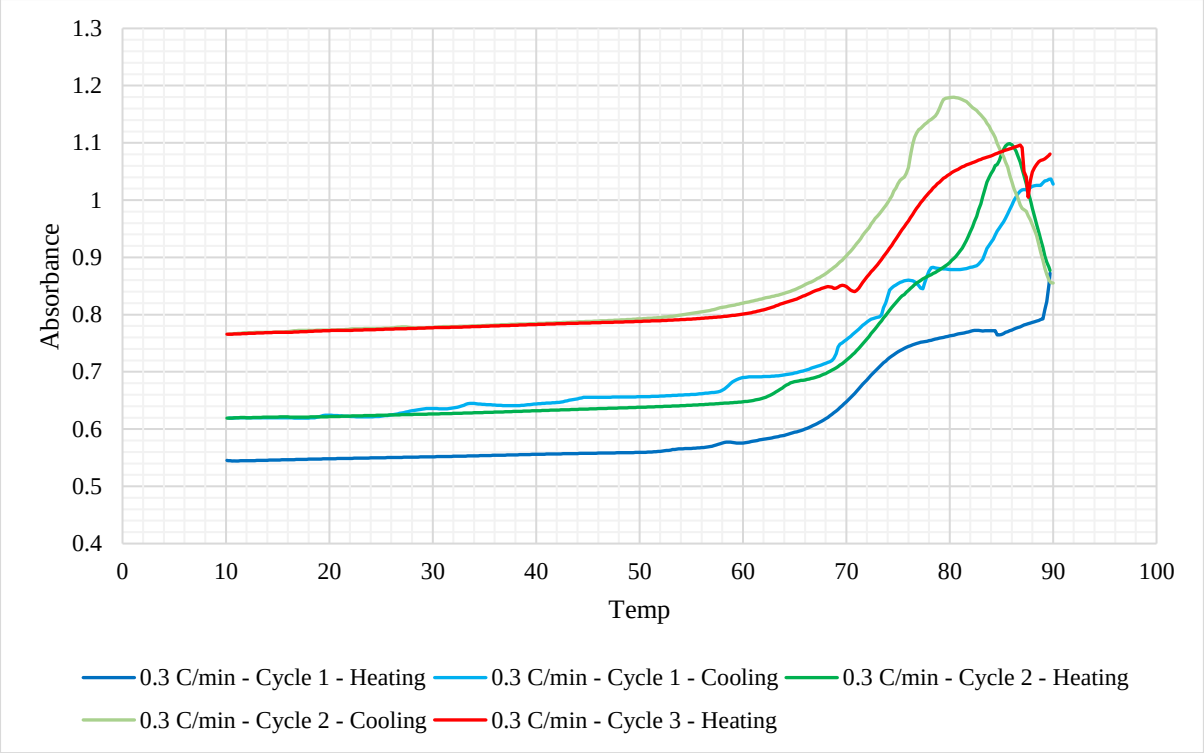


Figure 23. The absorbance profile of Inclisiran in 95/5 water/methanol and 50 mM HFIP, without addition of any silicone oil on top of the cuvettes.

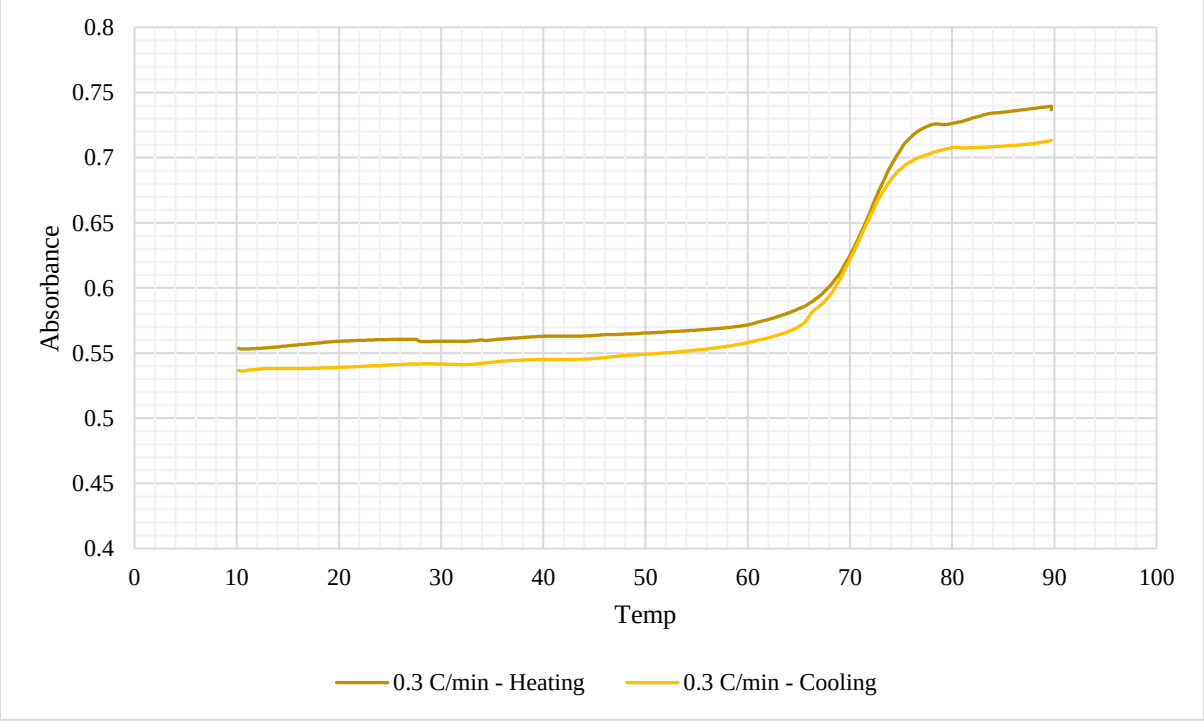


Figure 24. The absorbance profile of Inclisiran in 95/5 water/methanol and 50 mM HFIP, with addition 10 µL silicone oil on top of the cuvettes.

As shown in these two graphs, the effect of the oil on preventing evaporation is evident. In the absence of the oil, even in the first cycle, the heating and cooling cycle's absorbance curves shift by around 0.1 absorbance unit. However, this difference in the presence of the oil is negligible. The more significant factor here is the shape of the curves. In the second experiment with oil, the curves are smooth and without any unexpected fluctuations. These fluctuations are notably amplified in the absence of oil, especially at higher temperatures. In conclusion, the clear superiority of the data with oil indicates that its inclusion in the experiment is necessary.

Different temperature ramps

The third studied factor was the temperature ramp during the Tm measurement. When setting this rate, there are two critical factors to be considered. The first factor is related to the need for thermal equilibration in the solution. Here, the aim is to apply the smallest possible changes to ensure that the entire solution is thermally homogeneous. This, of course, depends on the construction of the heating block in this instrument and the heat resistance of the cuvettes being used. However, typically, a slower rate of temperature change provides more time for equilibration and is therefore desired. On the other hand, there is an opposing requirement regarding the evaporation of the solutions. This necessitates running the experiments as quickly as possible and avoiding prolonged exposure of the solutions to high-temperature ranges.

Therefore, a trade-off exists between these two factors, in which the identification of an optimal point where both requirements can be satisfied is necessary. According to the manual from Agilent Technologies, the recommended temperature ramp using these quartz cuvettes is up to 0.5°C/min. In this experimental section, three different rates of 0.1, 0.3, and 0.5°C/min were chosen to assess their performance. These rates were explored using three samples: 1) 5/95 methanol/water (Fig. 25), 2) 5/95 methanol/water with 50 mM HFIP (Fig. 26), and 3) a blank sample of 5/95 methanol/water + 50 mM HFIP, without any Inclisiran (Fig. 27). Graphs showing these experiments are presented below.

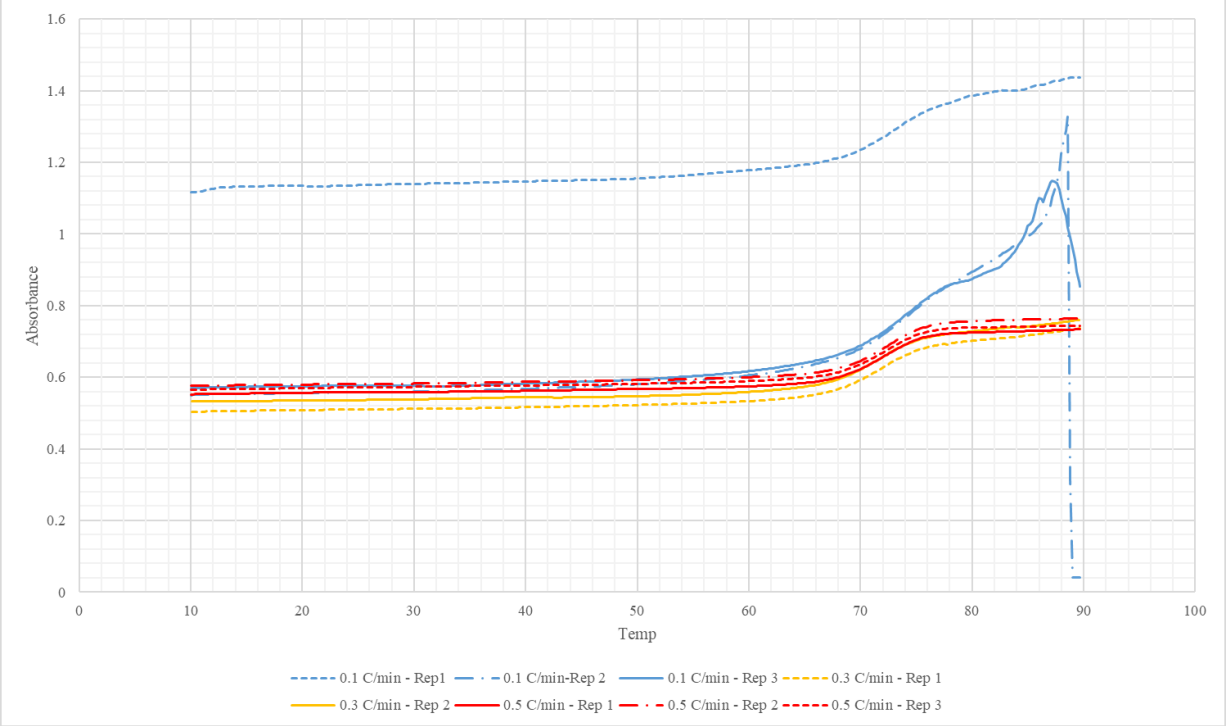


Figure 25. Absorbance profile of Inclisiran in 5/95 methanol/water in three consecutive thermal cycles.

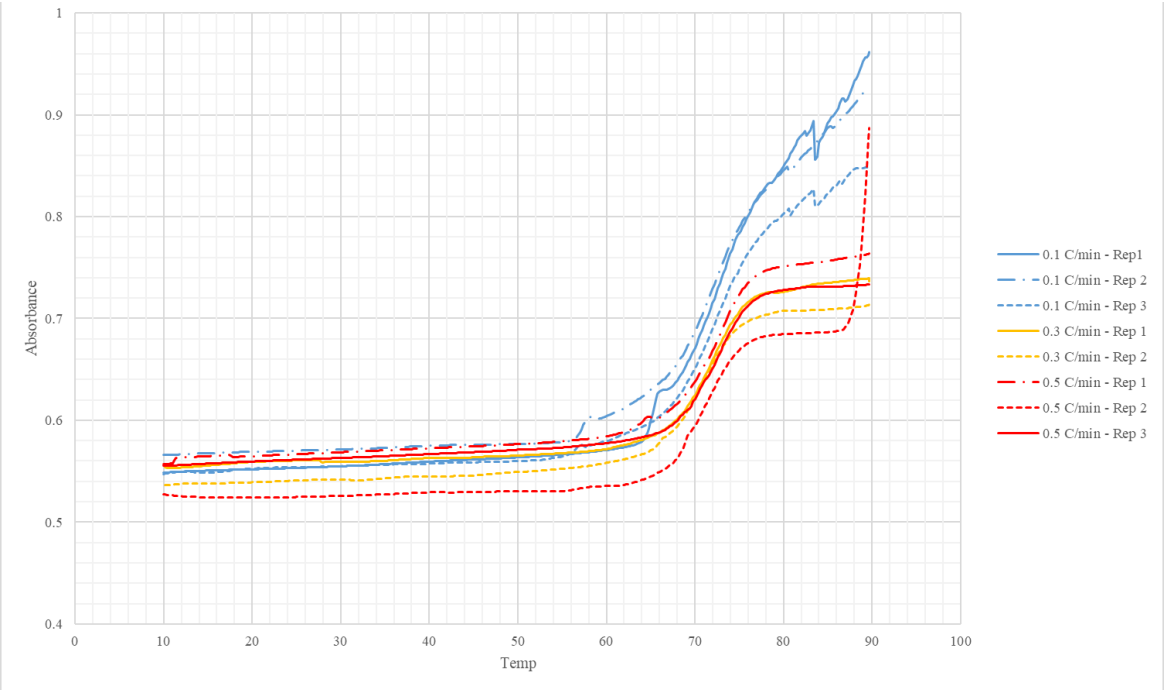


Figure 26. Absorbance profile of Inclisiran in 5/95 methanol/water + 50 mM HFIP in three consecutive thermal cycles.

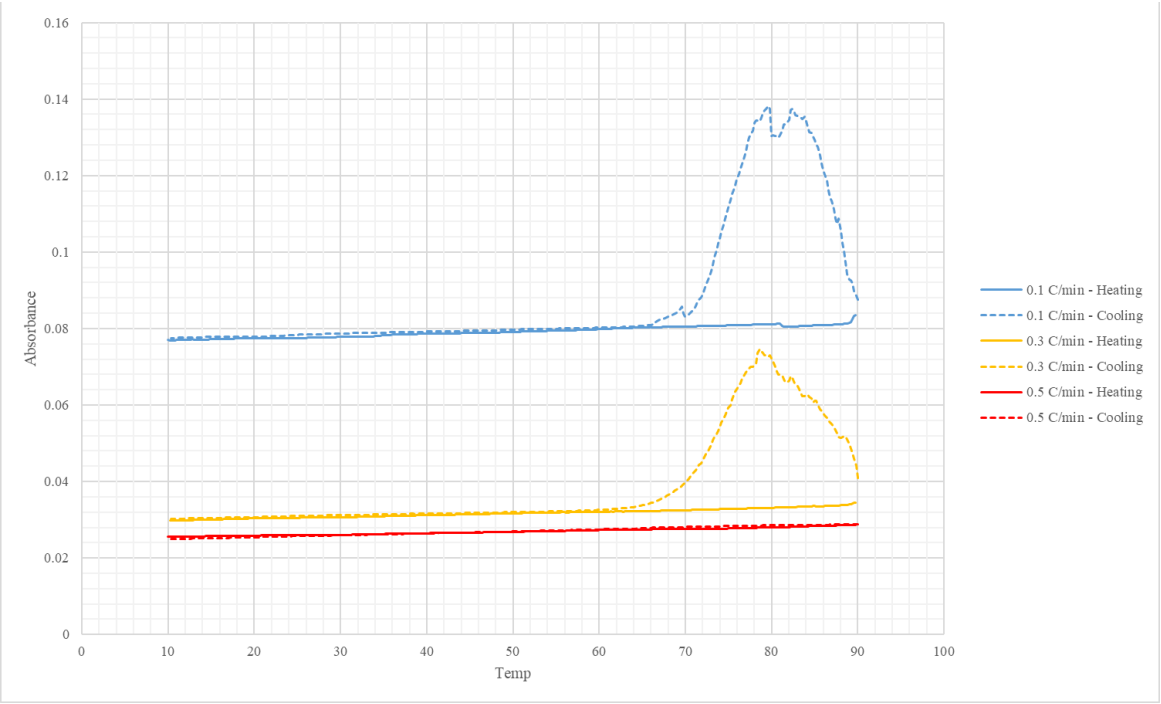


Figure 27. Absorbance profile of blank samples containing 5/95 methanol/water + 50 mM HFIP, and without Inclisiran.

As evident in these three experiments, signs of evaporation, particularly in temperature ranges above 70°C, can be clearly seen with the 0.1°C/min rate. Additionally, a substantial amount of solution was observed to have evaporated after this experiment, as depicted in Fig. 28.

Figure 28. Sever evaporation of solutions inside cuvettes after the experiment with 0.1°C/min rate.

However, both the 0.3 and 0.5 rates demonstrate acceptable results, with nearly identical absorbance patterns observed in Figures 25 and 26. It is noteworthy that when examining the absorbance curves for these three rates in the blank samples, although the changes are minimal, both the 0.3 and 0.1 rates exhibit mismatched absorbance curves in their cooling and heating cycles.

Additionally, the time spent by our samples within the high temperature range was calculated. It was assumed that the majority of evaporation occurs within the temperature range of 80 to 90°C. Based on this assumption, the duration for the cuvettes to remain in that range was examined. With a temperature ramp set to 0.1°C/min, the solution would be subjected to that high temperature range for 3.36 hours. For the 0.3 and 0.5 rates, these durations were reduced

to 1.14 and 0.7 hours, respectively. Consequently, it is highly likely that at lower rates, cuvettes containing 90 µL of the aqueous solution would be prone to evaporation.

Overall, it can be concluded that there is no considerable difference in the melting curves between the two rates of 0.3 and 0.5°C/min, as both demonstrate that thermal equilibration is achieved in the experiments. Therefore, the prevention of evaporation becomes more crucial, favouring a higher rate. Consequently, for both preventing the evaporation of the solution and conducting experiments at a faster rate, the 0.5 rate is preferred.

Optimized Method for Tm measurement

In summary, based on these findings, the optimized method for measuring the melting temperature (Tm) can be determined as outlined below.

- Number of thermal cycles: 1
- Number of replicates: 3
- Presence of silicone oil: Necessary, 10 µL is sufficient
- Temperature ramp: 0.5°C/min

3.4.2 Testing the Stability of Inclisiran in TEA/HFIP System

In this section, a series of experiments were conducted to assess the stability of Inclisiran in the TEA/HFIP system. This involved varying the concentration of either TEA or HFIP and observing their respective effects on the recorded Tm. To achieve this objective, an experimental setup was devised as outlined below in Table 11.

Table 11. Experiments with different concentrations of TEA and HFIP to determine their effects on siRNA stability.

Experiment	Ion pairing reagent	Diluent
1	5 mM TEA + 50 mM HFIP	5% MeOH/95% water
2	15mM TEA + 100mM HFIP	5% MeOH/95% water
3	100mM TEA + 100mM HFIP	5% MeOH/95% water
4	50 mM HFIP	5% MeOH/95% water
5	-	5% MeOH/95% water
6	-	100% water

These experiments were subsequently executed following the optimized method outlined in the previous section. The results obtained from these experiments are presented in Table 12, which includes the averages and relative standard deviations of the data.

Table 12. Results of experiments 1 to 6 on Tm measurement with the average and relative standard deviation (RSD) values.

Experiment	Measured Tm (°C)	Average Tm (°C)	%RSD
1 (5 mM TEA + 50 mM HFIP)	70.6 70.0 70.4 70.0 70.8 70.0	70.3	0.501
2 (15mM TEA + 100mM HFIP)	70.0 68.8 70.2 68.8 72.4 69.6	70.0	1.900
3 (100mM TEA + 100mM HFIP)	56.2 62.2 56.4 60.6 56.4 60.6	58.7	4.587
4 (50 mM HFIP)	72.8 73 68.8 70.8 72.8 71.0	71.5	2.310
5 (Only methanol)	72.2 70.8 72.4 70.8 72.4 71.2	72.1	1.094
6 (Only water)	72.6 72 72.4 71.4 72.6 71.4	71.6	0.779

From the results obtained, several important conclusions can be drawn. Firstly, it appears that the concentration of TEA has no significant impact on the stability of siRNAs at concentrations of 5 and 15 mM. Only when this concentration is increased to 100 mM is an adverse effect observed on the stability of Inclisiran, resulting in an 11.6°C reduction in the Tm compared to the 5 mM concentration.

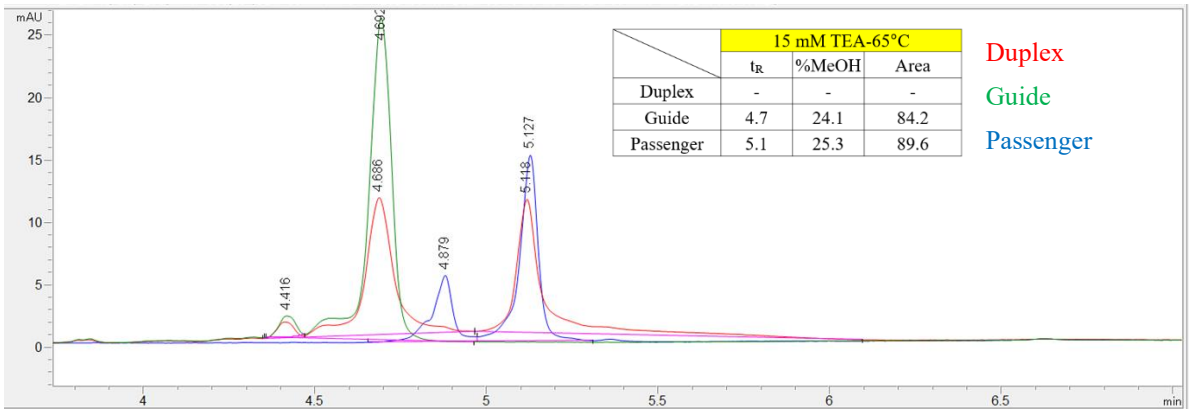
Furthermore, upon comparing Exp. 4 and 5, where the only difference is the addition of 50 mM HFIP, it can be concluded that HFIP does not significantly affect the Tm. This observation also holds true for 100 mM of HFIP, as evidenced by the minimal change in the Tm between Exp. 1 and 2 (only 0.3°C).

Overall, the only factor that significantly altered the Tm of the Inclisiran in this setup is the 100 mM concentration of TEA. The other experiments show Tm values within a very close range of temperature.

One potential approach for future investigation could involve testing the Tm at higher concentrations of the organic phase (methanol or acetonitrile). Donegan et al. [55] demonstrated that for an oligo with a length of 20mer, with 5 mM TEA/50 mM HFIP, 6.43% acetonitrile, and by 10 mM TEA/50 mM HFIP, 7.76% acetonitrile is required for elution at 60°C oven temperature. Therefore, a possible extension of this study could be to align the concentration of the organic phase with those values. However, a critical consideration in this regard is the risk of evaporation associated with increasing the organic solvent concentration.

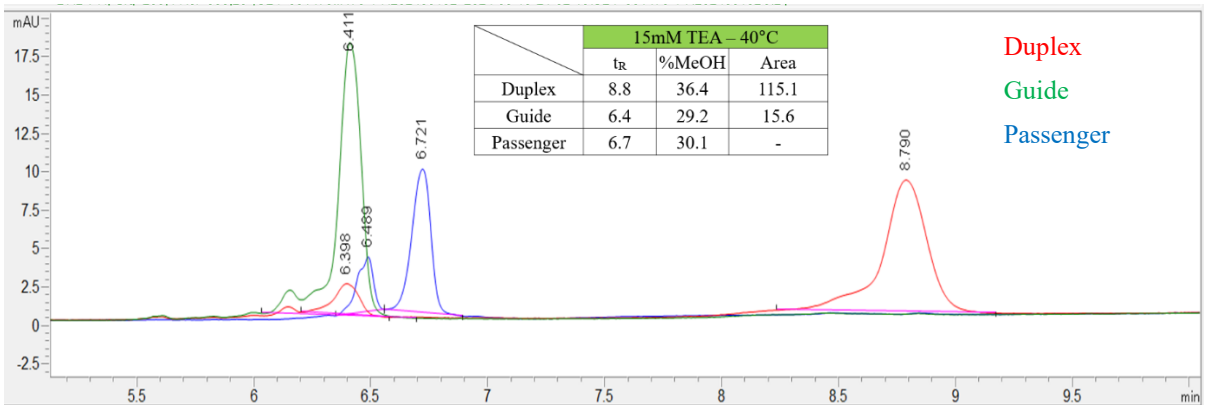
3.4.3 Three Test Runs on LC with Different Temperatures

In the final phase of this study, the separation of Inclisiran on IPRP-LC with the TEA/HFIP system was investigated. In these experiments, mobile phase A (aqueous) was prepared to contain 15 mM TEA and 100 mM HFIP in pure water, while mobile phase B (organic) consisted of pure methanol. The separation was studied at three different temperatures: 65°C, 40°C, and 25°C. Additionally, a gradient elution of 10-70% of mobile phase B over 20 minutes was applied. As detailed in the method section, the chromatography system comprised an Agilent 1260 Infinity II along with an AdvanceBio Oligonucleotides C18 (120 Å, 2.7 µm, 2.1 x 50 mm) column. The resulting chromatograms for 65°C (Fig. 29), 40°C (Fig. 30), and 25°C (Fig. 31) are presented below. It should be noted that here *guide* refers to the antisense strand, while *passenger* denotes the sense strand.



	15 mM TEA-65°C		
	t_R	%MeOH	Area
Duplex	-	-	-
Guide	4.7	24.1	84.2
Passenger	5.1	25.3	89.6

Figure 29. Chromatogram for separation of single and double strands of Inclisiran at 65°C.



	15mM TEA – 40°C		
	t_R	%MeOH	Area
Duplex	8.8	36.4	115.1
Guide	6.4	29.2	15.6
Passenger	6.7	30.1	-

Figure 30. Chromatogram for separation of single and double strands of Inclisiran at 40°C.

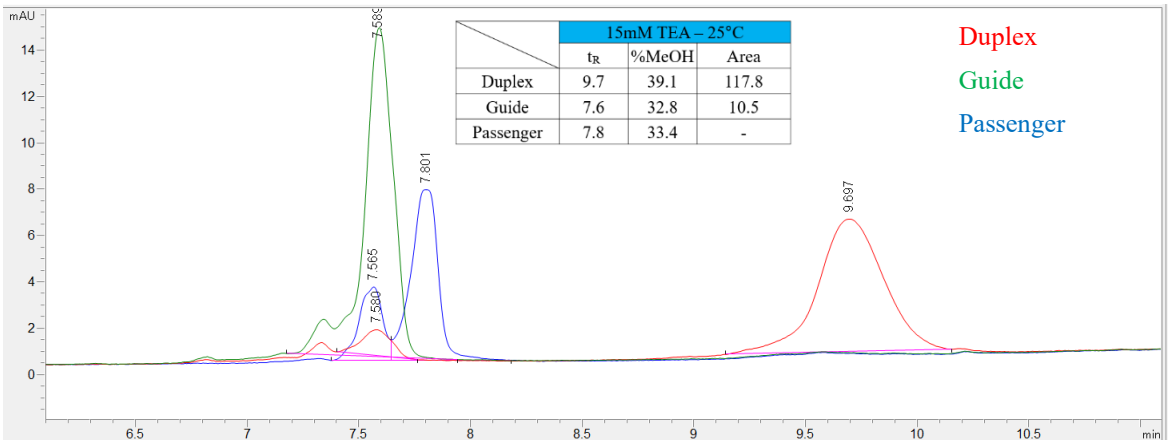


Figure 31. Chromatogram for separation of single and double strands of Inclisiran at 25°C.

Based on these results, complete denaturation is observed only at 65°C, while at the other two temperatures, Inclisiran remains intact. Comparing these findings with the Tm experiments reveals two important factors. Firstly, although the Tm of this system in Exp. 2 of the previous section was recorded as 70.0°C, as seen in Fig. 29, denaturation occurs at 65°C during LC. This difference may be attributed to other factors involved in LC that can destabilize the duplex. The second factor pertains to the methanol required for eluting the oligos. As shown in Fig. 29, at least 25% methanol is necessary to elute samples from the column at 65°C. This underscores the importance of conducting experiments with higher percentages of organic solvents in the Tm experiments

As can be seen in Fig. 30 and 31, at 40°C and 25°C, the duplex remains intact. However, based on the area of the peak observed in the 40°C experiment, we suspect a minor percentage of the duplex may have undergone denaturation, as indicated by the ratios of the peak areas.

3.5 Conclusion

The focus of this study was to develop a robust experimental procedure for investigating the stability of Inclisiran, a commercial siRNA utilized for reducing low-density lipoprotein cholesterol (LDL-C).

Based on the detailed exploration of experimental parameters in measuring the melting point (Tm), several key conclusions were drawn. Firstly, by considering the number of thermal cycles in this experiment, it became evident that only data from the first cycle could be reliable due to evaporation effects observed in subsequent cycles. Secondly, the addition of silicone oil proved to be crucial in preventing evaporation and ensuring the stability of the samples throughout the experiment. The addition of 10 µL of silicone oil effectively minimized fluctuations in absorbance curves, especially at higher temperatures. Thirdly, it was observed that the choice of temperature ramp significantly impacts the reliability of Tm measurements. While slower temperature ramps allow for better thermal equilibration, faster ramps are essential for minimizing evaporation effects. Through experimentation, it was determined that a temperature ramp of 0.5°C/min strikes a balance between these requirements, providing both thermal equilibration and minimal evaporation.

Additionally, according to the experiments conducted to assess the stability of Inclisiran in the TEA/HFIP system, it is clear that the concentration of TEA does not significantly impact the stability of siRNAs at concentrations of 5 and 15 mM. However, an adverse effect is observed at a concentration of 100 mM TEA, resulting in an 11.6°C reduction in Tm compared to the 5 mM concentration. Furthermore, the addition of HFIP, even at concentrations of 50 mM and 100 mM, does not notably affect the Tm of Inclisiran.

Finally, the LC separation experiments provided valuable insights into the behaviour of Inclisiran under chromatographic conditions. While a complete denaturation of Inclisiran was observed at 65°C, at lower temperatures of 40°C and 25°C, Inclisiran remained intact, with minor indications of denaturation at 40°C.

Future studies could explore the effects of higher concentrations of organic solvents on Inclisiran stability and optimize chromatographic conditions to enhance separation efficiency. Building upon these findings, we anticipate future advancements in the separation capability of oligonucleotides, ultimately contributing to the more effective development of these therapeutics.

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