Accurate Quantitation of Deamidated Peptides by Mass Spectral Accuracy

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Overview

- The mAb digests were analyzed by LC/MS and deamidated peptides were selected to evaluate the quantitation of the peptides by spectral acacuracy.
- Peak shape calibration was performed for eight pairs of native and deamidated peptides and followed by spectral accuracy calculation for native peptides only and the mixture of native and deamidated peptides.
- Relative quantitation of deamidated peptides was determined.

Introduction

Deamidation of therapeutic proteins converts Asparagine into Aspartic Acid or Isoaspartic Acid. This byproduct from production of recombinant proteins may compromise efficacy of the biologicals or pose a safety risk for patients. According to guidelines of regulatory agency, it is required to quantify the deamidated proteins. Due to their similar hydrophobicity and small m/z value difference about 1 amu, deamidated and unmodified peptides are difficult to separate by HPLC or mass spectrometry. They appear almost always as a mixture in HPLC/MS analysis and are challenging to quantify. Conventional approach to quantitation of deamidated peptides or proteins relies on either ion chromatographic separation or 2D separation including both HPLC in the 1st dimension and ERLIC (electrostatic repulsion–hydrophilic interaction chromatography) in the 2nd (1). While providing accurate quantitation results, these time domain quantitation methods are time consuming and labor intensive. Alternative quantitation for such complex mixtures was reported on deamidation quantities of several γS-crystallin peptides (2) based on mass spectral data through spectral deconvolution on overlapped spectra of native and deamidated peptides. We propose a new mathematical way to separate the mixture by spectral accuracy to achieve highly accurate quantitation of deamidated peptides.

Methods

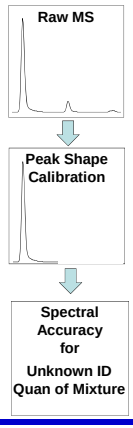
- **Samples:** Intact mAb purchased from Waters was reduced and alkylated (7mM DTT and 14 mM Iodoactemide, Sigma). Lys-C (Wako) was added 1:20 ratio.
- **HPLC**; Agilent 1100 binary pump, Vydac column (218TP52). Mobile phase; A (Water 0.1% TFA) and B (ACN 0.1%TFA). Gradient; 0% B to 100% B in 170 minutes.
- **Data Acquisition:** All LC/MS and LC/MS/MS data were acquired in a profile mode with a mass range from 400 to 2070 and resolving power of 30K in a Thermo Orbitrap MS.
- **MassWorks data processing:** All the MS spectra were exported from Xcalibur to MassWorks to perform peak shape calibration through sCLIPS (self Calibrated Lineshape Isotope Profile Search) based on monoisotope peak. Through the peak shape calibration, spectral accuracy can be calculated and utilized to perform isotope pattern matching between calibrated spectra and theoretically calculated spectra for the purpose of quantitation of deamidated peptides.

Results and Discussion

As recently presented in a cover page article in Analytical Chemistry (3), by applying the concept of spectral accuracy to mass spectrometric data, a unique mass spectral calibration for high resolution data can be performed to greatly improve mass spectral peak shapes. The spectral accuracy for peak shapes is achieved by mathematically fitting an observed mass signal to its theoretically calculated and thereby perfectly shaped counterpart. In this work we will be presenting a novel approach for direct and quantitative determination of deamination by calculating the goodness of fit of the overlapping isotopic distributions of measured spectra to the isotope pattern of theoretical spectra.

Eight pairs of native and deamidated peptides as shown in Fig 1 were selected for quantitative analysis by MassWorks through sCLIPS (Fig 2). For each pair of the peptides, spectral accuracy calculation was performed for both the spectra from native unmodified peptides only and the spectra from overlapped spectra of the native and deamidated peptides. Overall higher spectral accuracy has been achieved when both native and deamidated peptides were taken into consideration for isotope pattern matching than only native peptides were used for spectral accuracy calculation. These results not only demonstrate deamidated peptides indeed overlapped with their native counterparts, but also show relative concentration levels varied from 4.6% to 53.6% as summarized in Table 1. Here is an example to illustrate how spectral accuracy was

Fig 2. Peak shape calibration



- Monoisotopic peak of the unknown itself as "the standards"
- No formula required of "the standard(s)"
- No actual external or internal standard(s)
- "The standards" as close in m/z to unknown as possible
- "The standards" as close in time to unknown as possible
- Peak-shape-only calibration w/o mass adjustment
- sCLIPS: self calibration

Fig 1. TIC of mAb Digests with Deamidated Peptides labeled

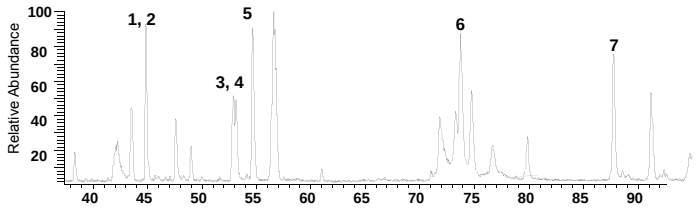


Table 1. Quantitative Analysis of Deamidated Peptides by Spectral Accuracy

Peak	Elemental Composition	Charge	Rt (min)	m/z [Da] Measured	Spectral Accuracy for Native Pepetide	Spectral Accuracy for mixture	Quan of Deamidated (%)
1	C68H112N20O20S1	2	44.4	780.4213	96.59	99.35	
	C68H111N19O21S1	2	45.5	780.9136			0.046
2	C92H147N29O37	2	44.5	1125.0514	87.29	98.77	
	C92H146N28O38	2	45.3	1125.5443			0.17
3	C87H130N20O30S1	2	52	983.4711	97.36	97.70	
	C87H129N19O31S1	2	53.7	983.9638			0.02
4	C109H170N33O39	3	52.3	855.0974	86.58	97.42	
	C109H169N32O40	3	53.2	855.4270			0.217
5	C124H184N39O41S1	3	55.7	969.1303	97.09	97.54	
	C124H183N38O42S1	3	56.8	969.4593			0.03
6	C144H220N37O44S1	3	72	1067.8852	72.05	98.82	
	C144H219N36O45S1	3	74.1	1068.2148			0.536
7	C246H390N66O79S2	4	88.8	1399.2306	96.01	98.37	
	C246H389N65O80S2	4	85.5	1399.4776			0.099

utilized to quantitatively determine deamidated peptides from overlapped spectra. In order to calculate only native peptides and their deamidated counterparts, the upper and lower bounds of elements are limited based on their unknown elemental composition as shown in Fig. 3. It is important to point out the unique feature provided by MassWorks to allow calculating overlapped spectra through input of possible modification, such as +O/-NH in this case for deamidated peptides. When this input was taken into consideration for spectral accuracy calculation, the calibrated spectra (measured spectra after peak shape Calibration) would match against the combination of native and deamidated peptides through least square fit.

Fig 3. Spectral Accuracy Calculation Parameters

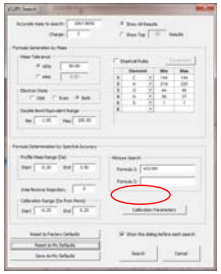
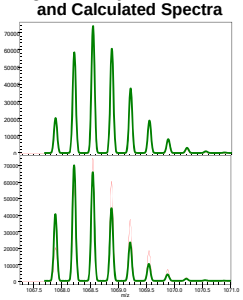


Fig 4. Overlays of Calibrated and Calculated Spectra



In this example, high spectral accuracy of 98.2% was achieved. This great fit provided relative quantitation of 46.4% native and 53.6% deamidated peptides respectively. On the other hand, when the input of +O/-NH was not considered, poor spectral accuracy of 72.1% was resulted. As shown in overlays in Fig.4, almost perfect match (Fig.4 top, red = calibrated and green = calculated) and obvious mismatch (Fig,4 botton) were found for the calculation including and excluding deamidation respectively.

Conclusions

- Spectral accuracy is a powerful tool to quantify deamidated peptides from overlapped mass spectra based on peak shape calibration technology
- Relative quantitation information of deamidated peptides can be obtained as low as 3% from the mixture of native and deamidated peptides according to current study.
- Spectral accuracy approach to quantitation of overlapped signals can apply to the quantitation of O18 labeled peptides and C14 labeled drug metabolites.
- Future work will include accuracy confirmation on quantitation of deamidated peptides through independent analytical methods such as ion exchange chromatography.

References

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(2) Surendra Dasari1, Phillip A. Wilmarth2, Ashok P. Reddy3, Lucinda J. G. Robertson2, Srinivasa R. Nagalla1,3, and Larry L. David2,* Quantification of Isotopically Overlapping Deamidated and 18O Labeled Peptides Using Isotopic Envelope Mixture Modeling. *J Proteome Res.* 2009 March 6; 8(3): 1263–1270.

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