Sensitive signature peptide quantification in a complex matrix using accurate mass spectrometry

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ABSTRACT

Conventional workflows for quantitative bioanalyses of peptides and proteins are increasingly adapted to LC-MS/MS platforms such as triple quadrupole mass spectrometers, due to their great quantitative performance and excellent sensitivity. More recently, suggestions to employ accurate mass spectrometry for quantitative bioanalysis have emerged because of better selectivity. However, accurate mass spectrometry platforms, such as traditional time-of-flight (TOF) systems, often lack sensitivity due to limited duty cycle in between TOF pulses. In this study, 3 signature peptides were quantified using a novel accurate mass spectrometer with greater MS/MS sampling efficiency to increase quantitative sensitivity in a complex matrix.

INTRODUCTION

Traditional workflows for quantitative bioanalysis of peptides and proteins, such as immunological assays, have been displaced by LC-MS/MS analysis using triple quadrupole mass spectrometers. While the triple quadrupole platform has been the gold standard for most bioanalytical workflows, offering great sensitivity and quantitative performance, accurate mass spectrometry has been increasingly suggested for quantitative bioanalysis.[1,2] However, accurate mass spectrometry platforms, such as traditional time-of-flight (TOF) systems, often lack sensitivity due to limited duty cycle in between TOF pulses.

With the introduction of the ZenoTOF 7600 system, the improvement in MS/MS sampling efficiency offers a robust and sensitive platform to support routine peptide and protein quantification. Here, the Zeno trap controls the ion beam from the collision cell which facilitates greater ion transmission to the TOF accelerator, improving the duty cycle to ≥90% (classical TOF is below 30%) (Figure 1). As a result, this enhances the overall MS/MS sampling efficiency enabling the ZenoTOF 7600 system to be highly advantageous for quantitative bioanalysis workflows that can benefit from the accessibility of the full product ion profile and the improvement in sensitivity using the Zeno trap.[3,4]

In this technical note, 3 peptides were selected as model analytes to evaluate the quantitative performance of signature peptides on the ZenoTOF 7600 system. Ultra-low LLOQs, ranging from 0.025 fmol/µL to 0.05 fmol/µL were achieved with an LDR greater than 4.3 orders of magnitude. The measured upper limit of quantification (ULOQ) ranged from 500 fmol/µL to 2000 fmol/µL.

MATERIALS AND METHODS

Sample preparation:
Plasma proteins were precipitated with cold methanol and the resulting pellet was solubilized in 200 mM ammonium bicarbonate in 10:90 (v/v) methanol/water after centrifugation. Digestion was performed using trypsin for 1 hour at 60ºC. The digestion was stopped by acidification using formic acid.[5] The digested plasma was diluted by 200x using 5:1:94 (v/v/v) acetonitrile/formic acid/water. The diluted plasma digest was used as a biological matrix for the assay. Synthesized peptides were spiked into the digested plasma solution followed by serial dilution in the matrix.

LC conditions:
The separation was performed at a flow rate of 0.4 mL/min using an ExionLC system. A Phenomenex bioZen Peptide XB-C18 column (2.1 x 50 mm, 2.6 µm, 100 Å) was used for separation. The column oven temperature was set to 40ºC. The mobile phase A consisted of 0.1% formic acid in water, while the mobile phase B was composed of 0.1% formic acid in acetonitrile. Gradient conditions are summarized in Table 1. A volume of 10 µL was injected for analysis. All samples were analyzed in triplicate.

Mass spectrometry conditions:
Data were acquired in positive mode using Zeno MRM^{HR} on a ZenoTOF 7600 system. Collision energy (CE), source and MS parameters were optimized for all the signature peptides. A summary of the source and MS parameters and the Zeno trap settings is displayed in Table 2. Additionally, the MRM^{HR} parameters and fragments used for quantification for each of the signature peptides are summarized in Table 3. Unit Q1 resolution was used for the analysis.

Table 1. LC gradient conditions.

Time (min)	Mobile phase B (%)	Mobile phase A (%)
0	97	3
1	97	3
4	60	40
4.1	10	90
5.1	10	90
6.1	97	3
8	97	3

Table 2. Source and MS conditions.

Parameter	Value	Parameter	Value
Curtain gas	30 psi	Source temp.	600˚C
Ion source gas1	50 psi	Ion source gas 2	60 psi
CAD gas	7	Ion spray voltage	5500 V
MS accumulation time	80 ms	MS/MS accumulation time	10 ms
TOF MS start mass (m/z)	600	TOF MS stop mass (m/z)	1200
TOF MS/MS start mass (m/z)	100/600	TOF MS/MS stop mass (m/z)	600/1200
Zeno threshold	20,000 cps		

Table 3. MRM^{HR} parameters and fragments used for quantification.

Peptide	Q1 mass (m/z)	Q3 mass (m/z)	DP (V)	CE (V)
FNWYVDGVEVHNAK (b2)	560.27	262.118	80	30
FNWYVDGVEVHNAK (y4)	560.27	469.252	80	30
FNWYVDGVEVHNAK (y6)	560.27	697.363	80	30
AGLIVAEGVTK* (y6)	533.32	612.344	80	32
LGLDFDSFR* (y5)	540.27	681.322	80	34
LGLDFDSFR* (y6)	540.27	796.351	80	34
LGLDFDSFR* (y8)	540.27	966.457	80	34

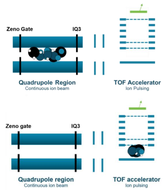

Figure 1. Zeno trap enables ion beam control from the collision cell prior to entrance into the TOF accelerator.

RESULTS

FNWYVDGVEVHNAK, AGLIVAEGVTK* and LGLDFDSFR* were used as model peptides to evaluate the quantification of signature peptides on the ZenoTOF 7600 system. The peptides were spiked into processed rat plasma at concentrations ranging from 0.025 fmol/µL to 2000 fmol/µL.

Quantification was performed using the following strategies: 1) using the most sensitive fragment ion and 2) using the sum of multiple highly abundant fragment ions. For the former method, fragment ions y6, y8 and y6 were used for the quantification of peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively.

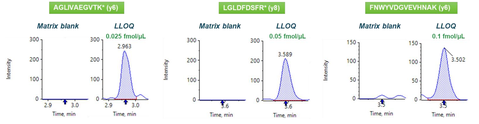

Figure 2. Extracted ion chromatograms (XICs) of the matrix blank and LLOQ using Zeno MRM^{HR}. Using the single fragment ion approach, an LLOQ of 0.025 fmol/µL, 0.05 fmol/µL and 0.1 fmol/µL was achieved for peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No matrix interferences were observed in the blank.

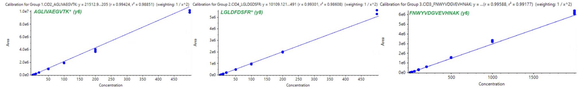

Figure 3. Calibration curves for the signature peptides using single fragment ion quantification. The linear range covered 0.025 fmol/µL to 2000 fmol/µL with an overall LDR of greater than 4 orders of magnitude.

Table 4. Calculated concentration, precision and accuracy for quantification using a single fragment ion.

	AGLIVAEGVTK*		LGLDGDSFR*		FNWYVDGVEVHNAK	
Concentration (fmol/µL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.025	101.81	13.12	NA	NA	NA	NA
0.05	98.26	14.60	107.89	10.46	NA	NA
0.1	95.82	5.26	114.93	8.42	103.95	13.79
0.2	98.11	7.86	100.17	6.97	NA	NA
0.5	105.30	9.69	100.96	10.42	84.57	12.94
1	99.68	2.91	95.81	1.40	96.83	10.51
2	101.97	3.42	101.73	4.07	91.76	8.17
5	109.58	0.72	99.81	1.44	92.60	4.49
10	104.35	2.45	96.09	7.08	99.28	1.90
20	103.73	4.36	97.55	1.58	102.25	2.49
50	96.32	4.19	92.26	1.74	101.98	1.44
100	94.53	0.81	92.91	4.23	103.62	3.11
200	93.93	4.38	95.87	1.20	103.57	1.77
500	96.61	2.16	104.02	3.42	104.99	0.79
1000	NA	NA	NA	NA	109.50	2.75
2000	NA	NA	NA	NA	105.10	2.56

The linear range for the single fragment ion approach covered concentrations from 0.025 fmol/µL to 2000 fmol/µL (Figure 3). An LDR greater than 4 orders of magnitude was observed for each of the calibration curves.

Calculated concentrations for each calibration point were within ±15% of the nominal value (Table 4). The overall accuracy of the LLOQ was within 8% of the nominal concentration, indicating a highly accurate quantification platform for peptides at low concentrations. As shown in Table 4, the %CV value was less than 15%, demonstrating high reproducibility.

For the latter strategy, the XIC responses from fragment ions y5, y6 and y8 were added for the quantification of peptide LGLDFDSFR*. For the quantification of peptide FNWYVDGVEVHNAK, the XIC responses from fragment ions b2, y4 and y6 were added.

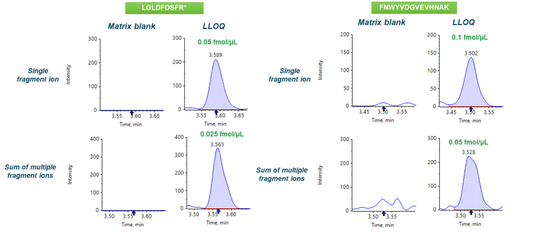

Figure 4. XICs of the matrix blanks and spiked peptides at the LLOQ using summation of multiple fragment ions. A 2-fold improvement in sensitivity was observed when multiple highly abundant fragment ions were summed for quantification. LLOQs of 0.025 fmol/µL and 0.05 fmol/µL were reached for the peptides LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No matrix interferences were observed in the blank.

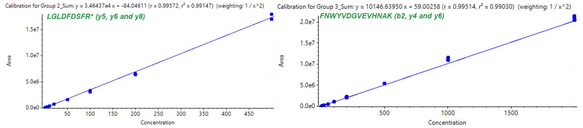

Figure 5. Calibration curves for the signature peptides using multiple fragment ion quantification. The linear range covered 0.025 fmol/µL to 2000 fmol/µL with an overall LDR of greater than 4.3 orders of magnitude.

Table 5. Calculated concentration, precision and accuracy for quantification using summation of multiple fragment ions.

	LGLDGDSFR*		FNWYVDGVEVHNAK	
Concentration (fmol/µL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.025	93.84	19.71	NA	NA
0.05	107.80	8.65	107.08	4.45
0.1	106.56	2.66	90.44	9.57
0.2	100.82	5.95	NA	NA
0.5	106.00	8.53	85.74	3.14
1	103.68	1.75	88.83	6.67
2	111.02	4.43	89.43	4.66
5	100.69	2.56	90.38	3.45
10	95.64	5.60	103.43	2.71
20	97.52	1.32	107.77	2.87
50	91.46	1.23	104.44	1.23
100	91.87	4.85	105.68	1.50
200	93.23	1.35	106.39	4.00
500	99.85	3.24	106.89	0.80
1000	NA	NA	110.21	2.56
2000	NA	NA	103.28	2.33

The linear range for the multiple fragment ion approach covered concentrations from 0.025 fmol/µL to 2000 fmol/µL (Figure 5). Strong linearity was observed for each of the calibration curves with an LDR of greater than 4.3 orders of magnitude.

A 2-fold improvement in LLOQ was achieved when multiple highly abundant fragment ions were summed for quantification (Figure 5). LLOQs of 0.025 fmol/µL and 0.05 fmol/µL were achieved for peptides LGLDFDSFR* and FNWYVDGVEVHNAK, respectively.

Calculated concentrations for each calibration point were within ±15% of the nominal value (Table 5). The overall accuracy was within 8% of the nominal concentration at the level of the LLOQ, indicating a highly accurate platform for low-level quantification. The overall %CV value for the acquired data, including the LLOQ, was less than 20%.

CONCLUSIONS

A highly sensitive signature peptide quantification workflow was developed using the ZenoTOF 7600 system. Low-amol/µL levels of quantification for signature peptides were reached using a Zeno MRM^{HR} workflow. Summation of multiple fragment ions enhances the LLOQ up to 2-fold with the availability of TOF MS/MS data and improvements in MS/MS sensitivity using the Zeno trap. GLP-level accuracy and precision for signature peptide quantification was achieved with greater than 4.3 orders of magnitude in LDR.

Overall, the assay demonstrated outstanding accuracy, precision and linearity, highlighting the robustness and performance of the developed method for the quantification of signature peptides.

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