

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

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## ABSTRACT

An analytical HPLC method was developed and validated for the quantitation of a therapeutic aptamer (TA) in both rat and monkey plasma. The method employs digestion of the plasma samples to liberate the aptamer from plasma proteins followed by analysis using ion chromatography and UV detection at 256 nm. The method is capable of quantitation of the parent compound to an LLOQ of 0.50 µg/mL with a linear range up to 200 µg/mL. The method also resolves the parent compound from its N-1 thru N-4 truncated species.

The aptamer is a PEGylated synthetically manufactured aptamer. The core aptamer is a 40-mer (MW ~13 kDa) phosphodiester DNA/RNA oligonucleotide composed of unmodified 2'-deoxy nucleotides, modified 2'-O-methyl-nucleotides (to minimize endonuclease digestion), a 3'-terminus “cap” (an inverted deoxythymidine nucleotide to minimize 3'-exonuclease digestion), and a single phosphorothioate linkage. The 20 kDa PEG group is conjugated at the 5'-terminus.

## Sample Preparation

To an aliquot (50 µL) of each standard, QC, blank and sample

- 25 µL of digestion buffer (60 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5% SDS w/v)
- 75 µL of proteinase-K solution (1.0 mg/mL)

were added. The samples were incubated at 55 ± 5 °C with shaking overnight for 16 ± 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

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

Column Temperature: 60 °C

Flow Rate: 0.5 mL/min

Injection Volume: 30 µL

UV Detector: 256 nm

Run Time: 31 minutes

RT: Approximately 15.0 min

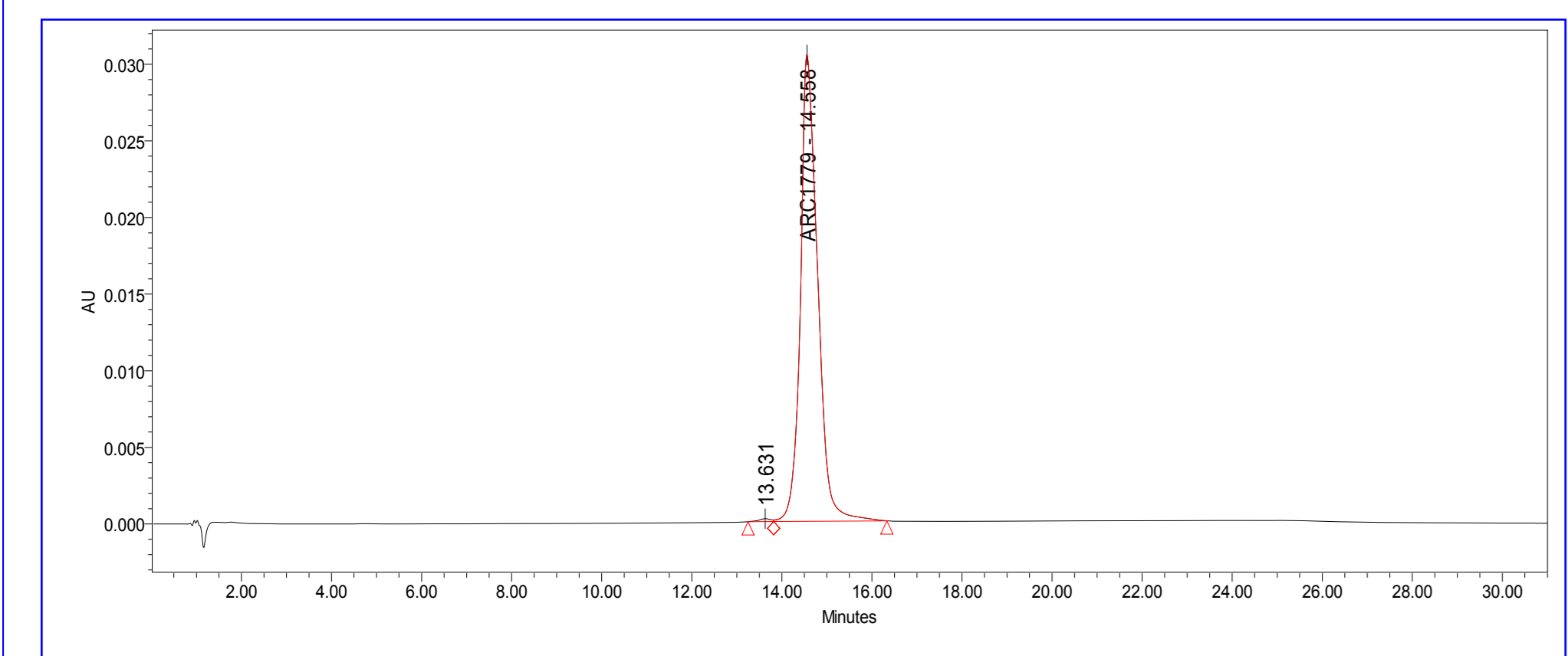
Injector Washing Solution: 75% 25 mM sodium phosphate dibasic in water (pH 7.4) and 25% Acetonitrile containing 400 mM NaClO<sub>4</sub>

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 200 mM NaClO<sub>4</sub>

| Time  | %A | %B |
|-------|----|----|
| 0.00  | 40 | 60 |
| 18.00 | 20 | 80 |
| 23.00 | 20 | 80 |
| 23.10 | 40 | 60 |
| 31.00 | 40 | 60 |

Figure 1. Example chromatogram of 10.0 µg/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

## Specificity

Figure 2. Blank chromatogram of Monkey Plasma. No interfering peaks were observed at the Retention Time of the TA with area greater than 20.0% of the LLOQ. Similar results observed in Rat Plasma.

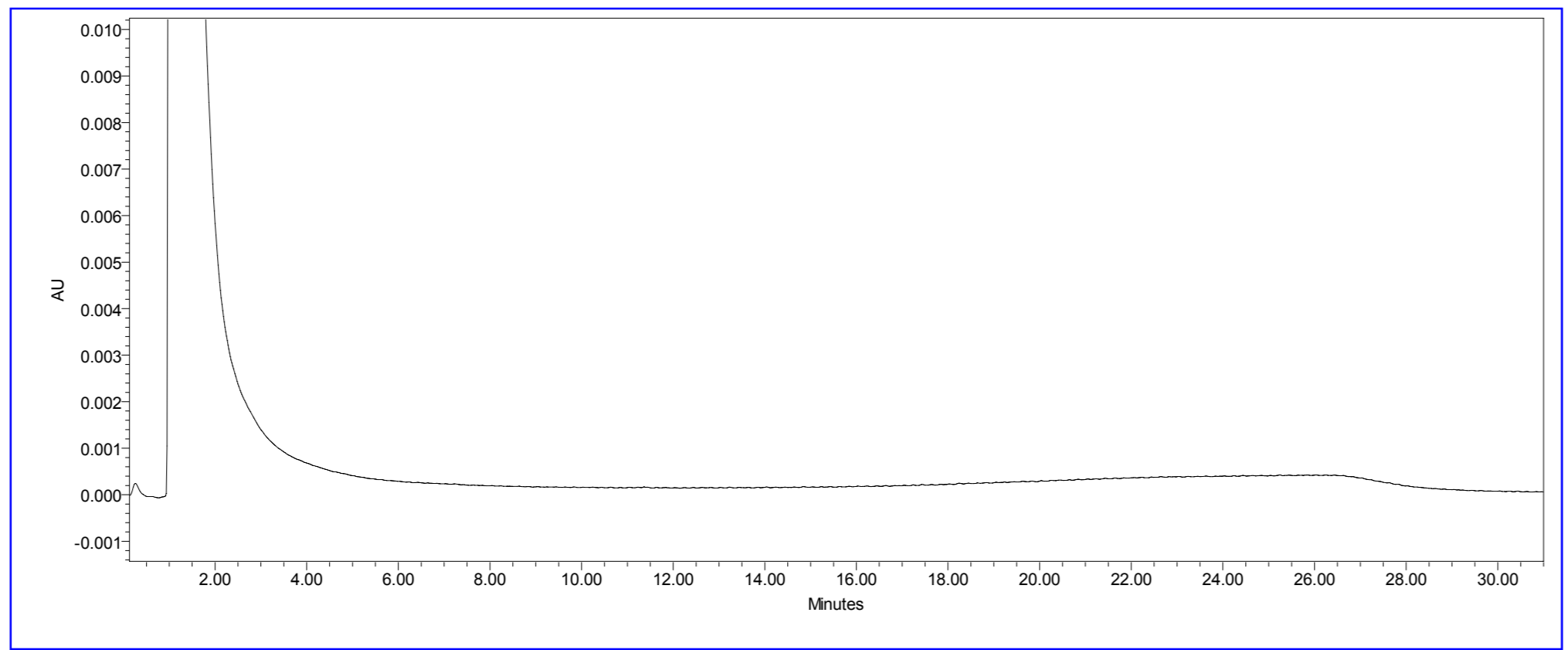


Figure 3. Spike of TA and truncated species N-1, N-2, N-3 and N-4 into Rat Plasma. The Resolution between TA and N-1 is 1.1. Similar results observed in Monkey Plasma.

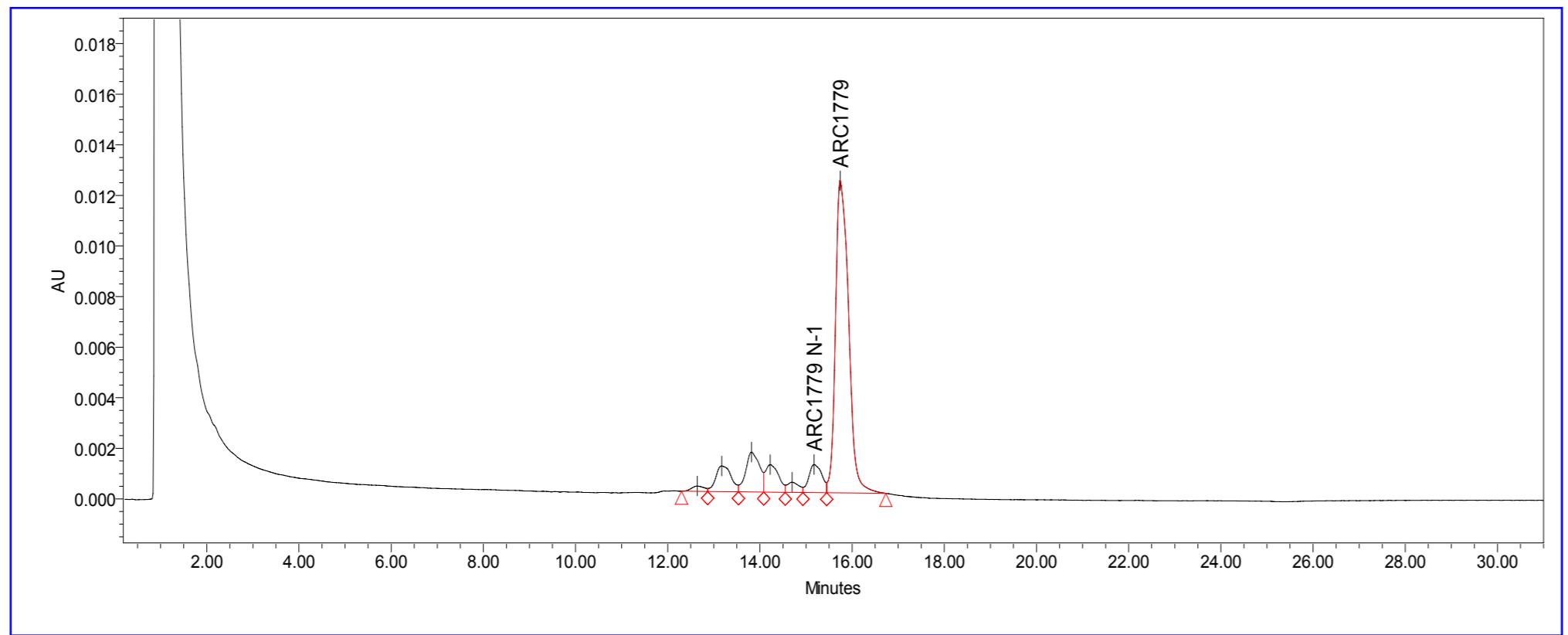
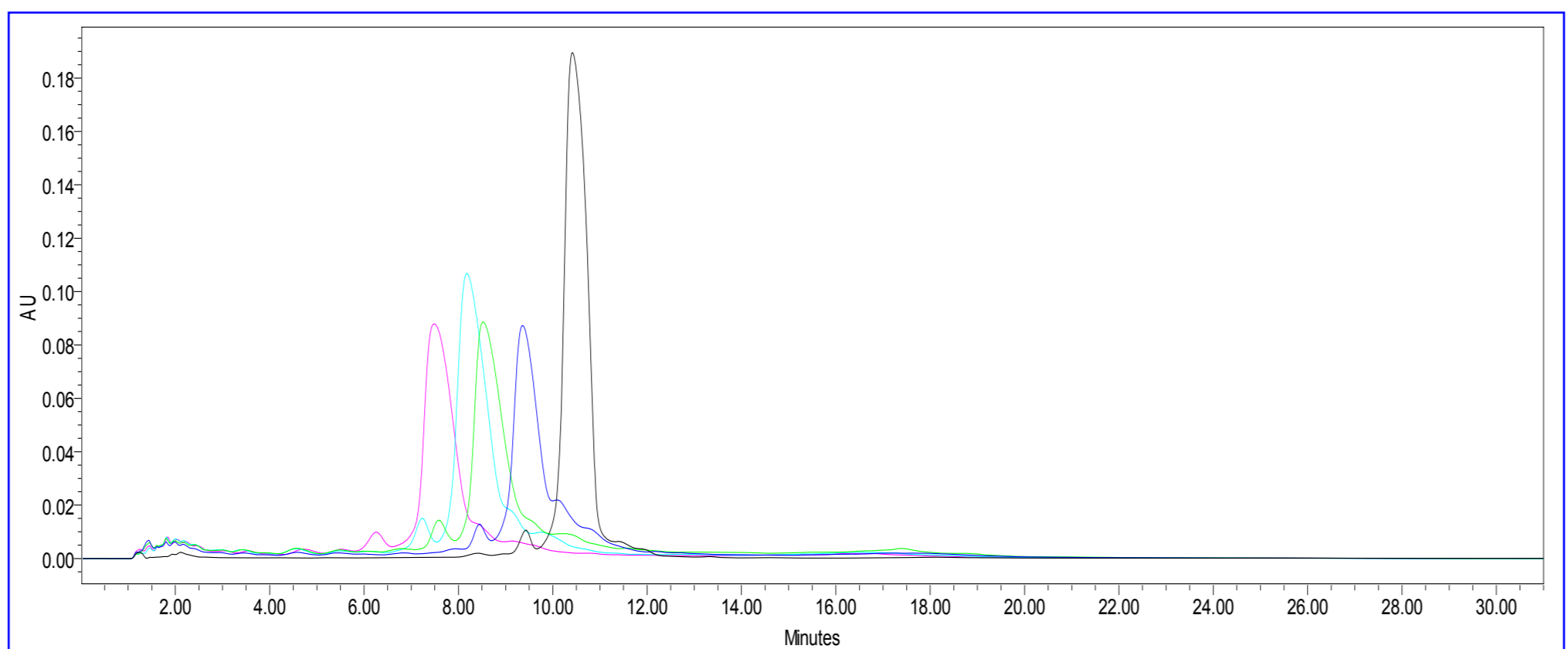


Figure 4. Overlaid chromatogram of TA, N-1, N-2, N-3 and N-4.



## Linearity

Two calibration curves were generated. The back calculated concentrations of 75% of the standards were within 15.0% of target concentration (except for the LLOQ which was within 20.0% of target). The correlation coefficient R<sup>2</sup> for all experiments was ≥ 0.95.

Figure 5. Representative Calibration Curve (Nominal Concentration 0.50 to 10.0 µg/mL)

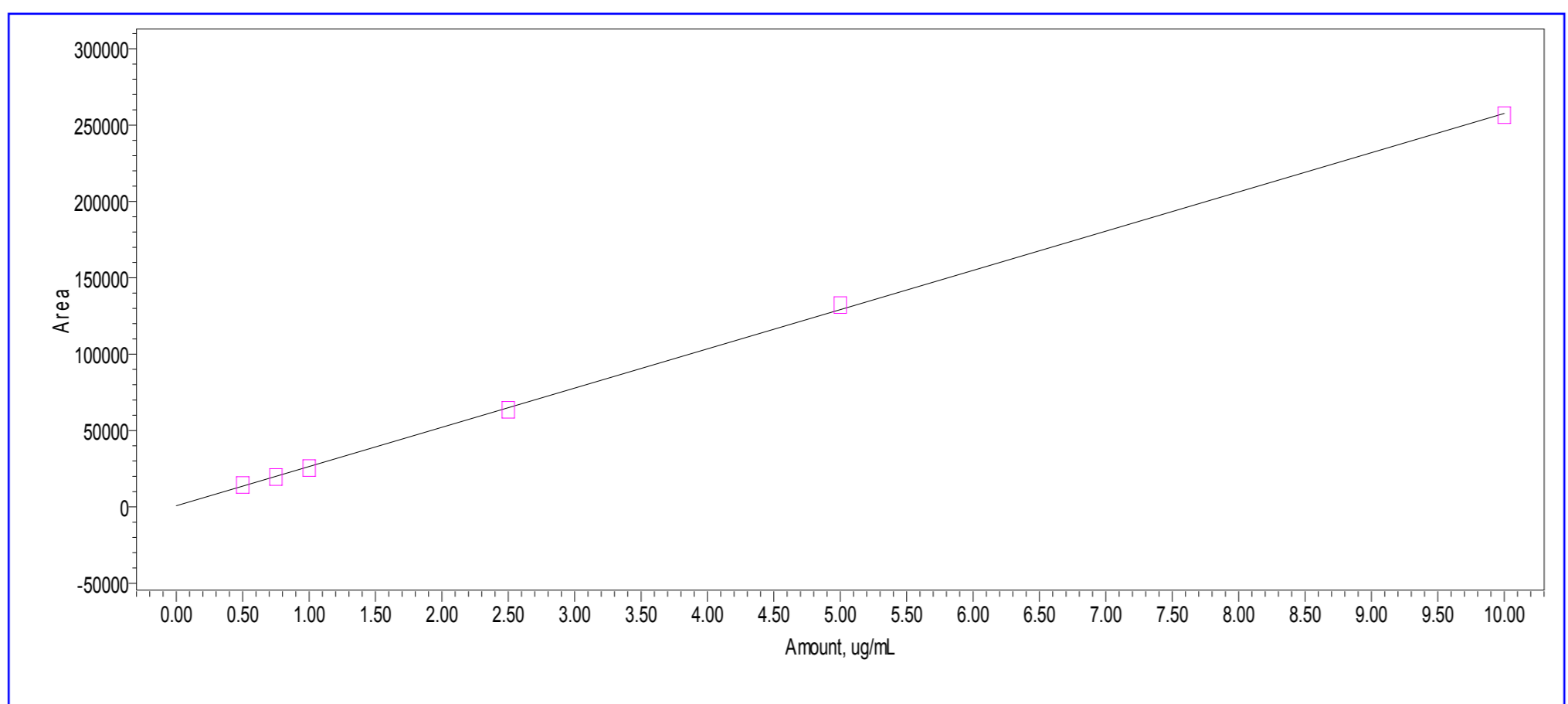
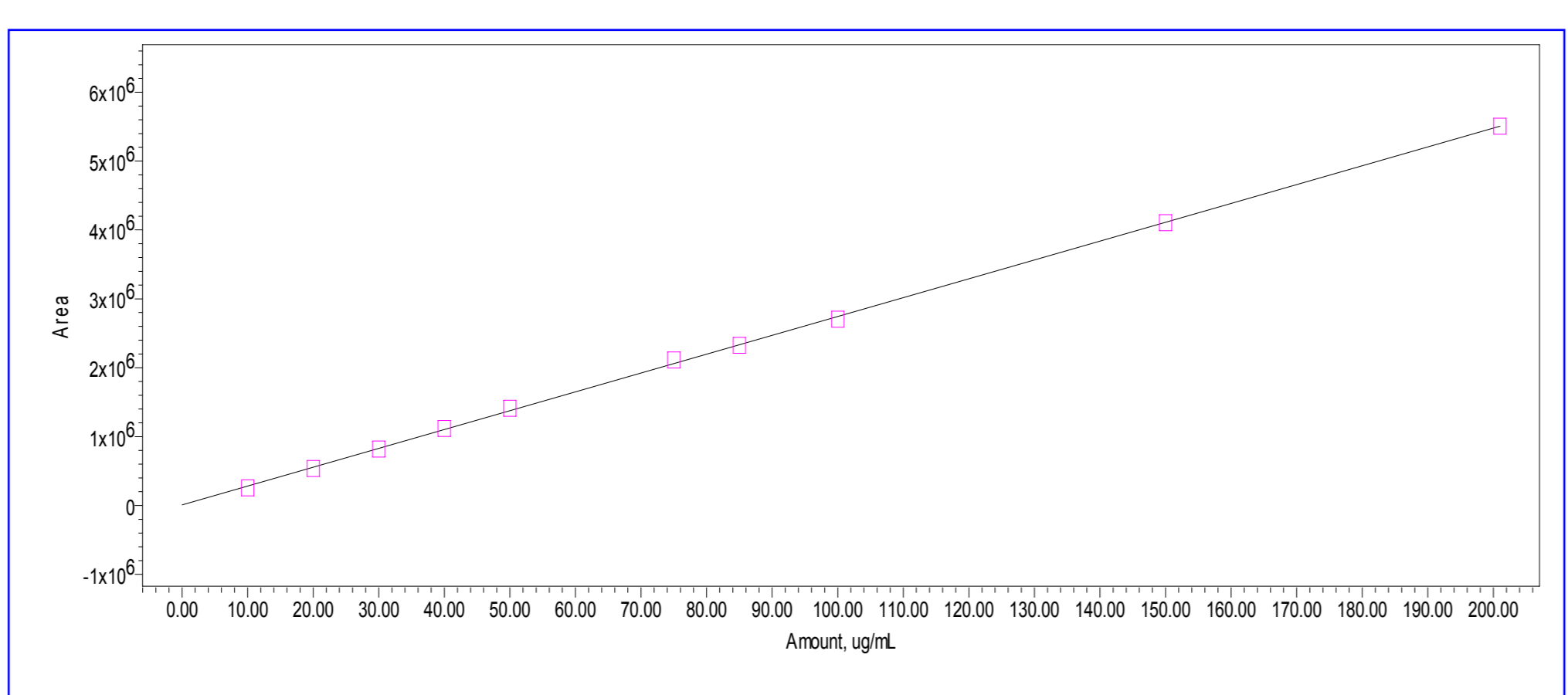


Figure 6. Representative Calibration Curve (Nominal Concentration 10.0 to 200 µg/mL)



## LLOQ (Sensitivity)

The analyte response at LLOQ was at least 5 times higher than the blank response in rat plasma and at least 8 times higher than the blank response in monkey plasma.

The mean recovery of six LLOQ samples analyzed against the calibration curve were within 20.0% of theoretical with % RSD ≤ 20.0% for both rat and monkey plasma.

Table 1. Lower Limit of Quantitation (Sensitivity) in Monkey Plasma. Similar results observed in Rat Plasma.

| Theoretical Concentration (µg/mL) | Observed Concentration (µg/mL) | %Recovery | Mean Recovery | SD  | %RSD |
|-----------------------------------|--------------------------------|-----------|---------------|-----|------|
| 0.50                              | 0.48                           | 96.0      | 93.7          | 1.5 | 1.6  |
|                                   | 0.47                           | 94.0      |               |     |      |
|                                   | 0.47                           | 94.0      |               |     |      |
|                                   | 0.46                           | 92.0      |               |     |      |
|                                   | 0.47                           | 94.0      |               |     |      |
|                                   | 0.46                           | 92.0      |               |     |      |

## Accuracy and Precision

For Intra-Assay Accuracy and Precision six replicates of the LLOQ-QC (0.50 µg/mL), LOW-QC (1.00 µg/mL), MID-QC (100 µg/mL) and HIGH-QC (150 µg/mL) were analyzed against a standard curve. 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 (0.50 µg/mL). Similar results observed in Rat Plasma.

| Theoretical Concentration (µg/mL) | Analysis | Measured Concentration (µg/mL) | %Recovery | Mean Recovery for Individual Analysis | Mean Recovery | SD  | %RSD |
|-----------------------------------|----------|--------------------------------|-----------|---------------------------------------|---------------|-----|------|
| 0.50                              | 1        | 0.48                           | 96.0      | 93.7                                  | 99.0          | 8.5 | 8.6  |
|                                   |          | 0.47                           | 94.0      |                                       |               |     |      |
|                                   |          | 0.47                           | 94.0      |                                       |               |     |      |
|                                   |          | 0.46                           | 92.0      |                                       |               |     |      |
|                                   |          | 0.47                           | 94.0      |                                       |               |     |      |
|                                   |          | 0.46                           | 92.0      |                                       |               |     |      |
|                                   | 2        | 0.61                           | 122.0     | 101.7                                 | 99.0          | 8.5 | 8.6  |
|                                   |          | 0.53                           | 106.0     |                                       |               |     |      |
|                                   |          | 0.52                           | 104.0     |                                       |               |     |      |
|                                   |          | 0.47                           | 94.0      |                                       |               |     |      |
|                                   |          | 0.46                           | 92.0      |                                       |               |     |      |
|                                   |          | 0.46                           | 92.0      |                                       |               |     |      |
|                                   | 3        | 0.58                           | 112.0     | 101.7                                 | 99.0          | 8.5 | 8.6  |
|                                   |          | 0.55                           | 110.0     |                                       |               |     |      |
|                                   |          | 0.48                           | 96.0      |                                       |               |     |      |
|                                   |          | 0.50                           | 100.0     |                                       |               |     |      |
|                                   |          | 0.49                           | 98.0      |                                       |               |     |      |
|                                   |          | 0.47                           | 94.0      |                                       |               |     |      |

Table 3. Accuracy and Precision Results in Monkey Plasma at 1.00 µg/mL. Similar results observed in Rat Plasma.

| Theoretical Concentration (µg/mL) | Analysis | Measured Concentration (µg/mL) | %Recovery | Mean Recovery for Individual Analysis | Mean Recovery | SD  | %RSD |
|-----------------------------------|----------|--------------------------------|-----------|---------------------------------------|---------------|-----|------|
| 1.00                              | 1        | 0.92                           | 92.0      | 91.7                                  | 92.7          | 3.8 | 4.1  |
|                                   |          | 0.93                           | 93.0      |                                       |               |     |      |
|                                   |          | 0.95                           | 95.0      |                                       |               |     |      |
|                                   |          | 0.92                           | 92.0      |                                       |               |     |      |
|                                   |          | 0.88                           | 88.0      |                                       |               |     |      |
|                                   |          | 0.90                           | 90.0      |                                       |               |     |      |
|                                   | 2        | 0.90                           | 90.0      | 90.5                                  | 92.7          | 3.8 | 4.1  |
|                                   |          | 0.93                           | 93.0      |                                       |               |     |      |
|                                   |          | 0.91                           | 91.0      |                                       |               |     |      |
|                                   |          | 0.90                           | 90.0      |                                       |               |     |      |
|                                   |          | 0.90                           | 90.0      |                                       |               |     |      |
|                                   |          | 0.89                           | 89.0      |                                       |               |     |      |
|                                   | 3        | 0.99                           | 99.0      | 95.8                                  | 92.7          | 3.8 | 4.1  |
|                                   |          | 0.93                           | 93.0      |                                       |               |     |      |
|                                   |          | 0.95                           | 95.0      |                                       |               |     |      |
|                                   |          | 0.99                           | 99.0      |                                       |               |     |      |
|                                   |          | 1.01                           | 101.0     |                                       |               |     |      |
|                                   |          | 0.88                           | 88.0      |                                       |               |     |      |

Table 4. Accuracy and Precision Results in Monkey Plasma at 100 µg/mL. Similar results observed in Rat Plasma.

| Theoretical Concentration (µg/mL) | Analysis | Measured Concentration (µg/mL) | %Recovery | Mean Recovery for Individual Analysis | Mean Recovery | SD  | %RSD |
|-----------------------------------|----------|--------------------------------|-----------|---------------------------------------|---------------|-----|------|
| 100                               | 1        | 95.1                           | 95.1      | 93.6                                  | 96.0          | 4.1 | 4.3  |
|                                   |          | 91.8                           | 91.8      |                                       |               |     |      |
|                                   |          | 93.7                           | 93.7      |                                       |               |     |      |
|                                   |          | 94.2                           | 94.2      |                                       |               |     |      |
|                                   |          | 92.8                           | 92.8      |                                       |               |     |      |
|                                   |          | 93.7                           | 93.7      |                                       |               |     |      |
|                                   | 2        | 93.0                           | 93.0      | 95.1                                  | 96.0          | 4.1 | 4.3  |
|                                   |          | 96.3                           | 96.3      |                                       |               |     |      |
|                                   |          | 93.3                           | 93.3      |                                       |               |     |      |
|                                   |          | 95.1                           | 95.1      |                                       |               |     |      |
|                                   |          | 95.4                           | 95.4      |                                       |               |     |      |
|                                   |          | 97.4                           | 97.4      |                                       |               |     |      |
|                                   | 3        | 103.8                          | 103.8     | 99.4                                  | 96.0          | 4.1 | 4.3  |
|                                   |          | 104.0                          | 104.0     |                                       |               |     |      |
|                                   |          | 92.2                           | 92.2      |                                       |               |     |      |
|                                   |          | 100.4                          | 100.4     |                                       |               |     |      |
|                                   |          | 103.6                          | 103.6     |                                       |               |     |      |
|                                   |          | 92.4                           | 92.4      |                                       |               |     |      |

Table 5. Accuracy and Precision Results in Monkey Plasma at 150 µg/mL. Similar results observed in Rat Plasma.

| Theoretical Concentration (µg/mL) | Analysis | Measured Concentration (µg/mL) | %Recovery | Mean Recovery for Individual Analysis | Mean Recovery | SD  | %RSD |
|-----------------------------------|----------|--------------------------------|-----------|---------------------------------------|---------------|-----|------|
| 150                               | 1        | 143                            | 95.3      | 94.6                                  | 96.1          | 3.4 | 3.5  |
|                                   |          | 138                            | 92.0      |                                       |               |     |      |
|                                   |          | 141                            | 94.0      |                                       |               |     |      |
|                                   |          | 143                            | 95.3      |                                       |               |     |      |
|                                   |          | 145                            | 96.7      |                                       |               |     |      |
|                                   |          | 141                            | 94.0      |                                       |               |     |      |
|                                   | 2        | 140                            | 93.3      | 95.0                                  | 96.1          | 3.4 | 3.5  |
|                                   |          | 145                            | 96.7      |                                       |               |     |      |
|                                   |          | 142                            | 94.7      |                                       |               |     |      |
|                                   |          | 142                            | 94.7      |                                       |               |     |      |
|                                   |          | 143                            | 95.3      |                                       |               |     |      |
|                                   |          | 143                            | 95.3      |                                       |               |     |      |
|                                   | 3        | 151                            | 100.7     | 98.7                                  | 96.1          | 3.4 | 3.5  |
|                                   |          | 155                            | 103.3     |                                       |               |     |      |
|                                   |          | 139                            | 92.7      |                                       |               |     |      |
|                                   |          | 154                            | 102.7     |                                       |               |     |      |
|                                   |          | 150                            | 100.0     |                                       |               |     |      |
|                                   |          | 140                            | 93.0      |                                       |               |     |      |

## Carry-Over

Table 6. Rat Matrix Blanks Results from early in the batch.

| Matrix Blanks after HIGH-QC | % Area in Matrix Blank comparing to LLOQ | % Area in Matrix Blank comparing to HIGH-QC |
|-----------------------------|--|---|
| Matrix Blank 1              | 23.8                                     | 0.1   |
| Matrix Blank 2              | 14.2                                     | <0.1  |
| Matrix Blank 3              | 13.9                                     | <0.1  |
| Matrix Blank 4              | 12.2                                     | <0.1  |
| Matrix Blank 5              | 10.5                                     | <0.1  |
| Matrix Blank 6              | 8.8                                      | <0.1  |

Table 7. Rat Matrix Blanks Results from late in the batch.

| Matrix Blanks after HIGH-QC | % Area in Matrix Blank comparing to LLOQ | % Area in Matrix Blank comparing to HIGH-QC |
|-----------------------------|--|---|
| Matrix Blank 7              | 22.5                                     | 0.1   |
| Matrix Blank 8              | 12.3                                     | <0.1  |
| Matrix Blank 9              | 11.5                                     | <0.1  |
| Matrix Blank 10             | 9.8                                      | <0.1  |
| Matrix Blank 11             | 10.2                                     | <0.1  |
| Matrix Blank 12             | 7.0                                      | <0.1  |

The assessment of carry-over did not meet the acceptance criteria.

Therefore the method was modified and reads as follows:

“To minimize the impact of carryover the following steps must be taken.

- Standards and QCs must be injected from lowest to highest. Immediately following the High Standard and the High QC, three injections of needle wash followed by a saline blank will be made.
- Samples from individual animals will be analyzed together in reverse order from collection (i.e. the last time point will be analyzed first).
- If samples from multiple animals are analyzed in one analysis, in between individual animal sets make three injections of needle wash followed by a saline blank.
- If all time points are being analyzed for a control group, the samples for the control group must be analyzed separately from any group dosed with active.
- If pre-dose samples are included in the study, the pre-dose samples should be analyzed with the samples from the control group.
- To account for carry-over, if a sample with a calculated concentration above 20.0 µg/mL is followed within 1 injection by a sample(s) with a concentration of less than 5% of its concentration, the result(s) for the lower concentration sample(s) are rejected and the sample(s) must be repeated.”

## Freeze/Thaw Stability

Table 8. Freeze/Thaw Stability Results in Rat Plasma.

The mean Recovery for each validation level was not greater than ± 15.0% of Time 0. Similar Results observed in Monkey Plasma

| QC Name  | % Target Time 0 | % Target after 3 freeze/thaw cycles | % Difference from Time 0 | Mean of % Difference from Time 0 |
|----------|-----------------|-------------------------------------|--------------------------|----------------------------------|
| LOW-QC1  | 104.5           | 110.7                               | 5.9                      | 1.8                              |
| LOW-QC2  |                 | 106.6                               | 2.0                      |                                  |
| LOW-QC3  |                 | 107.4                               | 2.8                      |                                  |
| LOW-QC4  |                 | 105.5                               | 1.0                      |                                  |
| LOW-QC5  |                 | 107.2                               | 2.6                      |                                  |
| LOW-QC6  |                 | 101.1                               | -3.3                     |                                  |
| HIGH-QC1 | 106.0           | 97.8                                | -7.7                     | -6.8                             |
| HIGH-QC2 |                 | 96.7                                | -8.8                     |                                  |
| HIGH-QC3 |                 | 102.3                               | -3.5                     |                                  |
| HIGH-QC4 |                 | 99.1                                | -6.5                     |                                  |
| HIGH-QC5 |                 | 98.3                                | -7.3                     |                                  |
| HIGH-QC6 |                 | 98.7                                | -6.9                     |                                  |

## Stability in Injection Medium

Table 9. Stability in Injection Medium in Monkey Plasma under Refrigerated Conditions. 14 Days Analysis.

The mean Recovery for each validation level was not greater than ± 15.0% of Time 0 for both 7 Days and 14 Days Analysis under both Ambient and Refrigerated Conditions. Similar Results observed in Rat Plasma.

| QC Name  | Observed Concentration (µg/mL) Time 0 | Observed concentration (µg/mL) 14 days Analysis | % Difference from Time 0 | Mean of % Difference from Time 0 |
|----------|---------------------------------------|---|--------------------------|----------------------------------|
| LOW-QC1  | 1.01                                  | 1.01  | 0.0                      | 1.4                              |
| LOW-QC2  | 1.03                                  | 0.99  | -3.9                     |                                  |
| LOW-QC3  | 0.96                                  | 0.94  | -2.1                     |                                  |
| LOW-QC4  | 0.95                                  | 0.94  | -1.1                     |                                  |
| LOW-QC5  | 0.95                                  | 0.96  | 1.1                      |                                  |
| LOW-QC6  | 0.92                                  | 1.05  | 14.1                     |                                  |
| MID-QC1  | 90.7                                  | 90.0  | -0.8                     | 2.0                              |
| MID-QC2  | 94.2                                  | 93.3  | -1.0                     |                                  |
| MID-QC3  | 90.8                                  | 91.6  | 0.9                      |                                  |
| MID-QC4  | 89.1                                  | 90.1  | 1.1                      |                                  |
| MID-QC5  | 85.6                                  | 90.1  | 5.3                      |                                  |
| MID-QC6  | 88.1                                  | 93.8  | 6.5                      |                                  |
| HIGH-QC1 | 130                                   | 133   | 2.3                      | 1.9                              |
| HIGH-QC2 | 130                                   | 133   | 2.3                      |                                  |
| HIGH-QC3 | 130                                   | 130   | 0.0                      |                                  |
| HIGH-QC4 | 130                                   | 134   | 3.1                      |                                  |
| HIGH-QC5 | 130                                   | 134   | 3.1                      |                                  |
| HIGH-QC6 | 132                                   | 133   | 0.8                      |                                  |

## Stability in Plasma Under Ambient Conditions

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

The mean Recovery for each validation level was not greater than ± 15.0% of Time 0. Similar Results observed in Monkey Plasma.

| QC Name  | % Target Time 0 | % Target after 24-hour storage | % Difference from Time 0 | Mean of % Difference from Time 0 |
|----------|-----------------|--------------------------------|--------------------------|----------------------------------|
| LOW-QC1  | 106.3           | 115.7                          | 8.8                      | 2.5                              |
| LOW-QC2  |                 | 110.3                          | 3.8                      |                                  |
| LOW-QC3  |                 | 109.1                          | 2.6                      |                                  |
| LOW-QC4  |                 | 107.1                          | 0.8                      |                                  |
| LOW-QC5  |                 | 106.8                          | 0.5                      |                                  |
| LOW-QC6  |                 | 104.5                          | -1.7                     |                                  |
| HIGH-QC1 | 101.1           | 96.9                           | -4.2                     | -3.1                             |
| HIGH-QC2 |                 | 97.6                           | -3.5                     |                                  |
| HIGH-QC3 |                 | 98.7                           | -2.4                     |                                  |
| HIGH-QC4 |                 | 97.8                           | -3.3                     |                                  |
| HIGH-QC5 |                 | 98.6                           | -2.5                     |                                  |
| HIGH-QC6 |                 | 98.5                           | -2.6                     |                                  |

## Stability in Plasma at -80 °C

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

The mean Recovery for each validation level was not greater than ± 15.0% of Time 0. Similar Results observed in Rat Plasma.

| QC Name  | Observed Concentration (µg/mL) Time 0 | Average of Observed Concentrations (µg/mL) Time 0 | Observed concentration (µg/mL) Day 49 | % Difference from Average at Time 0 | Mean of % Difference from Time 0 |
|----------|---------------------------------------|---|---------------------------------------|-------------------------------------|----------------------------------|
| LOW-QC1  | 0.9                                   | 0.96  | 0.98                                  | 2.1                                 | 2.1                              |
| LOW-QC2  | 0.98                                  |   | 0.96                                  | 0.0                                 |                                  |
| LOW-QC3  | 1.06                                  |   | 0.95                                  | -1.0                                |                                  |
| LOW-QC4  | 0.9                                   |   | 0.95                                  | -1.0                                |                                  |
| LOW-QC5  | 0.94                                  |   | 1.05                                  | 9.4                                 |                                  |
| LOW-QC6  | 0.97                                  |   | 0.99                                  | 3.1                                 |                                  |
| HIGH-QC1 | 149                                   | 149   | 140                                   | -6.0                                | -8.5                             |
| HIGH-QC2 | 140                                   |   | 129                                   |                                     |                                  |