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Residual metals cause variability in methionine oxidation measurements in protein pharmaceuticals using LC-UV/MS peptide mapping

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ABSTRACT

Methionine oxidation has been demonstrated to play an important role in protein stability *in vitro* and *in vivo*. It may also cause changes in biological activity and immunogenicity profile of therapeutic proteins. Therefore, it is critical to monitor methionine oxidation in biopharmaceuticals during process and formulation development, as well as long-term stability studies. A common analytical method for methionine oxidation determination is peptide mapping analysis of protein enzymatic digests using UV detection with or without mass spectrometric detection. The quantitation of oxidation is performed based on the UV or extracted ion chromatographic peak areas of the oxidized and non-oxidized peptides. This method was found to be susceptible to significant variability over long-term use. Major factors leading to this variability included presence of low levels of metal ions, especially iron, in the digestion buffer, chromatographic column, LC injector, and other sample contact surfaces. Careful control of metal ion levels generally leads to less variability and long-term consistency of peptide mapping methods for oxidation determination.

1. Introduction

Methionine is commonly believed to be one of the amino acids most susceptible to oxidation in recombinant proteins and monoclonal antibodies [1–3]. Methionine oxidation was reported to play an important role in affecting the *in vitro* and *in vivo* protein stability by introducing conformational changes to the protein structure [4–7]. Liu et al. studied humanized IgG1 antibodies expressed in both *E. Coli* and CHO cell systems with oxidized methionines in the CH_2 domains. It was found that the oxidized methionines led to changes in the secondary and tertiary structures of the CH_2 domain, which affected stability of the antibody molecules [8]. Methionine oxidation could also lead to the decrease or total loss of biological activity of proteins [9]. Moreover, it has been linked to increased aggregation [4] and increased immunogenicity of protein drugs [10]. Therefore, it is critical to monitor the oxidation levels in biopharmaceuticals through long-term stability. The therapeutic protein production process and protein formulation should assure consistent levels and no or low increase in oxidation levels over the intended storage or shelf life.

A large array of analytical methods have been developed to analyze protein oxidation level, including amino acid analysis, electrophoresis [11], immunoassays, HPLC-UV/MS of intact proteins using reversed phase, ion-exchange [12] or hydrophobic interaction chromatography [13] and HPLC-UV/MS of the protein digest after full or limited proteolysis of proteins [14–16]. The method based on proteolytic digestion and separation of non-modified and oxidized peptides by LC, and quantitation of oxidation using UV or mass spectrometric detection appears to be the most common method for monitoring oxidation levels in protein biopharmaceuticals. Using this method, the protein molecule is first cleaved into peptides, typically by enzymes with high specificity in protein cleavage. The resulting digest is usually separated using reversed phase chromatography with UV detection. Mass spectrometry is also often used for detection of peptides with greater sensitivity and specificity.

HPLC-UV/MS methods are used in our laboratory for monitoring methionine oxidation for biopharmaceutical proteins at various stages of development, from pre-clinical to commercial. However, these methods were found susceptible to large variability during long-term use. The major factors affecting this variability were attributed for the most part to trace levels of residual metals present during sample preparation and analysis. The digestion buffers, the components of LC systems and analytical columns were all found to contribute to inducing oxidation of peptides which led to variability in the oxidation measurements using the LC-UV/MS peptide mapping approach.

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In order to reduce method variability, we evaluated various compositions of digestion buffers in order to minimize artificial oxidation of peptides during protein digestion. Two common digestion buffers, containing either sodium phosphate (pH 7.2) or Tris (pH 7.5), were compared for their impact on the protein oxidation level as determined using lysyl endopeptidase (Lys-C) peptide mapping. Phosphate buffer, found to contain higher level of residual metals, induced more oxidation during protein digestion than Tris buffer. EDTA, when added to phosphate and Tris buffer, led to more consistent oxidation measurements. Analytical LC columns could also be potential sources of method variability due to different levels of residual metal ions on new columns or trapped on-column over time during column usage. Washing the columns with diluted EDTA solution restored the measured oxidation values to the expected levels. The stainless steel injection needle in the LC system could potentially introduce an increase of oxidation level in peptides following multiple injections of the digest from the same vial, presumably due to the introduction of iron into the sample *via* the exposure of the needle to the acidified digest sample [13]. In addition to the exposure of proteins or peptides to residual metals at various stages of sample handling, oxidation might be affected by trace levels of detergent in the LC system. Cleaning solutions, containing detergents with low levels of peroxide, should be avoided for laboratory glassware to minimize the artificial oxidation due to residual peroxide.

2. Materials and methods

Biogen Idec produces the recombinant proteins or monoclonal antibodies (rmAb), which were used in this work and all the chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

2.1. Lys-C digestion and comparison of digestion buffers

A volume equivalent to 100 μg of protein was diluted to 4 mg/mL in digestion buffer, *e.g.* 50 mM sodium phosphate buffer, pH 7.2. The sample was mixed with 90 μL of 8 M guanidine HCl (Pierce, Rockford, IL) and 5 μL 100 mM DTT, which was then incubated at 25 °C for 30–60 min for denaturation and reduction. The sample was further diluted by adding 235 μL digestion buffer to bring the guanidine concentration below 2 M. The digestion was conducted at 25 °C for 18–20 h using 12 μL of 0.8 μg/μL Lys-C (Wako, Richmond, VA). The final enzyme-to-substrate ratio is approximately 1:10.

The following digestion buffers were compared for their effect on the methionine oxidation using multiple IgG1 and IgG4 antibody molecules: 50 mM sodium phosphate, pH 7.2; 5 mM EDTA in 50 mM sodium phosphate, pH 7.2; 5 mM methionine in 50 mM sodium phosphate, pH 7.2; 50 mM Tris, pH 7.5; 5 mM EDTA in 50 mM Tris, pH 7.5; and 5 mM methionine in 50 mM Tris, pH 7.5. Digestion was performed in each respective buffer in the same manner as previously described. For one of the IgG1 molecules, duplicate preparations of the samples using the above digestion buffers were made on two different days to examine the reproducibility of the findings.

2.2. LC–MS peptide mapping

After digestion, 90 μL of the protein digest, corresponding to approximately 160 pmol of protein, was transferred to the injection vial with addition of 10 μL of 100 mM DTT to maintain a reducing environment in the sample until the analysis. The rest of the samples were stored at −70 °C for future use.

The peptide mapping was performed using an Agilent 1100 (Agilent Technologies, Foster City, CA) coupled with either a LCQ Deca XP (Thermo Electron, San Jose, CA) or an API 4000 (Applied Biosystems, Foster City, CA). In the former system, 90 μL of the protein digest was injected for each analysis using a YMC ODS-A, S5 column, 2.0 mm × 250 mm, 120 Å. A 3 h gradient was usually used for the separation and the LCQ Deca XP was set up to scan in the m/z range of 400–2000 amu in the positive mode. In the latter system, 10–20 μL of the protein digest was used for each analysis using a YMC ODS-A, S5 column, 2.0 mm × 150 mm, 120 Å and the API4000 was set up to scan the narrow m/z windows surrounding the theoretical m/z of at least two different charge-state ions of the targeted peptides. A shorter gradient lasting 30–50 min was usually used for a focused separation of the oxidized and the non-oxidized peptides. Both methods used mobile phases containing 0.1% (v/v) TFA in water (A) and 0.1% (v/v) TFA in 90% acetonitrile (B). The LC flow rate was 0.3 mL/min and the columns were heated to 50–55 °C during the separation.

2.3. Time-course study of the digestion process

Multiple preparations of an IgG4 antibody were reduced and denatured as previously described and pooled together after dilution using the digestion buffer. Digestion buffers for comparison in this study included 50 mM sodium phosphate, pH 7.0, with or without 10 mM EDTA. Aliquots of 100 μL of the pooled samples were placed in injection vials and kept in the Agilent 1100 autosampler tray compartment with the temperature set at 25 °C. The peptide mapping was performed using an LC-UV method and injections were made interchangeably from samples in the two digestion buffers for analysis. Altogether, 28 analyses were performed in sequence, 14 each from preparations in the two different digestion buffers. This analysis lasted approximately 30 h.

2.4. ICP-MS analysis

All reagents used in the protein digestion were analyzed by ICP-MS for metal content. Samples were diluted 1:100 by adding 100 μL of sample to 9.9 mL of 2% nitric acid. A 40 ppb instrument calibration standard was prepared by diluting a 100 ppm stock of ICP-MS Instrument Calibration Standard 2 (Perkin Elmer) 1:2500 through adding 4 μL of standard to a 10 mL volumetric flask and diluting to the volume with 2% nitric acid. Previously prepared assay controls containing 50 ppb and 1000 ppb of Instrument Calibration Standard 2 were analyzed to ensure consistency and accuracy of the method. Elemental concentrations from each sample were determined using a "TotalQuant" method on a Perkin Elmer ELAN DRC2 ICP-MS by injecting 4 mL of each sample into the nebulizer.

2.5. EDTA column-wash

An individual column producing higher than typical oxidation result for an IgG1 protein was washed with a solution of 10% ACN, 20 mM EDTA, at 0.3 mL/min overnight (approximately18 h), followed by equilibration with mobile phase A for approximately 6 h at 0.3 mL/min using the starting gradient before use. The relative percentage of oxidation measured for a Lys-C digest of an IgG1 was compared before and after EDTA column-wash. A second column, which showed lower relative percentage of oxidation without any column treatment, was used as an experiment control.

3. Results and discussion

Lys-C digestion of a protein followed by LC-UV/MS analysis is a common method used in our lab for oxidation determination. However, this approach has been found to produce variable results during long-term usage, which impedes the ability to establish trends and susceptibility of a protein to oxidation in stability

Table 1
Methionine oxidation measured for a peptide in an IgG1 protein digest prepared using digestion buffers with EDTA or methionine as additives.

Digestion buffers	% Met oxidation			Absolute difference (%)	Relative difference (%)
	1st prep[a] (%)	2nd prep[a] (%)	Average (%)		
50 mM phosphate	7.4	8.0	7.7	0.6	7.7
5 mM EDTA in 50 mM phosphate	5.1	5.4	5.3	0.3	5.0
5 mM Met in 50 mM phosphate	5.7	6.2	6.0	0.5	9.1
50 mM Tris	4.3	4.3	4.3	0.0	0.3
5 mM EDTA in 50 mM Tris	3.9	3.9	3.9	0.1	1.6
5 mM Met in 50 mM Tris	4.2	4.4	4.3	0.2	5.7

[a] The 1st and 2nd preparations of the protein digest were performed on two different days. Each preparation was made in duplicate and the reported values in this table are average of the duplicate measurements.

studies, as well as reduces the value of such an approach for protein characterization and comparability studies. The variability was suspected to result from low levels of residual metals, which are present during sample preparation and analysis. In order to minimize this variability, as well as confirm the source being the residual metals, the components of the protein digestion buffers, LC system and LC column were examined for their potential contribution to methionine oxidation measurement variability.

3.1. Protein digestion buffers

In order to determine the potential effect of trace metal ion levels on protein oxidation during sample preparation, two buffer systems, commonly used for protein digestion in our laboratory, phosphate buffer (50 mM sodium phosphate buffer, pH 7.2) and Tris buffer (50 mm Tris HCl buffer, pH 7.5) have been examined. Lys-C digests of two IgGs and a recombinant protein were prepared in these digestion buffers containing different additives such as EDTA or methionine. The resulting protein digest samples were analyzed using peptide mapping by HPLC with UV and mass spectrometric (MS) detection. Methionine oxidation levels were determined by relative quantitation of the methionine-containing peptides and their oxidized counterparts using either UV or extracted ion chromatographic (XIC) peak areas of the oxidized and non-oxidized peptides, as described in Section 2.

Table 1 shows the methionine oxidation results measured for the most oxidation-susceptible Lys-C peptide from an IgG1 molecule, prepared using different combinations of additives in either phosphate or Tris buffer. It is clearly demonstrated that switching from phosphate to Tris buffer leads to a decrease of the oxidation level for this peptide. In addition, the inclusion of EDTA or methionine in both phosphate and Tris buffers reduced oxidation levels, indicating that the addition of a metal chelating reagent or an antioxidant both inhibited extra oxidation otherwise occurring during sample preparation. EDTA works as a chelating reagent, forming a chelating complex with the metal ions in the solution to minimize metal ion catalyzed oxidation. Methionine is a common antioxidant [17], which acts through competing with the methionine residues in peptides for oxidation due to its higher susceptibility to oxidation. Between EDTA and methionine, EDTA reduces oxidation in each group more effectively. Addition of 5 mM EDTA in phosphate buffer decreases the oxidation levels, however not to the same degree as 50 mM Tris buffer alone, indicating that 5 mM EDTA is not sufficient to fully remove the effect of metals present in the phosphate buffers used for protein digestion.

Day-to-day variability of oxidation determination using different digestion buffer systems was evaluated using the same buffer preparations on two different days. As shown by the results in Table 1, the digestion performed in phosphate buffer was found to have more day-to-day variability in terms of oxidation, while the preparations using Tris buffer showed reproducible oxidation measurements on two different days. All the observations led to the conclusion that 5 mM EDTA in 50 mM Tris buffer, pH 7.5, would be an appropriate buffer system for the Lys-C digestion of proteins for the purpose of methionine oxidation determination using peptide mapping approach.

In order to clearly understand the contribution of the metal ions to the induced oxidation occurring during the Lys-C protein digestion, all the reagents used in Lys-C digestion of proteins were analyzed by ICP-MS for metal quantitation. It was found that three metal elements, Cr, V and Fe, were present at the highest levels compared to all the other elements tested. Fig. 1 shows concentration of these three elements in the analyzed reagents. Almost all the reagents were found with Fe present at significantly higher level than Cr or V except guanidine HCl, which contained comparable levels of the three. Tris buffer stock (1 M), 20-fold diluted to make the 50 mM Tris buffer used for digestion, contained Fe at the level of 1149 ppb, which corresponds to 57 ppb of Fe in the final 50 mM Tris buffer. HPLC water (Fisher, Fairlawn, NJ) was used for all the dilution with a known total evaporation residue of no more than 1 ppm. In comparison, the 50 mM phosphate buffer contained 734 ppb of Fe, over 10-fold higher level as compared to 50 mM Tris buffer. Although the intact protein sample in its formulation buffer contained a relatively high level of Fe at 1319 ppb prior to dilution into the digestion buffer, the introduction of Fe from protein sample to the digest was minimal as only a few microliters of the sample was eventually diluted in more than 300 μl of the digestion buffer, as initial protein concentration in the formulation buffer is commonly at 20–100 mg/mL.

Combined with Fe introduced by the protein formulation buffer and the reagents used for protein digestion, the level of Fe reached 1911 ppb in the protein digest samples prepared in phosphate buffer. Main contributors to the Fe level in the protein digest samples were guanidine and digestion buffer, which accounted for 1/4 and 2/3 of the final volume of the protein digest, respectively. As the Tris buffer contained a significantly lower level of Fe compared to phosphate buffer, the protein digest prepared in Tris buffer should contain much less Fe in the final digest. The data shown in Table 1 demonstrated that inclusion of a chelating reagent such as EDTA effectively prevented Fe in the digest mixture from causing artificially elevated oxidation of the Met-containing peptides. The weak chelating ability of Tris might have also contributed to reduction of the Fe-catalyzed oxidation in the protein digest in Tris buffer without EDTA addition [18]. It is therefore confirmed that the Fe ions constitute a major cause of induced protein oxidation occurring during protein digestion. The use of Tris buffer containing lower levels of Fe, in addition to its chelating ability have resulted in minimizing oxidation induced by the presence of residual metals.

As shown in Table 1, inclusion of 5 mM EDTA in phosphate buffer was not adequate to eliminate the effect of residual metals on the elevated methionine oxidation. A separate experiment was performed for an IgG1 to find out if addition of higher levels of EDTA to the 50 mM phosphate buffer could eliminate artificial oxidation of peptides to same level as 5 mM EDTA does in 50 mM Tris buffer. The results of this experiment are shown in Table 2. It was found

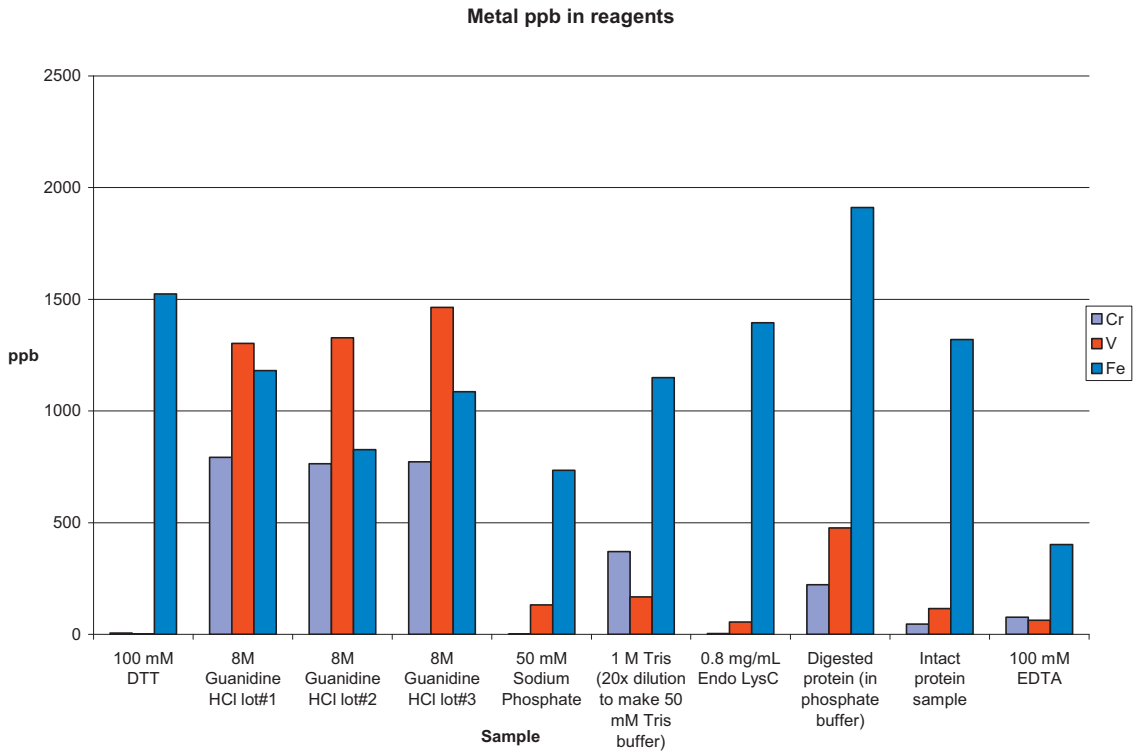

Fig. 1. ICP-MS analysis of metal levels in digestion reagents. The levels of three major metal contents found in the Lys-C digestion reagents are shown in this figure. Sodium phosphate buffer was found to contain 10-fold higher Fe content than the Tris buffer.

that addition of 10 mM EDTA to 50 mM phosphate buffer resulted in similar oxidation levels measured for all the peptides compared to 5 mM EDTA in 50 mM Tris buffer, pH 7.5. This result indicates an opportunity of using either one of these two buffer systems for a peptide mapping method for methionine oxidation determination.

In order to confirm the applicability of 10 mM EDTA in 50 mM phosphate buffer as the digestion buffer for an oxidation peptide mapping method, a time-course study was performed using a LC-UV method monitoring one Met-containing peptide and its oxidized form for an IgG4 antibody molecule. Altogether 28 analyses were performed in an alternative sequence for the protein digest, prepared respectively in 50 mM phosphate buffer with or without 10 mM EDTA and the results are shown in Fig. 2. The LC-UV method variability is consistent between the tests done for digest in the two different buffers as the samples from both buffers were analyzed alternatively in the same sequence run. The data demonstrated that the methionine oxidation level of this peptide kept increasing during the digestion process in 50 mM phosphate buffer from 0.8% after 5 h to 3.5% at the end of 30 h digestion. In comparison, the methionine oxidation showed much lower rate of increase during the digestion, from 0.5% to 0.7% within the same period of time, in the EDTA-containing phosphate buffer. The change between 0.5 and 0.7% may reflect the variability of using the LC-UV method for oxidation determination. The increase from 0.8% to 3.5% for protein digest shows the artificial oxidation introduced during the digestion in phosphate buffer without EDTA.

Although 10 mM EDTA in 50 mM phosphate buffer, pH 7.2 and 5 mM EDTA in 50 mM Tris buffer, pH 7.5 both demonstrated comparable and consistent performance for the methionine oxidation quantitation, it was found that the use of Tris buffer system at pH 7.5 resulted in increased deamidation levels on some Asparagine-containing peptides compared to the phosphate buffer system. As shown in Tables 2 and 3, Asn-containing peptides from IgGs and a recombinant protein all showed increased deamidation when the protein digestion was performed in Tris buffer system. At pH above 7, the deamidation rate of Asn is believed to be proportional to the hydroxide ion concentration of the solution [19]. Buffer catalysis effect was also observed for both the phosphate and the Tris buffer when pH was above 7 [20]. As Tris (pK_a = 8.1 at 20 °C) buffer is typically used at slightly higher pH such as 7.5 compared to the 50 mM sodium phosphate (pK_a = 7.2 at 20 °C) buffer at 7.2 in our laboratory, slightly higher concentration of hydroxide ions in Tris buffer and the catalytic effect from Tris may have led to the higher deamidation levels in these peptides. In order to use a common procedure for analysis of both methionine oxidation and asparagine deamidation for characterization of biopharmaceutical proteins, it is necessary to use a buffer system that introduces the least artifacts in both modifications.

Table 2
Methionine oxidation measured for protein digest prepared using different digestion buffers.

Buffers	Peptides from an IgG1 protein						
	Oxidation					Deamidation	
	H1[a] (%)	H16[a] (%)	H31[a] (%)	L1[a] (%)	L2[a] (%)	H18-19[a] (%)	H28[a] (%)
50 mM phosphate	4.8	9.1	6.7	3.6	6.4	3.8	3.7
10 mM EDTA in 50 mM phosphate	1.0	6.8	3.9	0.9	2.7	4.0	3.9
5 mM EDTA in 50 mM Tris	0.6	5.7	4.3	0.6	3.1	11.1	11.5

[a] Designations for the different peptides monitored for methionine oxidation or asparagine deamidation in the tested proteins.

Table 3
Increased deamidation of peptides found from protein digest prepared in Tris buffer.

Buffers	IgG1 protein		Recombinant protein	
	H30[a] (%)	L1[a] (%)	L27-28[a] (%)	L17[a] (%)
50 mM sodium phosphate, pH 7.2	0.0	0.4	22.4	0.3
50 mM Tris, pH 7.5	2.5	7.7	22.5	3.6

[a] Designations for the different peptides monitored for asparagine deamidation in the tested proteins.

In this work, only Lys-C enzyme has been evaluated, which does not require the presence of any metal to maintain its activity. Other enzymes, such as Asp-N or Lys-N metalloendopeptidase, which require the presence of metals for their activity, will most likely not work properly in digestion buffer containing EDTA. When using these enzymes to perform digestion, caution should be taken for the presence of excess metal ions in the solution to avoid introduction of artificial oxidation to the peptides. One option is inclusion of antioxidants in the digestion buffer, such as methionine, to suppress induced oxidation in the targeted peptides. Tris buffer, possessing a weak chelating ability, has worked well for Asp-N digestion in our hands, which may also be used if oxidation needs to be determined from an Asp-N peptide map.

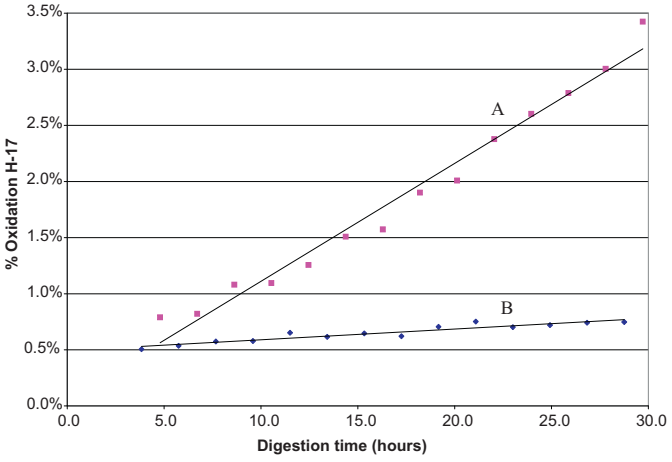

Fig. 2. Digestion time-course monitoring of methionine oxidation from an IgG4 antibody in 50 mM sodium phosphate buffer without (A) and with (B) 10 mM EDTA. Time-course study of methionine oxidation measured using a LC/UV peptide mapping method from an IgG4 Lys-C digest prepared in 50 mM sodium phosphate buffer, pH 7.0 with and without 10 mM EDTA. An injection was made every hour from the protein digest while the digestion was proceeding on the autosampler tray. The oxidation was quantitated from the UV peak areas of the oxidized and non-oxidized peptides, which was found to increase during the digestion process much more apparently in 50 mM sodium phosphate buffer without EDTA. In the specified time range for digestion, 18–24 h, the oxidation value measured from digest prepared in 50 mM sodium phosphate buffer without EDTA ranged from 1.9 to 2.6%, and ranged from 0.6 to 0.7% from the digest prepared in 10 mM EDTA in 50 mM sodium phosphate buffer. This indicated that a more reproducible and robust protein digest can be prepared in presence of EDTA in the phosphate buffer for oxidation measurement using peptide mapping method.

3.2. Oxidation induced by LC columns and injection systems

In addition to metal impurities present in the reagents used for the protein digestion; they are also present in devices and on surfaces to which the protein digest is exposed during the analysis.

Examination of column-to-column reproducibility is one component of method qualification for measurement of Met oxidation by peptide mapping in our laboratory. It has been observed that the same type of columns from different lots can lead to different oxidation values from the same protein digest. Fig. 3 shows overlaid XIC chromatograms of an IgG1 Lys-C digest analyzed using two columns from different lots before and after an EDTA wash. Table 4 summarizes the oxidation values measured under these different conditions. The peptide monitored by this method contained two methionines and oxidation of the two methionines led to two separate singly oxidized peptide peaks, peak 1 and peak 2. Both peaks showed peptide masses with an additional 16 Da to the mass of the intact peptide. Peak 1 was a doublet peak, suspected to have resulted from the separation of the R- and S- isomer pairs formed by the methionine oxidation [21]. The identification of peaks 1 and 2 is described in Supplemental material.

The relative oxidation level for peak 1 was elevated from 3.2% (Fig. 3A, solid trace, column 2) to 5.0% (Fig. 3A, dotted trace, column 1). At the same time, peak 2 oxidation levels increased from 1.2% to 3.1%. Data are summarized in Table 4. Peak 3 corresponds to the non-oxidized peptide and peak 4 represents the same peptide containing one deamidated asparagine. Peaks 3 and 4 did not have a significant change of their relative intensity between the two columns. Fig. 3B shows the overlaid chromatograms obtained after column 1 was washed overnight with mobile phase containing 20 mM EDTA. After the EDTA wash, the oxidation level determined using column 1 (Fig. 3B, dotted trace, column 1) became comparable with the value measured from column 2 (Fig. 3B, solid trace, column 1). The peak 1 oxidation level was found to be 3.9% (column 1) and 3.7% (column 2). Meanwhile, the peak 2 oxidation level was found to be 1.1% using column 2 and 1.5% when column 1 was used post the EDTA wash, as shown in Table 4. The overnight wash using EDTA-containing solution has most likely removed the residual metal contents from the solid phase packing in column 1 and therefore eliminated the on-column metal-catalyzed oxidation during chromatographic separation.

Metal ions introduced from any surface in contact with the protein digest can result in artificial increase of oxidation levels. For example, elevated oxidation was observed when the same Lys-C digest was injected repeatedly from the same vial for LC-UV/MS

Table 4
Oxidation measured for a peptide from an IgG antibody using columns cleaned by EDTA.

Columns	Before EDTA wash		After EDTA wash	
	Column lot 1 (%)	Column lot 2[b] (%)	Column lot 1 (%)	Column lot 2[b] (%)
% Oxidation (peak 1)[a]	5.0	3.2	3.9	3.7
RSD	3.7	4.5	2.2	3.5
% Oxidation (peak 2)[a]	3.1	1.2	1.5	1.1
RSD	13.2	6.6	10.2	8.0

[a] Each %oxidation was an average of 6 replicate injections.
[b] Column lot 2 was not treated with EDTA wash and used as a control in the experiment.

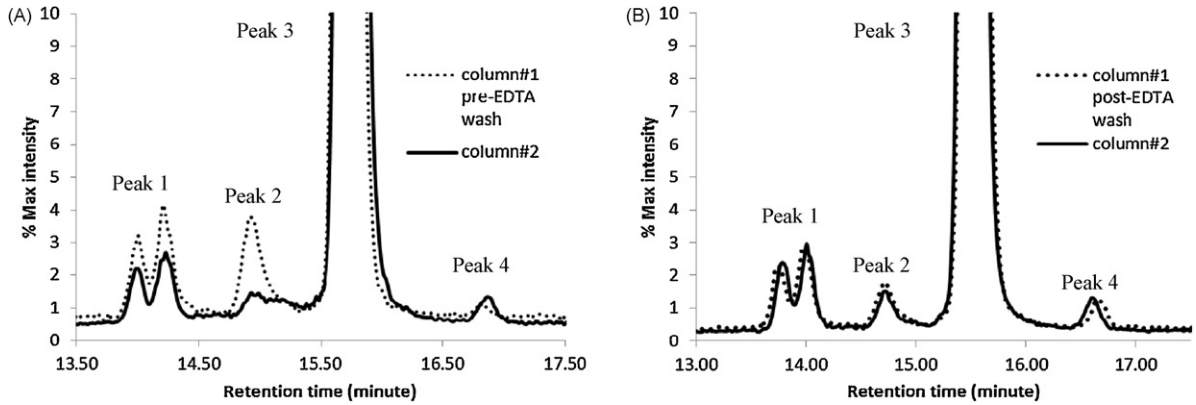

Fig. 3. Total ion chromatograms of an oxidation peptide map from a column before (A) and after (B) an overnight EDTA wash. The peptide monitored in this method contains two methionines, single oxidation of which resulted in two separate oxidation peptide peaks, peaks 1 and 2. Peak 3 is the non-oxidized peptide peak and peak 4 is the deamidation peptide peak. The chromatograms were normalized by peak 3, the most abundant peak in the chromatograms. The levels of oxidation indicated by peaks 1 and 2 were found to be artificially elevated when analyzing the same protein digests using column 1 (dotted trace) in comparison with column 2 (solid trace), shown in (A). Column 1 (dotted trace) after overnight wash with EDTA-containing mobile phase led to a decrease of the oxidation level of peaks 1 and 2 to comparable levels as column 2, which was not treated with EDTA wash (dotted trace) and used as a control in the experiment, shown in (B).

analysis. It has been suggested that low amount of acid added to Lys-C digest at the end of the digestion to stop the digestion process aids in a transfer of trace levels of metal ions from the stainless steel autosampler injection needle and a subsequent increase of oxidation for later injections (data not shown). In order to avoid this phenomenon, the stainless steel injection needle could be replaced with a PEEK injection needle; alternatively, multiple injections from the same vial should be avoided.

It is also useful to note that detergents used for washing the mobile phase containing bottles may contain small amounts of peroxide [22], which can result in additional peptide oxidation during the chromatographic separation (data not shown). Therefore, it is recommended to avoid washing laboratory glassware used for peptide mapping analysis with detergents potentially containing residual peroxide.

4. Conclusions

A LC-UV/MS peptide mapping approach for protein oxidation measurement is susceptible to method variability introduced by multiple sources, including presence of low levels of metal ions such as Fe in digestion reagents, chromatography columns, and injection needles.

Digestion buffer components may vary significantly in their metal content, and therefore induce variable and inconsistent levels of oxidation. Phosphate buffer was found to generally contain higher metal content compared to Tris buffer, which led to higher oxidation values measured in phosphate buffer system. At the same time, Tris buffer at pH 7.5 induced increased deamidation levels on selected asparagine-containing peptides in our study. Addition of 10 mM EDTA to 50 mM phosphate buffer, pH 7.2 was found to be a good compromise for analysis of pharmaceutical proteins, which allowed eliminating the induced increase of peptide oxidation and deamidation occurring during protein digestion. This buffer was successfully utilized for Lys-C digestion procedure and is recommended for characterization and quantitative analysis, such as stability studies, of IgGs and recombinant proteins. However, if metal-catalyzed enzymes are desired for protein digestion, separate peptide mapping preparations in different buffer systems might have to be used for separate oxidation and deamidation measurements, or anti-oxidants may have to be added to suppress artificial oxidation during sample preparation. Care must be taken to reduce potential metal impurities in new HPLC columns by, for example, using chelating column washes in cases where higher than expected oxidation measurements were obtained and a chromatographic column is a suspect. Used LC columns may be exposed to different levels of metals during the column life-time and application of different mobile phases, which might result in variable measurements of methionine oxidation; in such cases chelating column washes may be helpful. Also, when stainless steel autoinjector needles are used for sample injection, only one injection per vial is recommended as the interaction between the metal needle and the sample can result in higher oxidation values in subsequent measurements, where the injections from the same vial have been performed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.03.016.

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