WIL RESEARCH

The analysis of a single stranded antisense oligonucleotide in plasma via lysation and hybridization with HPLC-fluorescence

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Introduction

The cytokine human transforming growth factor beta (TGF-β) plays an important role in key pathways such as cell proliferation, cell differentiation, immune response and tissue modeling. Because TGF-β is chronically elevated in many diseases, including ophthalmic and fibrotic diseases and cancer, and involved in their pathophysiologies, it is an extremely versatile drug target.

Antisense oligonucleotides like ASPH_0047, are single-stranded DNA- or RNA- sequences of 13- to 25-nucleotides, that induce the cleavage of homologous stretches of mRNA sequences and subsequent inhibition of target protein expression of TGF-β.

The analysis of ASPH_0047 was based on a fluorescent- peptide nucleic acid / locked nucleic acid (PNA/LNA) duplex with HPLC-fluorescence using a proprietary method developed by Axolabs.

The sample processing is performed in two stages.

Sample preparation:

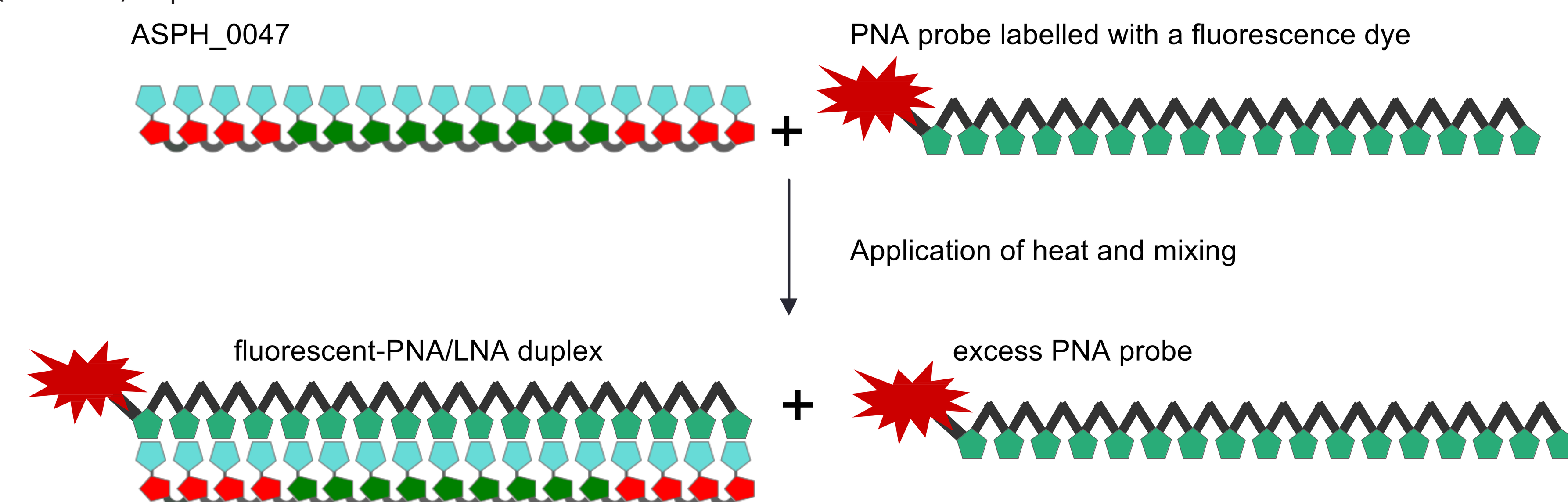
Stage One :

- Buffer with Proteinase and EDTA was mixed with 50 µl of sample in an 96 wells PCR plate.
- Mix on a thermo-mixer for 60 minutes at 65°C.
- Cool on ice for 5 minutes.
- Centrifuge at 3761g for 5 minutes This first stage is needed to digest the proteins into smaller fragments that do not interfere with the assay.

The second stage is the hybridization stage (see also Figure 1):

- Hybridization mix (PNA probe with acetonitrile and buffer) is added to the 96 well PCR plate resulting from the first stage.
- Mix on a thermo-mixer for 15 minutes at 70°C.
- Cool on ice for 5 minutes.
- Injected on the LC- fluorescence system.

Figure 1 Schematic overview of ASPH_0047 undergoing a hybridization (Stage 2) with a complementary PNA probe labelled with a fluorescence dye. PNA probe is added in excess resulting in free PNA and fluorescent- peptide nucleic acid / locked nucleic acid (PNA/LNA) duplex.

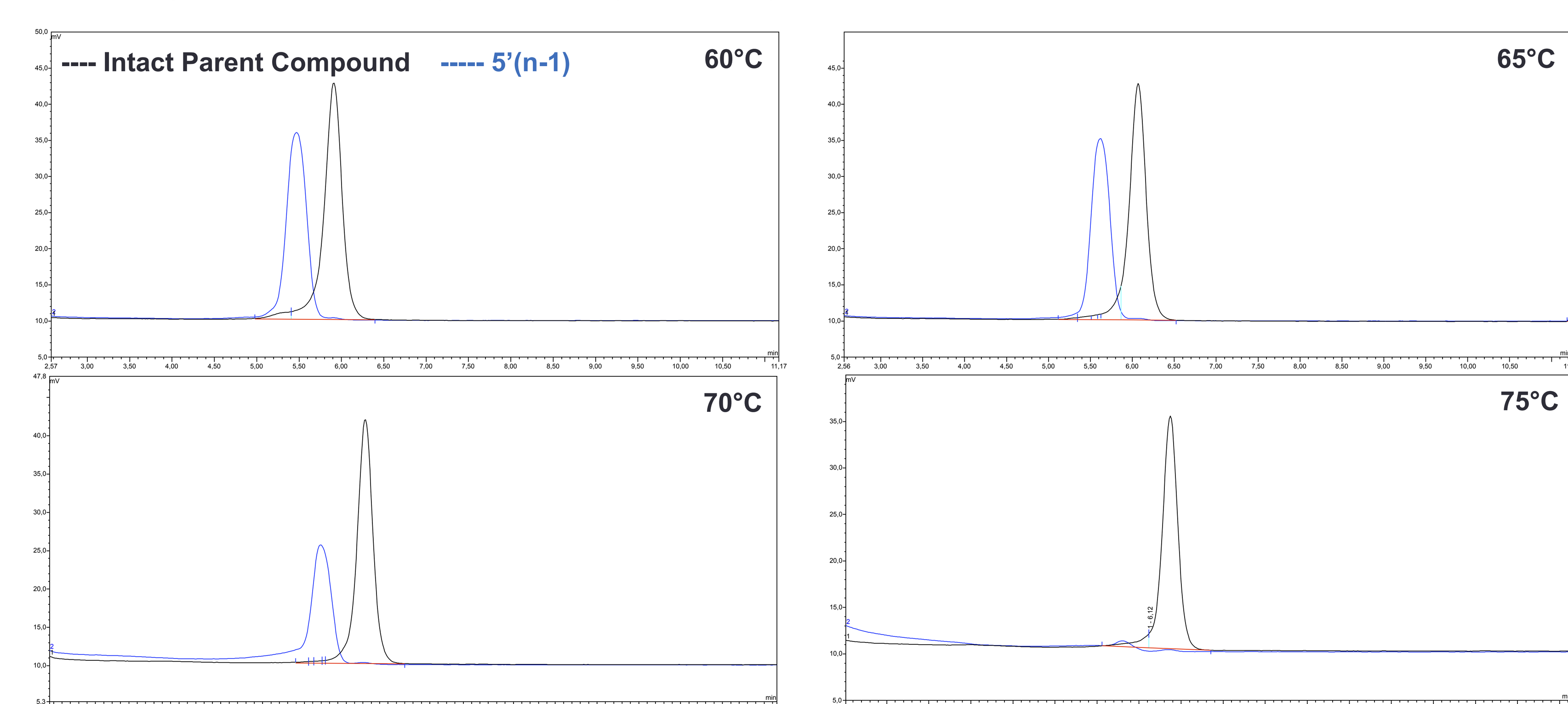


Instrumental Set-Up

Instrument	Acquity® UPLC® System (Waters®)
Detector	Acquity UPLC Fluorescence Detector (Waters)
Column	DNA Pac PA200, 4x250 mm (Dionex®)
Inline filter/guard column	Inline filter ASSY frit, 2.1 mm id., 0.2 µm (Waters)
Column temperature	73°C
Autosampler temperature	Room temperature
Injection volume	50 µL
Eluent A	30/70 (v/v) acetonitrile/25 mM Tris-buffer pH 8 with 1mM EDTA
Eluent B	800 mM $NaClO_4$ in Eluent A
Eluent C	4M $NaClO_4$ in 10/90 (v/v) acetonitrile/water
Flow	1.0 mL/min
Detection	Excitation at 436 nM
	Emission at 484 nM

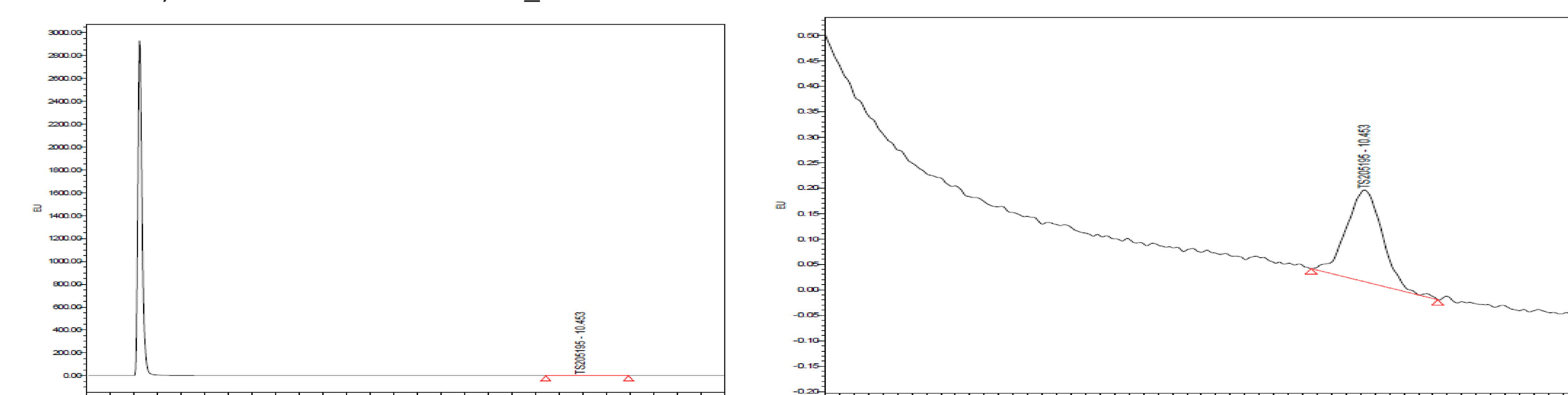
Eluent C is used for the cleaning of the chromatographic system after each injection to prevent carry-over to the next injection.

Figure 2 By variation of the column temperature, it is possible to differentiate between the peak of interest and compounds which differ just slightly in the base or nucleotide composition. The duplex is more resilient against higher temperatures due to the perfect fit of the compound and the PNA probe as compared to duplexes from different DNA.



The concentration range of the method is a compromise between the tailing of the excess of the PNA probe eluting in the initial part of the chromatogram and the detectability of small peaks at the LLOQ level eluting in the tailing. The concentration range was established by increasing the PNA probe concentration and observing the effect of this increase on LLOQ level. In figure 3 the final result is presented. The PNA probe concentration was added in excess of around 120% of the ULOQ concentration. The established concentration range was 5.00 – 2000 ng/mL.

Figure 3 Chromatogram of the LLOQ (5 ng/mL) in rat plasma. The chromatogram at the left is the total chromatogram with the excess PNA probe at the t= 1.1min and duplex with ASPH_0047 at 10.4 minutes. The chromatogram at the right is an enlargement of the area of interest, the retention time of ASPH_0047.



The dip seen in the right chromatogram at 13.50 minutes is the cleaning of the chromatographic system. This cleaning step is necessary to prevent carry-over of the system. The total runtime per injection was 18 minutes. This had an impact on the sample throughput. An entire run including QCs and calibration curve and 72 study samples takes 32 hours.

Method Validation

The method was validated for Cynomolgus monkey and rat plasma. The results are presented below.

Analytical performance obtained for rat and monkey plasma QC's during method validation

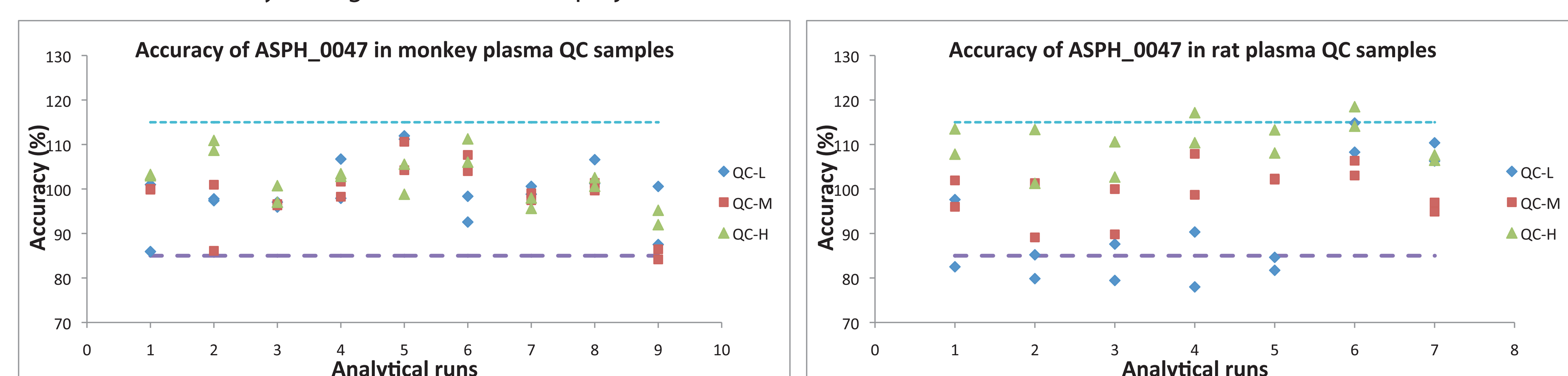
	Wistar Han Rat				Cynomolgus Monkey			
	Accuracy		Precision		Accuracy		Precision	
QC-level	Within batch (%)	Between batch (%)	Within batch (CV%)	Between batch (CV%)	Within batch (%)	Between batch (%)	Within batch (CV%)	Between batch (CV%)
LLOQ 5 ng/mL	90-99	93	4	5	103-120	110	6	8
Low 15 ng/mL	89-94	91	3	3	96-97	96	4	1
Mid 100 ng/mL	100-101	100	2	1	96-102	99	2	3
High 1500 ng/mL	110-112	111	2	1	101-108	104	2	3

For validation in both species the 3 freeze/thaw cycles, dilution (up to 200 times), plasma stability at room temperature (26 hours), selectivity (no interfering peaks) and stability in the matrix in storage and in processed samples were well within the acceptance criteria. The method was successfully validated according FDA and EMA guidelines.

Sample Analysis

The assay was used for sample analysis of 4-week intravenous (slow bolus) toxicity studies in rat and monkey and effects of ASPH_0047 on cardio-respiratory function, electrocardiogram and body temperature study in monkey. Incurred Sample Reanalysis was performed on the 4-week studies and were accepted with 85% and 88% passed results for rat and monkey, respectively.

Figure 4 The performance of the method presented in a Shewhart chart of monkey and rat plasma QCs of ASPH_0047 demonstrating the method stability during the course of the project.



Conclusions

- Specific sample preparation, fluorescent tagging and UPLC with fluorescence detection is a highly suitable method for the measurement of antisense oligonucleotides like ASPH_0047 in plasma.
- The developed method was successfully validated according to FDA and EMA guidelines. Incurred sample analysis was accepted for all plasma sample analysis studies.
- Currently, sample throughput is limited to 72 study samples in 32 hours but the system can run uninterrupted for several weeks when solvents are replenished at regular intervals.
- The concentration range of the method is a compromise between the tailing of the excess of the PNA probe eluting in the initial part of the chromatogram and detectability of small peaks at the LLOQ level eluting in the tailing observed from the PNA probe.

Acknowledgment

WIL Research likes to thanks Isarna Therapeutics for the cooperation and Axolabs for the training and support in development of the method.

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