Multiple Isotopes Improve Precision for Surrogate Peptide Quantitation Mass Spectrometry

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ABSTRACT

The precision of surrogate peptide quantitation mass spectrometry can be improved through the use of multiple isotopic internal standards. We present our analysis of the impact on precision measurement of C-reactive protein associated with increasing the number of isotopic internal standards compared to a single isotopic internal standard that demonstrates the reduction in coefficients of variation.

THE APPROACH

Surrogate peptide quantitation provides a useful means to quantify proteins by mass spectrometric read out[1]. Intact proteins are enzymatically fragmented down to peptides, known quantities of isotope labelled peptide standards are added to the digest, and the signals from naturally occurring target peptide along with the internal standards are simultaneously measured. The ratio between these two signals are used to calculate the unknown quantity of the endogenous peptide from the known quantity of the standard peptide. This approach enjoys the benefit of the ease associated with synthesizing isotopic peptides compared with the difficulty associated with engineering isotopic labeled intact proteins for internal standardization.

Given the ease of synthesizing isotopes of the naturally occurring surrogate peptide, it is plausible to synthesize standards comprising multiple C13/N15 labelled amino acids. Such standards can be spiked into the sample at various levels which results in internal reference points distributed throughout the relevant range of the naturally occurring surrogate peptide. These multiple signals may be useful in decreasing errors associated with quantifying the naturally occurring surrogate peptide just by one reference peptide.

[1] Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., Gygi, S. P., Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proceedings of the National Academy of Sciences of the United States of America 2003, 100, 6940-6945

METHODS

In this study, we used one, or three times isotopically labelled peptides to quantify C-reactive protein in plasma by a mass spectrometry-based immunoassay. Characterized C-reactive protein containing plasma and low C-reactive protein plasma was mixed in multiple ratios to generate a broad range of C-reactive protein-containing samples. We explored various calculation methods to compare single versus multiple internal standard approach. These methods included 1) extrapolating the unknown quantity from the isotope standard with the nearest signal intensity to the signal intensity of the naturally occurring surrogate peptide, and 2) extrapolating the unkown quantity from the two isotopes with the nearest higher and lower signal intensity to the signal intensity of the naturally ocurring surrogate peptide unless there is not a higher or lower in which case the isotope with the nearest signal intensity is extrapolated.

CONCLUSIONS

The use of three differentially-labelled peptide standards for protein revealed better performance in terms of accuracy and precision compared to a one-standard approach. Using more than one internal peptide standards as references improves accuracy and precision. Two differentially labelled standards were adequate to cover a C-reactive protein-concentration range of three orders of magnitude. However, the spiking of three standards –high, medium and low - allows additional process control, since the signal ratios of the three standards could be used as performance controls for the mass spectrometer. By this approsch all samples itself become quality control samples and additional quality might become redundant.

RESULTS

		ug/ml	m/z	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Mean	Std Dev	Coefficients of Variation % Single isotope	Coefficients of Variation % Multi isotope
Sample 1															
	endogenous	0.120	568.785	1.25E+06	1.34E+06	1.46E+06	1.23E+06	1.22E+06	1.39E+06	1.29E+06	1.25E+06	1.30E+06	8.76E+04		6.72%
	isotope	0.501	572.792	3.77E+06	3.79E+06	3.54E+06	3.20E+06	3.08E+06	3.43E+06	3.21E+06	3.26E+06	3.41E+06	2.69E+05		7.89%
	isotope	2.003	577.805	2.14E+07	2.05E+07	2.08E+07	2.25E+07	1.95E+07	2.14E+07	2.15E+07	2.09E+07	2.11E+07	8.88E+05		4.21%
	isotope	12.520	581.314	1.00E+08	9.35E+07	9.82E+07	8.63E+07	1.06E+08	1.14E+08	8.40E+07	8.39E+07	9.57E+07	1.09E+07		11.41%
				0.166	0.177	0.207	0.192	0.198	0.203	0.201	0.192	0.192	0.014		7.21%
	endogenous	0.120	568.785	1.43E+06	1.23E+06	1.00E+06	1.27E+06	1.18E+06	1.31E+06	1.84E+06	1.71E+06	1.37E+06	2.80E+05	20.43%	
	isotope	2.003	577.805	2.22E+07	2.14E+07	1.70E+07	2.02E+07	2.04E+07	2.02E+07	2.16E+07	2.11E+07	2.05E+07	1.58E+06	7.71%	
				0.130	0.115	0.118	0.125	0.116	0.130	0.171	0.163	0.133	0.022	16.13%	
Sample 2															
	endogenous	0.245	568.785	2.01E+06	1.89E+06	1.90E+06	2.33E+06	1.96E+06	2.18E+06	1.69E+06	1.70E+06	1.96E+06	2.21E+05		11.28%
	isotope	0.501	572.792	3.40E+06	3.44E+06	3.29E+06	3.41E+06	3.47E+06	3.75E+06	3.56E+06	3.44E+06	3.47E+06	1.37E+05		3.94%
	isotope	2.003	577.805	2.05E+07	2.04E+07	2.01E+07	2.02E+07	2.20E+07	2.37E+07	2.21E+07	2.09E+07	2.12E+07	1.25E+06		5.90%
	isotope	12.520	581.314	9.56E+07	8.43E+07	8.34E+07	8.96E+07	9.61E+07	1.22E+08	8.92E+07	1.06E+08	9.58E+07	1.29E+07		13.44%
				0.296	0.275	0.289	0.342	0.283	0.291	0.237	0.247	0.282	0.032		11.42%
	endogenous	0.245	568.785	2.50E+06	2.59E+06	2.51E+06	2.64E+06	2.80E+06	2.50E+06	2.52E+06	2.42E+06	2.56E+06	1.15E+05	4.51%	
	isotope	2.003	577.805	2.31E+07	2.13E+07	2.11E+07	2.36E+07	2.29E+07	2.09E+07	2.35E+07	2.12E+07	2.22E+07	1.17E+06	5.27%	
				0.217	0.243	0.239	0.224	0.245	0.240	0.215	0.228	0.231	0.012	5.10%	
Sample 3															
	endogenous	0.746	568.785	6.96E+06	6.22E+06	6.11E+06	6.42E+06	5.77E+06	6.52E+06	6.21E+06	6.24E+06	6.31E+06	3.47E+05		5.50%
	isotope	0.501	572.792	3.32E+06	3.61E+06	2.99E+06	3.31E+06	3.19E+06	3.60E+06	3.36E+06	3.17E+06	3.32E+06	2.12E+05		6.39%
	isotope	2.003	577.805	2.43E+07	2.27E+07	1.79E+07	2.07E+07	1.95E+07	2.24E+07	1.99E+07	2.15E+07	2.11E+07	2.04E+06		9.66%
	isotope	12.520	581.314	9.91E+07	8.76E+07	8.57E+07	8.94E+07	8.48E+07	8.49E+07	8.25E+07	8.96E+07	8.79E+07	5.11E+06		5.81%
				1.050	0.863	1.024	0.971	0.905	0.907	0.927	0.988	0.954	0.064		6.75%
	endogenous	0.746	568.785	6.85E+06	5.82E+06	7.37E+06	7.49E+06	7.78E+06	8.06E+06	7.65E+06	8.68E+06	7.46E+06	8.50E+05	11.39%	
	isotope	2.003	577.805	2.15E+07	2.02E+07	2.01E+07	1.98E+07	2.06E+07	2.00E+07	2.03E+07	2.11E+07	2.04E+07	5.80E+05	2.84%	
				0.640	0.578	0.734	0.758	0.757	0.808	0.756	0.823	0.732	0.083	11.33%	
Sample 4															
	endogenous	3.250	568.785	2.82E+07	3.09E+07	2.61E+07	2.88E+07	2.59E+07	2.60E+07	3.05E+07	2.90E+07	2.82E+07	1.99E+06		7.06%
	isotope	0.501	572.792	3.50E+06	4.20E+06	3.02E+06	3.82E+06	3.22E+06	3.33E+06	3.84E+06	3.29E+06	3.53E+06	3.93E+05		11.15%
	isotope	2.003	577.805	2.25E+07	2.51E+07	1.95E+07	2.47E+07	2.14E+07	2.11E+07	2.48E+07	2.40E+07	2.29E+07	2.08E+06		9.07%
	isotope	12.520	581.314	9.24E+07	1.03E+08	7.68E+07	9.99E+07	8.69E+07	8.27E+07	9.78E+07	8.69E+07	9.08E+07	9.12E+06		10.04%
				2.509	2.468	2.687	2.338	2.428	2.473	2.468	2.417	2.474	0.100		4.06%
	endogenous	3.250	568.785	3.29E+07	3.70E+07	3.30E+07	3.49E+07	3.49E+07	3.52E+07	3.39E+07	3.61E+07	3.47E+07	1.43E+06	4.13%	
	isotope	2.003	577.805	1.77E+07	2.24E+07	1.91E+07	2.07E+07	2.17E+07	1.99E+07	1.96E+07	2.13E+07	2.03E+07	1.54E+06	7.58%	
		3.435		3.720	3.307	3.465	3.381	3.216	3.542	3.461	3.392	3.435	0.153	4.44%	
Sample 5															
	endogenous	25.159	568.785	1.91E+08	1.63E+08	1.89E+08	2.01E+08	1.63E+08	1.86E+08	1.96E+08	1.47E+08	1.80E+08	1.93E+07		10.75%
	isotope	0.501	572.792	3.34E+06	2.83E+06	3.36E+06	3.40E+06	3.05E+06	3.36E+06	3.62E+06	2.79E+06	3.22E+06	2.94E+05		9.14%
	isotope	2.003	577.805	2.05E+07	1.91E+07	2.20E+07	2.45E+07	2.09E+07	2.25E+07	2.45E+07	2.01E+07	2.18E+07	1.99E+06		9.15%
	isotope	12.520	581.314	9.45E+07	8.00E+07	9.39E+07	1.01E+08	7.92E+07	8.88E+07	8.80E+07	7.84E+07	8.80E+07	8.27E+06		9.40%
				25.323	25.553	25.242	24.965	25.763	26.246	27.902	23.499	25.561	1.240		4.85%
	endogenous	25.159	568.785	2.38E+08	2.54E+08	2.65E+08	2.32E+08	2.45E+08	2.45E+08	2.58E+08	1.71E+08	2.38E+08	2.94E+07	12.34%	
	isotope	2.003	577.805	2.09E+07	2.34E+07	2.43E+07	1.94E+07	2.08E+07	2.05E+07	2.29E+07	1.95E+07	2.14E+07	1.85E+06	8.60%	
		22.253		22.795	21.779	21.819	23.938	23.629	23.955	22.587	17.526	22.253	2.097	9.42%	
	Summary													9.29%	6.86%

C-reactive protein depleted plasma was digested with trypsin and variously isotopic labled peptides of C-reactive protein were spiked into aliquots to prepare a range of samples of 5 different C-reactive protein concentrations with 2 different isotope combinations of isotopic labels; 1) single isotopic label and 2) a multi isotopic label. These 10 separate samples were each assayed in 8 replicates for a total of 80 assays. The assays of the samples with the single isotopic label were quantified based on the single isotopic label. The assays of the samples with the multi isotopic labels were quantified based on the isotopic label with the signal intensity closest to the endogenous signal intensity.

Each of these 80 assays were quantified using the signals from the internal isotopic labels. Coefficients of variation were calculated for each of the 10 samples and analyzed. The coefficient of variation for the single labeled assays is 9.29%. The coefficient of variation for the multi labeled assay is 6.86% representing a 26% improvement.