An in-sample calibration evaluation approach for the quantification of peptides using an LC-MS/MS workflow

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ABSTRACT

Protein and peptide therapeutics are becoming increasingly important biologics, given their high target specificity. As a result, there is a strong demand for the generation of highly accurate and sensitive bioanalytical methods to ensure proper testing for efficacy throughout the biopharmaceutical development process. Current methods for the bioanalysis of peptide therapeutics are often met with challenges, including limited sensitivity and presence of matrix interferences. Here, an in-sample calibration approach was used to evaluate specificity and sensitivity for the quantification of peptides using an LC-MS/MS platform.

INTRODUCTION

Given their high target specificity, protein and peptide therapeutics are becoming increasingly important therapeutic products. As a result, there is a strong demand for the generation of highly accurate and sensitive bioanalytical methods to ensure proper quantitative measurement throughout the biopharmaceutical development process. Current methods for the bioanalysis of peptide therapeutics are often met with challenges involving limited sensitivity and the presence of matrix interferences.

Herein, a SCIEX 7500 system was used for the quantification of a series of peptides in matrix. Multiple hardware improvements on the ion source and the front-end of the mass analyzer significantly boosted the systems sensitivity and %CV for low-level quantification.[1,2] An in-sample calibration approach was applied to evaluate specificity and sensitivity for quantifying peptides using a LC-MS/MS platform.

MATERIALS AND METHODS

Sample preparation:

The reference set (6x5 LC-MS/MS peptide reference mix, Promega) contains 6 peptides with a mixture of 5 isotopologues for each peptide. The isotopologues are introduced into the mixture with a 10-fold molar increase. Therefore, with each sample injection, a linear range of 4 orders of magnitude was examined for each of the 6 peptides.

Samples were prepared by spiking the 6x5 peptide mix into a trypsin digest of BSA. The BSA digest was prepared at a concentration of 60 fmol/µL in 0.1% formic acid with 2% acetonitrile in water. Two samples were prepared, with the final concentration of the peptides ranging from 1 amol/µL to 10 fmol/µL and 10 amol/µL to 100 fmol/µL, each in 60 fmol/µL BSA digest.

LC conditions:

An ExionLC system was used for analyte separation. Gradient and other chromatographic conditions for the assay are summarized in Tables 1 and 2. A volume of 1 µL or 5 µL of the sample was loaded for analysis. The flow rate was set to 0.6 mL/min.

Mass spectrometry conditions:

A SCIEX 7500 system with the OptiFlow Pro ion source operated in MRM mode. All source and MS parameters were optimized and reported in Tables 3 and 4. The dwell time was set to 20 ms for optimal assay sensitivity.

Samples were analyzed in 6 replicates, using both 1 and 5 µL injection volumes.

Table 1. Chromatographic conditions.

Parameter	Setting
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*Acetonitrile*
Analytical column	*2.1 x 50 mm, 1.8 µm, 100 Å, Acquity UPLC HSS T3*
Analytical column temperature	*60°C*
Injection volume	*1 and 5 µL*

Table 2. LC method.

Time (min)	Mobile phase %A	Mobile phase %B
0.0	*98*	*2*
0.5	*98*	*2*
5.50	*65*	*35*
5.51	*2*	*98*
7.00	*2*	*98*
7.01	*98*	*2*
8.00	*98*	*2*

Table 3. Source conditions for the SCIEX 7500 system.

Parameter	Setting
Polarity	*Positive*
Curtain gas	*45 psi*
Gas 1	*45 psi*
Gas 2	*60 psi*
CAD gas	*12*
Ion spray voltage	*2000 V*
Source temperature	*400°C*

Table 4. MRM transitions and MS parameters for analysis of the 6x5 peptide mix on the SCIEX 7500 system. The measured precursor ions had a +2 charge. The CXP and EP values used for the analysis were 20 V and 10 V, respectively.

Peptide	Isotopologue number	Q1 mass (m/z)	Q3 mass (m/z)	CE (V)
VTSGSTSTSR	*1*	*496.7*	*792.4*	*22.6*
VTSGSTSTSR	*2*	*499.7*	*792.4*	*22.6*
VTSGSTSTSR	*3*	*502.3*	*792.4*	*22.6*
VTSGSTSTSR	*4*	*504.8*	*797.4*	*22.6*
VTSGSTSTSR	*5*	*509.8*	*806.4*	*22.6*
LASVSVSR	*1*	*414.7*	*644.4*	*18.5*
LASVSVSR	*2*	*417.7*	*650.4*	*18.5*
LASVSVSR	*3*	*420.8*	*656.4*	*18.5*
LASVSVSR	*4*	*424.3*	*656.4*	*18.5*
LASVSVSR	*5*	*428.3*	*660.4*	*18.5*
YVYVADVAAK	*1*	*553.8*	*844.5*	*21.5*
YVYVADVAAK	*2*	*556.8*	*850.5*	*21.5*
YVYVADVAAK	*3*	*559.8*	*856.5*	*21.5*
YVYVADVAAK	*4*	*562.8*	*856.5*	*21.5*
YVYVADVAAK	*5*	*566.8*	*864.5*	*21.5*
VVGGLVALR	*1*	*447.3*	*695.4*	*20.1*
VVGGLVALR	*2*	*450.3*	*695.4*	*20.1*
VVGGLVALR	*3*	*453.3*	*695.4*	*20.1*
VVGGLVALR	*4*	*456.3*	*701.5*	*20.1*
VVGGLVALR	*5*	*459.8*	*708.5*	*20.1*
LLSLGAGEFK	*1*	*521.8*	*816.4*	*23.9*
LLSLGAGEFK	*2*	*525.3*	*816.4*	*23.9*
LLSLGAGEFK	*3*	*528.8*	*816.4*	*23.9*
LLSLGAGEFK	*4*	*532.3*	*823.4*	*23.9*
LLSLGAGEFK	*5*	*537.3*	*833.5*	*23.9*
LGFTDLFSK	*1*	*518.3*	*922.5*	*23.7*
LGFTDLFSK	*2*	*521.8*	*922.5*	*23.7*
LGFTDLFSK	*3*	*525.3*	*929.5*	*23.7*
LGFTDLFSK	*4*	*530.3*	*939.5*	*23.7*
LGFTDLFSK	*5*	*535.3*	*949.5*	*23.7*

- The peptide reference matrix contained the 6 peptides VTSGSTSTSR, LASVSVSR, YVYVADVAAK, VVGGLVALR, LLSLGAGEFK and LGFTDLFSK. For each peptide, a mixture of 5 isotopologues were introduced
- Each of the isotopologues were distinguished by mass through the incorporation of stable labeled amino acids. For example, peptide VTSGSTSTSR had 5 heavy labelled isotopologues each labeled from heavy 1 to heavy 5 with distinguishable masses (Table 4)
- The isotopologues were mixed with a 10-fold molar increase enabling an analysis of a wide linear range within a single sample injection

RESULTS

Significant challenges can arise when quantifying lower abundant peptides in matrix. Improved front-end capabilities of the SCIEX 7500 system present better ion generation and transmission, which facilitates quantification of low-level peptides present in matrix.

Peptide quantification was performed using MRM mode on the SCIEX 7500 system. Each calibration point was measured in 6 replicates. The quantitative criteria for %CV was less than 20% and accuracy was within ±20% of the nominal concentration at the level of the LLOQ. For the remaining concentrations, the %CV was required to be less than 15%, while the accuracy was required to be within ±15% of the nominal concentration.

As shown in Figure 1, the on-column LLOQ for peptides VTSGSTSTSR, LASVSVSR, YVYVADVAAK, VVGGLVALR, LLSLGAGEFK and LGFTDLFSK were 100 amol, 5 amol, 10 amol, 1 amol, 10 amol and 50 amol, respectively. Injection volumes were either 1 µL or 5 µL and are labeled in the LLOQ XICs in Figure 1.

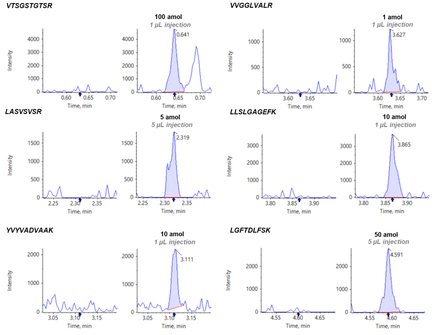

Figure 1. XICs of the peptides at the LLOQ level and the matrix blank on the SCIEX 7500 system. The LLOQs for the peptides VTSGSTSTSR, LASVSVSR, YVYVADVAAK, VVGGLVALR, LLSLGAGEFK and LGFTDLFSK were 100 amol, 5 amol, 10 amol, 1 amol, 10 amol and 50 amol, respectively.

Table 5. Accuracy and precision at each concentration level on the SCIEX 7500 system. Samples were analyzed in 6 replicates. The accuracy and precision values are shown in combination from both injection volumes of 1 µL and 5 µL.

	VTSGSTGTSR		LASVSVSR		YVYVADVAAK		VVGGLVALR		LLSLGAGEFK		LGFTDLFSK	
Concentration (amol)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
1	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*15.9*	*99.7*	*N/A*	*N/A*	*N/A*	*N/A*
5	*N/A*	*N/A*	*17.4*	*94.6*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*
10	*N/A*	*N/A*	*13.9*	*89.8*	*N/A*	*N/A*	*3.2*	*97.6*	*9.4*	*99.9*	*N/A*	*N/A*
50	*N/A*	*N/A*	*3.4*	*98.8*	*7.0*	*98.4*	*3.4*	*98.8*	*6.0*	*97.1*	*11.0*	*111.6*
100	*19.7*	*88.1*	*3.6*	*107.4*	*7.1*	*106.5*	*3.7*	*101.1*	*6.3*	*100.0*	*7.8*	*91.6*
500	*N/A*	*N/A*	*1.5*	*102.8*	*2.1*	*107.8*	*1.5*	*102.8*	*2.7*	*102.5*	*5.6*	*93.1*
1000	*4.1*	*113.1*	*1.2*	*103.2*	*2.4*	*102.1*	*1.6*	*101.7*	*1.7*	*100.1*	*4.1*	*94.6*
5000	*2.1*	*113.7*	*1.8*	*104.2*	*1.5*	*102.3*	*1.8*	*104.2*	*1.5*	*100.4*	*3.1*	*95.3*
10000	*2.6*	*98.8*	*1.2*	*99.6*	*1.2*	*99.7*	*2.6*	*99.8*	*1.3*	*100.0*	*1.9*	*96.9*
50000	*1.5*	*98.5*	*1.7*	*99.6*	*2.1*	*99.7*	*1.7*	*99.6*	*2.1*	*99.9*	*2.9*	*100.0*
500000	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*2.9*	*100.1*

Accurate and reproducible quantification was achievable by loading as low as 1 amol on-column, facilitating the quantification of low-abundant peptides. The overall %CV was less than 20%, with accuracy within ±15% of the nominal concentration (Table 5). This demonstrates the overall assay sensitivity where low amol-level concentrations were detected and quantified with excellent accuracy and precision.

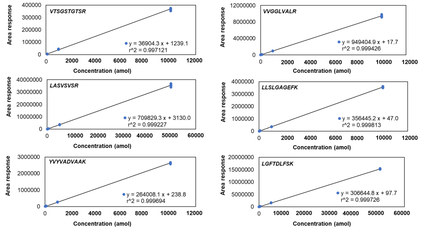

Figure 3. Calibration curves based on the XIC areas of the peptides on the SCIEX 7500 system. Strong linearity was achieved across the peptide concentrations examined. Since 2 injection volumes were used for the analysis, the injection volume enabling the most sensitive quantification of peptides was selected to display overall linearity of the assay. Linearity was plotted using the data from 1 µL, 5 µL, 1 µL, 1 µL, 1 µL and 5 µL injections for peptides VTSGSTSTSR, LASVSVSR, YVYVADVAAK, VVGGLVALR, LLSLGAGEFK and LGFTDLFSK, respectively.

Strong linearity across the peptides was achieved with an overall LDR up to 4.7 orders of magnitude across the linear range tested (Figure 3). The hardware improvements on the SCIEX 7500 system provided significant sensitivity gains for peptide quantification facilitating quantification of low-abundant peptides.

CONCLUSIONS

- Peptide quantification as low as 1 amol on-column was demonstrated with front-end improvements enabling greater ion generation and transmission on the SCIEX 7500 system
- Low-amol on-column LLOQs for peptide quantification were achieved with GLP accuracy, precision and linearity
- Superior sensitivity was observed for peptide quantification with greater ion generation and transmission provided by the improved front-end technology of the SCIEX 7500 system

REFERENCES

1. Enabling new levels of quantification. SCIEX technical note, RUO-MKT-02-11886-A.
2. High sensitivity MRM workflow for signature peptide quantification. SCIEX technical note, RUO-MKT-02-11882-A.

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