Stability Indicating Ion-Pair Reversed-Phase Liquid Chromatography Method for Modified mRNA

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ABSTRACT: Modified messenger RNA (mRNA) represents a rapidly emerging class of therapeutic drug product. Development of robust stability indicating methods for control of product quality are therefore critical to support successful pharmaceutical development. This paper presents an ion-pair reversed-phase liquid chromatography (IP-RPLC) method to characterise modified mRNA exposed to a wide set of stress-inducing conditions, relevant for pharmaceutical development of an mRNA drug product. The optimised method could be used for separation and analysis of large RNA, sized up to 1000 nucleotides. Column temperature, mobile phase flow rate and ion-pair selection were each studied and optimised. Baseline separations of the model RNA ladder sample were achieved using all examined ion-pairing agents. We established that the optimised method, using 100 mM Triethylamine, enabled the highest resolution separation for the largest fragments in the RNA ladder (750/1000 nucleotides), in addition to the highest overall resolution for the selected modified mRNA compound (eGFP mRNA, 996 nucleotides). The stability indicating power of the method was demonstrated by analysing the modified eGFP mRNA, upon direct exposure to heat, hydrolytic conditions and treatment with ribonucleases. Our results showed that the formed degradation products, which appeared as shorter RNA fragments in front of the main peak, could be well monitored, using the optimised method, and the relative stability of the mRNA under the various stressed conditions could be assessed.

Keywords: Modified mRNA, Ion-Pair Reversed-Phase Liquid Chromatography, Stability indicating, RNA ladder, Ribonucleic acid, Nucleotides, Chemical degradation, Degradation study

1 Introduction

Nucleic acid-based therapeutics constitutes a class of medicines that has progressed rapidly during the last years. Innovations improving intracellular stability and translational efficiency of modified nucleic acids into therapeutic proteins along with an improved safety profile have led to the development of several novel drug products that are currently on the market and many more are currently being tested in clinical studies worldwide [1-9]. AstraZeneca has recently reported in-human studies of this modality type – with the AZD8601 compound [10, 11]. In addition, messenger RNA (mRNA) has shown excellent potential for the development of vaccine therapies. This has been well exemplified in the rapid progression of the commercially available mRNA-based vaccine therapeutics in the response to the SARS-CoV-2 pandemic [12-15]. The increased focus towards this new type of drug product has highlighted the need for effective and reliable characterisation methods, which can be used during development of mRNA drug products [16].

Stability studies are critical components of pharmaceutical development processes and require a range of robust and reliable analytical methods. During manufacture and storage of both drug substance and drug product, mRNA can rapidly degrade through exposure to heat, light, hydrolytic and oxidative conditions, as well as through contamination with ribonucleases [12, 17]. For therapeutic use, mRNA is typically modified to be more resistant to *in vivo* degradation compared to unmodified mRNA. While processes for *in vivo* degradation of mRNA have been widely studied, in contrast the chemical degradation pathways relevant for pharmaceutical quality testing are sparsely described, and the number of reported stability indicating methods is low. To validate the stability indicating power of an analytical method, the stress test conditions for the stability testing samples containing the

active pharmaceutical ingredient should be more severe than those used for long term stability studies. The European Medicines Agency, (CPMP/ICH/2736/99) states that "*Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved."* [18]. It should be noted that due to the novelty of this emerging area, the regulatory requirements for quality control of mRNA drug products are not yet fully established [19]. For mRNA vaccine drug products, the regulatory guidelines for vaccines apply, while non-vaccine mRNA-based drug products falling within the definition of a gene therapy that are regulated as an advanced medicinal product within the EU [20, 21].

The utility of Ion-Pair Reverse-Phase Liquid Chromatography (IP-RPLC) for separation of large RNA compounds, up to 8000 nucleotides, has previously been demonstrated [22-28]. Retention of polar mRNA analytes is facilitated by ion-pairs that are formed between the negatively charged mRNA backbone and a positively charged ion-pairing agent added to the mobile phase. The mRNA-ion-pair complex displays an increased affinity to the stationary phase compared to mRNA alone, resulting in increased retention on reverse phase columns. In addition, the ion-pairing agents from the mobile phase may cover the stationary phase to different degrees and therefore may cause variation in the analyte retention mechanisms. Ion pair agents with shorter alkyl chain lengths (e.g. TEAA) only partially cover the stationary phase and may allow direct interactions between the mRNA analyte and the stationary phase to take place. This can therefore facilitate separations based both on mRNA length and base composition. Longer ion pair agents, which fully cover the stationary phase have been found to predominantly exhibit sized-based separations [26, 29]. Dickman *et al.* suggests that IP-RPLC can offer the ability to analyse a wide range of RNA compounds through modification of chromatographic conditions, including choice of ion-pairing agents, temperature and mobile phase compositions [25]. Commonly used ion-pair combinations for analysis of large RNA include: Triethylammonium Acetate (TEAA), Hexylammonium Acetate (HAA) and Dibutyl ammonium Acetate (DBAA).

Chromatographic separation of high molecular weight RNA has a number of challenges. Slow mass transportation, dispersity of the RNA compounds, and the influence of variable secondary and tertiary RNA structures can all impact the separation performance. In addition, for analysis of large RNA, insufficient column pore sizing can impact separation, restricting mass transport through the column, as well as reducing available surface area for ion-pair interactions. This can limit the ability to optimise method parameters, such as mobile phase gradient, flow rate and composition, to achieve the desired separation [26]. Lloyd *et al.* have shown that large pore sizes (4000Å) are typically needed to resolve nucleotides greater than 500 base pairs. Stationary phases based on polystyrene divinylbenzene (PS-DVB) polymer matrices are available with such sufficiently large pore sizes, and promising separations of RNA compounds and RNA ladders have been demonstrated [30]. Azarani, Georgopoulos, and Dickman *et al.* have used columns based on alkylated PS-DVB co-polymeric stationary phases, such as DNASep columns from Transgenomic for separations of large RNAs [24, 25, 27]. Yamauchi *et al*. have presented separations of large RNA ladders up to 8000 nucleotides using a PLRP-S column, from Polymer Labs, which has a monolithic non-alkylated PS-DVB stationary phase, with a 4000 Å pore size. In their findings, it is suggested that one of the key factors in enabling high resolution separations for large RNA fragments, using IP-RPLC, is selecting an appropriate column pore size for the analyte size of interest [22]. In recent publications a wide-pore DNAPac RP from ThermoFisher Scientific has been used [31-36].

Additionally, column temperature plays an important role in improving peak shape and resolution during RNA analyses. Waghmare *et al*. have previously demonstrated that high resolution separations of ssRNA, up to 1000 nucleotides, can be achieved in IP-RPLC using high temperature column conditions [37]. Similarly, Roussis *et al*. report that analysis of oligonucleotides at elevated temperatures, using IP-RPLC, improved peak resolution [38]. Higher column temperatures are suggested to help facilitate denaturating conditions in the presence of an organic co-solvent, i.e acetonitrile (MeCN) [39]. Thus, the impact of secondary/tertiary RNA structures on the analyses can be reduced.

Azarani *et al.* have previously established that IP-RPLC methods can be utilised to detect and assess RNA transcript degradation [24]. The study reports analysis of a transcription reaction product, showing that smaller and/or degraded RNA fragments could be distinguished from the full-length transcript. The study by Kanavarioti has demonstrated the most informative approaches to characterize large RNA, using both an ion exchange LC (IEX) and an IP-RPLC method to quantify amount of RNA fragments, including modified mRNA [31]. The study demonstrated that these types of methods could potentially be exploited for quality control and purity determination purposes. Additionally, Fekete *et al*. has explored both IEX and IP-RPLC methods and combinations thereof [40, 41]. Detailed descriptions of IP-RPLC methods using mixtures of TEAA and DBAA, and TEA with 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) for mRNA purity determination by UV detection was presented by Packer and Patel *et al*. [32, 42]. Recently, the United States Pharmacopeia (USP) released a Draft Chapter on Analytical Procedures for mRNA Vaccine Quality for mRNA based vaccines [43]. We propose that an IP-RPLC method is preferable for stability indication of modified mRNA of size about 1000 nt, due to the ability to operate at a lower pH than IEX methods. Hydrolysis of mRNA is one of the main degradation pathways and high pH

conditions can induce on-column analyte stability. Additionally, the use of IP-RPLC allow use of mass spectrometry for detection/identification of shorter RNA fragments and degradation products [41, 44-46].

It should also be noted that additional types of mRNA degradation products may require alternative techniques and set-ups to be monitored [17]. The most prominent RNA degradation pathways result in the formation of shorter RNA fragments. However, degradation pathways which result in modification of the nucleotides, for example oxidation, depurination and deamidation require complementary/orthogonal methods for analysis [47-51].

To the best of our knowledge, to date, there are few reports describing development of stability indicating methods applicable for pharmaceutical development of large mRNAs using liquid chromatography. The aim of this study was to identify and evaluate key separation parameters to consider when developing stability indicating IP-RPLC UV methods for mRNA. Various aspects for developing IP-RPLC methods of large mRNA are discussed. Baseline separation of all peaks of an RNA ladder comprising of seven fragments (100, 200, 300, 400, 500, 750 and 1000 nucleotides) is demonstrated using optimized IP-RPLC method conditions. The stability indicating power of the optimized IP-RPLC method is verified by analysis of eGFP modified mRNA that has undergone stressed degradation.

2 Experimental

2.1 Materials

Acetonitrile (99.9% Purity) and Hydrochloric Acid (37%) (12 M) were purchased from Merck (Darmstadt, Germany). Triethylamine (TEA) (99.5% purity), Dibutylamine (DBA) (99.5% purity), Hexylamine (HA) (99.5% purity) and Sodium Hydroxide (98% purity) were all purchased from Sigma-Aldrich (St. Louis, USA). Acetic Acid (glacial) (99.7% purity) was purchased from Alfa Aesar (Kandel, Germany). Distilled water for mobile phase preparation was prepared through a Millipore Milli-Q Direct Ultrapure water system, while RNase-free water for sample preparation, was filtrated through a Millipore Biopak Polisher (Darmstadt, Germany). Ambion Century Plus single-stranded RNA markers (100, 200, 300, 400, 500, 750 and 1000 nucleotide fragments) were purchased from Invitrogen ThermoFisher Scientific (Vilnius, Lithuania). The RNA markers were received as 50 µL of 1.0 mg/mL RNA in 0.1 mM EDTA. Clean-Cap enhanced green fluorescent protein (eGFP) modified mRNA (996 nucleotides) and mRNA coding for Cas9 (4521 nucleotides), with a fully substituted 5-Methoxy-Uridine base composition was purchased from TriLink Biotechnologies (San Diego, USA). The model mRNA sample was delivered as 1 mL of 1.0 mg/mL mRNA in 1 mM sodium citrate buffer (pH 6.4).

2.2 Sample Preparation

Prior to analysis, mRNA and RNA ladder samples were stored in a freezer at -80°C. As required, samples were thawed at room temperature and carefully mixed by repeated pipetting. For samples used to establish suitable separation parameters, the RNA ladder samples were diluted to 0.05 mg/mL by addition of RNase-free water. For ion-pair comparison and stress testing studies, both the RNA ladder samples and the mRNA samples were diluted to 0.5 mg/mL through addition of RNase free water.

Stress testing of eGFP mRNA was carried out under hydrolytic conditions, treatment with RNase A, and prolonged exposure to high temperature conditions respectively:

High/Low pH: 100 µL sample vials were prepared containing 50 µL eGFP mRNA at 1.0 mg/mL and either 50 µL of 0.01 M HCl or 50 µL of 0.02 M NaOH, resulting in a solution of 0.5 mg/mL mRNA in corresponding high or low pH media.

RNase treatment: 5 µL of 0.1 µg/mL RNase A solution was added to a vial containing 50 µL 1.0 mg/mL eGFP mRNA and 45 µL RNase-free water. These samples were stored in the autosampler at 25°C and tested at intervals until significant degradation was observed.

Heat: eGFP mRNA (1.0 mg/mL) was heated at 85°C at time intervals up to 240 minutes and immediately placed on ice for 5 minutes. mRNA samples were then diluted to 0.5mg/mL by addition of RNase free water before use. Autosampler was kept at 10°C to minimise further degradation.

2.3 Instrumentation

All testing was carried out using an Agilent 1100 and 1200 HPLC system (Santa Clara, USA). The system consisted of a binary solvent pump with degasser unit, a G1315C UV diode array detector (DAD), a temperature-controlled auto sampling unit and temperature-controlled column housing. Agilent ChemStation Software was used for data collection, peak integration and data analysis.

A ThermoFisher Scientific DNAPac Reversed Phase column (Waltham, USA) containing 4µm polymeric particles with proprietary wide pore sizing was used (Dimensions: 2.1 x 100 mm).

2.4 Method

All chromatograms were recorded at a wavelength of 260 nm. Separations were carried out using a sample injection volume of 4 µL.

The mobile phases were prepared by dissolving the alkylamine into acetonitrile at the selected concentration before the addition of deionised water. When using TEAA, the mobile phase A did not contain any MeCN. Consequently, the TEA was added directly into the deionised water. Mobile phases were produced at selected alkylamine concentrations - 15mM, 25mM, 100mM for hexylamine, dibutylamine and triethylamine, respectively. Acetate ion-pair combinations were formed by adjusting the mobile phase pH 8.5 by titration, through addition of glacial acetic acid. Mobile phase compositions and gradients are detailed in full within the supplementary

material (Supplementary Table 1 and 2). Mobile phases not in use were stored in the refrigerator at 5 °C. Typically, 3 blank injections were performed before any sample injections in order to allow the column to reach equilibrium.

An analysis time window for elution of the RNA ladder was set at 15 minutes. A target retention time for the 100-nucleotide peak was set at 3 minutes, while for the 1000-nucleotide peak it was set to 14 minutes. For initial tests, a steep mobile phase gradient was utilised in order to evaluate an approximate range for RNA ladder sample retention. The mobile phase gradient was then adjusted to meet the analysis window criteria. Half peak height method was used to determine resolution values for the fragment sizes of interest. Details of resolution calculation method can be found in the supplementary material (Equations 1-6). Optimal mobile phase flow rate was 0.2 mL/min, while column temperature was 80 °C.

Analysis was then performed on the eGFP mRNA sample using the optimal gradient to determine whether any further adjustment was required. For the final optimized method for eGFP mRNA, utilising the DNAPac column (0.2 mL/min flow rate at 80°C), mobile Phase A consisted of 100 mM TEA in H2O (pH 8.5) and mobile Phase B consisted of 100 mM TEA in 40% MeCN (pH 8.5). Elution within the analysis time window was achieved with 8.6-13.7% MeCN in 15 min, 13.7-8.6% MeCN in 1 min and 8.6 % MeCN for 4 minutes. Total run time was 20 minutes.

3 Results and Discussion

3.1 Model Compounds

In this study, we evaluate the performance of three alkylamines, commonly used for separation of large RNA, in combination with acetate, as potential ion-pairing agents in a stability indicating method for modified mRNA. During initial method development, analyses of an RNA ladder, with fragment sizes ranging from 100-1000 nucleotides, were performed to evaluate key separation parameters. The two largest fragments in the RNA ladder were 750 and 1000 nucleotides. We aimed to maximise the resolution between these fragments to improve method performance for eGFP modified mRNA (996 nucleotides). The eGFP mRNA compound was selected as a model compound in this study as it is widely used for development of various RNA drug delivery systems, owing to the fluorescent properties of its expressed protein. Explorative tests were carried out using Cas9 mRNA with 4521 nt to represent a longer mRNA. Optimization of methods for longer mRNA in addition to other modification types would be of interest in future work.

3.2 Development of a stability indicating method

3.2.1 Method Run Time

The run time was fixed to 15 minutes to enable a fast and reliable stability indication method. Given that mRNA can be susceptible to rapidly degrade under certain conditions a short analysis method is preferred. Visualizing the effect of changing chromatographic parameters required all fragment sizes across the RNA ladder range to be separated within the 15 minutes analysis time window. In addition, the majority of the degradation products resulting from the mRNA do not have a defined size length. Therefore, optimization of the method parameters on a similarly sized RNA ladder supports the monitoring of degradation products from the mRNA during stress testing.

3.3 Column / Stationary Phase

The wide-pore DNAPac RP column was selected for our method development process. This column has previously demonstrated high resolution separations for the analysis of large RNA using reversed phase ion-pair chromatography [31-33].

3.4 Effect of Column Temperature on RNA Separation

To assess the effect of column temperature on separations of large RNA, separations of RNA marker were performed at both 50°C and 80°C with 100 mM TEAA, (Supplementary Material Figure 1). The gradient slope, and the analysis time were the same for both analyses. At increased column temperature, we observed improved resolution between the 750/1000 nucleotide fragments. At 50°C and 80°C, the chromatographic resolution of these fragments was calculated to be 1.9 and 2.2, respectively. Higher column temperature also contributed to slightly improved peak shapes across the marker size range. An additional benefit from using higher column temperature was a small reduction in overall analysis time. As high temperature column conditions did not appear to impact the stability of the analyte, we opted to fix the column temperature at 80°C throughout the method development process.

3.5 Effect of Mobile Phase Flow Rate on RNA Separation

The effect of mobile phase flow rate on the separation was examined by performing RNA marker analyses at 80°C, using 100 mM TEAA, under different column flow rates with a fixed gradient (Supplementary Material Figure 2). The highest calculated resolution value, between the 750/1000 nucleotide fragments, was found at a mobile phase flow rate of 0.6 mL/min (Supplementary Material Table 3). However, it was observed that mobile phase flow rate at 0.2 mL/min resulted in the highest column efficiency (N) for the 750 and 1000 nucleotide fragments peaks (Supplementary Material Table 4).

At mobile phase flow rates above 0.2 ml/min, retention time of the 100 nucleotide fragment peak was shown to be less reproducible, and the separation between the system peak and the 100-nucleotide fragment peak decreased. This would limit quantification of RNA fragments shorter than 100 nucleotides. That is a disadvantage for the stability studies of modified mRNA compounds, where degradation products could potentially fall in this 1-100 nucleotide range.

A flow rate of 0.2 mL/min was found to be most suitable for all sample analyses. Potentially there is an opportunity

to enable more informative separation analyses of large RNA through further manipulation of the mobile phase flow rate. This would be an aim for an additional investigation and could include modulation of mobile phase flow rates across a single analysis run.

3.6 Evaluation of Ion-Pairing Agents

Several parameters related to the ion-pairing mechanism can affect the chromatographic retention of RNA, including alkylamine concentration, chemical properties and water solubility of the alkylamine, as well as the mobile phase pH. The interplay between these parameters can make method development challenging [52].

In this study, we have attempted to perform representative best-case separations for each of the selected ion-pairs, using only linear gradients. Stepwise linear gradients have previously been used to separate both small and large RNA fragments [24, 26]. However, as part of our evaluation strategy, linear gradients enabled us to limit the number of parameters to test and optimise. All alkylamine concentrations were fixed to proposed optimal values as reported in the literature (Table 1) [53-55].

Table 1: Proposed Optimal Mobile Phase Alkylamine Concentrations

Alkylamine	Concentration (mM)
Hexylamine	15
Dibutylamine	25
Triethylamine	100

All mobile phases were adjusted to pH 8.5 through addition of glacial acetic acid. This pH value was selected as it is well below that of the pKa values for the chosen alkylamines, thus ensuring complete ionisation within the mobile phases [55]. Additionally, pH 8.5 was not shown to impact the stability of the model RNA compounds during analysis.

During our method development process, we observed that both MeCN starting concentration and gradient slope had to be optimised for each IP combination to enable elution within the set analysis time for the RNA marker sample. MeCN starting concentrations for both HAA and DBAA were approximately similar, 32.3% and 34.6% respectively. However, in order to achieve the desired elution profile, DBAA required a steeper gradient (0.80% min^{-1} MeCN) than HAA (0.53% min^{-1} MeCN). The RNA marker sample was found to be less retained when using TEAA, requiring both a lower starting MeCN concentration (8.6%) and a flatter gradient (0.27% min^{-1} MeCN) to obtain separations within the 15 minute analysis time window.

Under the respective chromatographic conditions for each ion-pair, full separation of the RNA marker fragments was achieved within the 15 minute analysis window. As RNA size length increases, we observed it becomes more challenging to separate similarly sized fragments from one another (Figure 1). This has previously been noted for IP-RPLC analyses as well as using orthogonal separation techniques for RNA, such as capillary gel electrophoresis and IEX [26, 56]. The lower separation power for the larger RNA will influence the development strategy for a stability indicating method. Non-linear/step-wise gradients in combination with longer analysis times could offer opportunity to improve separation performance.

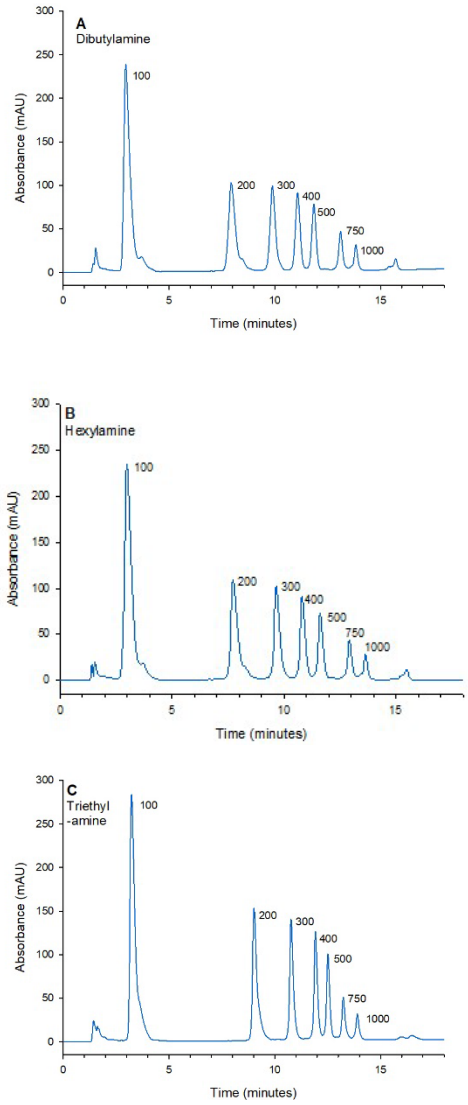

Figure 1: Comparison of different ion-pair agents for the separation of a single stranded RNA ladder 100-1000 nucleotides using the polymer based DNAPac RP column.
(A) 25 mM DBA (34.6% - 46.6% MeCN) (B) 15 mM HA (32.3% - 40.3% MeCN) (C) 100 mM TEA (8.6% - 12.6% MeCN)

Separations obtained using HAA and DBAA, resulted in similar peak patterns and retention times at the chosen alkylamine concentrations. However, the calculated resolution values showed that DBAA offered improved separation power for the larger fragment sizes (Table 2). The highest resolution was achieved using TEAA, which enabled a resolution of 2.5 between the 750 and 1000 nucleotide RNA marker fragments. In samples analysed using either HAA or DBAA, it was possible to detect related impurities alongside the 100 and 200 nucleotide fragment peaks (Figure 1 A/B). In contrast, for TEAA

these impurities seem to co-elute with the 100 and 200 RNA fragment peaks. This highlights the potential opportunity to combine ion-pairs for improved separation performance across a wider fragment size range.

Table 2: Resolution Values Between 750/1000 Nucleotide RNA Ladder Sample Fragments for Selected Alkylamines

Alkylamine	Resolution
Hexylamine	2.1
Dibutylamine	2.3
Triethylamine	2.5

The method parameters developed for the RNA ladder sample, using each of the selected alkylamines, were subsequently applied to analyse the model mRNA compound – modified eGFP mRNA (996 nucleotides) (Figure 2). When utilising DBAA and HAA, retention times for the main mRNA peak corresponded to that of the

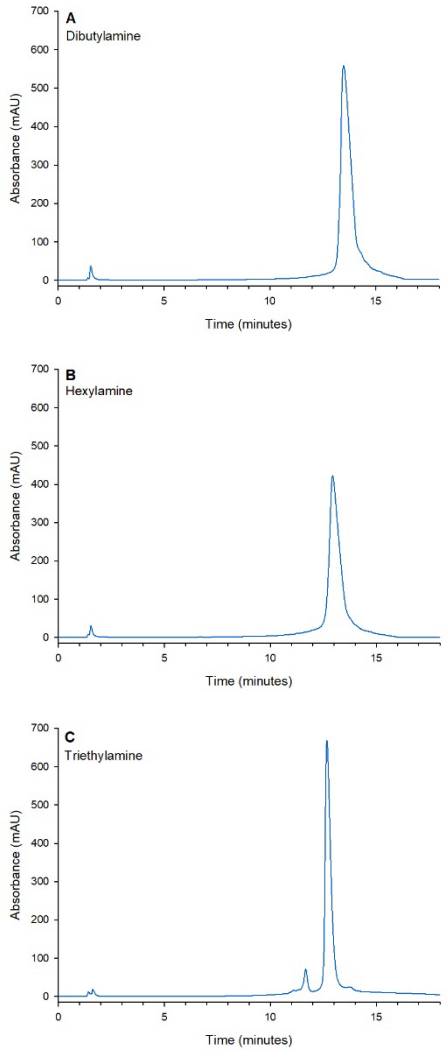

Figure 2: Comparison of different ion-pair agents for the separation of eGFP mRNA with 996 nucleotides using the polymer based DNAPac RP column. (A) 25 mM DBA (34.6% - 46.6% MeCN) (B) 15 mM HA (32.3% - 40.3% MeCN) (C) 100 mM TEA (8.6% - 13.7% MeCN).

1000 nucleotide sized fragment from the RNA marker sample. However, the peak shapes using these ion-pair agents were not optimal for the eGFP mRNA sample– displaying a broader main peak (Table 3).

Table 3: eGFP mRNA Main Peak Half Height Width Using the Selected Alkylamines (Optimised Method Parameters Within 15 Minute Analysis Time Window)

Alkylamine	eGFP mRNA Half Height Peak Width (Minutes)
Hexylamine	0.51
Dibutylamine	0.57
Triethylamine	0.27

This would likely impact quantification of any longmer/shortmer impurities similar in size to the intact mRNA, potentially limiting the usability of the method for compounds of this type.

Using TEAA, the gradient endpoint was adjusted to 13.7% MeCN (0.34% min^{-1} MeCN) to ensure that the eGFP mRNA eluted within the 15 minute analysis time window. As a consequence of this adjusted gradient, a slightly narrower mRNA main peak is observed (Supplementary Material Figure 3). Additionally, there is a separated pre-main eGFP peak. This was also reported by Kanavarioti for eGFP mRNA [31]. The lack of pre-main peak indicates that DBAA and HAA were both unable to resolve the main peak from pre-existing shortmer fragments at the applied separation parameters. The improved separation performance for the eGFP mRNA, using TEAA, is concurrent with the superior resolution that was obtained between the largest sized fragments of the RNA ladder sample, using this ion-pair.

Close *et al.* have previously reported separation of RNA fragments based on both size and base composition when using TEAA. They propose that short chain ion-pairing agents, such as TEAA, only partially cover the stationary phase and may in fact allow direct reversed phase interactions between the RNA strands and the stationary phase [26]. The interaction of the analyte with the partially covered stationary phase could therefore be impacted by the specific analyte structure. The shift in retention time between the 1000-nucleotide RNA fragment, in the ladder sample, and the similarly sized eGFP mRNA, could therefore be a product of differing structure/base composition between these two analytes. This was an important finding, showing that the method optimized for the RNA ladder sample was not directly applicable for analysis of the eGFP mRNA. We propose that separation methods, for other mRNAs, can be developed using a similar approach as described above. However, additional optimization of the method parameters may require to be suitable for the size and sequence of the analyte. For example, method development tests with a longer mRNA, Cas9 (4521 nt), showed that longer analysis time, 60 minutes instead of 15 minutes, was needed to enable a better separation of shortmers from the main peak

(Supplementary Figure 4). Further optimization of a method for Cas9 mRNA was outside scope of this study.

Analysis of eGFP mRNA, using our newly optimised method conditions using 100mM TEA in the mobile phase, was carried out in triplicate, displaying excellent repeatability (Supplementary Material Figure 5).

3.7 Evaluation of Method Stability Indicating Power

To further evaluate the stability indicating power of the IP-RPLC methods we analysed eGFP modified mRNA, after exposure to different stress conditions.

To our knowledge, there are few reports about the major degradation pathways and resulting degradation products with respect to pharmaceutical development of mRNA-based therapeutics. Stress testing conditions selected were exposure to heat, hydrolytic conditions, and addition of ribonucleases. The potential degradation products could comprise of fragments similar in size to the main component, down to single nucleotide fragments. When analysing the stressed samples we expected to observe decreased main peak area as well as an increased percentage of pre-main peak area and increased number of smaller fragment peaks.

TEAA was earlier shown to offer superior separation capabilities compared to the other ion-pairs for RNA ladder and intact mRNA. Furthermore, chromatograms of stressed eGFP mRNA samples using TEAA displayed better separation of the shorter fragments from the intact main peak (Figure 3-4), compared to DBAA and HAA (Supplementary Figure 6 and 7).
Stress inducing hydrolytic conditions, applied directly to the mRNA sample, were shown to result in rapid degradation, visible as an increase in the pre-main peak areas (Figure 3). At both high and low pH, the main peak decreased by more than 50% within 30 minutes, indicating that the mRNA was unstable under both sets of conditions (Figure 3B and C).

Decrease in main peak height however, is more pronounced in the acidic conditions, in comparison to the basic environment. Under both sets of conditions, the degradation products from hydrolysis appear in the same retention time window as the existing impurities in the untreated mRNA.

Exposure to RNase A, at the chosen concentration, led to a prolonged degradation process, – extending to over 12 hours, facilitating the analysis of samples with varying degrees of degradation. The RNase A degradation pathway appears markedly different to that of the other stress tested conditions, with the pre-main peak appearing far less prominently (Figure 3D). More notably, at similar main peak heights, the earlier eluting peak (2 minutes) is significantly larger than in either hydrolysis analyses. This suggest that the RNA digested by RNase forms smaller fragments. This is to be expected as the enzyme breaks the polynucleotide chain at specific and defined intervals, through endonuclease cleavage. The distinctive peak pattern observed is therefore likely related to the mRNA base composition and structure. Analysis of 0.005 ng/µL RNase A solution without RNA, confirmed that the earlier eluting peak corresponded only to smaller less well/unretained RNA fragments rather than any RNase A residues.

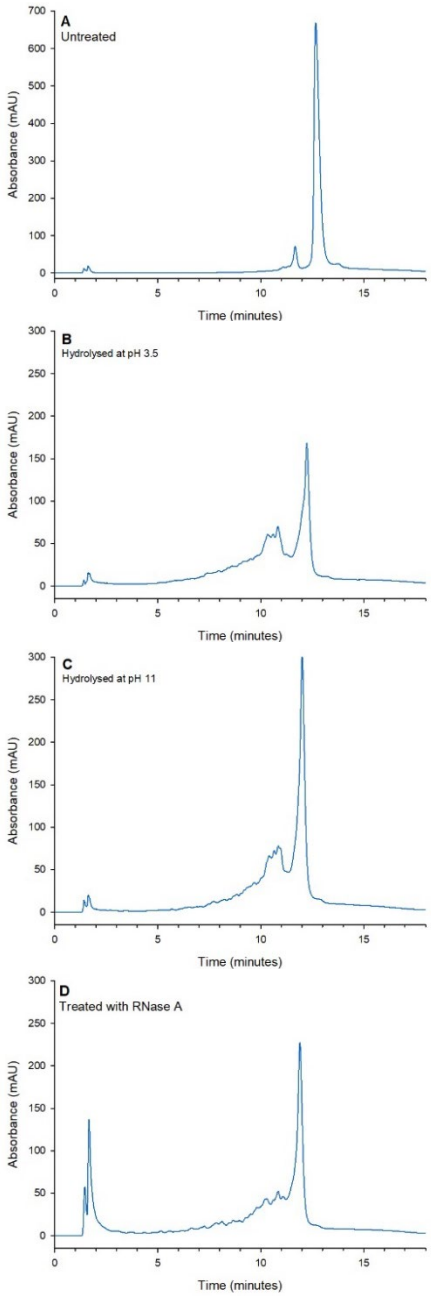

Figure 3: Analysis of the stress-tested model compound, modified eGFP mRNA (996 nucleotides), using optimized method conditions. Separation with DNAPac RP column with TEAA in the mobile phase (8.6% - 13.7% MeCN). Further experimental details are provided in the Experimental Section. (A) Untreated eGFP mRNA (B) Hydrolysis at pH=3.5, 30 min after preparation, (C) Hydrolysis at pH=11, 30 min after preparation (D) Exposed to RNase A, 12 hours after preparation.

Degradation through exposure of the model mRNA to elevated temperatures could also be well visualised using our optimised IP-RPLC method (Figure 4). A reduction in percentage main peak area is observed with increasing

sample exposure time to high temperature conditions (Table 4).

Table 4: eGFP mRNA Main Peak and Pre-Main Peak Percentage Areas Upon Increasing Exposure Time at 85°C

Sample Exposure Time at 85°C (Minutes)	Percentage Main Peak Area (%)	Percentage Pre-Main Peak Area (%)
15	85	15
30	85	15
90	69	31
150	66	34
210	42	58
240	36	64

Sample exposed for 15 minutes at 85°C did not appear to show any heat induced degradation effects (Supplementary Material Figure 8A). This confirms that high temperature column conditions should not impact the stability of the eGFP mRNA, during the time of the analysis method. Moreover, only minor temperature-induced mRNA degradation can be observed after 30 minutes sample exposure time, at 85°C (Supplementary Material Figure 8B). These effects start to become more pronounced after 90 minutes exposure time, indicating that modified mRNA, eGFP was relatively stable upon exposure to high temperature conditions.

Additionally, a longer mRNA, Cas9, with expected lower stability was studied. The Cas9 sample was exposed to 85°C for 45 minutes, and the heat stressed sample was analysed at 15, 30 and 60 minutes time windows. Results indicated that 60 minutes time window was needed to enable at least some separation of the main peak from the pre-main peak area. Due to the longer analysis time, on-column degradation of the longer Cas9 mRNA compound could not be excluded. Further optimization is needed to ensure stability indication power of the analysis methods of Cas9 mRNA (Supplementary figure 9).

It should be noted that the stress conditions applied may also influence the conformational structure of the analyte and thus its resulting chromatographic behaviour. We observed that treatment of the sample under high temperature conditions resulted in small changes of the post-main peak area (Supplementary Figure 10). The main peak retention time remains consistent throughout analyses, providing indication that limited conformational changes take place (Figure 4E).

4 Conclusion

In this study, a robust stability indicating IP-RPLC method has been successfully developed for modified eGFP mRNA. Through evaluation of key chromatographic parameters the method was optimised to enable high resolution of large RNA fragments sized up to 1000 nucleotides. High column temperature was shown to improve the peak resolution. In addition, we found that the mobile phase flow rate was an important parameter to control the elution profile of the RNA analytes. All of our

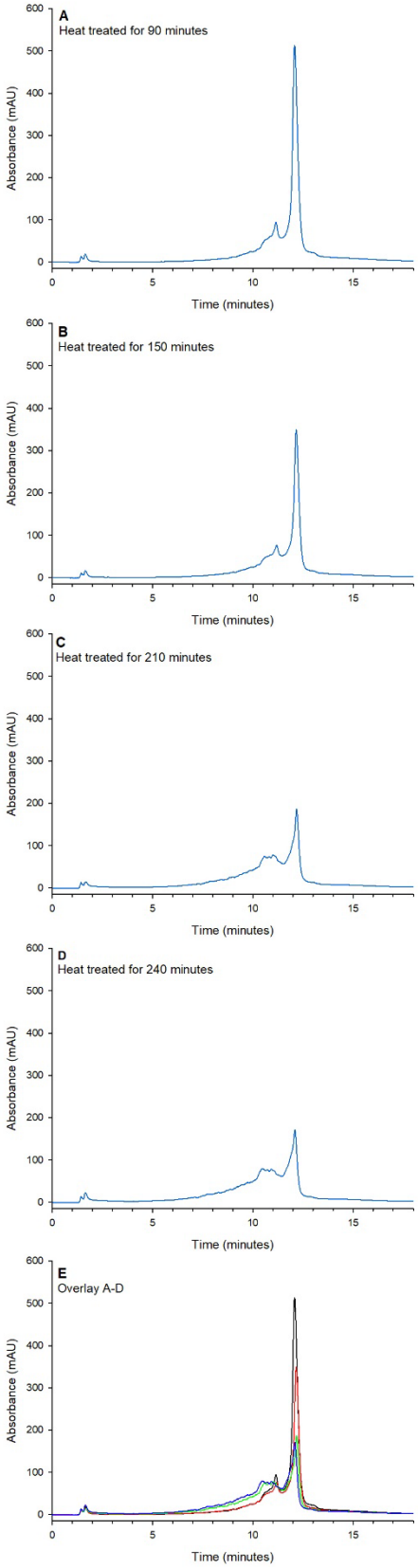

Figure 4: Analysis of the heat-treated model compound, modified eGFP mRNA (996 nucleotides), at 85°C using optimized method conditions. Separation with DNAPac RP column with TEAA in the mobile phase (8.6% - 13.7% MeCN). Further experimental details are provided in the Experimental Section. (A) Sample heat treated 90 min at 85°C (B) Sample heat treated 150 min at 85°C, (C) Sample heat treated 210 min at 85°C (D) Sample heat treated 240 min at 85°C and (E) Overlay of sample heat treated 90, 150, 210 and 240 min at 85°C.

chosen ion-pairing agents were able to successfully baseline separate the fragments in our chosen RNA ladder sample, within a 15 minute analysis time window. However, we found 100 mM Triethylamine in the mobile phase to offer better separation power for the largest RNA sample fragments, as well as for the eGFP modified mRNA. A key finding was that the optimised method could be used to study the degradation of the mRNA under several stress-inducing conditions (heat, hydrolytic conditions and exposure to ribonucleases). Our results showed that the amount of degradation products eluting before the mRNA main peak could be quantified and the relative stability of the mRNA analyte determined. The methodology discussed within this work can therefore be used to guide method development strategies for the assessment of mRNA drug product/substance stability.

4.1 Associated Content

4.2 Supplementary Information

The supplementary material contains tables of mobile phase compositions and method conditions for each analysis run, equations detailing the resolution calculation method and supporting chromatographs and tables.

5 Author Contributions

Jonathan Currie: Conceptualisation, Methodology, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing and Visualisation.
Jacob R. Dahlberg: Conceptualisation, Methodology, Formal Analysis, Investigation, Writing – Review & Editing.
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Fritz Schweikart: Conceptualisation, Methodology, Formal Analysis and Writing – Review & Editing.
Gunilla A. Nilsson: Conceptualisation and Writing – Review & Editing.
Eivor Örnskov: Conceptualisation, Methodology, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualisation and Supervision.

6 Acknowledgements

The authors would like to thank, Nadim Akhtar, Anders Karlsson, and Stephen Wren, from AstraZeneca, for valuable discussions regarding the work and their assistance in reviewing the manuscript.
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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