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Quantification of protein posttranslational modifications using stable isotope and mass spectrometry. II. Performance

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ABSTRACT

In this report, we examine the performance of a mass spectrometry (MS)-based method for quantification of protein posttranslational modifications (PTMs) using stable isotope labeled internal standards. Uniform labeling of proteins and highly similar behavior of the labeled vs nonlabeled analyte pairs during chromatographic separation and electrospray ionization (ESI) provide the means to directly quantify a wide range of PTMs. In the companion report (Jiang et al., Anal. Biochem., 421 (2012) 506–516.), we provided principles and example applications of the method. Here we show satisfactory accuracy and precision for quantifying protein modifications by using the SILIS method when the analyses were performed on different types of mass spectrometers, such as ion-trap, time-of-flight (TOF), and quadrupole instruments. Additionally, the stable isotope labeled internal standard (SILIS) method demonstrated an extended linear range of quantification expressed in accurate quantification up to at least a 4 log concentration range on three different types of mass spectrometers. We also demonstrate that lengthy chromatographic separation is no longer required to obtain quality results, offering an opportunity to significantly shorten the method run time. The results indicate the potential of this methodology for rapid and large-scale assessment of multiple quality attributes of a therapeutic protein in a single analysis.

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Therapeutic proteins represent one of the fastest growing markets in biotechnology [1–4]. High-throughput identification and accurate quantification of all posttranslational modifications (PTMs)[1] are essential for assessing the product quality attributes of a therapeutic protein. Currently, chromatography- or capillary electrophoresis (CE)-based methods with UV or fluorescence detection are commonly used to monitor and quantify the modifications in a protein.

Mass spectrometry (MS) has become an essential analytical tool for the characterization of therapeutic proteins, and has been widely used in virtually all phases of product and process development, including clone selection, cell culture process development, purification and formulation development, stability, and comparability studies [1–5]. MS-based "label-free" approaches can be used for relative and absolute quantification. Relative quantification can be achieved by comparing the peak area of modified and unmodified species under the selected ion chromatogram (SIC) in the same LC-MS run. As an example, large-scale identification and quantification of modifications in therapeutic proteins was reported recently by Zhang [6]. In a single LC-MS/MS analysis of a tryptic digestion of an IgG2 monoclonal antibody, 227 modifications were identified and quantified using a high-resolution mass spectrometer. Alternatively, if a well-characterized "external standard" is available and analyzed in parallel with the samples, absolute levels of modification can be determined by comparing the peak area of the modified species in the external standard with the peak area of the same modified species in the sample [6–8]. However, the accuracy of the label-free approaches might be compromised due to varying detector responses, differential ionization yields for different substances, run-to-run variability, and other factors.

Internal standards provide unique benefits for accurate and precise quantification, since all sample handling variability is minimized as the internal standard is introduced into the sample for analysis prior to any sample handling steps, such as proteolytic

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[1] *Abbreviations used:* mAb, monoclonal antibody; LC-MS, liquid chromatography-mass spectrometry; PTMs, posttranslational modifications; SILIS, stable isotope-labeled internal standard; SILAC, stable isotope labeling by amino acid in cell culture; TFA, trifluoroacetic acid; ACN, acetonitrile; ESI, electrospray ionization; TOF, time of flight; NEM, N-ethylmaleimide; TCEP, tris(2-carbosyethyl)phosphine; DTT, dithiothreitol; IAM, iodoacetamide; SIC, selected ion chromatogram; SRM, selected reaction monitoring; CID, collision induced dissociation; TBHP, tert-butylhydroperoxide; LC, light chain; HC, heavy chain.

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digestion and chromatographic separation, etc. In separate reports, we have described *in vivo* metabolic labeling of proteins with ^{15}N-enriched media (manuscript in preparation), and the strategy and principle of the MS-based quantification method using stable isotope labeling [9]. In this report, we examined the performance of our method in terms of precision, accuracy, and linearity on ion-trap, TOF, and triple quadrupole instruments. When compared with the MS-based label-free approach, our method demonstrates improved precision, accuracy, and linearity over a broad concentration range on all three instrument platforms.

Experimental

Materials

Stable isotope-labeled recombinant proteins, an IgG2 monoclonal antibody (mAb) and an Fc fusion protein [10], were produced *in vivo* by metabolic labeling (manuscript in preparation). In brief, stable isotope-labeled recombinant mAb was expressed in CHO cell lines using exclusively ^{15}N-labeled amino acids (Cambridge Isotope Laboratories, Inc., Andover, MA) at all N positions. The ^{15}N-labeled mAb was subsequently purified by protein A chromatography and buffer-exchanged into 10 mM sodium acetate solution, pH 5.0. In order to label the Fc fusion protein expressed in *Escherichia coli*, ^{15}N-enriched Celtone media (Cambridge Isotope Laboratories, Inc.) was used for cell culture. The protein was expressed as insoluble inclusion bodies. After harvest, the inclusion bodies were washed, oxidized, solubilized, and refolded to form the correct conformation. The refolded Fc fusion protein was then purified by cation ion-exchange chromatography followed by size exclusion chromatography.

Trypsin was obtained from Roche Diagnostics (Penzberg, Germany), and endoproteinase Lys-C (Lys-C) was from Wako Chemicals, Inc. (Richmond, VA). Guanidine hydrochloride and urea were obtained from ICN Biomedicals Inc. (Aurora, OH). *N*-Ethylmaleimide (NEM), acetonitrile (ACN), tris(2-carboxyethyl) phosphine (TCEP), dithiothreitol (DTT), iodoacetic acid sodium salt (IAA), 2-aminobenzamide (2-AB), and tris(hydroxymethyl)aminomethane (Tris) base were obtained from Sigma–Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) was from Pierce (Rockford, IL). NAP™-5 columns were from GE Healthcare (Pittsburgh, PA). Peptide-N-Glycosidase F (PNGase F) was obtained from QA-Bio (Palm Desert, CA).

2-AB-labeled RP-HPLC glycan map

Fluorescence-based RP-HPLC analysis of *N*-glycans from a recombinant mAb was performed as described previously [11]. In brief, N-linked glycans were enzymatically released from the protein by PNGase F and labeled with 2-AB. The 2-AB-labeled N-glycans were then separated on a C18 RP-HPLC column and quantified based on the fluorescence signal.

Enzymatic digestion of reduced ^{15}N-labeled and nonlabeled recombinant mAbs

Enzyme digestion of reduced samples was prepared as described in our companion report [9]. In brief, ^{15}N-labeled and nonlabeled proteins were reduced with DTT and alkylated with IAA. The buffer was then exchanged to 50 mM Tris buffer, pH 7.5, to remove salts and reagents. The proteolytic enzyme was added to the samples to achieve a protein:enzyme ratio of approximately 20:1 (w/w) and incubated at 37 °C overnight. The mixture was acidified with 5% TFA to quench the digestion. The digests were separated on a C5 RP-HPLC column (Phenomenex Jupiter C5, 2 × 250 mm, 300 Å pore size, 5 μm particle size) with a long gradient (hold at 2% B for 5 min, 2–22% B for 25 min, then 22–42% B within 95 min) or a short gradient (hold at 2% B for 5 min, 2–42% B for 15 min). Mobile phase A was 0.1% (v/v) TFA in water, and mobile phase B was 0.1% (v/v) TFA and 90% (v/v) ACN in water.

Enzymatic digestion of nonreduced and reduced ^{15}N-labeled and nonlabeled recombinant Fc-fusion proteins

As described in our companion report [9], the protein samples were alkylated with NEM and diluted into digestion buffer to achieve a final concentration of 4 M urea, 100 mM NH_2OH HCl, and 100 mM phosphate, pH 7.5. Lys-C (1:20 (w/w)) was added to digest the samples at 37 °C for 5 h. Half of the digestion mixture was reduced with TCEP. Both nonreduced and reduced digestion mixtures were then acidified with 5% TFA to quench the reaction. The digests were separated on a C4 column (ACQUITY UPLC BEH300 C4, 2.1 × 450 mm, 300 Å pore size, 1.7 μm particle size, Waters, Milford, MA) with a long (hold at 2% B for 5 min, 2–22% B for 25 min, then 22–42% B within 95 min) or a short gradient (hold at 2% B for 5 min, 2–42% B for 15 min).

LC-ESI-MS/MS analysis

The on-line LC-ESI-MS/MS analyses were performed using an Agilent 1200 HPLC system directly coupled with a Thermo Scientific (San Jose, CA) LTQ XL ion-trap mass spectrometer equipped with an electrospray ionization source. The solvent peaks were diverted to waste before the flow entered the ESI source. The ESI source voltage of the LTQ was set at 4.5 kV, and the capillary temperature was set at 275 °C. For MS experiments, mass spectra were acquired from 300 to 2000 *m/z* in the positive mode, followed by a data-dependent ultrazoom scan to determine the charge state of the most intense ion and a MS/MS scan to identify the sequence of the peptide precursor ion. In the MS/MS scan, the precursor ions were fragmented by collision-induced dissociation (CID) with 35% relative collision energy. Peptides were identified using MassAnalyzer, [12] an in-house developed software application, which correlates the experimental tandem mass spectra with the theoretical tandem mass spectra generated from known peptide sequences. The selected ion chromatogram (SIC) of the peptides of interest was used for quantitative analysis. In a SIC, one or more *m/z* values representing one or more analytes of interest are extracted from the entire chromatographic data set. The peak intensity within an *m/z* window is plotted against the retention time. Therefore, the quantitative results of modifications on an amino acid can be determined by comparing the peak areas under the SIC of the ^{15}N-labeled peptides and their nonlabeled counterparts using Qual Browser of Xcalibur software from Thermo Scientific.

The performance of our approach was also examined on a single stage Agilent 6224 TOF (Agilent) mass spectrometric instrument and on a TSQ Quantum Ultra Triple Stage Quadrupole (Thermo) mass spectrometer equipped with an ESI source. The ESI source voltage for the Agilent 6224 TOF MS was set at 3.5 kV, and the gas temperature was 350 °C. The analysis was performed in positive mode in which mass spectra were acquired with *m/z* range of 200–3100. The raw data were processed using Agilent MassHunter software.

For analyses using TSQ triple quadrupole mass spectrometer, selected reaction monitoring (SRM) was used to monitor precursor to product ion transition. In SRM mode, the mass of the parent peptide is first selected while other coeluted ions are filtered away. The parent peptide ion is then fragmented in the gas phase and a specific fragment ion is monitored. This experiment has very high specificity because the SRM chromatogram represents only ions of a particular mass that fragment in a manner that produce a very