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International Journal of Pharmaceutics 322 (2006) 136–145
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Glycation of interferon-beta-1b and human serum albumin in a lyophilized glucose formulation Part III: Application of proteomic analysis to the manufacture of biological drugs☆

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Received 13 April 2006; received in revised form 22 June 2006; accepted 26 June 2006
Available online 1 July 2006

Abstract

Glycation of interferon-beta-1b and human serum albumin was identified in a lyophilized glucose formulation by a sensitive LC–MS approach. The extent of glycation was measured by a label-free quantitation strategy. The glycation sites were determined by the accurate mass (FTICR MS) with MS/MS measurements on the corresponding tryptic peptides. The extent of glycation was measured by the ratio of the peak intensity between the glycated and the average value for three non-glycated peptides in the same run. Residues lysine 18 of interferon-beta-1b, and lysine 51, lysine 233, and lysine 545 of human serum albumin were more prone to be glycated than other sites in this lyophilized glucose formulation. Residues of lysine 51 and lysine 233 but not lysine 545 of human serum albumin are highly accessible to solvent as found in a solution storage study by Lapolla et al. The extent of glycation of both proteins and the number of glycation sites of human serum albumin were increased with the storage time at 25 °C. In total, two glycation sites of interferon beta-1b and 17 glycation sites of human serum albumin were identified in the lyophilized glucose formulation with a storage time at 25 °C of 35 days. Among the 17 glycation sites, only lysine 525 of human serum albumin has been found *in vivo* in diabetic patients by Shaklai et al. As expected, there was no glycation found on both interferon-beta-1b and human serum albumin in the control samples (similar lyophilized formulation but using mannitol instead of glucose).

Keywords: Glycation; Interferon-beta-1b; Human serum albumin; Lyophilized drug product; Proteomic analysis; LC-MS; Fourier transform mass spectrometry; Quantitation

1. Introduction

Appropriate formulation of therapeutic protein drugs is an important step in the manufacturing process as related to drug stability and safety. An ideal formulation can prevent proteins from degradation or modification, which is frequently the key concern in biopharmaceutical product development (Chloupek et al., 1989; Chen et al., 1994; Shahrokh et al., 1994; Li et al., 1996). Lyophilized drugs often have longer stability profile (i.e. shelf life) than drugs in liquid formulation. To prevent protein aggregation or degradation during lyophilization process, sugar-related excipients are often used as lyophilization protectants (Li et al., 1996). However, reaction of the excipients with the protein drugs can cause safety concerns and should be avoided as much as possible.

Native beta interferon (IFN-beta) is a hydrophobic protein with 166 amino acids (Houghton et al., 1980; Lin, 1998). Several pharmaceutical companies have manufactured recombinant interferon beta to treat relapse-remitting multiple sclerosis, such as Biogen's Avonex™ (interferon-beta-1a) and Berlex's Betaseron™ (interferon-beta-1b). Recombinant interferon-beta-1b from Betaseron is an analogue of native interferon-beta-1b, which lacks the methionine at the N-terminus of native interferon-beta-1b and has serine rather than

☆ For the first and the second papers in the series see references Wang et al. (2005) and Wang et al. (2006b).

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doi:10.1016/j.ijpharm.2006.06.038

cysteine at position 17 (Lin, 1998). The substitution at position 17 was made to eliminate the free sulfhydryl of cysteine to obtain a product that is more stable upon storage.

Since beta interferon is hydrophobic in nature, a carrier protein such as albumin and an excipient such as mannitol are used in the product formulation. Lately, a generic version of this product using glucose instead of mannitol as the excipient for the formulation was developed (Uribeta as the brand name). Since glucose but not mannitol has a reducing end carbonyl group, it is important to study the stability of this generic drug (interferon beta-1b) even it contains the same carrier protein (human serum albumin).

In this study, we will focus on the glycation modification of interferon beta-1b caused by the Maillard reaction. In theory, the Maillard reaction will take place if proteins containing lysine residues are stored with sugars that contain a reducing carbonyl group such as glucose (Schalkwijk et al., 2002). Glycated human serum albumin (gHSA) and glycated interferon-beta-1b are potentially risky compounds. For example, gHSA is associated with diabetes: extra glucose in human blood produces gHSA *in vivo* leading to immunological problems like retinopathy (Schalkwijk et al., 2002).

Proteomics is the systematic analysis of protein expression patterns including analyzing protein modifications and protein quantity change (Peng and Gygi, 2001). There are two general approaches to analyze a proteome: one is two-dimensional gel electrophoresis coupled with mass spectrometry and the other is reversed phase liquid chromatography coupled with ion-trap tandem mass spectrometry analysis (nanoLC–ESI–MS/MS) of the corresponding tryptic digest (Link et al., 1999). The new generations of mass spectrometers with high detection sensitivity have been used to study protein or peptide modifications extensively using nanoLC–ESI–MS/MS (Ficarro et al., 2002; Wu et al., 2005; Wang et al., 2006a). In this study, we adopted our nanoLC–ESI–MS/MS approach to identify and quantify the existence and location of glycation (162 Da) formed in generic interferon-beta-1b in a high throughput manner. Another important aspect of this study is the use of the new generation mass spectrometers, namely LTQ-FT MS. The glycation site could be determined by combining the accurate mass measurement (FTICR MS) and sequence assignment (MS/MS measurement in LTQ MS) of the corresponding tryptic peptides. In addition, quantitation of selected peptides in the samples with different storage conditions can be achieved via the measurement of peptide ion intensity after normalization with intrinsic internal standards to study the extent of glycation (Chelius and Bondarenko, 2002; Chelius et al., 2003; Steen et al., 2005).

2. Experimental

2.1. Materials

Interferon-beta-1b, the active drug ingredient, was in a lyophilized formulation containing human serum albumin and glucose (Uribeta as the brand name). The drug product in this lyophilized vial contained 300 μg of interferon-beta-1b, 15 mg of human serum albumin, and 15 mg of glucose. In the control sample, interferon-beta-1b was in a similar formulation but using mannitol instead of glucose (Betaseron as the brand name). In this drug product, the lyophilized vial contained 300 μg of interferon-beta-1b, 15 mg of human serum albumin, and 15 mg of mannitol. Both drug products (Uribeta and Betaseron, three vials per product) were stored at the following conditions: 4 °C for 2 months, which was set as starting time point (T0), 25 °C for 7 days (after 2 months at 4 °C), which was designed as T7, and 25 °C for 35 days (after 2 months at 4 °C), which was designed as T35. Every storage condition comprised three vials from three different lots. Trifluoracetic acid (TFA), formic acid (FA), and ammonium bicarbonate were obtained from ICN Biomedicals (Aurora, OH). MALTI-TOF matrix, α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma (St. Louis, MO). The CHCA matrix was recrystallized before use. Microcon YM-10 and Microcon YM-3 Centrifugal Filter Unit were purchased from Millipore (Bedford, MA). LC–MS grade water was purchased from JT baker (Phillipsburg, NJ). HPLC grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ). Wide-pore C4 (250 mm × 4.6 mm, 5 μm) column was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

2.2. Equipment

The HPLC for intact protein separation was performed on a Shimadzu VPSeries HPLC system (Shimadzu, Norwell, MA). Intact protein measurement by MALDI TOF MS analysis was performed on a 4700 Proteomics Analyzer (Applied Biosystems Framingham, MA). The nanoLC for tryptic peptide separation was performed on an Ultimate 3000 nanoLC pump (Dionex, Mountain View, CA) and the online peptide detection was carried out by LTQ and LTQ-FT mass spectrometers (Thermo Electron, San Jose, CA).

2.3. Chromatography

The lyophilized vials (from different storage conditions) were reconstituted with 1.2 mL of the sterile diluent (0.54% sodium chloride) provided from the manufacturers. An aliquot of 100 μL was loaded on a wide-pore C4 (4.6 mm × 25 cm, 5 μm particle) reversed-phase column (Baker, Phillipsburg, NJ) for the separation of intact human serum albumin and interferon-beta-1b. The gradient was started at 30% B (A: water with 0.1% TFA; B: acetonitrile with 0.1% TFA) for 10 min, and linearly increased to 70% B in 60 min. To avoid the possible carryover of human serum albumin, the same C4 reversed-phase column was used to re-chromatograph the collected interferon-beta-1b fraction with a three step linear gradient, starting from 0% to 42% B in 22 min, then increasing to 55%B in 23 min, and then further increasing to 100% B in 5 min.

2.4. Mass spectrometry

For intact protein measurement, an aliquot of 1 μg of interferon-beta-1b was mixed with 5 μL of matrix solution (7 mg/mL CHCA, 0.1% (v/v) TFA in 50% (v/v) ACN/water), and the mixture was then deposited onto a stainless steel MALDI plate for MS measurement. The MS spectrum was obtained from

a total of 1000 laser shots across the entire spot (50 laser shots at each of 20 random positions within the spot).

For tryptic peptide measurement, a 100 μg amount of interferon-beta-1b (or human serum albumin) was reconstituted with 500 μL of 6 M guanidine hydrochloride containing 100 mM ammonium bicarbonate, reduced with 5 mM DTT for 30 min at 37 °C and alkylated with 20 mM of IAA in the dark for 90 min at room temperature. Then the reduced and alkylated protein fraction was transferred to a Microcon centrifugal filter with a 3 kDa (or 10 kDa for human serum albumin) molecular weight cut-off (MWCO) to remove guanidine hydrochloride and excess DTT and IAA and buffer exchanged to trypsin digestion buffer (100 mM ammonium bicarbonate, pH 8). Trypsin (1:100 w/w) was added to the protein solution for 8 h at 37 °C, and further incubated with another dose of trypsin (1:100 w/w) for an additional 12 h at room temperature. The trypsin-digested mixture (~1 μg) was loaded on a capillary column (75 μm × 150 mm) packed with Magic C18 (3 μm, 200 Å pore size) (Michrom Bioresources, Auburn, CA). The analytical separation was carried out by using a three step linear gradient, starting from 2% B to 40% B in 40 min (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid), increased to 60% B in 10 min, and then increased to 80% B in 5 min. The flow rate was maintained at 200 nL/min after splitting. The LTQ-FT MS was operated as in the following conditions: a full precursor ion measurement using FTICR mass analyzer (scan from 400 to 2000 *m/z* at 100,000 resolution) followed by fragmentation of the eight most abundant precursor ions using collision-induced dissociation in the linear ion trap mass analyzer (data-dependent MS/MS scan with 28% normalized collision energy) throughout the entire 55 min separation.

2.5. Protein identification and quantitation

Peptide sequences were first determined by BioWorks3.2 imbedded with Sequest (Thermo Electron, San Jose, CA) from a human database (http://ncbi.nih.gov/blast.db/FASTA on 01/15/2006) to include interferon-beta-1b, which lacks the signal sequence and replace the cysteine at position 17 of interferon with serine. Peptide Prophet probability software (Institute of System Biology, Seattle, WA), was then utilized to screen peptides with >95% confidence, followed by confirmation from accurate mass assignment (within 10 ppm). The peak areas from the extracted ions (i.e. glycated peptides) were used for comparison.

3. Results and discussions

Protein drugs in biotechnology manufacturing are often complex in nature. Recently, the advent of generic or 'follow-on' biologicals may challenge the need of well defined manufacturing processes even for producing identical protein drugs. It is clear that in this context proteomics is a very powerful technology, which is capable of measuring many protein changes and can evaluate the production of safe generic drugs. Recently we systematically applied proteomic technologies to each stage of the manufacturing process, ranging from raw materials, the fermentation, recovery and formulation process. We have published one study using proteomics tools to better understand the growth of *E. coli* on glucose in high density, fed-batch cultures and response to the overexpression of plasmid-encoded 6-phosphogluconolactonase (PGL) (Wang et al., 2005), and one study on using proteomics tools to monitor specific changes in individual proteins in the fermentation medium (Zheng et al., in press). In this study, we used proteomic tools to study the protein modification, glycation, arising from the formulation process.

The major goal of this study is to detect and locate non-enzymatic glycation products in two different interferon beta-1b (IFN-beta-1b) lyophilized formulations. One formulation used glucose and the other used mannitol and both formulations included the same amount of IFN-beta-1b and human serum albumin, and the same pH after reconstitution with the same diluent. The workflow used to study the two slightly different formulations was as follows: reconstitute the lyophilized vials, separate the constituent proteins, and then analyze the separated fractions.

3.1. Protein separation

Because the ratio of human serum albumin and IFN-beta-1b is 50:1 in both formulations, it is necessary to separate them first to avoid the high abundance of human serum albumin masking the detailed study of IFN-beta-1b. In addition, the IFN-beta-1b fraction is usually re-chromatographed to eliminate any carryover from human serum albumin. Fig. 1A shows the protein separation profile. Human serum albumin (eluted between 14 and 22 min) was eluted significantly earlier than IFN-beta-1b (eluted between 33 and 38 min) even though the molecular weight of human serum albumin (~66 kDa) is three times bigger than that of IFN-beta-1b (~19 kDa). It is clear, therefore, that IFN-beta-1b is a more hydrophobic protein than human serum albumin. As shown in Fig. 1A, the separation profiles are very similar for these two formulations, except that the one related to the glucose formulation has a shoulder in the IFN-beta-1b peak, which may indicate some modification. We then used MALDI-TOF MS to study the two IFN-beta-1b peaks.

3.2. Glycation identification—interferon-beta-1b

As shown in Fig. 1B, the IFN-beta-1b fraction (in glucose formulation) was revealed to contain two species by MALDI-TOF MS, 19,833 Da and +162 Da species (see the zoom-in spectrum in the insert). In contrast, the IFN-beta-1b fraction in the mannitol formulation was shown with only one species with a molecular weight approximately 19,830 Da by MALDI-TOF MS (data not shown). The molecular weight difference of 162 Da indicated a possible glucose addition to IFN-beta-1b formulated with glucose. To further confirm this observation, a detailed peptide sequence analysis was performed.

As shown in Fig. 2A, the tryptic peptide sequence analysis revealed the glycation site at K18 (Note: This glycation site was observed for samples stored at 4 °C. Other glycation sites found at different storage conditions were discussed later.). This result was consistent with the result obtained from MALDI-TOF

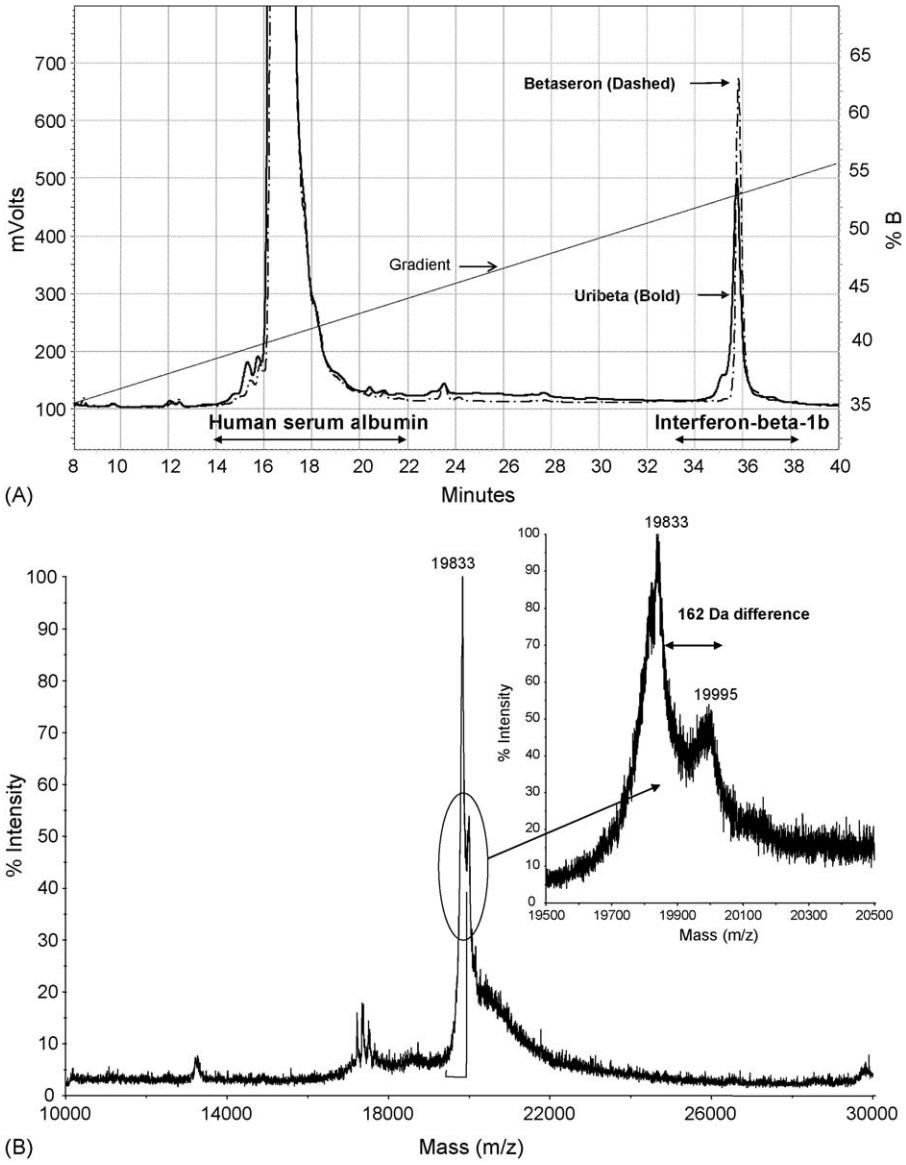

Fig. 1. (A) Separation of the constitute proteins in Betaseron and Uribeta formulation. Each drug product (Betaseron and Uribeta) was separated by a C-4 reversed phase column (2.1 mm i.d. × 25 cm) using a water and acetonitrile gradient. As indicated in the figure, fractions corresponding to human serum albumin (eluted between 14 and 22 min) and beta-interferon (eluted between 34 and 38 min) were collected. The glucose (or mannitol) fraction eluted at the beginning of the gradient (not shown) was not collected. The two separation profiles (one for Betaseron and the other for Uribeta) were overlaid for comparison. (B) Analysis of interferon-beta-1b from the glucose formulation (Uribeta) by MALDI TOF MS. Aliquots of intact beta-interferon from Uribeta, collected from the C-4 column first and re-chromatograph with C-4 column again, was analyzed by MALDI-TOF MS. The molecular weight of 19833 Da and +162 Da species (zoom-in spectrum in the insert) were shown. The intact beta-interferon from Betaseron did not have the additional (+162 Da) species (data not shown).

MS analysis of intact IFN-beta-1b. Also in this analysis, the expected sequence for recombinant interferon, which did not have the methionine at the N-terminus and also the substitution of cysteine with serine at position 17, was confirmed. The majority of IFN-beta-1b sequence was identified except very small tryptic fragments (as indicated by arrows) and resulted in >95% sequence coverage. It is interesting to note that one peptide, ^{52}EDAALTIYEMLQNIFAIF**R**QDSSSTGWNETIVENLLANVYHQINHLK^{98}, eluted with approximately 60% B, found to be mainly an incomplete tryptic fragment (noticed the residue **R** is in the middle of the sequence). This large peptide has the observed charge state from 4+ to 7+ (with 5+ as the most intense charge state), which can be easily overlooked by use of mass spectrometers without high resolution (Wu et al., 2005). The hydrophobicity of this peptide is very similar to the intact protein of IFN-beta-1b (based on the % organic solvent at elution), which may contribute to the low trypsin accessibility of this **R** residue. In addition, hydrophobic peptides are hard to elute from a reversed phase C18 column, which may result in low peptide recovery without careful optimization of the separation.

To confirm the above assignment, a hybrid linear ion trap FTMS instrument (LTQ-FT MS) was then used in this study. As shown in Fig. 3A, the glycation site with its corresponding tryptic peptide sequence (SSNFQSQ**K#**LLWQLNGR) was identified by accurate mass measurement of the peptide (hav-

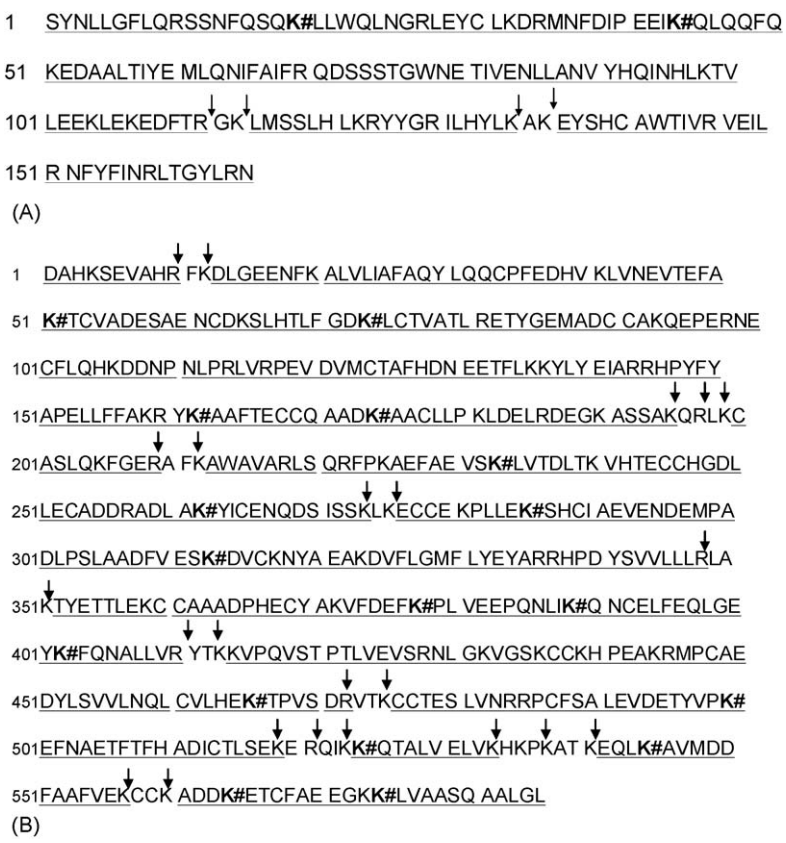

Fig. 2. Summarized results of LC–MS/MS analysis on the trypsin-digested interferon-beta-1b and human serum albumin in the glucose formulation (Uribeta). A majority of interferon-beta-1b sequence (2A) and human serum albumin sequence (2B) were identified except the very small tryptic fragments (as indicated by arrows). The glycation sites were labeled as **K#** in the sequence. The recombinant interferon sequence, which does not have the methionine at the beginning and also cysteine replaced with serine at position 17, was also confirmed. Note: sequence identified (with underline), not identified (without underline), **K#**: glycated site, and ↓: indicated as tryptic sites.

ing one glucose addition with water loss from the peptide), and MS/MS measurement was used for the sequence assignment (the fragmentation pattern of y and b ions were used to deduce the sequence and locate the site of glucose addition). The peptide was detected as a triply charged ion with a high confidence MS/MS matching (Xcorr of 3.86) and mass accuracy of 5 ppm.

In total, there are 11 lysine residues in this protein. The reason for the high reactivity of this lysine (K18) is unclear at this time. One possible explanation is that the nature of the proximal amino acids may strongly affect the lysine reactivity towards glucose (Mennella et al., 2006).

3.3. Glycation identification—human serum albumin

In theory, the possibility of the lysine residues in human serum albumin reacting with glucose will be larger than that of IFN-beta-1b because the amount of human serum albumin is 50 times that of IFN-beta-1b in the formulation. As shown in Fig. 2B, three glycated sites (K51, K233, and K545) in human serum albumin (glucose formulation) were identified for the 4 °C samples. No glycated peptides were detected in human serum albumin with the mannitol formulation (data not shown). Similar to the IFN-beta-1b study, the majority of the human serum albumin sequence was identified except the very small tryptic fragments (as indicated by arrows). This high sequence coverage is very important to search for all potential lysine glycation sites. The same analytical protocol was used as for the IFN-beta-1b assignment with the hybrid linear ion trap FTMS instrument (LTQ-FT MS). The accurate mass measurement and the MS/MS spectra of the three glycated peptides are shown in Fig. 4A (for K51), 4B (for K233), and 4C (for K545).

It was also found in a solution storage study by Lapolla et al. (2004) that human serum albumin was preferentially glycated at residues K51 and K233 but not at K545 sites. The glycation reaction in a lyophilized powder in this study, in which the IFN-beta-1b and human serum albumin are in intimate contact with glucose, may contribute to the preferential glycation at K545 site. Others also found that protein drugs stored in a solid state (i.e. with less than 4% water moisture content) often have a different degradation profile than stored in a solution state (Chen et al., 1994; Li et al., 1996). In the future, the studies of secondary and tertiary structure of the protein following lyophilization, and the determination of glass transition temperature of the lyophilized cakes may aid the understanding of the difference.

3.4. Glycation quantitation—interferon-beta-1b

Since the glycation was found in the lyophilized glucose formulation even at the storage temperature of 4 °C (as shown in

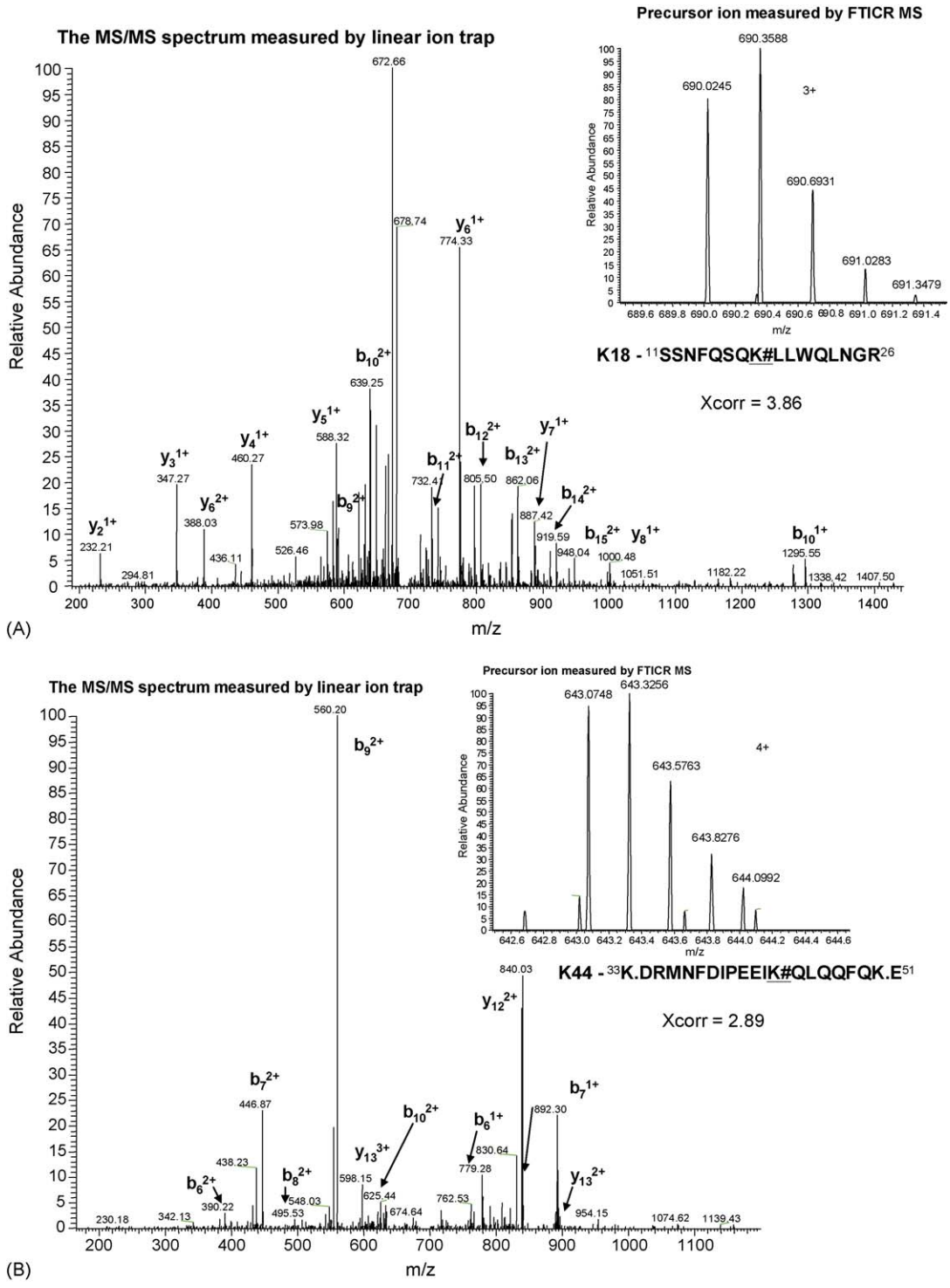

Fig. 3. Mass spectrum assignment for the 2 glycated peptides of interferon-beta-1b (Uribeta). The glycation site with its corresponding tryptic peptide sequence was identified by the accurate mass measurement as shown in the insert (having one glucose addition with water loss from the peptide), and the MS/MS measurement as shown in the figure (the fragmentation pattern of y and b ions locate the site of the glucose addition). The peptides with glycation site at K18 (3A), and K44 (3B) were shown.

above), it might indicate that the glycation could have occurred during the lyophilization process. It is of interest to determine if the amount of glycation can increase with longer storage under elevated temperature. Since this product is likely exposed at room temperature (or 25 °C) before injecting into patients, we monitored the glycation profile over three time points—T0, T7, and T35 at 25 °C (T0 represented the drug powder which was stored at 4 °C, T7 at 25 °C for 7 days, and T35 at 25 °C for 35 day). In addition to the glycation site at K18 of IFN-beta-1b, another glycation site at K44 (Fig. 3B) was also found but to a much less extent in the stability study where the samples were stored at 25 °C for 35 days. The

structural information for tryptic peptide containing the glycation on K18 (SSNFQSQ**K#**LLWQLNGR) and K44 (DRMNFDIPEEI**K#**QLQQFQK) were shown in Table 1. Although the glycated peptide at the K18 site eluted with slightly different retention times in the nanoLC–MS system in different analyses, the accurate mass measurement was consistent for the assignment. The variable retention time was attributed to inter-day differences in the LC–MS system. Also noted, the glycated peptides were found mainly with incomplete tryptic fragments. On the other hand, the same tryptic peptides without glycation were found mainly with complete tryptic fragments, which could be the result of glycation at **K** preventing the cleavage by trypsin.

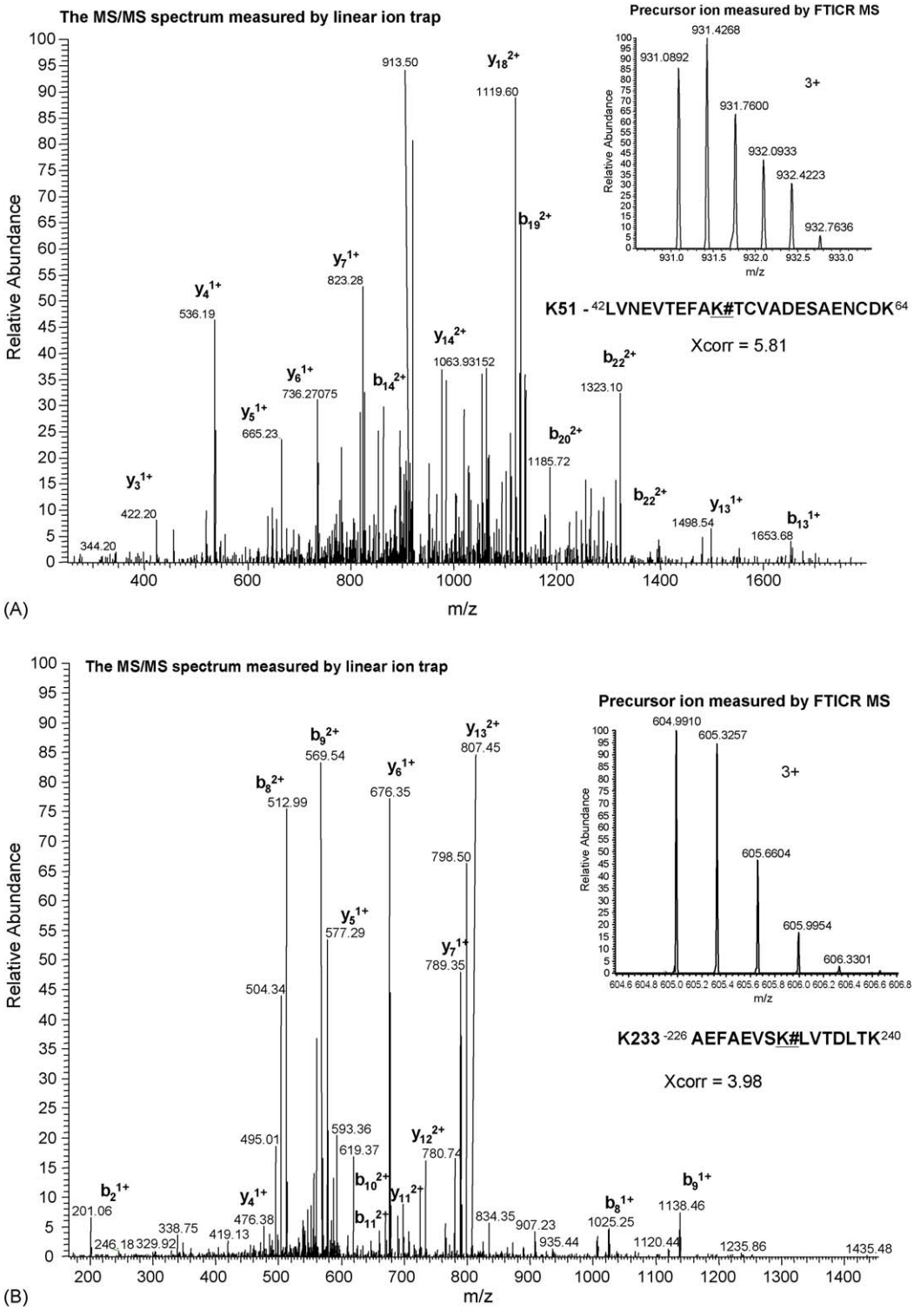

Fig. 4. Mass spectrum assignments for the three glycated peptides of human serum albumin (Uribeta). Similar to the assignment as shown in Fig. 3, the glycation sites with its corresponding tryptic peptide sequences were illustrated in (4A) (for K51 site), (4B) (for K233 site), and (4C) (for K545 site).

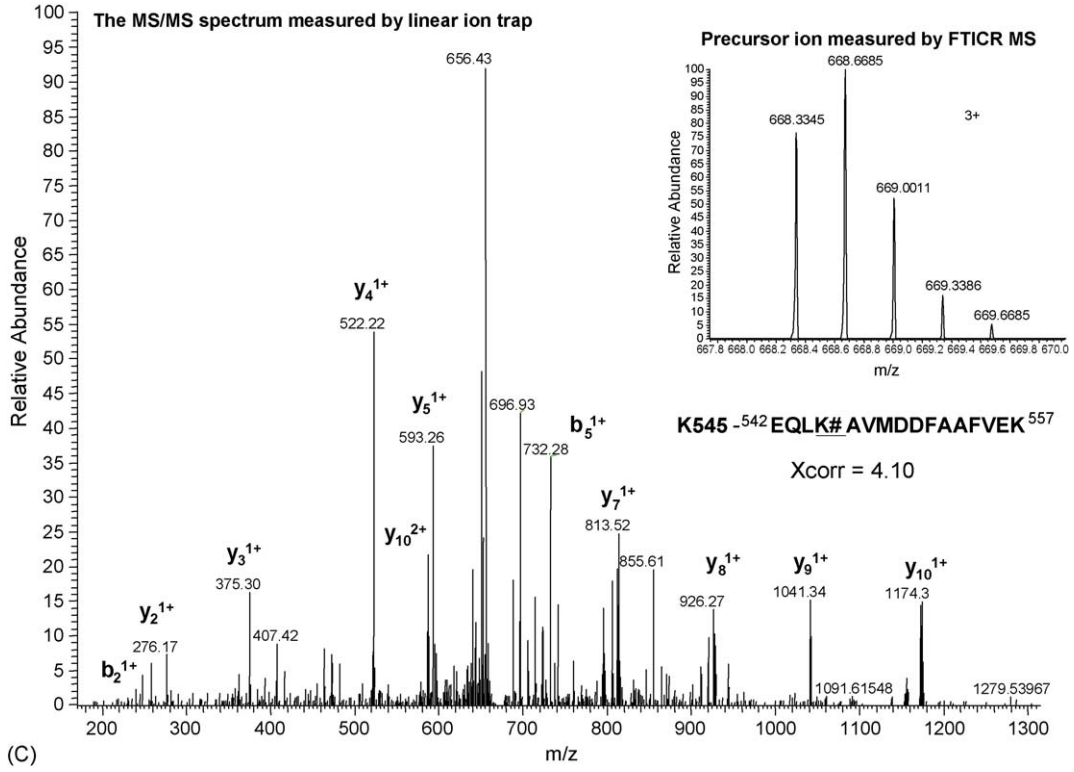

Fig. 4. (*Continued*).

The extent of glycation at K18 increased one-fold after every 7 days (up to six-fold over 35 days) as shown in Fig. 5. The *x*-axis of Fig. 5 is the storage time (in days) at 25 °C and the *y*-axis is the ratio of the peak intensity between the glycated and the average value for three non-glycated peptides. Since the peptide intensity may vary from run to run (due to either an inconsistent sample loading or mass spectrometry response factor), normalization of any inconsistencies over the time points of the experiment is important. Here, we achieved normalization by selecting three intrinsic internal standards from IFN-beta-1b, in which, the extracted ion chromatogram (peak area) of the specific glycated peptide is divided by the average peak area of three representative tryptic peptides that were not modified with glycation at each time point. The three representative tryptic peptides were chosen from the three different retention times near the elution of the glycated peptide to normalize any potential difference in % organic solvent. The high sequence coverage of IFN-beta-1b provides up to 20 specific peptides from IFN-beta-1b for normalization. We, therefore, choose three representative peptides in the specified retention window. This normalization approach has the advantage of correcting for differences due to LC sample loading or mass spectrometry response factor from run to run. The percentage of glycation on this site can be obtained if we have synthetic standards (i.e. glycated and non-glycated species). Assuming the response factors between glycated and non-glycated forms are not greatly different, the percent glycation of beta-interferon at K18 site from T0 to T35 time points are estimated as 2–12%, respectively. Interestingly, we also found that the estimated percentage was similar to the percentage of the shoulder to the main peak in Fig. 1A, assuming the shoulder peak was mainly the glycated variant.

Table 1
Identification of glycated interferon-beta-1b in glucose formulation (Uribeta) at different storage times by LC–MS analysis

Retention time (min)	Sequence	Modification site	Theoretical $[M+H]^+$	Observed $[M+H]^+$ in FTICR MS
Glycated peptide sequences at 4 °C				
30.44	R.SSNFQSQ**K#**LLWQLNGR.L	K18	2068.0463	2068.0577
Glycated peptide sequences at 25 °C for 7 days				
32.03	R.SSNFQSQ**K#**LLWQLNGR.L	K18	2068.0463	2068.0570
Glycated peptide sequences at 25 °C for 35 days				
29.53	R.SSNFQSQ**K#**LLWQLNGR.L	K18	2068.0463	2068.0561
35.45	K.DRMNFDIPEEI**K#**QLQQFQK.E	K44	2569.2608	2569.2755

Glycation sites (K18 and K44) with their corresponding tryptic peptide sequences, eluted at different retention times with observed and theoretical molecular weights, are shown.

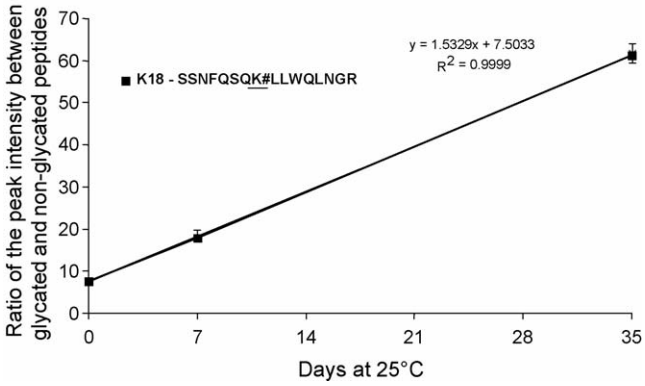

Fig. 5. The stability study of the glycation of interferon-beta-1b in glucose formulation (Uribeta). The extent of glycation increased one-fold after every 7 days (up to six-fold for 35 days). The *y*-axis is the ratio of the peak intensity between the glycated and the average of three non-glycated peptides, and the *x*-axis is the storage time (in days) at 25 °C.

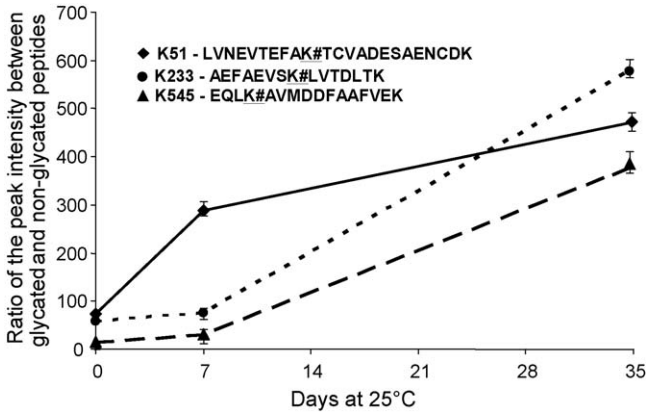

Fig. 6. The stability study of the glycation of human serum albumin in glucose formulation (Uribeta). The three different glycation sites had different profiles, two (K233 and K545) increased rapidly and the other one (K51) leveled off at later time point. The *y*-axis is the ratio of the peak intensity between the glycated and the average of three non-glycated peptides, and the *x*-axis is the storage time (in days) at 25 °C.

3.5. Glycation quantitation—human serum albumin

Similar to the IFN-beta-1b stability study, the extent of glycation of human serum albumin was also monitored under the same storage conditions. The number of glycation sites of human serum albumin was increased with longer storage time (3 at T0, 6 at T7, and 17 at T35) as shown in Table 2. The retention time listed in Table 2 was for peptides found from the T35 time point. The peptides in bold with underlining were found in all three of the storage conditions. The peptides without underlining were only found in the last two storage conditions, T7 and T35. Similar to the IFN-beta-1b study, the glycated peptides of human serum albumin were found mainly with incomplete tryptic fragments. No evidence of glycation at R residues was observed in these mild storage conditions. Seven of these 17 glycation sites were also found in a solution storage study by others (Lapolla et al., 2004). Among them, K525 was found to be glycated in diabetic patient in vivo (Garlick and Mazer, 1983; Shaklai et al., 1984; Iberg and Fluckiger, 1986). As shown in Table 2, only three glycation sites of human serum albumin were found in all three time points. These three glycation sites had a different extent of glycation profiles (as shown in Fig. 6). Two variants (K233 and K545) increased rapidly and the other one (K51) leveled off at later time point. Similar to beta-interferon, the percent glycation of human serum albumin for these sites are estimated as 1–3% for K51, 2–16% for K233, and 0.2–3% for K545.

Table 2
Identification of glycated human serum albumin in glucose formulation (Uribeta) at different storage time by LC–MS analysis

Retention time (min)	Sequence	Location of modification	Theoretical $[M+H]^+$	Observed $[M+H]^+$	T0	T7	T35
18.21	K.ADD**K#**ETCFAEEGK.K	K564	1661.6852	1661.6870			x
20.32	**R.YK#AAFTECCQAADK.A**	**K162**	**1824.7784**	**1824.7865**		x	x
24.31	**K.K#QTALVELVK.H**	**K525**	**1290.7520**	**1290.7544**		x	x
27.09	**R.ADLAK#YICENQDSISSK.L**	**K262**	**2103.9756**	**2103.9824**		x	x
28.92	K.AAFTECCQAADK#AACLLPK.L	K174	2287.0408	2287.0538			x
28.04	K.K#LVAASQAALGL.-	K574	1303.7476	1303.7590			x
29.29	***K.LVNEVTEFAK#TCVADESAENCDK.S***	**K51**	**2791.2290**	**2791.2518**	x	x	x
30.73	K.SLHTLFGDK#LCTVATLR.E	K73	2094.0905	2094.1000			x
29.29	K.VFDEFK#PLVEEPQNLIK.Q	K378	2207.1487	2207.1626			x
36.13	K.SHCIAEVENDEMPADLPSLAADFVESK#DVCK.N	K313	3638.6188	3638.6455			x
36.26	***K.AEFAEVSK#LVTDLTK. V***	**K233**	**1812.9482**	**1812.9572**	x	x	x
36.56	R.RPCFSALEVDETYVPK#EFNAETFTFHADICTLSEK.E	K500	4313.9899	4314.0208			x
36.65	K.QNCELFEQLGEYK#FQNALLVR.Y	K402	2761.3507	2761.3737			x
37.47	K.PLLEK#SHCIAEVENDEMPADLPSLAADFVESK.D	K286	3716.7293	3716.7651			x
37.52	K.PLVEEPQNLIK#QNCELFEQLGEYK.F	K389	3080.5137	3080.5410			x
37.75	***K.EQLK#A VMDDFAAFVEK. C***	**K545**	**2002.9683**	**2002.9877**	x	x	x
40.02	K.RMPCAEDYLSVVLNQLCVLHEK#TPVSDR.V	K466	3491.6973	3491.7209			x

Glycation sites with their corresponding tryptic peptide sequences, eluted at different retention times with observed and theoretical molecular weights, are shown. The increase in number of glycation sites with longer storage time at 25 °C (under T7 and T 35) is indicated by the number of x terms, in which, T0 represents the sample stored at 4 °C, followed by T7 (at 25 °C for 7 days), and T35 (at 25 °C for 35 days).

4. Conclusions

The LC–MS approach successfully identified glycation sites (K18 and K44) of interferon-beta-1b, which was lyophilized in a formulation containing human serum albumin and glucose (Uribeta as brand name). The glycation site was determined by accurate mass measurement (FTICR MS) and sequence determination (MS/MS measurement) of the corresponding tryptic peptide. The extent of glycation was measured by the ratio of the peak intensity between the glycated and the average value for three non-glycated peptides in the same run. In this lyophilized formulation (Uribeta), the extent of beta-interferon glycation increased proportionally with the storage time at 25 °C. In addition, many glycation sites of human serum albumin were also found. The number of glycation sites and the extent of glycation of human serum albumin increased with the storage time at 25 °C. In contrast, only 2 glycation site for beta-interferon were found. The glycation profile for the lyophilized product (solid state with less than 4% water moisture content), in which the beta-interferon and human serum albumin are in intimate contact with glucose, may have different glycation profile than for a liquid formulation. Nevertheless, except at the K545 site, several of these sites (K51, K233, K313, and K378) have been found by others to be preferentially glycated under solution storage (Lapolla et al., 2004). The glycation found at K525 site in this study was also reported in diabetic patients in vivo (Garlick and Mazer, 1983; Shaklai et al., 1984; Iberg and Fluckiger, 1986). As expected, there was no glycation found on both interferon-beta-1b and human serum albumin in the control sample (using mannitol instead of glucose). In conclusion, the development of generic biopharmaceuticals can benefit from the application of the power of proteomics approaches based on the new generation mass spectrometers.

Acknowledgement

The authors thank BRIMS center of Thermo Electron (Cambridge, MA) for the equipment support. Contribution Number 881 from the Barnett Institute.

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