Internal Standardization Approaches for the Quantification of a 25 kDa Fusion Protein to Support Early Stage Drug Development by LC-MS

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OVERVIEW

- **Purpose**
  - Evaluation of cost-effectiveness of readily available internal standards for the quantification of the therapeutic fusion protein VCTR4.0 by LC-MS/MS.
- **Method**
  - VCTR4.0 samples were prepared by the pellet digestion approach using trypsin.
  - Analog protein, stable labeled synthetic peptide and analog synthetic peptide were evaluated for internal standardization approaches.
  - Quantification of VCTR4.0 surrogate peptide was performed by LC-MS/MS.
- **Results**
  - The analog protein TRP4.0 as internal standard was considered adequate since it compensated both for extraction and ionization variation.
  - This method was successfully used for the quantification of VCTR4.0 in mouse plasma sample during pre-clinical studies.

INTRODUCTION

Over the last several years, LC-MS has proven to be complementary to LBA for the quantification of therapeutic proteins. Moreover, it offers numerous advantages such as faster method development and the use of internal standards to compensate for analytical variability. Based on analytical performances, the use of SIL-protein internal standards is the preferred choice in LC-MS. However, SIL-proteins are expensive to produce and are rarely available during early stage drug development. In this study, alternative internal standardization approaches for the LC-MS quantification of a novel therapeutic fusion protein currently in pre-clinical development will be evaluated, including the use of synthetic tryptic peptides and protein analogs.

Figure 1: Partial Amino Acids Sequence of the 25kDa Fusion Protein VCTR4.0 and Internal Standards

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VCTR4.0                  NH2-…KSRVVRPLGIAGERKRK…-COOH
VCTR4.0 SIL peptide      NH2-…KSRVVRPLGIAGER*KR…-COOH
VCTR4.0 analog peptide   NH2-…KSRVVRPLGIVGERKRK…-COOH
TRP4.0 protein analog    NH2-…RRRVVRPLGLAG-EVAA…-COOH

R*: (13C, 15N) Labeled Arginine
The peptide generated following tryptic digestion is
underlined. Shared sequence is highlighted in gray.
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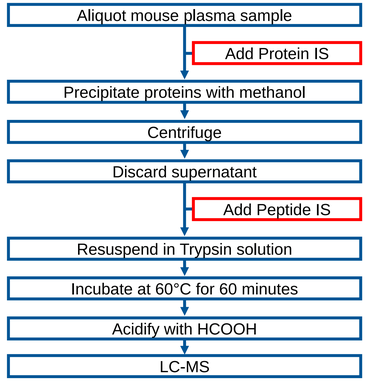
METHODS

Different internal standards were evaluated for the quantification of VCTR4.0, a novel therapeutic fusion protein. Due to time constraints and high costs, SIL-proteins were not considered a valuable option. However, as it is often the case in drug development, multiple compounds developed in the same program shared sequence homology with VCTR4.0 and were available. The first choice of protein analog, TRP4.0, had a tryptic peptide similar to VCTR4.0 signature peptide. Additionally, two peptides IS flanked by trypsin cleavage sites were synthesized: one analog and SIL-Peptide (**Figure 1**).

EXTRACTION

VCTR4.0 sample preparation was performed by the pellet digestion approach. The extraction scheme is shown below.

Figure 2: Pellet Digestion Sample Extraction Procedure



CHROMATOGRAPHY

- Agilent Technologies Series 1100 pumps and autosampler
- Zorbax 300SB-C18, 50x2.1mm, 3.5µm
- Gradient of 0.1% HCOOH in H_2O and ACN in 3.5 minutes

DETECTION

- AB SCIEX QTRAP®5500
- MRM mode ESI(+)
- The $[M+3H]^{3+}$ was monitored for the VCTR4.0 surrogate peptide and the internal standards

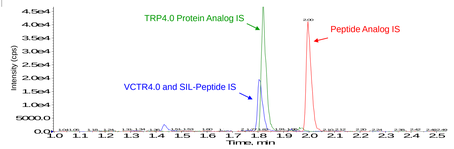
RESULTS

The ability of the three IS to compensate for ionization and extraction variability was evaluated. Matrix factor was performed on 6 lots of mouse plasma using all three internal standards (**Table 1**). Both the SIL-Peptide IS and the protein analog IS were adequate to compensate for matrix effect. However, more variability was observed when using the analog peptide IS, especially at low concentration. This could be explained since the analog peptide IS elutes in a distant region from VCTR4.0 and could be more susceptible to matrix interference (**Figure 3**).

Table 1: Internal Standard Normalized Matrix Factor for VCTR4.0 Using 3 Different Internal Standards (6 lots, 3 replicates)

	Low QC (0.750 µg/mL)		
	Peptide Analog	SIL-Peptide	Protein Analog
Mean (n=18)	1.046	1.0512	1.0063
STDEV	0.1239	0.0616	0.0333
%CV	11.8%	5.9%	3.3%
	High QC (75.000 µg/mL)		
	Peptide Analog	SIL-Peptide	Protein Analog
Mean (n=18)	1.0353	1.0090	1.0062
STDEV	0.0743	0.0561	0.0305
%CV	7.2%	5.6%	3.0%

Figure 3: Chromatogram of VCTR4.0 signature peptide and internal standards



The ability of the IS to compensate for extraction variability was evaluated (**Table 2**). In this case, the protein analog IS was more efficient to compensate for extraction variability since it can be added at the beginning of the sample preparation procedure.

Table 2: VCTR4.0 Extraction Reproducibility Using 3 Different Internal Standards

	Low QC (0.750 µg/mL) – Area Ratio		
	Peptide Analog	SIL-Peptide	Protein Analog
Mean Ratio (n=10)	0.0684	0.1668	0.1042
STDEV	0.0113	0.0230	0.0048
%CV	16.5%	13.8%	4.6%
	High QC (75.000 µg/mL) – Area Ratio		
	Peptide Analog	SIL-Peptide	Protein Analog
Mean Ratio (n=10)	6.5098	16.9918	10.5928
STDEV	0.7820	2.3450	0.3864
%CV	12.0%	13.8%	3.6%

Using TRP4.0 as a protein analog IS, VCTR4.0 calibration curve was linear (weighted $1/x^2$) from 0.25 to 100.00 µg/mL with good coefficient of correlation (>0.99). The between-run (n=3) accuracy for the LLOQ, low, mid and high QC samples ranged from 91.9% to 99.5% while the precision stayed between 3.1% and 11.2%.

Figure 4: Calibration Curve for VCTR4.0 in Mouse Plasma Using TRP4.0 Protein Analog Internal Standard

Calibrant	Nominal µg/mL	Calculated µg/mL	Deviation %
P1	0.250	0.236	-5.7
P2	0.500	0.537	7.4
P3	1.000	1.097	9.7
P4	2.500	2.409	-3.6
P5	7.500	7.409	-1.2
P6	15.000	14.795	-1.4
P7	30.000	30.089	0.3
P8	50.000	49.667	-0.7
P9	85.000	80.864	-4.9
P10	100.000	100.017	0.0

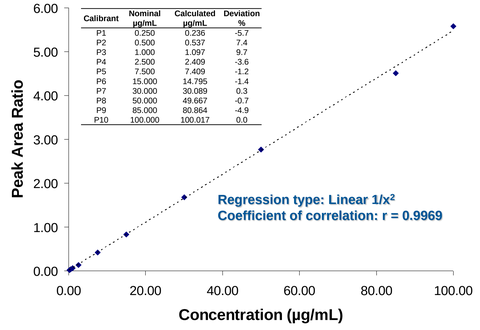


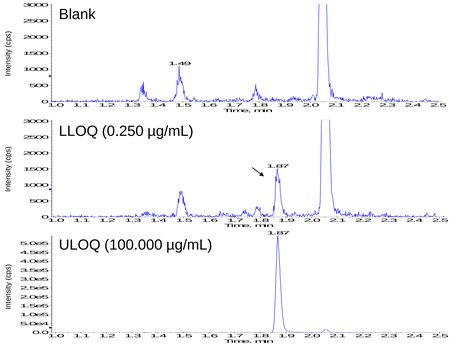
Table 3: Between Run (n=3) Precision and Accuracy for VCTR4.0 Using TRP4.0 Protein Analog Internal Standard

	Concentration (µg/mL)			
	LOQ QC	Low QC	Mid QC	High QC3
	0.250	0.750	25.000	75.000
Mean (n =18)	0.230	0.743	23.876	74.660
S.D.	0.026	0.048	0.746	3.202
% C.V.	11.2	6.4	3.1	4.3
% Nominal	91.9	99.0	95.5	99.5

Table 4: Summary of VCTR4.0 Bioanalytical Method

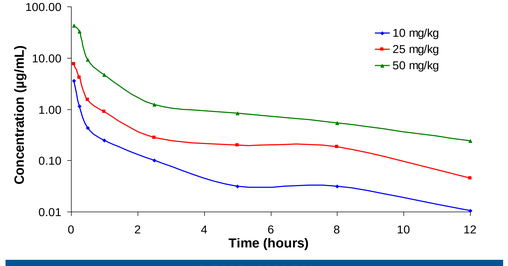
Evaluation	Results
Specificity	Acceptable for 6 lots of mouse plasma
Sensitivity	S/N at least 5 at LLOQ (0.250 µg/mL)
Linearity	Linear (weighted $1/x^2$) regression from 0.250 µg/mL to 100.000 µg/mL
Matrix Factor	Acceptable for 6 lots of mouse plasma, including lipemic and hemolysed
Whole Blood Stability	30 minutes at 4° C nominal in mouse blood
Short-Term Stability	6 hours at 4° C nominal in mouse plasma
Freeze-Thaw Stability	3 F/T cycles (-80° C to 4° C) in mouse plasma

Figure 5: Chromatograms of Extracted VCTR4.0 Samples



This LC-MS/MS assay for large molecule quantification was successfully used in preclinical studies to determine the pharmacokinetic profile of VCTR4.0 in the mouse following intravenous administration (**Figure 6**).

Figure 6: Pharmacokinetic Profiles of VCTR4.0 in the Mouse Following Intravenous Administration



CONCLUSION

This study demonstrated that the use of a protein analog internal standard could represent an interesting alternative for reliable and high throughput therapeutic proteins quantification. They are cost effective, readily available during early stage drug development and allow the development of bioanalytical methods within only a few days of lab work.

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