APPLICATION NOTE

Liquid Chromatography

AUTHORS

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HPLC: Analysis of mRNA with PDA Detection

Introduction

The recent emergence of mRNA-based vaccines and therapies has made clear the need for reliable analytical methods that can successfully identify, characterize, and quantify mRNA. The innate fragility of the mRNA strand leads to rapid degradation by phosphodiester bond cleavage and the formation of smaller strands as degradation products.[1] This chemical instability demands a deeper understanding of the mechanisms by which mRNA degrades, which can inform the development of formulations, manufacturing methods and storage conditions that maintain the desired biological activity for patients.

The analysis of mRNA chemical integrity can be performed by a number of methods, however to date there is no consensus regarding the most effective one. Chemical integrity can be assessed indirectly by functional characterization using *in vitro* translation experiments or reverse transcription quantitative real time polymerase chain reaction (RT-qPCR). However, these methods do not provide information on the sites of cleavage of the main strand or the degradation products formed. In addition, they are prone to interferences due to contamination. UV detection via the determination of A_{260}/A_{280} is useful to estimate total mRNA concentration, but does not differentiate strands of different sizes,[2] an issue that also applies to RNA-specific fluorescence-based methods. In contrast, capillary electrophoresis-based (CE) analysers have shown utility in separating mRNA and its degradants, and in detecting mRNA integrity with very low limits of detection (LOD) in a high-throughput format. CE analyzers are particularly well-suited to qualitative analysis of mRNA and degradant size and can also be used for quantitation with appropriate standards. Orthogonal methods with the potential to provide reliable mRNA quantitation are needed to provide a holistic view of mRNA integrity and to ensure the safety and purity of mRNA therapeutics.

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This application note reports data obtained using the PerkinElmer LC 300 HPLC System with a photodiode array (PDA) detector to analyze mRNA samples produced via *in vitro* transcription (IVT) using an ion pairing reversed phase assay. Additionally, stressed stability experiments were used to investigate the possibility of using HPLC methods to assess mRNA degradation over time, which would be invaluable in mRNA formulation and process development.

Experimental

Hardware/Software

Chromatographic separation was achieved using a PerkinElmer LC 300 HPLC System, consisting of an LC 300 HPLC Pump, LC 300 HPLC Autosampler, and an LC 300 Column Oven. Detection was achieved using an LC 300 Photodiode Array (PDA) detector. Instrument control, analysis and data processing were performed using the PerkinElmer SimplicityChrom™ Chromatorgraphy Data System (CDS) Software.

Method Parameters

The LC parameters are shown in Table 1.

Table 1. LC Parameters.

LC Parameters	
Column	Thermo Scientific™ DNAPac™, 100 x 4.1 mm
Mobile Phase	A: 100 mM Triethylamine-acetate (TEAA) pH=7 (aq.) B: 75:25 100 mM Triethylamine-acetate pH=7 (aq.): Acetonitrile Gradient methods shown in Table 2.
Flow Rate	0.3 mL/min
Oven Temperature	60 °C (unless otherwise stated)
Sample Temperature	Ambient
Injection Volume	20 µL
PDA Wavelength	254 nm

PerkinElmer LC 300 HPLC System.

Materials

mRNA encoding for firefly luciferase and green fluorescent protein (GFP) were prepared using the plasmids pKT305 and pCMV T7 EGFP (AddGene), respectively, after linearization with restriction enzyme AscII (Thermo Scientific FastDigest SgsI restriction enzyme) as the DNA template. *In vitro* transcription (IVT) was carried out using the Jena Bioscience mRNA synthesis kit. Triethylamine, acetic acid and HPLC-grade water were purchased from Fisher Scientific.

Methods

The method was adapted from that described by Vanhinsberg *et al.* (2022).[3] The gradient used in the experiments was varied to result in longer runs with higher resolution or shorter runs and quicker elution (Table 2). For individual runs, the mRNA concentration was set at approximately 40 µg/mL. A calibration curve was developed using mRNA standards with concentrations of 50, 25, 12.5, 6.25 and 3.125 µg/mL using method A and relating the area under the main mRNA elution peak to concentration (noise threshold 5 and bunching factor 20). Each standard was injected three times. The limit of detection (LOD) was calculated with the formula 3.3σ/S, where σ is the standard error of the response and S is the slope of the calibration curve. The limit of quantification (LOQ) was calculated using the formula 10σ/S. In experiments in which the effect of column temperature was investigated, method A was used. In mRNA degradation experiments, the sample (20 µg/mL) was incubated at 50 °C for up to four hours, and an injection was made every hour, starting from t=0 h. For stability experiments, Method D was used.

Table 2. Methods used in the experiments.

Gradient	Method A	Method B	Method C	Method D
22% B	0 min	0 min	0 min	0 min
27% B	2 min	2 min	2 min	2 min
27 → 62% B	9 min	13 min	17 min	22 min
62 → 27% B	11.5 min	15.5 min	19.5 min	24.5 min
27% B	12 min	16 min	20 min	25 min

Results and Discussion

Initially, luciferase-encoding mRNA (~ 1.7 kb) was used to evaluate the effects of varying the gradient of the ion-pairing mobile phase. Shorter runs with steeper gradients (Figure 1A) resulted in sharper, more intense peaks, as expected. For simple detection and identification of mRNA, method A appears to be the most time-effective. The effect of column oven temperature was also assessed (Figure 1 B); The highest temperature tested (60 °C) yielded the sharpest peak with shorter retention time (8.64 min). Despite the elevated temperature and the propensity of mRNA to undergo thermal degradation, no fragments were observed; fragments are expected to appear as small peaks at earlier retention times. Higher temperatures were not tested.

Similarly, GFP-encoding mRNA (~ 1 kb) was also analyzed using three methods of different duration (Fig. 2A). Although the peak-sharpening effect observed for luciferase-encoding mRNA (Fig. 1) was also observed for GFP-encoding mRNA (Fig. 2) with Method A, there were also notable differences in chromatograms for the two mRNAs. In particular, GFP-encoding mRNA showed a low intensity peak with very short retention time (1.1 min) and a shouldering effect, especially for Method C (Fig. 2A). The early eluting peak may correspond to unreacted nucleotides or short degradation products whereas the shoulder could indicate the formation of degradants similar in length to the native mRNA. As with luciferase-encoding mRNA, running Method A at higher column temperatures produced a more intense peak with shorter retention time (Fig. 2B).

In order to assess the resolution that can be achieved in terms of mRNA size, both mRNA types were mixed in the same vial and analyzed using Methods A, B and C and a

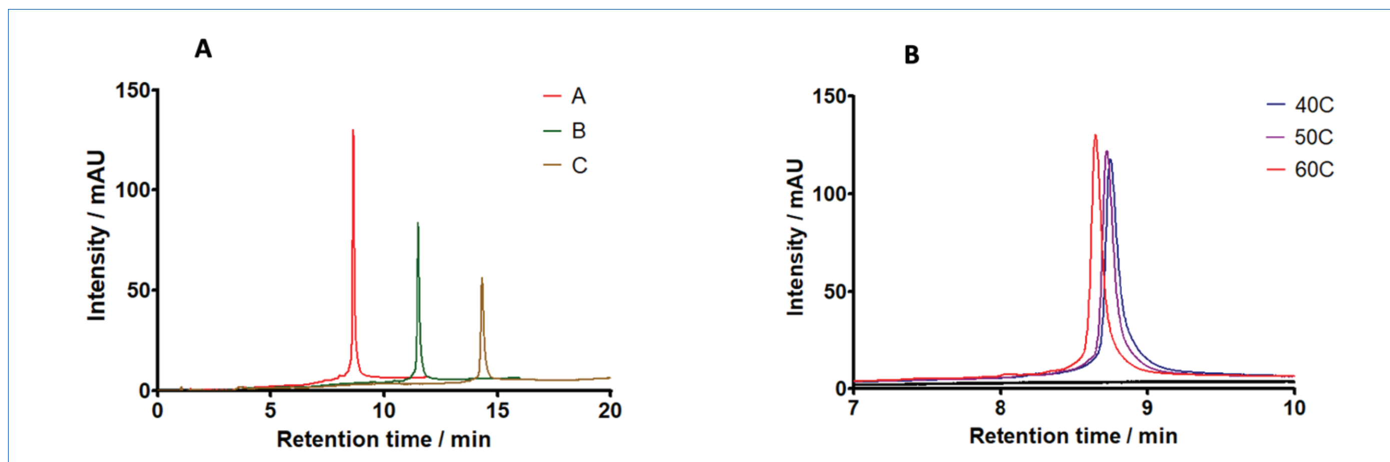

Figure 1. Chromatograms of luciferase-encoding mRNA by three different gradients (A) and at different column temperature using method A (B). See Table 1 for gradients.

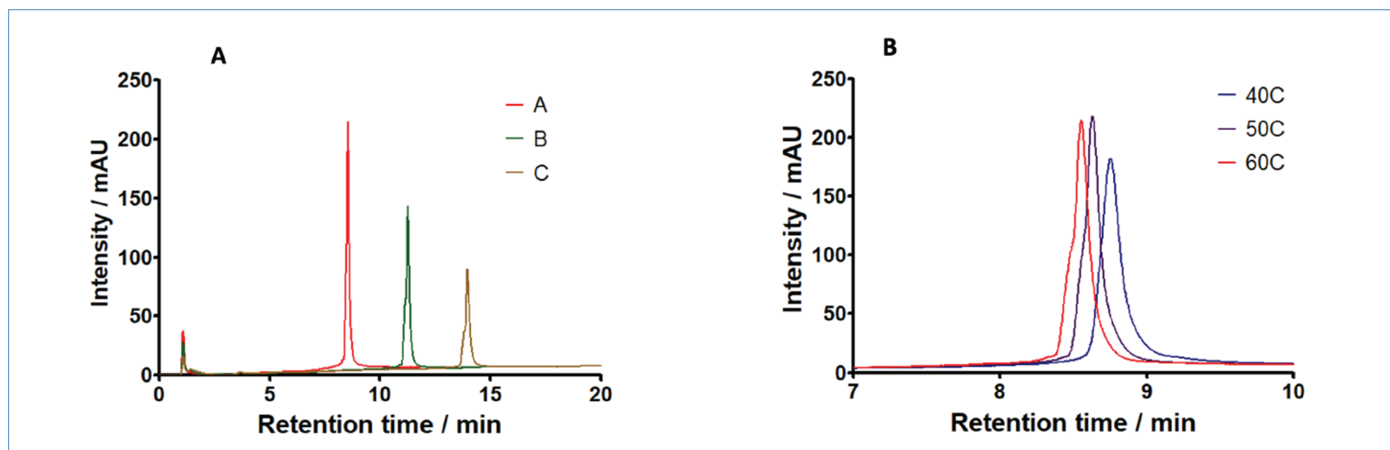

Figure 2. Chromatograms of GFP-encoding mRNA ran by three different methods (A) and by varying column temperature while using method A (B).

column temperature of 60 °C (Figure 3). The analytes were resolved using Methods A and B, but only Method C yielded near baseline resolution. This experiment also sheds light on the most likely retention times of shorter mRNA strands derived from degradation. For example, the shoulder for on the GFP-mRNA peak cannot belong to a species that differs by more than ~100 nucleotides from the main peak, since the difference in retention time of GFP- and luciferase encoding mRNAs is less than 2 minutes (Method C, Figure 3) and the retention times of the shoulder and main peak differ by less than 0.5 minutes (Method C, Figure 3).

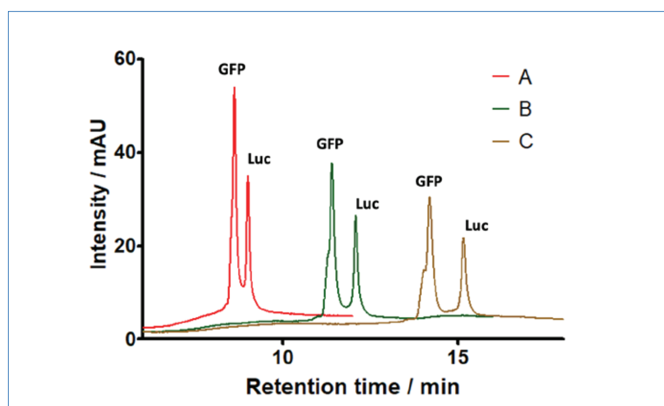

Figure 3. Chromatograms of mixtures of GFP- and luciferase-encoding mRNA ran by three different methods.

In the development of mRNA therapeutics, the quantification of native mRNA species is important for quality control and correct dosage. Therefore, we also investigated the relationship between the area of the main mRNA chromatographic peak and the mRNA concentration in various standards. Figure 4A shows representative chromatograms for each of the standards used, and the areas calculated under the curves were used to plot the calibration curve in Fig. 4B. A linear fit with good correlation (R^2 = 0.9999) was obtained. Using the regression parameters, the LOD was calculated as 0.42 ± 0.02 µg /mL and the LOQ was calculated as 1.27 ± 0.03 µg/mL. Using this calibration plot, it is possible to estimate the mRNA concentration in a given sample that has undergone partial degradation. Fig. 5 shows the chromatograms of a sample of luciferase-encoding mRNA that was incubated at 50 °C and analyzed every hour for 4 hours. By tailoring the peak detection feature of SimplicityChrom with recommended values of the bunching factor and noise threshold, it is possible to observe the decreasing concentration of the main mRNA peak during the time course of incubation (Fig. 5B). In this case, Method D was used so that the slower gradient would result in better resolved fragments, which are seen as smears eluting before 11.6 min.

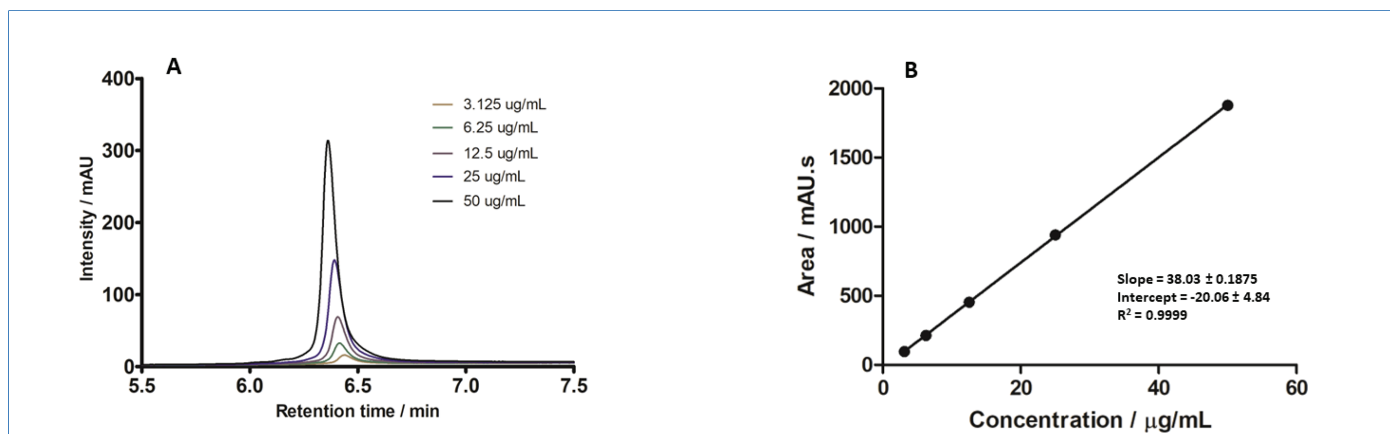

Figure 4. Chromatograms of standards of luciferase-encoding mRNA (A) and calibration curve using the area under the peak with retention time ~6.4 min (B).

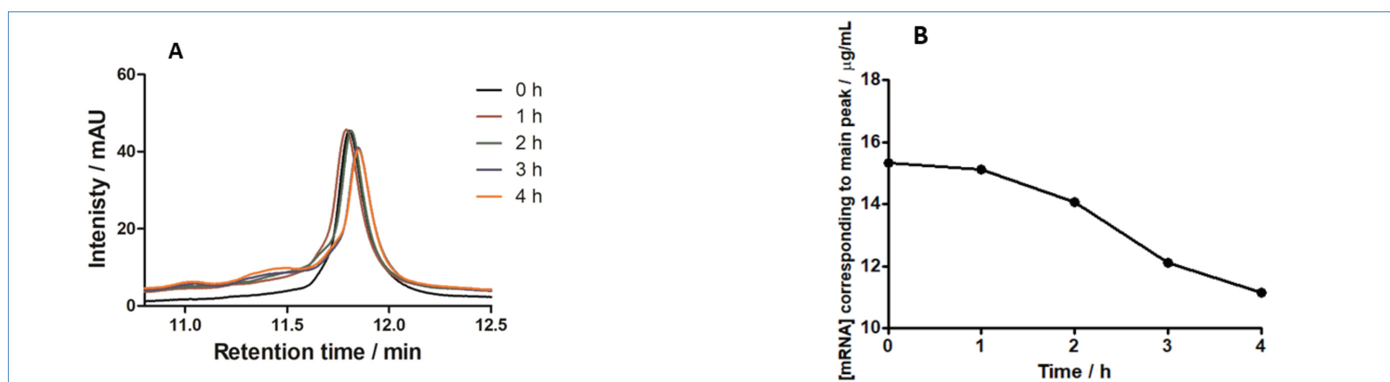

Figure 5. Chromatograms of a sample of luciferase-encoding mRNA injected every hour while being incubated at 50 °C (A) and degradation profile of mRNA peak (B).

Conclusion

For the analysis of mRNA samples using ion-pair reverse-phase (IP-RP) chromatography, the PerkinElmer LC 300 HPLC System equipped with an external column oven was used. Optimisation of gradients and temperature allowed improved sample analysis and quantification down to the low µg/mL range. mRNA degradation was detected and quantified in stability studies, suggesting the utility of the approach in the development of formulations and manufacturing processes for mRNA therapeutics.

References

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