

# LC-FAIMS-MS Validated Quantitation Method for a Peptide in Rat Serum

Tobias Klassen, Ph.D. and Axel Roemer, Ph.D., A&M Labor, Bergheim, Germany

## Key Words

- Bioanalysis
- FAIMS
- Interference Removal
- Peptide
- Quantitation

## Goal

To validate an LC-FAIMS-MS bioanalytical method for a peptide in rat serum according to FDA guidelines.

## Introduction

Bioanalytical method development of peptides poses special problems for conventional LC-MS/MS technology. During the ionization step, the ion current is distributed among several charge states owing to the fact that there are multiple basic sites for protonation. This leads to many multiply charged species in a distribution envelope. At the outset of development, the method has less absolute signal than is often seen with singly charged small molecules.

During tandem MS, the method becomes increasingly selective but because the peptide bonds are nearly equal in energy, the ion current gets distributed among many equally low abundance fragments. To prevent this low response, researchers will often try Selected Ion Monitoring (SIM). Although SIM is a sensitive technique due to the high ion current observed, it is not very selective. As a consequence the complex matrix always contributes interferences at the  $m/z$  for the analyte, often resulting in high chemical background and co-eluting interferences.

FAIMS (high-Field Asymmetric waveform Ion Mobility Spectrometry) increases the selectivity of an assay based on ion mobility prior to mass analysis. In this study, FAIMS was used in combination with SIM at unit resolution (0.7 u FWHM) to validate a quantitative method for peptide analysis in rat serum according to GLP standards.

## Methods

Rat serum samples were precipitated by addition of trichloroacetic acid. The supernatant was injected onto a C18 column (30 x 2.1 mm, 3  $\mu$ m). Gradient elution was accomplished using 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. Ionization was performed by heated electrospray (400 °C) in the positive ionization mode. FAIMS conditions were set to the standard values of 5000V dispersion voltage, a carrier gas stream of 4 L/min (equimolar nitrogen and helium), and the inner and outer electrode temperatures of 70 °C and 90 °C, respectively. The compensation voltage parameter was optimized at +35 V. The quintuply charged peptide was detected by SIM at 1277.3 u. As internal standard an [<sup>15</sup>N]-labeled analogue was used (1292.7 u).

Chromatographic peak areas for the standard calibration samples were integrated in Thermo Scientific LCQUAN 2.5.5. Area ratios (analyte divided by the IS) were plotted vs. nominal concentration. Linear regression with 1/[concentration]<sup>2</sup> weighting was performed. Back-calculated concentrations for the standards and QC samples were reported.

Standard calibration samples (10–2000 ng/mL) were analyzed in one replicate each except at the LLOQ and ULOQ (two replicates each). Validation quality control samples were analyzed in six replicates at the LLOQ, 3x LLOQ, mid-range and ULOQ concentrations. The confirmation of LLOQ experiment was conducted with control plasma from six different individuals. Validation samples at the LLOQ concentration were prepared and analyzed together with unfortified “blank” samples from the same individuals.

## Results and Discussion

LC-SIM analysis resulted in chromatograms with numerous background peaks at the retention time of the peptide. As shown in Figure 1, at the lower level of quantitation (LLOQ) an integrable peak was detected but there were nearly co-eluting interferences that adversely affected the peak integration. Even for the internal standard (IS) there was a raised baseline indicating chemical background.

The assay could very nearly be validated at this level by normal LC-MS as shown in Tables 1-3 (Without FAIMS results, Batch 1). Unfortunately, a problem arose during the confirmation of LLOQ experiment as shown in Figure 2 and quantified in Table 4 (Without FAIMS results). Control plasma appeared to have greater than 20% of the LLOQ signal. According to the FDA guidance criteria, blank matrix samples should have analyte signal less than 20% relative to the LLOQ samples. The signal was not a result of endogenous analyte due to the synthetic, exogenous nature of the analyte. Thus we investigated the use of FAIMS to remove these interferences.

## Without FAIMS

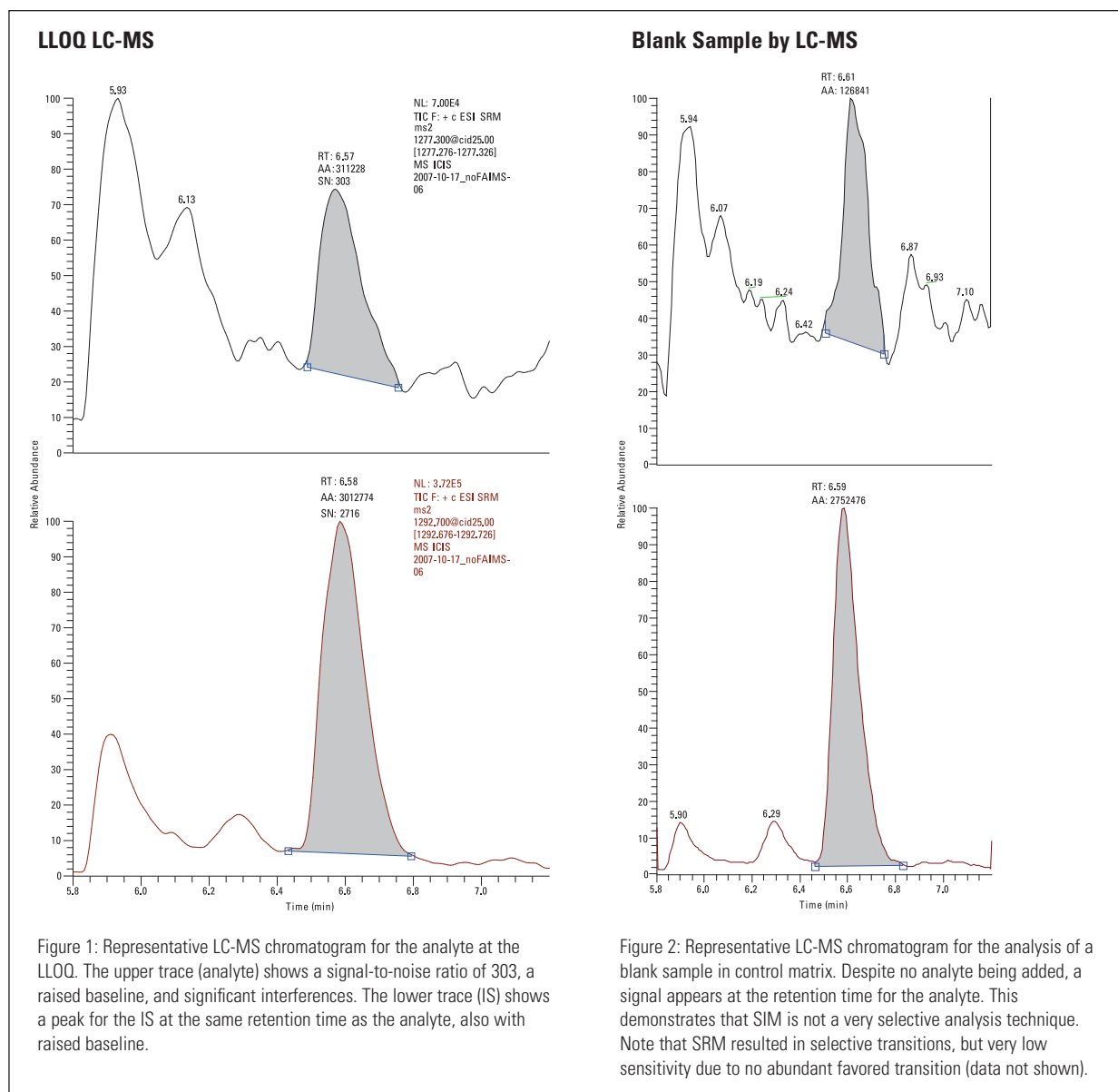


Figure 1: Representative LC-MS chromatogram for the analyte at the LLOQ. The upper trace (analyte) shows a signal-to-noise ratio of 303, a raised baseline, and significant interferences. The lower trace (IS) shows a peak for the IS at the same retention time as the analyte, also with raised baseline.

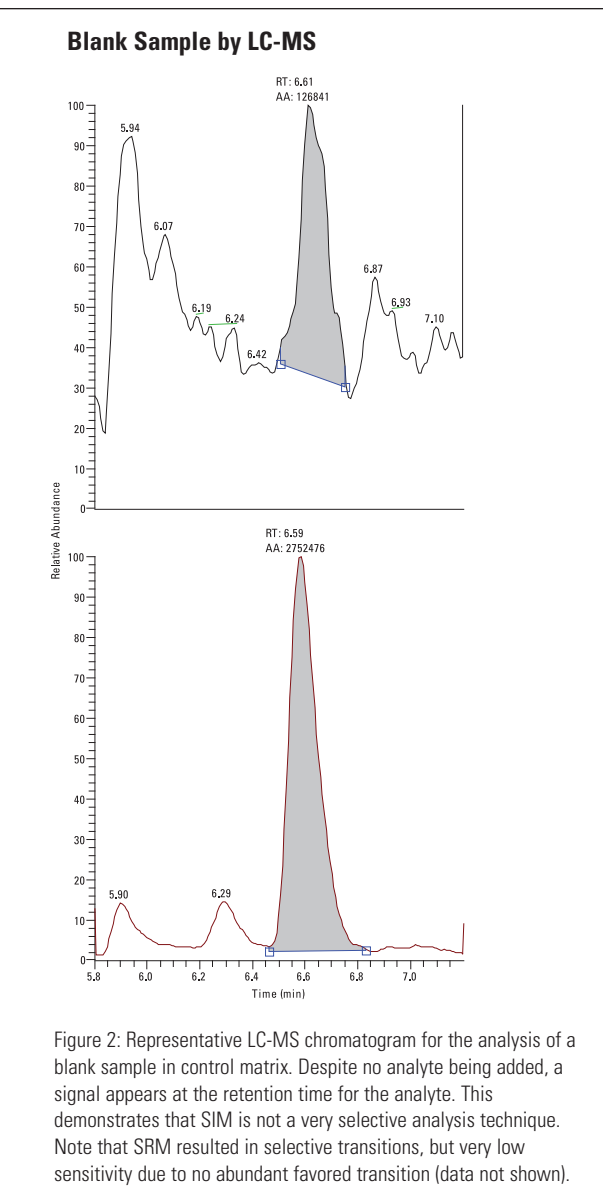


Figure 2: Representative LC-MS chromatogram for the analysis of a blank sample in control matrix. Despite no analyte being added, a signal appears at the retention time for the analyte. This demonstrates that SIM is not a very selective analysis technique. Note that SRM resulted in selective transitions, but very low sensitivity due to no abundant favored transition (data not shown).

## Calibration (Standard Samples)

### Without FAIMS

Conc. [µg/L]	K10 (10.0)	K30 (30.0)	K100 (100)	K250 (249)	K1000 (1000)	K2000 (1998)
Batch 1	10.3 9.67	29.9	105	250	977	1917 2032
Mean	9.98	29.9	105	250	977	1974
Accuracy	-0.204	-0.225	5.07	0.277	-2.35	-1.18

### With FAIMS

Conc. [µg/L]	K10 (10.0)	K30 (30.0)	K100 (100)	K250 (249)	K1000 (1000)	K2000 (1998)
Batch F	9.62 10.5	29.0	102	257	1027	1861 2029
Mean	10.0	29.0	102	257	1027	1945
Accuracy	0.396	-3.37	2.05	3.10	2.72	-2.65

Table 1: Calibration standard samples without and with FAIMS. There is essentially no difference between the %-deviation from theoretical.

## Calibration Regression Statistics

### Without FAIMS

Batch	Slope	Intercept	Coefficient of Determination (R <sup>2</sup> )
Batch 1	0.00700	0.0370	0.999

### With FAIMS

Batch	Slope	Intercept	Coefficient of Determination (R <sup>2</sup> )
Batch F	0.0109	0.0248	0.998

Table 2: Calibration regression statistics without and with FAIMS. The slope of the regression line with FAIMS appeared higher than without FAIMS, suggesting greater detector sensitivity to changing concentrations. With FAIMS, a lower y-intercept confirms that fewer interferences obstructed accurate and precise quantitation. The coefficient of determination was similar for both analyses.

With FAIMS

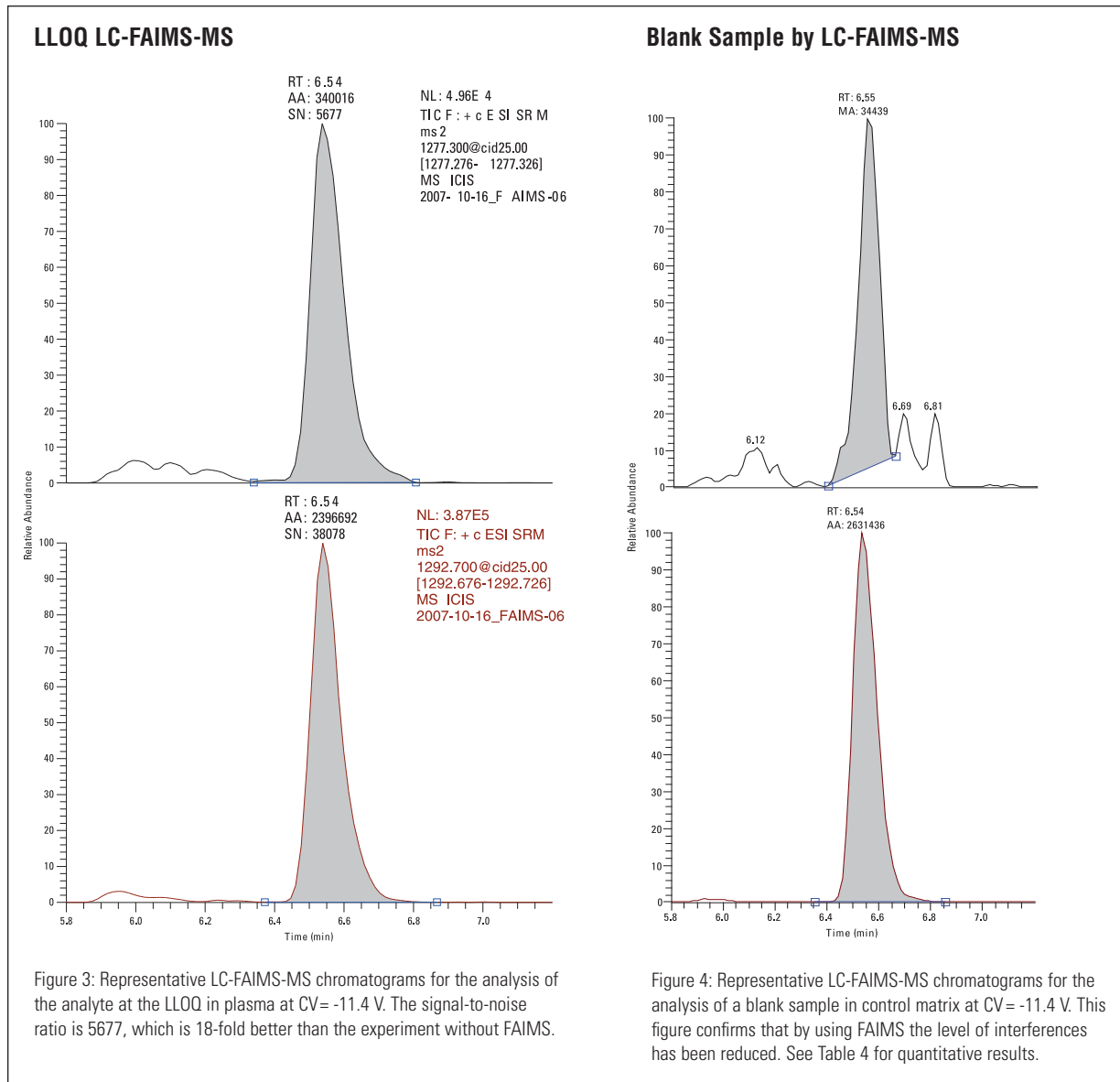


Figure 3: Representative LC-FAIMS-MS chromatograms for the analysis of the analyte at the LLOQ in plasma at CV= -11.4 V. The signal-to-noise ratio is 5677, which is 18-fold better than the experiment without FAIMS.

Figure 4: Representative LC-FAIMS-MS chromatograms for the analysis of a blank sample in control matrix at CV= -11.4 V. This figure confirms that by using FAIMS the level of interferences has been reduced. See Table 4 for quantitative results.

Validation Levels (Quality Control Samples)

Without FAIMS

Conc. [µg/L]	V10 (10.1)	V30 (29.6)	V500 (499)	V2000 (1995)
Batch 1	8.14	26.3	508	2015
	8.44	29.7	488	2026
	10.3	28.1	470	2085
	7.65	26.8	482	2061
	9.02	29.1	458	2033
	0.3	24.9	488	2089
Mean	8.96	27.5	482	2051
SD	1.10	1.81	17.3	31.3
CV[%]	<b>12.3</b>	<b>6.59</b>	<b>3.58</b>	<b>1.53</b>
Acc.[%]	<b>-11.3</b>	<b>-7.19</b>	<b>-3.35</b>	<b>2.83</b>

With FAIMS

Conc. [µg/L]	V10 (10.1)	V30 (29.6)	V500 (499)	V2000 (1995)
Batch F	9.44	30.9	544	2057
	10.5	29.7	559	1958
	8.84	27.8	514	1995
	8.50	30.3	512	1977
	9.00	28.4	538	2046
	10.8	29.6	537	1990
Mean	9.51	29.4	534	2004
SD	0.925	1.16	18.3	39.2
CV[%]	<b>9.73</b>	<b>3.95</b>	<b>3.42</b>	<b>1.96</b>
Acc.[%]	<b>-5.87</b>	<b>-0.573</b>	<b>7.03</b>	<b>0.439</b>

Table 3: Validation levels for quality control. Precision was comparable for both methods, but accuracy was better using FAIMS.

## Confirmation of LLOQ

### Without FAIMS

Conc. [µg/L]	V0 (areas*)	V10 (areas)
Batch 1	78623	289681
	105511	298043
	120187	358959
	96876	282550
	147404	322797
	107077	334798
Mean	109280	314471
% area of LLOQ	<b>34.8</b>	

### With FAIMS

Conc. [µg/L]	V0 (areas*)	V10 (areas)
Batch F	35405	369222
	34406	391589
	16812	371375
	16191	320791
	16470	323218
	30316	357612
Mean	24933	355635
% area of LLOQ	<b>7.01</b>	

\*Internal Standard contaminated by analyte (about 1%, is about 1 ng/mL)

Table 4: Confirmation of LLOQ (10 ng/mL) experiment. Without FAIMS blank serum extracts showed greater than 20% of the LLOQ signal. Because FAIMS could remove the interferences, the remaining signal is only 7% of the LLOQ. This remaining signal could be attributed to unlabeled material present in the synthetically prepared IS.

Figure 3 shows representative LC-FAIMS-MS chromatograms in which the chemical background is greatly reduced in comparison to without FAIMS (Figure 1). In addition, there are significantly fewer co-eluting peaks for both the analyte and IS channels. The signal-to-noise ratio was improved 18-fold.

As shown in Table 1, calibration standard samples with (Batch F) and without (Batch 1) FAIMS resulted in essentially no difference in the % deviation from theoretical (maximum 5.07% deviation from theoretical). Calibration regression statistics revealed a significantly higher slope with FAIMS (0.0109 with and 0.00699 without FAIMS, respectively) suggesting the assay with FAIMS provided greater detector sensitivity to changing concentrations (Table 2). With FAIMS, a lower y-intercept was obtained (0.0248 with and 0.0370 without FAIMS, respectively) confirming that fewer interferences obstructed accurate and precise quantitation. The coefficient of determination was larger than 0.998 for both analyses. In Table 3, the validation (quality control) samples showed better accuracy with FAIMS than without (7.03% and 11.3%, respectively). Precision, as defined by the coefficient of variation (CV) was comparable for both methods (9.73% with FAIMS and 12.3% without FAIMS, respectively).

Control blank plasma samples also contained fewer interference peaks as demonstrated in Figure 4 and quantified in Table 4 (FAIMS results). In the confirmation of the LLOQ (10 ng/mL) experiment without FAIMS, blank serum extracts in six matrix sources demonstrated greater than the FDA guideline level of < 20% relative to the LLOQ. The determined value was 34.8% area relative to the LLOQ. In comparison, using FAIMS nearly all of this interference was removed resulting in an acceptable level of 7.01% area relative to the LLOQ in the six matrix sources. This result confirmed by synthesis that the IS was contaminated by an acceptable trace of unlabeled material.

## Conclusions

A peptide quantitation assay was validated using FAIMS. Endogenous interferences which prevented validation in a traditional LC-MS assay, were successfully removed, resulting in an 18-fold increase in signal-to-noise ratio. The confirmation of LLOQ experiment was acceptable because FAIMS removed the interferences.

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