

# “Development and Validation of an HPLC Method with UV Detection for Quantitative Determination of a Therapeutic Aptamer in Rat and Monkey Plasma”

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## ABSTRACT

NU172 is a DNA aptamer (26 nucleotides) that is directed against the human coagulation factor,  $\alpha$ -thrombin. NU172 binds to the fibrinogen-binding exosite 1 of thrombin with high affinity and inhibits thrombin-mediated effects on coagulation and platelet aggregation. NU172 is being developed as an alternative anticoagulant during cardiac surgical procedures, including cardiopulmonary bypass and elective or emergent percutaneous coronary intervention.

The purpose of this study was to validate a method for the determination of NU172 in rat and monkey plasma by high performance liquid chromatography (HPLC). The method was developed to quantify NU172 with a method that has the specificity to separate NU172 from its 3'-truncated forms (NU172 truncated species N-1, N-2, N-3, N-4, N-5 and N-6), where a truncated form of NU172 is NU172 with a nucleotide deleted sequentially from the 3'-end of the parent compound NU172.

The validation included specificity, linearity, lower limit of quantitation (sensitivity), intra-assay precision and accuracy, inter-assay precision and accuracy, carry-over (for two different HPLC systems), freeze/thaw stability, stability in injection medium under refrigerated conditions, stability in plasma prior to digestion under ambient conditions, stability in plasma at -80°C and stock standard solution stability. The HPLC method for the determination of NU172 in monkey plasma was validated with an LLOQ of 0.16  $\mu$ g/mL and a quantitation range of 0.16 to 500  $\mu$ g/mL.

## Sample Preparation

To a 50  $\mu$ L aliquot of each standard, QC, blank and sample

- 25  $\mu$ L of digestion buffer (60 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5% SDS w/v)

- 75  $\mu$ L of proteinase-K solution (1.0 mg/mL)

were added. The samples were incubated at 55  $\pm$  5°C with shaking overnight for 16  $\pm$  2 hours. Following incubation, samples were centrifuged at approximately 10,000 rpm for approximately 10 minutes. The supernatant was transferred into HPLC vials and analyzed by HPLC-UV.

## HPLC conditions

Equipment: HPLC system equipped with an autosampler, column-temperature-controller and UV detector

Column: Dionex DNAPac PA-100 (2 x 250mm) with Guard (2 x 50mm)

Column Temperature: 40°C

Flow Rate: 0.5 mL/min

Injection Volume: 30  $\mu$ L

UV Detector: 256 nm

Run Time: 31 minutes

NU172 Retention Time: Approximately 10 min

Note: There are different injector washing solutions, depending on the model of HPLC used for analysis

Injector Washing Solution (Waters HPLC): 75% 25 mM sodium phosphate dibasic in water (pH 7.4) and 25% Acetonitrile containing 400 mM NaClO<sub>4</sub>

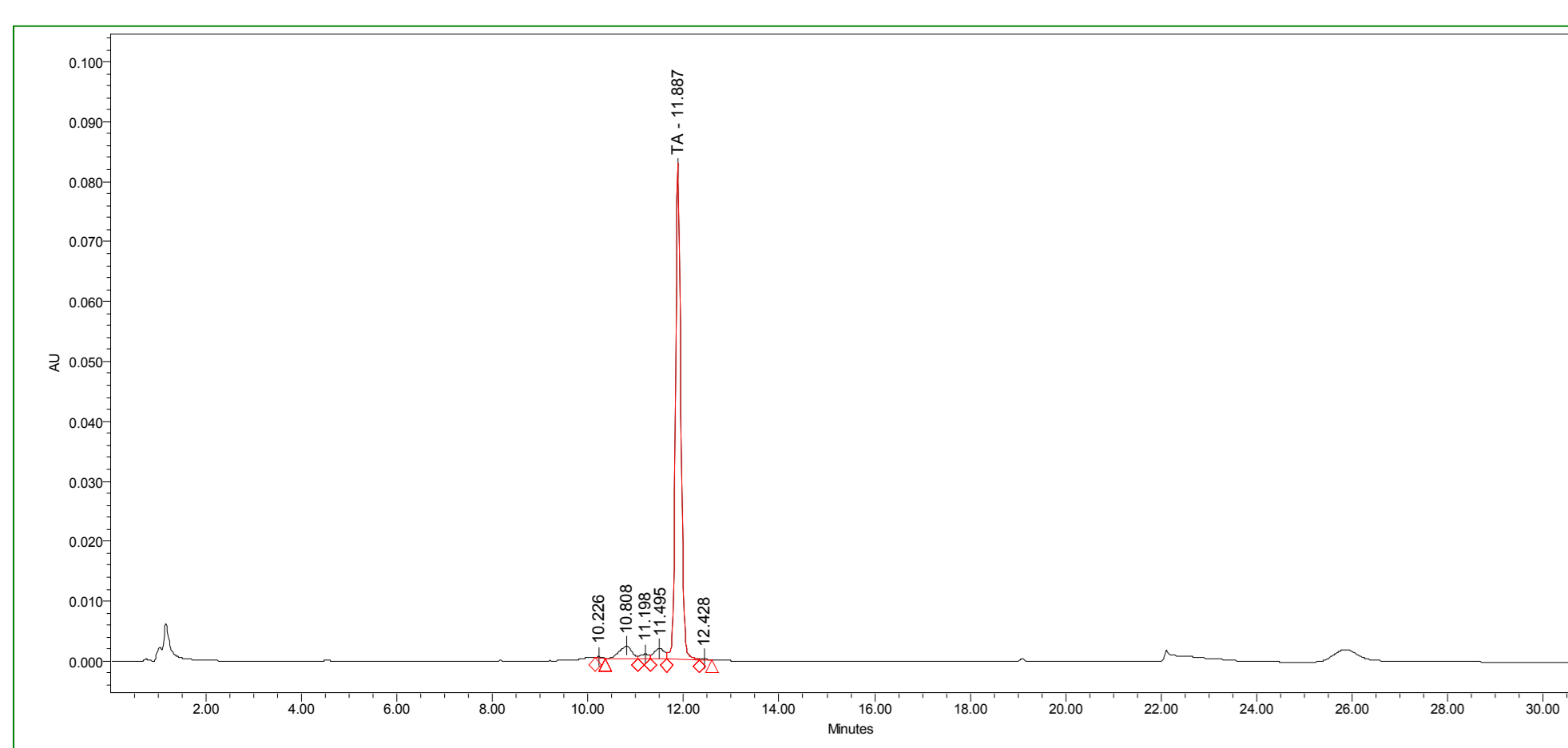
Injector Washing Solution (Shimadzu HPLC): 75% DI Water, 25% Acetonitrile

Mobile Phase: A: 75% 25 mM sodium phosphate dibasic buffer (pH 7.4) and 25% Acetonitrile.

B: 75% 25 mM sodium phosphate dibasic in water (pH 7.4) and 25% Acetonitrile containing 400 mM NaClO<sub>4</sub>

Time	%A	%B
0.00	80	20
20.00	60	40
20.01	0	100
23.00	0	100
23.01	80	20
31.00	80	20

Figure 1. Example chromatogram of 10.0  $\mu$ g/mL of TA in water



## Validation and Stability parameters

- Specificity
- Linearity
- LLOQ (Sensitivity)
- Intra-Assay Accuracy and Precision
- Inter-Assay Accuracy and Precision
- Carry-over
- Freeze/Thaw Stability
- Stability in Injection Medium
- Stability in Plasma Under Ambient Conditions
- Stability in Plasma at -80 °C
- Stock Standard Solution Stability

## Specificity

All six lots of blank rat plasma showed no interfering peaks at the retention time of NU172 (response < 20.0% of the LLOQ standard). The replicates (n=6) of MID-QC in 6 different lots of rat plasma had a mean %Recovery of 103.5%. The sample containing NU172 and the metabolites (NU172 truncated species N-1, N-2... and N-x) had a resolution of 1.7 between NU172 and the N-1 truncated species. The acceptance criteria with respect to specificity were met. Similar results observed in Monkey Plasma.

Figure 2. Blank chromatogram of Rat Plasma.

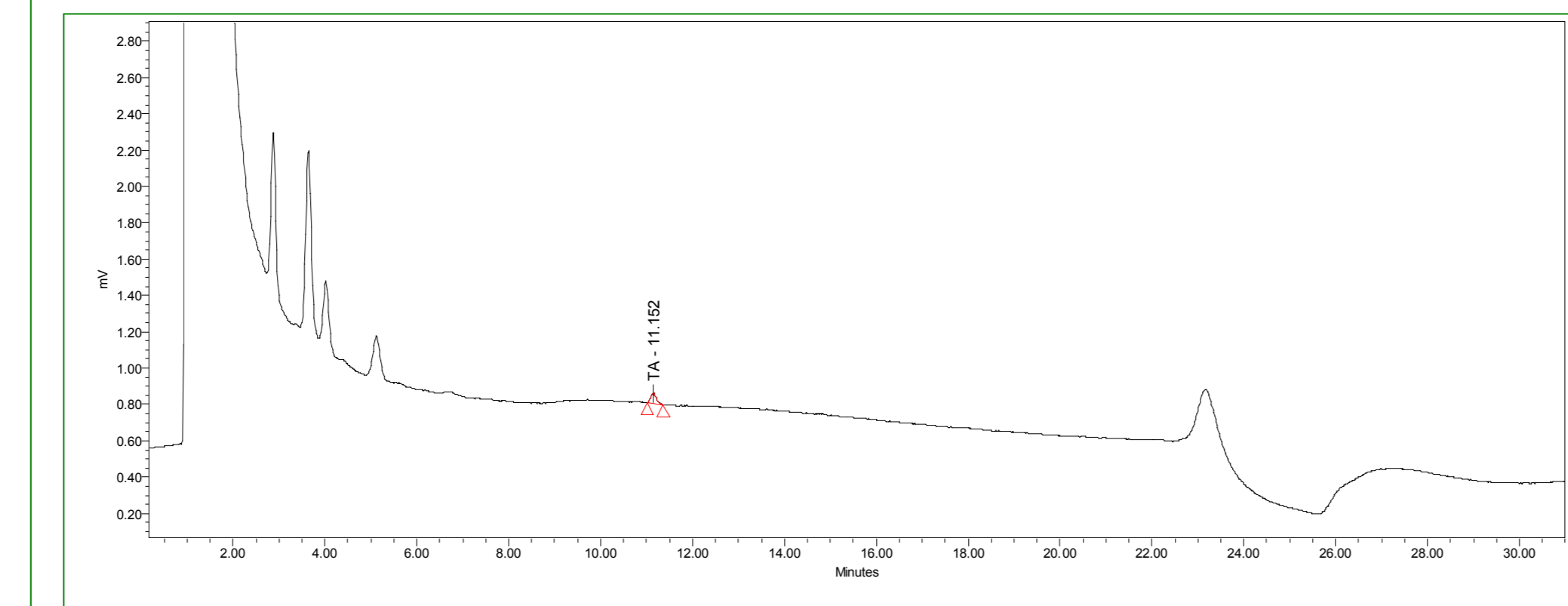
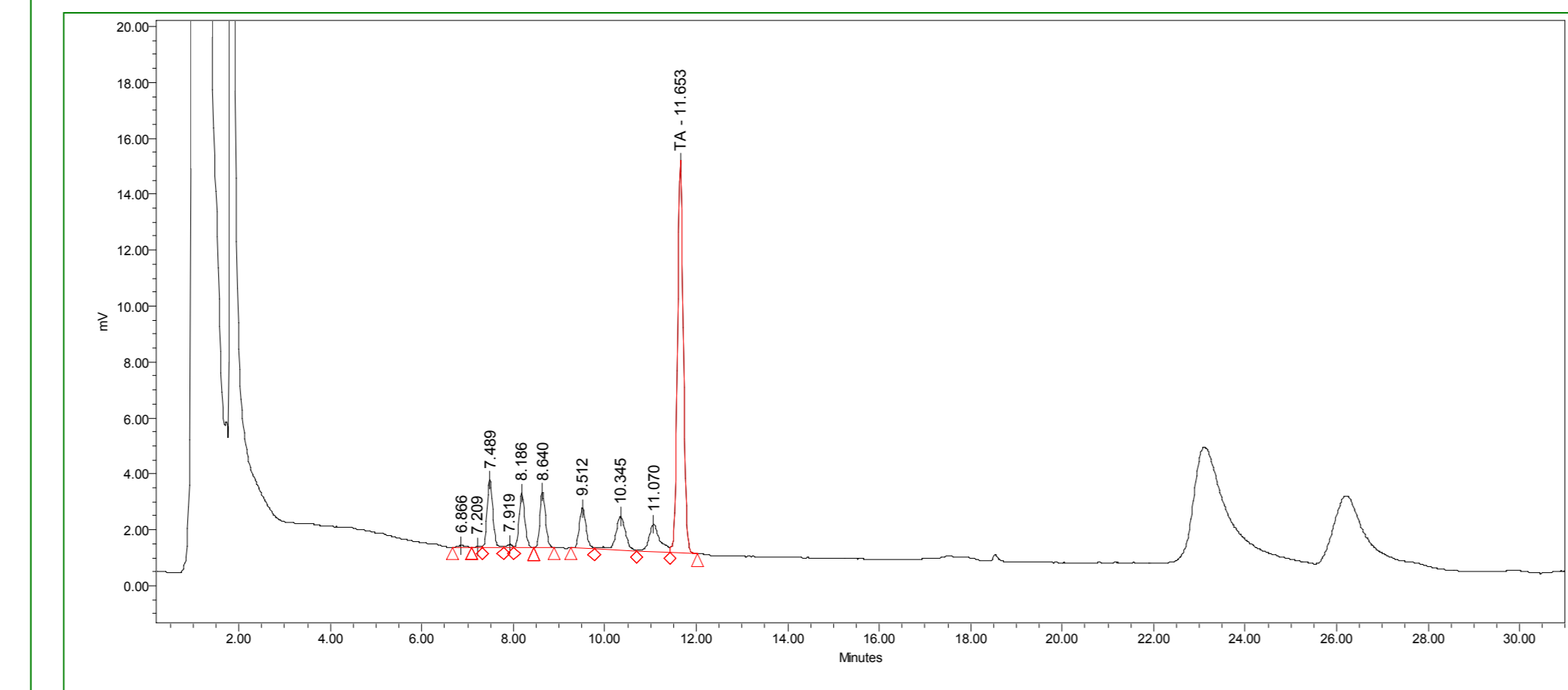


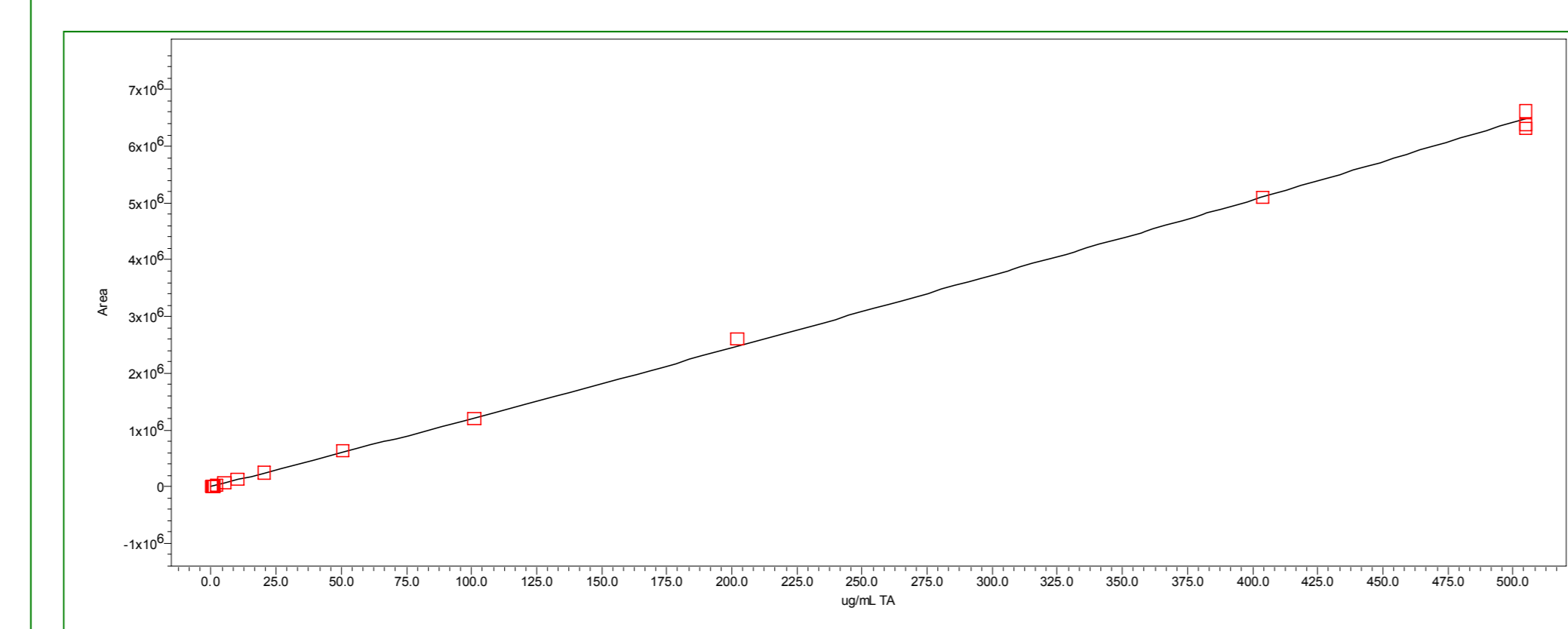
Figure 3. Spike of TA and truncated species N-1, N-2, N-3, N-4, N-5 and N-6 into Monkey Plasma.



## Linearity

A calibration curve was generated using 13 levels of standards and evaluated based on the recoveries of those standards. Individual back-calculated concentrations for all 13 levels of standards, including the LLOQ, were between 89.3 and 111.4% of target. The correlation coefficient (R<sup>2</sup>) value was 0.997. Acceptance criteria with respect to linearity were met. Similar results observed in Rat Plasma.

Figure 4. Representative Calibration Curve (Nominal Concentration 0.16 to 500  $\mu$ g/mL)



## LLOQ (Sensitivity)

The mean recovery of all six LLOQ samples was 92.7  $\pm$  2.4 % with 2.6% RSD. The analyte response at the LLOQ was more than 5 times higher than the blank response at the retention time of NU172. Acceptance criteria with respect to LLOQ were met. Results are presented in Table 1.

Table 1. LLOQ (Sensitivity) in Rat Plasma

Theoretical Concentration ( $\mu$ g/mL)	Observed Concentration ( $\mu$ g/mL)	%Recovery	Mean %Recovery	SD	%RSD
0.16	0.15	95.3	92.7	2.4	2.6
	0.15	94.6			
	0.14	90.4			
	0.14	89.7			
	0.15	91.7			
	0.15	94.3			

## Accuracy and Precision

For Intra-Assay Accuracy and Precision six replicates of the LLOQ-QC (0.16  $\mu$ g/mL), LOW-QC (0.25  $\mu$ g/mL), MID-QC (50  $\mu$ g/mL) and HIGH-QC (400  $\mu$ g/mL) were analyzed against a standard curve including a blank. The average, standard deviation and %RSD for all QC levels were calculated.

For Inter-Assay Accuracy and Precision in three separate analyses, six replicates of LLOQ-QC, LOW-QC, MID-QC and HIGH-QC were analyzed. A second analyst performed one of the three analyses. The Intra-assay accuracy and precision evaluation was used as one of the separate analyses. The grand mean, standard deviation and %RSD for all QC levels from all analyses were calculated.

Table 2. Accuracy and Precision Results in Monkey Plasma at LLOQ (0.16  $\mu$ g/mL). Similar results observed in Rat Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Analysis	Observed Concentration ( $\mu$ g/mL)	%Recovery	Grand Mean %Recovery	SD	%RSD
181 (10X Dilution from 1809)	1	0.22	134.7	102.6	11.4	11.2
		0.19	118.1			
		0.18	113.4			
		0.17	105.2			
		0.17	104.0			
		0.17	103.4			
	2	0.15	94.2			
		0.15	93.4			
		0.17	105.1			
		0.16	98.3			
		0.14	86.7			
		0.16	102.8			
	3	0.15	96.2			
		0.15	93.1			
		0.16	99.9			
		0.15	91.4			
		0.15	95.1			
		0.18	111.2			

Table 3. Accuracy and Precision Results in Monkey Plasma at 0.25  $\mu$ g/mL. Similar results observed in Rat Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Analysis	Observed Concentration ( $\mu$ g/mL)	%Recovery	Grand Mean %Recovery	SD	%RSD
0.25	1	0.30	118.6	103.3	10.1	9.8
		0.28	113.4			
		0.29	117.5			
		0.28	112.4			
		0.28	112.8			
		0.27	109.1			
	2	0.23	90.4			
		0.22	89.2			
		0.24	96.1			
		0.22	89.1			
		0.22	89.2			
		0.23	91.3			
	3	0.26	104.3			
		0.27	107.7			
		0.27	106.1			
		0.26	104.0			
		0.26	103.5			
		0.26	102.1			

Table 4. Accuracy and Precision Results in Monkey Plasma at 50  $\mu$ g/mL. Similar results observed in Rat Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Analysis	Observed Concentration ( $\mu$ g/mL)	%Recovery	Grand Mean %Recovery	SD	%RSD
50.5	1	50.20	99.4	100.8	3.5	3.4
		51.00	101.0			
		50.89	100.8			
		49.43	98.1			
		52.00	103.0			
		50.25	99.5			
	2	49.73	98.5			
		49.34	97.7			
		49.52	98.1			
		48.19	95.4			
		48.08	95.2			
		49.59	98.2			
	3	52.28	103.7			
		52.89	104.9			
		53.84	106.4			
		52.66	104.5			
		52.68	104.5			
		52.81	104.8			

Table 5. Accuracy and Precision Results in Monkey Plasma at 400  $\mu$ g/mL. Similar results observed in Rat Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Analysis	Observed Concentration ( $\mu$ g/mL)	%Recovery	Grand Mean %Recovery	SD	%RSD
404	1	457.44	113.2	102.8	8.7	8.5
		457.37	113.2			
		446.34	110.5			
		468.40	115.9			
		464.46	115.0			
		468.69	116.0			
	2	381.93	94.5			
		383.42	94.9			
		369.79	91.5			
		376.20	93.1			
		385.69	95.5			
		379.55	93.9			
	3	404.62	100.2			
		404.90	100.2			
		403.62	99.9			
		402.62	99.7			
		415.96	103.0			
		403.87	100.0			

## Dilution

To address plasma samples in which the NU172 concentration is above that of the high standard in the calibration curve, six replicates of the Dilution-QC were diluted with plasma, prior to digestion, to a concentration within the standard curve and then analyzed.

Table 6. Dilution Results in Rat Plasma. Similar results observed in Monkey Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Observed Concentration ( $\mu$ g/mL)	%Recovery	Mean Recovery	SD	%RSD
181 (10X Dilution from 1809)	192.16	106.2	103.6	1.4	1.4
	186.04	102.8			
	186.01	102.8			
	185.93	102.7			
	185.92	102.7			
	188.44	104.1			

## Carry-Over

Results of previous experiments suggested that carry-over may be observed. Therefore additional LLOQ-QC samples were included to evaluate the extent of carry-over. Replicates of the LLOQ-QC (n=12) and the HIGH-QC (n=2) were analyzed against a calibration curve including a blank. One of the HIGH QC samples was positioned early in the batch, and the second towards the end of the batch (the latter to check that there was no build up of analyte in the assay system). The order of sample injection on the HPLC was as follows: HIGH QC, LLOQ-QC #1, LLOQ-QC #2, LLOQ-QC #3, LLOQ-QC #4, LLOQ-QC #5, LLOQ-QC #6... HIGH QC, LLOQ-QC #1, LLOQ-QC #2, LLOQ-QC #3, LLOQ-QC #4, LLOQ-QC #5, LLOQ-QC #6.

The assessment of carry over did not meet acceptance criteria. The %recovery in the first LLOQ-QC injected following the HIGH-QC early in the batch on the Shimadzu HPLC was equivalent to 135.5% of target. It was demonstrated that one injection of needle wash followed by one injection of the digestion buffer and a saline blank was sufficient to eliminate any significant injector carry-over in the Shimadzu HPLC as all 12 replicates of the LLOQ-QC were within the acceptance criteria of  $\pm$ 20.0% of target.

Table 7. Rat Matrix Blanks Results Using a Shimadzu HPLC. Similar Results observed in Monkey Plasma

Wash Procedure	Sample	Target Concentration ( $\mu$ g/mL)	Observed Concentration ( $\mu$ g/mL)	% Recovery
No Wash	LLOQ QC - 1	0.16	0.22	135.5
	LLOQ QC - 2	0.16	0.16	101.0
	LLOQ QC - 3	0.16	0.16	97.2
	LLOQ QC - 4	0.16	0.15	95.3
	LLOQ QC - 5	0.16	0.15	96.7
	LLOQ QC - 6	0.16	0.15	92.2
	LLOQ QC - 7	0.16	0.18	113.5
	LLOQ QC - 8	0.16	0.15	96.7
	LLOQ QC - 9	0.16	0.14	90.2
	LLOQ QC - 10	0.16	0.16	97.4
	LLOQ QC - 11	0.16	0.15	95.5
	LLOQ QC - 12	0.16	0.14	87.4
1 Injection of Needle Wash followed by 1 Injection of Digestion Buffer and a Saline Blank	LLOQ QC - 1	0.16	0.15	95.2
	LLOQ QC - 2	0.16	0.15	93.4
	LLOQ QC - 3	0.16	0.15	95.7
	LLOQ QC - 4	0.16	0.15	95.2
	LLOQ QC - 5	0.16	0.16	98.0
	LLOQ QC - 6	0.16	0.14	90.0
	LLOQ QC - 7	0.16	0.15	93.8
	LLOQ QC - 8	0.16	0.15	95.6
	LLOQ QC - 9	0.16	0.15	93.7
	LLOQ QC - 10	0.16	0.15	92.2
	LLOQ QC - 11	0.16	0.15	91.0
	LLOQ QC - 12	0.16	0.15	93.6

## Freeze/Thaw Stability

Replicates (n=6) of the LOW-QC and HIGH-QC samples were analyzed after 3 freeze/thaw cycles. Samples were stored at approximately -80°C for at least 12 hours between thaw cycles and thawed under ambient conditions and mixed gently. On the day of preparation and after the three freeze/thaw cycles the samples were analyzed against a standard curve and a blank.

Table 8. Freeze/Thaw Stability Results in Monkey Plasma. Similar Results observed in Rat Plasma

Theoretical Concentration ( $\mu$ g/mL)	Time-0 %Recovery	Time-0 Mean %Recovery	Freeze/thaw %Recovery	Freeze/thaw Mean %Recovery	% Difference from Time 0
0.25	99.1	97.0	192.0*	94.4	-2.7
	95.7				
	96.8				
	95.3				
	96.0				
	99.1				
403	98.1	98.6	95.5	95.3	-3.3
	98.9				
	97.5				
	98.1				
	98.9				
	100.2				

\*This value was rejected as an outlier with 90% confidence. The mean value was calculated with the remaining 5 values

## Stability in Injection Medium

Two sets of replicates (n=6) of the LOW-QC, MID-QC and HIGH-QC, along with calibration curves and blanks, were digested. Following digestion one set was stored under ambient conditions and the other was stored under refrigerated (5°C  $\pm$  3°C) conditions in the LC vials intended to be used during routine analysis. At the following time points the samples and the blank were analyzed against the standard curve: Initial, 7 days and 14 days. After each analysis, the caps on the LC vials were replaced.

Table 9. Stability in Injection Medium in Monkey Plasma under Ambient and Refrigerated Conditions.

Theoretical Concentration ( $\mu$ g/mL)	Storage Condition	Time-0 Mean %Recovery	Day-7 Mean %Recovery	Day-7 %Difference from Time-0	Day-14 Mean %Recovery	Day-14 %Difference from Time-0
0.25	5°C $\pm$ 3°C	111.7	106.2	-4.9	104.1	-6.8
50.2		109.5	105.3	-3.8	105.3	-3.8
402		102.1	102.2	0.1	101.9	-0.2
0.25	ambient	108.4	106.1	-2.1	104.3	-3.8
50.2		109.2	104.8	-4.0	102.3	-6.3
402		102.5	102.3	-0.2	105.1	2.5

## Stability in Plasma Under Ambient Conditions

Replicates (n=6) of the LOW-QC and HIGH-QC were stored under ambient conditions for at least 24 hours. Aliquots were taken between 6 and 8 hours as well as after 24 hours and analyzed against a freshly prepared standard curve and a blank. The mean recovery for each validation level must be  $\pm$  15.0% of time zero.

Table 10. Stability in Plasma Under Ambient Conditions in Monkey Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Time-0 Mean Recovery	8-hour Mean Recovery	8-hour %Difference from Time 0	26-hour Mean Recovery	26-hour %Difference from Time 0
0.25	101.9	92.5	-9.2	87.5	-14.1
402	103.4	102.7	-0.7	100.5	-2.8

## Stability in Plasma at -80°C

Replicates of the LOW-QC and HIGH-QC samples were stored at -80°C. On the day of preparation and after approximately 45, 90, 120 and 180 days, replicates (n=6) for each level were removed from storage and analyzed against a freshly prepared standard curve and a blank. Two consecutive time-points failed to meet the acceptance criteria and were trending in the same direction (i.e. both time-points failed low) therefore stability assessment was terminated.

Table 11. Stability in Plasma at -80°C in Monkey Plasma.

Theoretical Concentration ( $\mu$ g/mL)	Time-0 Mean Recovery	Day-49 Mean Recovery	Day-49 %Difference from
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