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An LC–MS/MS Method was Developed and Validated for the Quantification of an Intact 9kDa Peptide in Animal Plasma Samples from a Toxicology Study

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Background

A novel peptide has been developed for the restoration of the activity of the protein phosphatase 2A (PP2A) tumor suppressor. PP2A is thought to play an important role in the deactivation of proteins that control proliferation and the programmed cell death. Cancer cells have developed mechanisms to inhibit PP2A so that uncontrolled growth can occur. One of these mechanisms is by making a protein known as SET in quantities that exceed the normal levels. The novel peptide is in clinical studies to determine if the peptide can deactivate the SET protein and restore the function of PP2A.

Purpose

- Develop an LC-MS/MS method for the quantification of a large intact peptide
- Overcome column secondary interactions by chromatography since the peptide had multiple basic residues
- Determine from 14 charge states which state had the best fragmentation response, and stabilize that charge state in solution

Methods

Samples were prepared with an aliquot of the compound and internal standard (IS) mixed with 200mM $HCOONH_4$ pH 3.0 w/HCOOH in each well. The plate was then vortexed for 1 min. Methanol was added to each well, vortexed, incubated for 20 min, followed by a partial drying step. The supernatant was transferred and filtered into a 96 well plate, then mixed with a reconstitution solution of 0.07% TFA. Reconstitution solution with HCOOH and TFA were tested and it was determined that a reconstitution solution of 0.07% TFA gave the best stability for the LLOQ quantification.

Samples were analyzed using reverse phase chromatography on Phenomenex, Aeris™ WIDEPORE C4, 200 Å, 50 x 2.1 mm, 3.6 µm, Waters Acquity UPLC® System equipped with a SCIEX Triple Quad™ 6500 mass spectrometer, and an ESI source.

Mobile Phase A:	90:10:0.07% ACN: H_2O: TFA
Mobile Phase B:	0.07% TFA
Infusion Mobile Phase:	80:15:5% ACN:CH_3COOH:H_2O
Flow Rate:	0.4 ml/min for all pumps
Gradient Initial:	5% Mobile Phase A
Gradient 5.5 min:	45% Mobile Phase A
Gradient Final:	5% Mobile Phase A

The peptide was monitored in multiple reaction monitoring (MRM) mode. To overcome TFA suppression of the response, a post-column infusion of mobile phase 80:15:5% ACN:CH_3COOH:H_2O was used. The overall amount of acetic acid to the source was 7.5%. Diverting the chromatographic eluent to waste, except during the elution of the peaks of interest, helped with overall instrument performance.

During the compound infusion, 0.1 amu fluctuations of a selected charge state were noticed. Summing the 0.1 amu response shifts from Q1 helped minimize fluctuation of the response.

Results

Table 1. Sample Stability.

Batch	LLOQ QC 0.250 µg/mL	QC A 0.750 µg/mL	QC B 1.40 µg/mL	QC C 5.70 µg/mL	DF = 10 QC D 30.0 µg/mL
33	0.246	0.728	1.40	5.72	29.3
	0.240	0.741	1.39	5.49	29.4
	0.236	0.704	1.38	5.53	28.3
	0.232	0.727	1.48	5.80	29.4
	0.258	0.679	1.45	6.00	28.2
	0.246	0.686	1.42	5.63	30.1
Mean	0.243	0.711	1.42	5.70	29.1
% CV	3.8	3.5	2.7	3.3	2.5
% Theoretical	97.2	94.8	101.4	100.0	97.0
n	6	6	6	6	6

The stability of processed (extracted) samples after being injected and analyzed with calibration standards, followed by storage in a polypropylene 96 well plate for 143 hr at 5°C and subsequent reinjection was evaluated using replicate LLOQ, low, medium, high, and dilution QC samples. The results indicate that samples may be extracted and stored at 5°C for up to 143 hr following extraction and then reinjected.

Table 2. Matrix Effect.

Batch	Lot#	LLOQ		High	
		0.250 µg/mL	% Dev.	5.70 µg/mL	% Dev.
28	1	0.248	-0.8	5.24	-8.1
	2	0.249	-0.4	5.20	-8.8
	3	0.274	+9.6	4.88	-14.4
	4	0.229	-8.4	5.12	-10.2
	5	0.262	+4.8	5.23	-8.2
	6	0.268	+7.2	5.01	-12.1
Mean		0.255		5.11	
% CV		6.4		2.8	
% Theoretical		102.0		89.6	
n		6		6	

Six different lots of plasma (EDTA) were fortified with the peptide at the LLOQ and high QC sample concentrations and then analyzed to evaluate potential matrix effects. No matrix effect was observed in any of the six acidified plasma lots that were fortified with the peptide at the concentration of the LLOQ or at the concentration of the high QC sample.

Table 3. Hemolyzed Sample Integrity.

Batch	Lot#	LLOQ		High	
		0.250 µg/mL	% Dev.	5.70 µg/mL	% Dev.
30	1	0.211	-15.6	4.96	-13.0
	2	0.195	-22.0	5.27	-7.5
	3	0.218	-12.8	5.13	-10.0
Mean		0.208		5.12	
% CV		5.7		3.0	
% Theoretical		83.2		89.8	
n		3		3	

The effect that hemolyzed plasma (fortified with 2% whole blood) has on the quantitation of the peptide was evaluated. Three previously screened lots of blank plasma (EDTA) were fortified with 2% whole blood. Each lot of hemolyzed plasma was then fortified with the peptide at the LLOQ and high QC sample concentration and acidified. Samples were frozen for a minimum of 24 hr, thawed, extracted and analyzed against calibration standards prepared in non-hemolyzed control matrix.

Figure 1. Mass Shifts During the Infusion of the Compound.

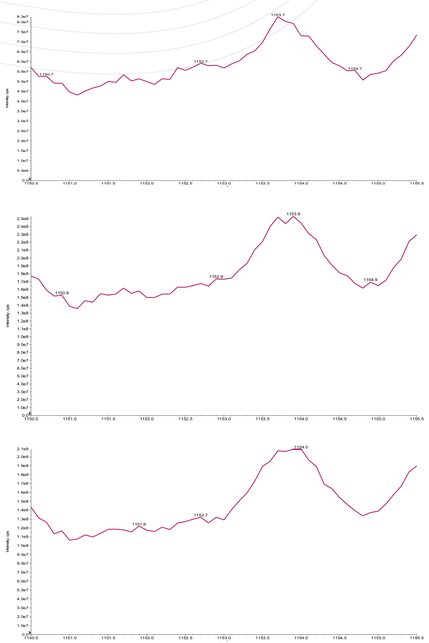

Figure 2. Calibration Curve of Standards.

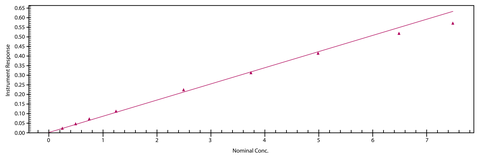

Figure 3. Representative Chromatogram of Peptide from an Extracted Blank Acidified Plasma (EDTA) Sample.

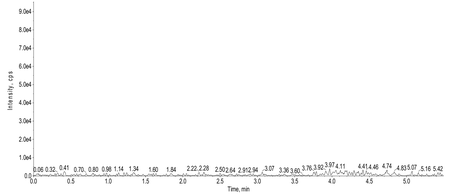

Figure 4. Representative Chromatogram of Peptide from Extracted Acidified Plasma (EDTA) LLOQ Sample.

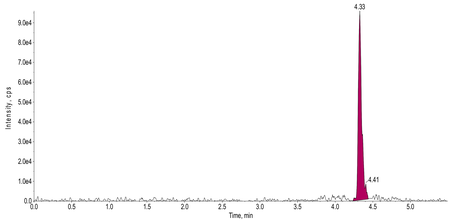

Figure 5. Representative Chromatogram of Peptide from Extracted Acidified Plasma (EDTA) ULOQ Sample.

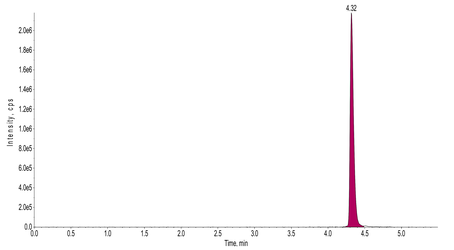

Table 4. Long Term Stability.

Batch	LTS LLOQ QC 0.250 ug/mL	DF = 10 LTS D 30.0 ug/mL
30,34	0.250	28.6
	0.211	29.4
	0.218	28.3
	0.212	30.9
	0.206	30.0
	0.222	30.2
Mean	0.220	29.6
% CV	7.2	3.4
% Theoretical	88.0	98.7
n	6	6

Analyte stability in frozen matrix was evaluated by analyzing replicate LLOQ and dilution QC stability samples. These samples were stored in polypropylene tubes at -80°C for 118 days (LLOQ QC) and 126 days (dilution QC) against freshly prepared calibration standards as part of Batches 30 and 34. Results from the long-term stability evaluation indicate that the peptide is stable under these storage conditions for 118 days (LLOQ QC) and 126 days.

Table 5. Post-Preparative Stability.

Batch	PPS A 0.750 ug/mL	PPS C 5.70 ug/mL
30	0.714	5.52
	0.733	5.58
	0.787	5.69
	0.818	5.84
	0.781	5.74
	0.780	5.72
Mean	0.769	5.68
% CV	5.0	2.0
% Theoretical	102.5	99.6
n	6	6

Post-preparative extract stability was evaluated, by analyzing replicate low and high-quality control samples that were extracted and stored in a polypropylene 96 well plate at 5°C for 88 hr, prior to being assessed against freshly extracted calibration standards.

Conclusions

The LC-MS/MS method for the determination of the peptide in acidified plasma (EDTA) met the requirements as specified in the validation protocol. Stability was demonstrated for the peptide in acidified plasma (EDTA) samples and solutions under varying conditions of storage. The method was validated using a calibration curve range of 0.250 to 7.50 µg/mL and a 0.0250 mL sample volume.

- An LC-MS/MS method was developed and validated for the quantification of a large basic peptide
- Column secondary interactions were eliminated with 0.07% TFA
- The lower response from the TFA in the mobile phase was minimized by infusion of acetic acid
- Summing of Q1 responses, and using a mobile phase divert flow helped with overall stability and response of the instrument

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