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TECHNICAL NOTE

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Pitfalls in Protein Quantitation Using Acid-Catalyzed O^{18} Labeling: Hydrolysis-Driven Deamidation

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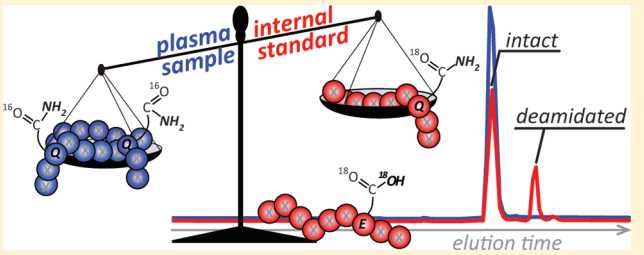
Supporting Information

ABSTRACT: Proteolysis combined with O^{18} labeling emerged recently as a powerful tool for quantitation of proteins for which suitable internal standards cannot be produced using molecular biology methods. Several recent reports suggested that acid-catalyzed O^{18} labeling may be superior to the commonly accepted enzymatic protocol, as it may allow more significant spacing between the isotopic clusters of labeled and unlabeled peptides, thereby eliminating signal interference and enhancing the quality of quantitation. However, careful examination of this procedure reveals that the results of protein quantitation assisted by acid-catalyzed O^{18} labeling are highly peptide-dependent. The inconsistency was found to be caused by deamidation of Asn, Gln, and carbamidomethylated Cys residues during prolonged exposure of the proteolytic fragments to the acidic environment of the labeling reaction, which translates into a loss in signal for these peptides. Taking deamidation into account leads to a significant improvement in the consistency of quantitation across a range of different proteolytic fragments.



The emergence and rapid proliferation of biopharmaceutical products[1] raises a spectrum of issues related to their analysis and characterization.[2,3] Pharmacokinetics of protein therapeutics[4,5] is one area where analytical technologies are critically important. While measurements of pharmacokinetic profiles of biopharmaceuticals have been traditionally carried out using bioactivity assays and immunoassays,[6] mass spectrometry (MS) based methods are being increasingly looked at as a very attractive alternative that may offer significantly higher precision and accuracy.[7,8] One factor that has prevented so far broader utilization of MS-based methods in pharmacokinetic profiling of protein therapeutics is their complexity (i.e., extensive glycosylation and the presence of multiple disulfide bridges), which makes it nearly impossible (or prohibitively expensive) to produce isotopically labeled forms of these species that can be used as internal standards for the purposes of quantitation.

An elegant way to solving this problem was proposed by Yao et al.,[9] who introduced a new method for stable isotope labeling at the peptide level by incorporating O^{18} atoms into the carboxyl termini of all tryptic peptides in the course of proteolysis. This set of peptides can be used as internal standards, as they coelute with their unlabeled counterparts in HPLC, while producing distinct ionic signals in MS due to a mass difference of 4 Da. Further modification of this technique by Niles et al.[10] sought to increase the spacing between the isotopic clusters of labeled and unlabeled peptides in mass spectra and reduce the occurrence of back exchange as a means of enhancing the accuracy of protein quantitation. This was accomplished by separating the labeling step and proteolysis, with the former carried out under acidic conditions,[10] taking advantage of the exchange of carboxylic oxygen atoms in peptides with the aqueous solvent at low pH.

While the carboxyl oxygen exchange reactions under acidic conditions occur much more slowly than the enzyme-catalyzed exchange (typically it takes over 10 days to reach the equilibrium), it results in almost complete labeling of all carboxylic oxygen atoms, including those located on the acidic side chains. As a result, peptides containing at least one acidic residue exhibit a mass shift of $4(n + 1)$, where n is the total number of acidic residues (Asp and Glu) in the peptide. Since the frequency of occurrence of these two amino acids in proteins is higher than the average for all common amino acids, a significant number of proteolytic peptides are expected to benefit from the acid-catalyzed O^{18} labeling by having better separation between the labeled and unlabeled ions in MS, thereby facilitating data analysis and enhancing quantitation. Furthermore, the back exchange of O^{18} labeled peptides during LC separation is negligible due to the extremely slow exchange rate of carboxyl oxygen atoms.

However, prolonged exposure of the sample to an acidic environment may also result in hydrolysis-driven chemical degradation. Amide bonds are the most frequent targets of hydrolysis, with the ensuing degradation processes leading either to the cleavages of polypeptide backbones or deamidation of certain side chains (e.g., asparagine[11]). Occurrence of either process during internal standard (IS) preparation would certainly have a negative impact on the quality of protein quantitation by inflating the apparent concentration of the protein (due to the diminished signal of the O^{18} labeled peptide ion used as an IS). In this work we report on a significant dependence of the protein quantitation

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results on the nature of the peptides used for quantitation, and this inconsistency is linked to the presence of amino acid residues bearing amide groups on their side chains (asparagine, glutamine, and modified cysteine). The occurrence of deamidation in such peptides is confirmed by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses. Once the side chain deamidation is taken into account, the consistency of protein quantitation results across a range of different proteolytic fragments is greatly improved.

MATERIALS AND METHODS

The recombinant form of human serum transferrin with an N-terminal His-tag[12] (hTf*) was provided by Prof. Anne B. Mason (University of Vermont College of Medicine, Burlington, VT); the amino acid sequence of hTf* is presented in the Supporting Information. Bovine serum, H_2O^{18} (97% purity), and proteomic-grade trypsin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals and solvents used in this work were of analytical grade or higher. A small amount of hTf* was added to 200 μL of unprocessed bovine serum to a final concentration of 10−200 nM. The spiked serum sample was denatured by incubating it at 37 °C for 2 h in 6 M of guanidine hydrochloride and then hTf* was extracted by running the denatured samples through a Ni affinity column (PerfectPro Ni-NTA Superflow, 5 PRIME, Inc. Gaithersburg, MD). The sample was reduced with TCEP and DTT (2 and 4 mM, respectively), at 50 °C for 20 min, followed by alkylation of cysteine residues with iodoacetamide (12 mM at 50 °C for 20 min in the dark) and digestion with trypsin using a published procedure[13] (buffer exchange to 50 μL of 100 μM ammonium bicarbonate, followed by addition of trypsin at a 20:1 substrate-to-enzyme ratio, and incubation at 37 °C for 24 h). A spin concentrator with a molecular weight cutoff of 10 000 Da was used as the reaction container for all steps. The tryptic digest was recovered by spinning down the analyte solution (50 μL of 100 mM ammonium bicarbonate buffer was added to wash the filter and then recovered and combined with the digestion solution).

The IS was prepared by digesting an aliquot of the stock solution of hTf* using the procedure outlined above followed by acid-catalyzed ^{18}O labeling. Briefly, the hTf* digestion solution was dried under a stream of nitrogen and then dissolved in 10 μL of DMSO and 89 μL of H_2O.[18] The labeling was initiated by adding 1 μL of trifluoroacetic acid to this solution, and the labeling products were sampled over 26 days by taking 1 μL of the solution every 2 days for LC/MS/MS analysis to check the completeness of labeling.

The digested sample was spiked with IS and then analyzed with LC/MS/MS using an LC Packings Ultimate (Dionex/Thermo Fisher Scientific, Sunnyvale, CA) nano-HPLC system coupled with a QStar-XL (AB SCIEX, Foster City, CA) hybrid quadrupole/time-of-flight mass spectrometer. The samples were first loaded onto a trap column (C18 PepMap 100, 5 μm, 100 Å, 300 μm i.d. × 1 mm) at a relatively high flow rate (30 μL/min) for preconcentration and cleaning. Then the peptides were resolved using a C18 column (Acclaim PepMap 100 C18, 3 μm, 75 μm i.d. × 15 cm) at a flow rate of 0.2 μL/min with a gradient as follows: 0−10% solvent B in 6 min, 10−50% solvent B in 34 min, 50−100% solvent B in 3 min, 100% solvent B in 5 min, followed by 0% solvent B in 12 min. The mobile phase A was 100% water with 0.1% formic acid. Mobile phase B was 100% acetonitrile with 0.1% formic acid. The nano-HPLC column was coupled to MS using a nanoelectrospray ionization source. The MS was operated in a data-dependent mode, where each MS1 scan was followed by two MS/MS scans, in which the two most abundant ions detected in the MS1 scan were fragmented by CAD. The precursor ion selection window was set at unit resolution in all MS/MS experiments.

RESULTS AND DISCUSSION

Analytical methods have always been critically important in pharmacokinetic studies of protein therapeutics, but until recently immunoassays and bioactivity assays were the two mainstays of the analytical support of pharmacokinetics of protein drugs, while MS-based methods were used only for a limited range of niche applications.[7] The explosive growth of proteomics in recent years, and the central role played by MS in this field[14] have resulted in a dramatic expansion of the scope of MS-based protein quantitation methods in pharmacokinetic studies of protein drugs.[8] Since a protein drug in circulation typically represents a very small fraction of the total plasma proteome, quantitation of a therapeutic protein in biological fluids with high sensitivity usually requires protein enrichment as a first step.

Quantitation of low-abundance proteins in plasma is usually carried out by first depleting the sample of a dozen or so of the most abundant proteins using commercially available kits.[15−17] Unfortunately, this approach cannot be readily applied for pharmacokinetic profiling of transferrin-based therapeutics, since endogenous transferrin is one of the most abundant plasma proteins and its removal from the sample using immunoprecipitation or related techniques cannot be carried out without affecting the levels of transferrin-based therapeutic agents. Our approach to circumventing this problem is based on incorporating a His-tag in the exogenous transferrin molecule, which allows selective enrichment of the low-abundance exogenous species using Ni affinity chromatography. Indeed, a proteomic analysis of bovine serum spiked with hTf* to a final concentration of 10 nM (3 orders of magnitude below that of the endogenous transferrin level) readily reveals the presence of exogenous transferrin following Ni affinity purification and proteolysis without the need for any additional steps to deplete the plasma of its most abundant proteins. At the same time, only endogenous transferrin could be detected in the same sample without metal affinity purification (see the Supporting Information for more detail).

Out of a great variety of MS-based methods developed for protein quantitation,[14] isotopic labeling is arguably the most appealing due to its simplicity and universality. Since transferrin and transferrin-based therapeutic products (as the vast majority of protein therapeutics) cannot be expressed in bacterial cells, peptide labeling with O^{18} during the proteolytic step[18] is an attractive alternative to labeling the entire protein due to the relative ease of its implementation. We modified this method by applying acid-catalyzed O^{18} labeling[10] to increase the spacing between the isotopic clusters of labeled and unlabeled peptides and reducing the occurrence of back exchange. Application of this modified O^{18} labeling procedure to the quantitation of exogenous hTf* in bovine plasma yielded good linearity over a wide concentration range; however, the slopes of the calibration curves appear to be highly dependent on the identity of the peptide selected for quantitation (Figure 1). Furthermore, even though the slope of the calibration curve should not exceed 1.0 (preparation of IS does not expose hTf* to the affinity

purification step, thereby eliminating the unavoidable protein loss), the apparent slope for one of the peptides shown in Figure 1 (SVIPSDGPSVAC*VK) exceeds that level.

Close examination of the data presented in Figure 1 reveals a common structural feature among all peptides that yield higher quantitation levels of exogenous hTf* in bovine serum: each of these peptides has at least one amino acid residue bearing an amide group on its side chain (Asn, Gln, and carboxamidomethylated cysteine, Cys*). Because of the susceptibility of amides to acid hydroloysis, deamidation was therefore suspected as a likely cause of the inconsistencies observed during quantitation. If any fraction of a peptide is degraded during the acid-catalyzed O^{18} labeling step, subsequent utilization of this peptide as an IS in protein quantitation would necessarily inflate the apparent level of exogenous hTf* in bovine serum.

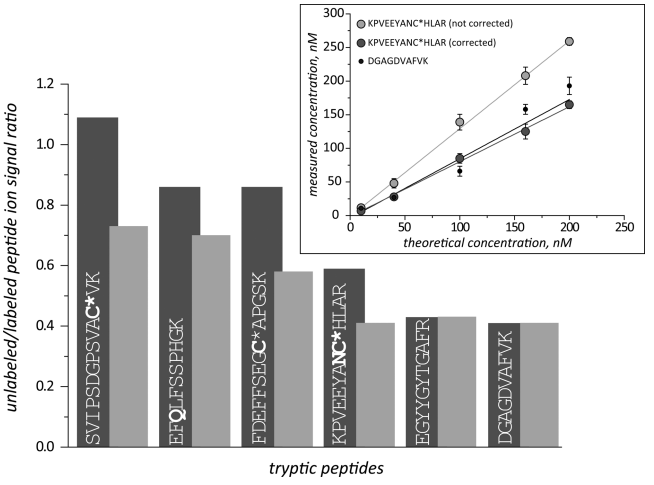

Figure 1. Quantitation of exogenous hTf* in bovine serum using acid-catalyzed O^{18} labeling. The black bars show the abundance ratio of unlabeled to labeled tryptic peptide ions for a sample in which the concentration of hTf* in bovine sample matches that of the IS. The gray bars show the same ratios after taking into account deamidation of IS tryptic peptides containing Asn, Gln, and Cys* residues. The inset shows the linearity of the calibration curves for two select peptides before and after correcting for IS deamidation.

An example of using acid-catalyzed O^{18} labeling to produce an IS for quantitation of exogenous hTf* in bovine serum is shown in Figure 2. Here the IS (O^{18} labeled tryptic digest of hTf* incubated in an acidic environment for 2 weeks) was mixed with the unlabeled digest of a Ni-affinity purified sample of bovine plasma spiked with hTf* and analyzed by LC/MS. Figure 2 presents the data for tryptic fragment KPV***EE***YANC*HLAR, where the presence of two acidic residues allows four additional O^{18} atoms to be incorporated in glutamic acid side chains in addition to the two O^{18} atoms that can occupy the C-terminal carboxylic group. This results in the complete separation of isotopic clusters of the two coeluting (labeled and unlabeled) peptides, with ionic species labeled I_0 and I_{12} representing the most abundant isotopic species of the unlabeled and labeled peptides, respectively (Figure 2D).

The presence of the low-abundance species I_{10} in Figure 2D (at m/z 532.9) is indicative of the incomplete labeling of IS (incorporation of a total of five out of six possible O^{18} atoms). Even such incomplete labeling does not prevent full separation of the isotopic clusters of the two peptides, and quantitation of exogenous hTf* can be easily carried out by relating the total intensity of ionic peaks I_0 through I_5 to that of I_{10} through I_{12} in Figure 2D. However, careful examination of the entire set of LC/MS data also reveals the presence of other ionic species that are related to tryptic fragment KPV***EE***YANC*HLAR. For example, the reconstructed ion chromatogram of the I_{15} species (seen as a minor isotopic peak in the distribution of the labeled peptide in Figure 2D) unexpectedly reveals two additional chromatographic peaks with slightly longer elution times compared to KPV***EE***YANC*HLAR (top two traces in Figure 2A). The first of these two peaks (elution time 25 min 10 s) is particularly

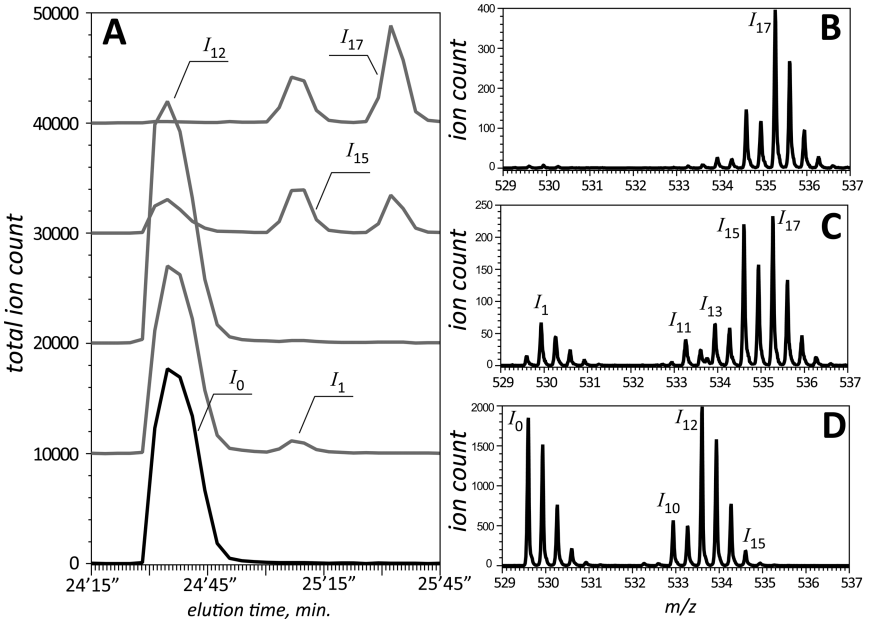

Figure 2. LC/MS analysis of a tryptic digest of hTf* Ni-affinity purified from bovine plasma and spiked with IS: extracted ion chromatograms of different ionic species related to tryptic fragment KPV***EE***YANC*HLAR (A) and mass spectra averaged across chromatographic peaks eluting at 25 min 30 s (B), 25 min 20 s (C), and 24 min 30 s (D).

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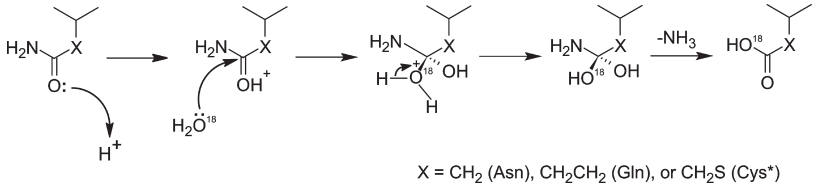
Scheme 1



interesting, as the two most abundant isotopic peaks in the mass spectrum averaged across this peak correspond to I_{15} and I_{17} species, with I_1, I_{11}, and I_{13} also being prominent (Figure 2C). While the latter three peaks resemble the pattern observed in Figure 2D shifted toward higher mass by 1 amu, the presence of I_{15} and I_{17} species in this mass spectrum is likely to suggest incorporation of O^{18} atoms by the I_{11} and/or I_{13} species. The peptide ion mass increase by 1 amu may signal its deamidation, which would also explain the ability of the peptide to incorporate up to two additional O^{18} atoms. Furthermore, while the abundant isotopic cluster in the m/z range 533−537 would be consistent with extensive deamidation of the IS under acidic conditions, the presence of the lower-abundance cluster at m/z 530 in Figure 2C is likely to highlight the occurrence of deamidation in hTf* prior to acid treatment.

The presence of the second chromatographic peak (elution time 25 min 35 s) in Figure 2A is likely to reflect the fact that KPV***EE***YANC*HLAR contains two deamidation targets (Asn and Cys*), and their modification would lead to two chemically different species, even though their masses would be indistinguishable from each other. The absence of the I_1 peak in the mass spectrum averaged across the late-eluting peak (Figure 2B) indicates that this peak corresponds to deamidation of Cys*-9, an event that is very unlikely to occur in exogenous hTf* extracted from bovine serum (since this residue was never exposed to acid following its alkylation). Therefore, the earlier-eluting chromatographic peak can be tentatively assigned as a product of deamidation of the Asn-8 side chain, which may be triggered by acid hydrolysis during the IS preparation but may also occur (albeit to a lesser extent) without deliberate acid treatment during protein production and storage (hence the appearance of I_1 species in the mass spectrum shown in Figure 2C).

Deamidation is one of the most common nonenzymatic post-transitional modifications occurring in proteins. Under near-physiological conditions, it targets primarily asparagine and proceeds via a succinimide intermediate, which can then form either an Asp or *iso*-Asp residue.[19] However, in an acidic environment deamidation can also proceed through a simple nucleophilic substitution (as shown in Scheme 1) and target Gln and Cys* in addition to Asn residues, which only produces the corresponding Glu, carboxymethylated Cys, and Asp residues.[20] While the analysis of LC/MS data in the preceding paragraph provides strong support to the notion of extensive deamidation affecting tryptic fragment KPV***EE***YANC*HLAR during the acid-catalyzed O^{18} labeling step, conclusive evidence can be obtained only from MS/MS experiments. Collisional activation of the triply charged ion corresponding to this peptide yields a fragmentation pattern that provides complete coverage of its sequence with a series of singly charged y-ions (Figure 3). Incorporation of O^{18} atoms into any amino acid side chain manifests itself by shifting the mass of the corresponding y-ion (and all larger *y*-ions), as can be clearly seen in the MS/MS spectrum of the I_{12} species, where masses of fragment ions y_2 through y_8 are increased by 4 amu (due to incorporation of two O^{18} atoms in the C-terminal of the peptide), followed by additional mass increases for y_9 (incorporation of two O^{18} atoms in the side chain of Glu-5) and y_{10} (incorporation of two more O^{18} atoms in the side chain of Glu-4).

The fragment ion mass spectrum of the I_{15} species does not show any additional mass shifts compared to I_8 for smaller fragments (up to y_5), suggesting that no modification is present in the last five residues (including Cys*-9). However, mass shifts of 3 and 5 amu are observed for all larger fragments, clearly suggesting deamidation of Asn-8 followed by incorporation of either one or two O^{18} atoms into the side chain of the newly created acidic residue. Finally, MS/MS analysis of the late-eluting I_{17} species (top right panel in Figure 3) provides conclusive evidence that in this species deamidation affects Cys*-9 residue (a mass shift of 9 amu at the y_5 fragment signals deamidation of the alkylated side chain of Cys*-9, followed by incorporation of two O^{18} atoms). Therefore, the assignments of deamidated species based on LC retention time and MS1 data analysis are completely confirmed by the results of MS/MS experiments.

Similar analyses (both LC/MS and LC/MS/MS) were carried out for other amide-containing peptides shown in Figure 1, and in all cases extensive deamidation was observed for IS prepared by acid-catalyzed O^{18} labeling (data not shown). Although the duration of labeling in these experiments was long (to ensure near-complete labeling of acidic side chains), noticeable deamidation was evident even on a much shorter time scale (data not shown). As a general trend, deamidation of Cys* appears to occur faster than that of Asn or Gln, most likely due to the excellent electron-withdrawing properties of the β sulfur atom in the Cys* side chain, which is likely to induce a higher partial positive charge on the amide carbon atom, making it a better target for the nucleophilic attack (first step in Scheme 1). The extent of deamidation varies among different peptides, but on average it is within 15−30% of the total peptide population following 2 weeks of acid-catalyzed labeling (the typical amount of time needed to achieve a near-complete labeling of the acidic side chains).

The loss of a significant fraction of an IS due to deamidation obviously leads to an artificial increase of the apparent (measured) level of the protein extracted from the biological matrix. Therefore, it is not surprising that the slopes of calibration curves based on peptides incorporating at least one side chain amide group are consistently higher compared to peptides lacking such groups. An attempt to remediate this problem by including all deamidated species in calculating the intensity of IS led to a significant narrowing of the spread of the quantitation results (see gray bars in Figure 1) but has not eliminated the differences completely.

One reason for this remaining discrepancy is the existence of a matrix effect when preparing the tryptic digests of the serum

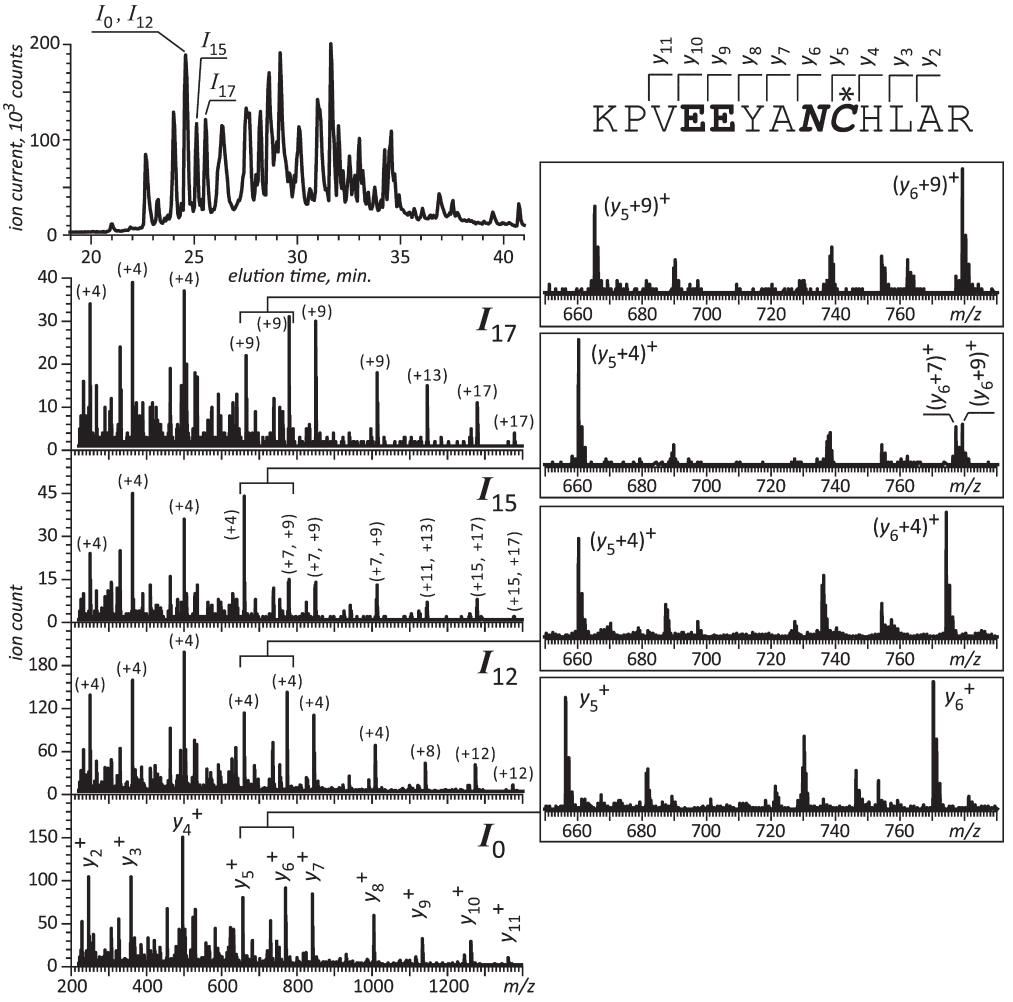


Figure 3. LC/MS/MS analysis of the structure of ionic species I_0, I_{12}, I_{15}, and I_{17} related to tryptic fragment KPV*EE*YANC*HLAR showing full views of fragment ions spectra (left column) and zoom views of m/z regions corresponding to y_5^+ and y_6^+ ions (right column). The retention times for the various parent ions are indicated in the total ion chromatogram (upper left panel).

sample and the IS, since the latter was prepared in the absence of bovine serum. Another possible reason is a possibility that in addition to the side chain amide hydrolysis, the extensive acid-catalyzed labeling may also lead to hydrolysis of backbone amides resulting in the backbone cleavage(s). The occurrence of such processes, however, could not be confirmed by screening all products of acid-catalyzed labeling of hTf* tryptic peptides, which yielded no peptides produced by dissociation of Asp−Pro bonds (the most common target of the polypeptide backbone acid hydrolysis). A more likely reason for the remaining discrepancy of the quantitation results across the peptide pool shown in Figure 1 is the inadvertent change of the ionization efficiency of deamidated peptides compared to their intact counterparts (which is caused not only by the alteration of their covalent structure but also by a change in the composition of the eluting solvent at longer retention time and a different matrix/ion suppression effect from species coeluting with the modified peptide).

CONCLUSIONS

Although acid-catalyzed O^{18} labeling offers several advantages over commonly accepted enzymatic labeling, consistent and reliable quantitation requires that careful attention be paid to the design of experiments and data processing. Extensive deamidation of all residues containing amide groups on their side chains results in a significant loss of IS, leading to inflated values of apparent (measured) protein concentration. Although this effect is mitigated to a large extent by taking into account all deamidated species within the IS when calculating the ratios of labeled and unlabeled species, some variation still remains. It is probably best to avoid using Asn-, Gln-, and Cys-containing peptides for protein quantitation.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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