Journal of Chromatography B, 877 (2009) 4065–4070

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Rapid online proteolytic mapping of PEGylated rhGH for identity confirmation, quantitation of methionine oxidation and quantitation of UnPEGylated N-terminus using HPLC with UV detection

Jeffrey Schneiderheinze*, Zara Walden, Rob Dufield, Charles Demarest

Pfizer Global Biologics Pharmaceutical Sciences, Analytical Research and Development, St. Louis Laboratories, 700 Chesterfield Parkway N, St. Louis, MO 63017, USA

ARTICLE INFO

Article history:
Received 1 June 2009
Accepted 13 October 2009
Available online 22 October 2009

Keywords:
PEGylated
Growth hormone
Online
Mapping
Chromatography

ABSTRACT

Proteolytic mapping is a widely used tool in the BioPharmaceutical Industry for the analysis of post-translation modifications as well as confirmation of protein identity by comparison to a well-characterized reference standard. This manuscript presents an integrated chromatographic approach which provides the ability to rapidly digest and analyze a PEGylated rhGH for methionine oxidation, identity confirmation and free (unPEGylated) N-terminal peptide by RP-HPLC using UV detection at 280 nm. This approach utilizes an *online* procedure in which the digestion step is integrated to the RP-HPLC analysis via an external column switching valve. A Poroszyme Trypsin cartridge is used in the digestion step, followed by delivery of the digested sample plug through a sample loop to an orthogonal RP-HPLC column for separation and quantitation of the resulting tryptic peptides. Oxidation of the methionine (met14) in the T2 tryptic fragment was quantified with a sensitivity of approximately 1.0% (peak area percent relative to parent T2). The RP-HPLC profile obtained with the integrated system was nearly identical to that obtained via traditional methods (e.g. batch digestion followed by RP-HPLC analysis). The integrated technique, however, represents a 10-fold reduction in total analysis time when compared to the optimized batch digestion procedure. In addition, the identity of the PEGylated rhGH compound could be confirmed as well as the percentage of free N-terminus in a single injection.

1. Introduction

Peptide mapping has long been recognized as a powerful tool for protein analysis [1,2]. Since the late 1970s, the technique has been providing researchers with valuable information regarding the detection of post-translational modification, identification of genetic variants, and the quality of genetically engineered protein products [3–5]. Proteolytic mapping typically involves digestion of the target protein in bulk solution followed by a chromatographic or electrophoretic separation of the digested peptides. Trypsin is widely used for proteolytic mapping due to its ability to quantitative cleave at the N-terminus of arginine and lysine residues of the protein that statistically yields peptides of useful number and size to efficiently characterize primary structure. In addition, RP-HPLC has proven to be the separation technique of choice, in part, due to its ability to separate the resulting peptides that can span a wide range of hydrophobicity.

Despite its popularity, batch digestion approaches to peptide mapping require extensive sample preparation and typically require 4–24 h to achieve complete digestion of the target protein [6]. This time is largely dependent on the size and structure of the intact protein, pH of the digestion solution, incubation temperature and enzyme:substrate ratio [6]. Additives can sometimes reduce the time required to achieve complete digestion but often the impact on the enzyme kinetics is minimal [6]. As a result, recent research in the field has given rise to immobilized enzyme reactors (IMERs) [7–10]. Typically, IMERs contain active enzyme that has been chemically attached to inorganic or polymeric solid supports (monolithic or particle based) or magnetic microparticles [7]. Packing of immobilized trypsin into continuous flow columns allows for online proteolysis [8]. This approach has been reported to reduce digestion times when compared to digestion in solution [7]. IMERs offer higher digestion efficiency, larger enzyme to substrate ratio, faster digestion times, reduced sample requirements and increased enzyme stability when compared to batch digestion [11–14]. Analysis time is even further reduced when IMERs are coupled to separation techniques in an integrated or multidimensional fashion [7,8]. Various configurations and equipment designs have been reported in the literature [7]. Frequently, these configurations

* Corresponding author. Tel.: +1 636 247 5767; fax: +1 636 247 5351.
E-mail address: jeffrey.m.schneiderheinze@pfizer.com (J. Schneiderheinze).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.10.015

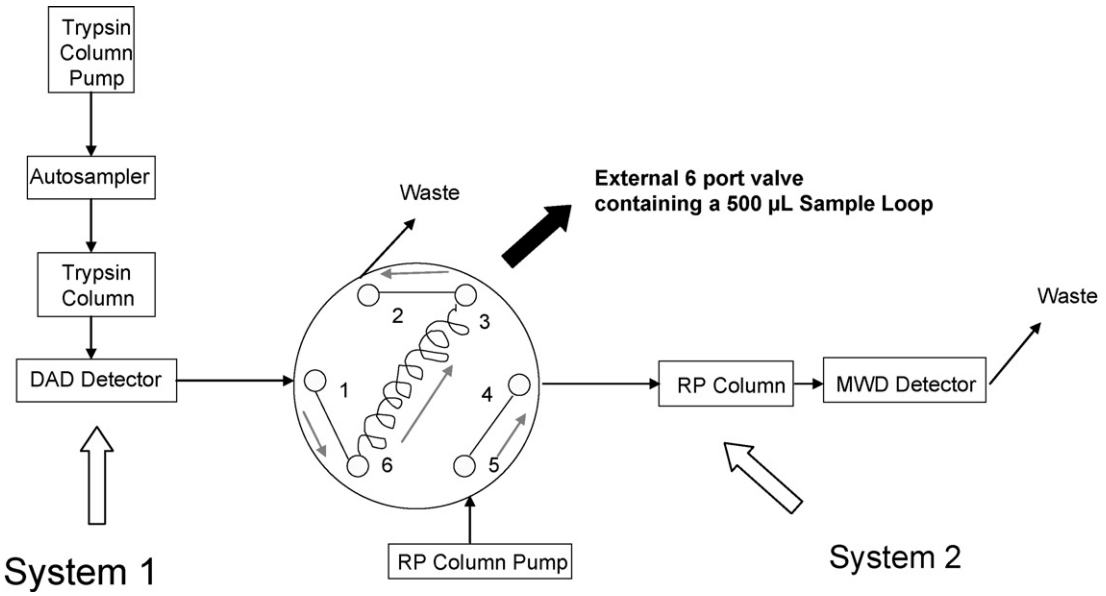

Fig. 1. Schematic which illustrates the configuration of the integrated HPLC system. The two HPLC systems operate independently. Sample eluent is delivered from system 1 to system 2 via a 6-port external switching valve.

include MS detection which provides the added benefit of peptide mass confirmation [7].

Much of the research in the field is focused on well characterized, model proteins. Little attention, however, has been given to employing IMERs based on proteolytic enzymes to in-depth characterization of a therapeutic protein. In the Biopharmaceutical industry, peptide mapping can yield powerful information regarding the identity of the protein, the presence of structural isomers as well as quantitative information about post-translational modifications. All of these are factors that can potentially impact the pharmacological profile and activity of the protein therapeutic agent. Much of the work around IMERs is still in the research stage and few applications have been reported so far. Subsequently, advances in the field, while promising have had limited applicability to characterization of biotherapeutics in the Pharmaceutical industry.

The test substrate of this study is a novel, PEGylated recombinant human growth hormone (rhGH). Recombinant hGH has been extensively studied in the literature and is well characterized. The amino acid sequence of rhGH, including the tryptic cleavage sites, was determined and previously published [15]. PEGylation is an approach to extend the pharmacokinetic half-life of a therapeutic agent via the chemical attachment of a polyethylene glycol (PEG) moiety. The PEG reagent is not pharmacologically active but serves to increase the circulating half-life of a therapeutic agent via several known mechanisms. These include an increase in the aqueous solubility of the protein, an increase in its resistance to enzymatic degradation and a reduction of its renal uptake due to the increase of the molecular weight [16]. This particular rhGH (approximately 22 kDa) is PEGylated selectively at the N-terminus with a 40 kDa, branched PEG moiety. The resulting PEGylated rhGH is approximately 62 kDa in molecular weight.

This article evaluates the ability of a commercially available Poroszyme IMER to improve sample cycle time for a novel PEG-rhGH while preserving data integrity and method reliability when compared to a traditional, batch digestion method of sample preparation. This study evaluates the ability of an integrated chromatographic approach (with an IMER in the first step) to provide identity, oxidation and free N-terminus data of the PEG-rhGH utilizing ultraviolet (UV) detection at 280 nm. In addition, the precision of the system using the test substrate will be evaluated in comparison to the analogous batch digestion method.

2. Materials and methods

2.1. Reagents and materials

Recombinant hGH was expressed and purified in house as previously described [15]. The 40 kDa linear PEG reagent was obtained from Necktar Therapeutics (Huntsville, AL). Conjugation of the PEG reagent to the rhGH protein and purification selectively at the N-terminus was performed in house. The PEG-rhGH was then purified using a Q Sepharose anion exchange column with a linear gradient from 95/5 (20 mM HEPES, pH 8.0; Sigma–Aldrich, St. Louis, MO) to 5/95 (20 mM HEPES, 125 mM NaCl, pH 8.0; Sigma–Aldrich, St. Louis, MO). The purified material was then collected, buffer exchanged and concentrated using an ultrafiltration/diafiltration step (using a Sartorius ultrafiltration system; Sartorius Stedim Biotech, France) to a concentration of approximately 25 mg/mL in 10 mM Sodium Phosphate, 3.7 mM glycine with a pH of 6.7. Ultrafiltration/diafiltration was originally described for use in protein purification by McPhie [17]. This formulated PEG-rhGH drug substance is the stock solution that is used in the mapping experiments.

The Poroszyme Immobilized Trypsin Cartridge (2.1 mm × 30 mm) was obtained from Applied Biosystems (Carlsbad, CA). The HPLC column used was a Vydac (Grace Vydac, Deerfield, IL) C18 Mass Spec (5 µm; 2.1 mm × 250 mm) 218MS52. The HPLC systems

Table 1
Gradient profile for the RP-HPLC analysis of the tryptic peptides.

Step	Run time (min)	Flow rate (mL/min)	Phase B (%)	Curve
1	0	0.5	0	
2	3	0.5	0	Step
3	18	0.5	15	Linear
4	38	0.5	19	Linear
5	38.1	0.5	22	Step
6	50	0.5	40	Linear
7	55	0.5	50	Linear
8	55.1	0.5	90	Step
9	60	0.5	90	Linear
10	60.1	0.5	0	Step

used were Agilent HP1100s (Santa Clara, CA) equipped with an external 6-port valve (Agilent, Santa Clara, CA). The external sample loop in the 6-port valve was a 500 μL Rheodyne PEEK loop (Idex Health and Science; Oak Harbor, WA). Calcium chloride, Tris-Trizma base, L-methionine and acetonitrile were obtained from Sigma (St. Louis, MO). Concentrated HCl and hydrogen peroxide were obtained from J.T. Baker (Phillipsburg, NJ). Trifluoroacetic acid was obtained from Pierce Chemical (Rockford, IL). Modified sequencing grade lyophilized trypsin was obtained from Promega (Madison, WI). Mobile phase and buffers were prepared using an in-house Millipore system (Billerica, MA).

2.2. Online and batch digestion HPLC system configuration

Two Agilent HP1100 systems were configured orthogonally as shown in Fig. 1. These systems were controlled and operated independently using Chemstation software. System 1 contained the trypsin cartridge and effluent from the system was detected at 280 nm by a diode array detector (DAD). The mobile phase used in this system consisted of 50 mM Tris, 10 mM $CaCl_2$, pH 8.0 and was delivered at 0.05 mL/min. The temperature of the trypsin cartridge was maintained at 37 °C. System 2 contained the Vydac HPLC column and effluent from the system was detected at 280 nm by a multi-wavelength UV detector (MWD). The column temperature of system 2 was maintained at 30 °C. The sample tray of system 1 (and system 2 in the batch digestion mode) was maintained at 5 °C. Mobile phase A for system 2 consisted of 0.1% TFA in water. Mobile phase B consisted of 0.085% TFA in acetonitrile. The tryptic peptides were separated by gradient elution at 0.5 mL/min. Table 1 below contains the gradient that was used. Experiments were performed to determine the optimum switching time for the external valve (data not shown). Based on the results of this experiment, the switching valve was programmed to change positions 8.4 min after the injection of sample on system 1. Batch digestion sample analysis was performed by disconnecting the external valve and operating system 2 as an independent HPLC system with the aforementioned operating parameters.

2.3. Sample preparation

For online analysis, a drug substance solution of PEG-rhGH (described in Section 2.1) was diluted to 1 mg/mL in the 50 mM Tris buffer described in Section 2.2 (approximately a 25-fold dilution from stock). After dilution, 60 μL of sample was injected onto system 1. After the peak eluted from the trypsin cartridge the column switching valve was programmed to change positions (optimized to 8.4 min after injection). In essence, this represented a 500 μL injection of digested peptides onto the RP-HPLC column. At this time, the gradient described in Table 1 was programmed to begin.

For batch digestion analysis, the lyophilized sequencing grade trypsin was reconstituted in 20 μL of the 50 mM Tris buffer which also contained 0.1 M methionine. The same stock solution of PEG-rhGH (described in Section 2.1) above was diluted to 1 mg/mL with the Tris buffer described above. 10 μL of the reconstituted trypsin solution was then added to the diluted PEG-rhGH sample and incubated at 37 °C for approximately 16 h. The digestion was quench by the addition of 10 μL of 1N HCl. 40 μL of the quenched digest was injected onto HPLC system 2 for analysis. For the force-oxidized sample, the PEG-rhGH stock solution was oxidized in the presence of 1% peroxide for 10 min.

2.4. Optimization of batch digestion conditions

Batch digestion conditions were optimized prior to the experiments with the integrated system. Optimization experiments were performed to assess optimal digestion temperature (ambient and 37 °C), enzyme to substrate ratio (1:30, 1:20, 1:12), and incubation time (data not shown). In order to evaluate the optimal digestion time, aliquots of the sample digest were taken at 2, 4, 6, 8, 16 and 24 h, quenched and analyzed by RP-HPLC. Although some digestion is observed as early as 2 h, optimal digestion efficiency was not obtained until the 16 h time point (data not shown). Therefore, 16 h was chosen as the incubation time for the batch digestion.

2.5. Confirmation of peak identification

The data and chromatograms presented in this manuscript were collected with a UV detector. Development of the RP-HPLC separation was performed prior to the experiments using the integrated system. During development of the separation, peak identification was performed using LC/MS and compared to the previously reported sequence and tryptic fragments of hGH [15]. An Agilent 1100 HPLC system was coupled to a Micromass LCT mass spectrometer and the digested rhGH, PEG-rhGH and oxidized PEG-rhGH were analyzed using the same conditions as described in Section 2.2. The mass spectrometer was calibrated with myoglobin prior to sample analysis and data was collected from 200–2500 Da based on the mass to charge ratio (m/z) and acquired by MassLynx software. In order to assign identities to the peaks in the RP-HPLC chromatogram, the masses obtained for each peptide were compared to the theoretical masses based on the reported sequence of rhGH and the known cleavage sites of trypsin. The experimental

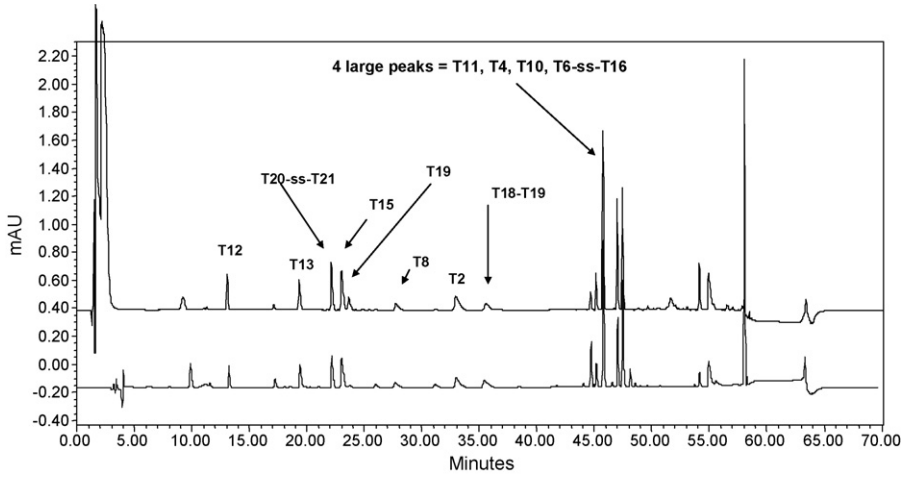

Fig. 2. Chromatographic overlay of a PEG-rhGH sample analyzed via batch digestion (top) and via the integrated system (bottom).

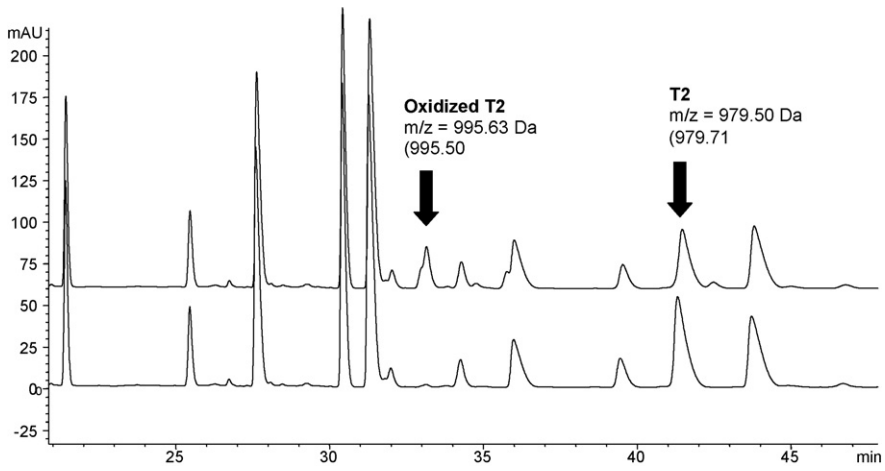

Fig. 3. Chromatographic overlay of oxidized PEG-rhGH (top) and the PEG-rhGH conjugate without peroxide treatment (bottom). These chromatograms have been expanded to the region of interest.

and theoretical masses are reported for each tryptic peak identified. Once the peak assignment was complete, a UV detector was used in the integrated system to demonstrate ease of implementation in a pharmaceutical research environment. Peaks were then assigned in the chromatograms generated from the integrated system based on retention time correlation with LC/MS characterized sample.

3. Results and discussion

3.1. Identity confirmation

Fig. 2 shows a chromatographic overlay of a peptide map obtained from a batch digestion compared to that obtained for the same sample using the integrated system with the Poroszyme IMER. The RP-HPLC conditions are the same in each case. The tryptic fragments labeled in the chromatogram are T12, T13, T20-ss-T21, T15, T19, T18-T19, T8, T2, T11, T4, T10 and T6-ss-T16. The previously reported theoretical masses (in Da) based on the reported sequence of rhGH for these peaks are 772.37, 692.39, 1399.62, 1488.68, 763.42, 1272.84, 843.48, 978.50, 1360.67, 2341.13, 2261.12 and 3760.77, respectively. The experimental masses (in Da) obtained for each of these fragments are 772.47, 692.48, 1399.82, 1488.86, 763.55, 1270.82, 843.61, 978.64, 1360.89, 2341.48, 2261.48 and 3761.58, respectively. Each of the experimentally determined masses agrees well with theoretical values for these fragments. Therefore, positive identity was established and the peaks in the chromatograms were labeled appropriately.

As can be seen in Fig. 2, the peak shape, resolution and profile of the peptide map is comparable in both chromatograms. The sample load is the same in both chromatograms, however, there appears to be a slight decrease in the sensitivity of the integrated system as all peaks are slightly smaller in area compared to those in the batch digestion chromatogram. This maybe indicative that the delivery of the sample plug to the second HPLC system requires further optimization. The tryptic peptides that are used for a positive identity confirmation are consistent across both chromatograms.

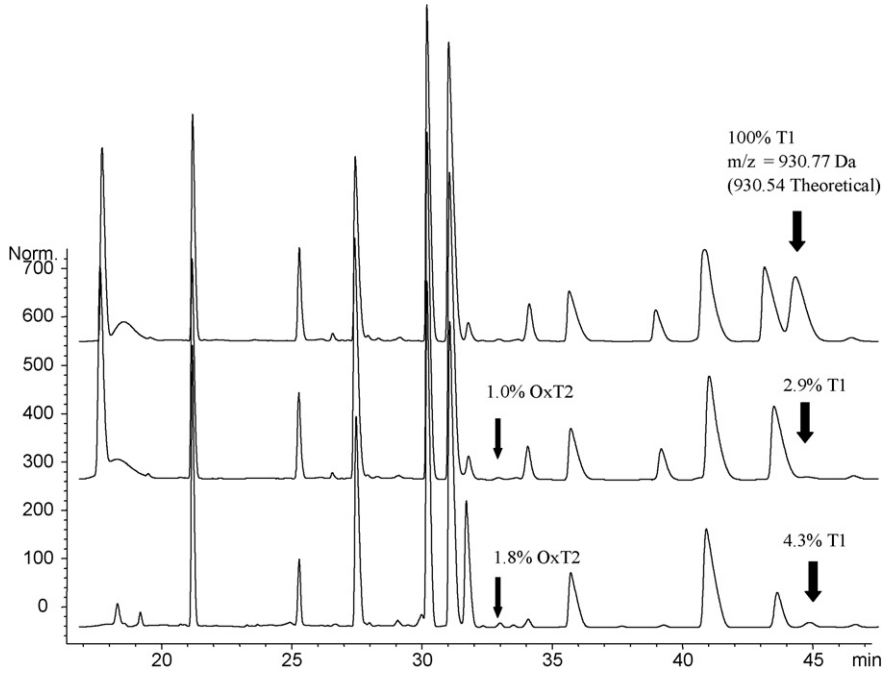

Fig. 4. Chromatographic overlays of unPEGylated rhGH (top), PEGylated rhGH analyzed with the 2D system (middle) and the same PEGylated rhGH sample analyze via offline digestion (bottom).

There are a few small peaks that are present in the integrated digest chromatogram that are not present in batch digest chromatogram. These peaks were confirmed to be partially digested fragments of the PEGylated rhGH compound based on comparison of the chromatogram with those generated in the incubation time optimization experiments (see Section 2.4). In addition, there are some minor hydrophobic peaks in the online chromatogram that were not identified.

3.2. Experimental confirmation of stability indication with respect to protein oxidation

In addition to confirming product identity, the integrated system was evaluated for its ability to quantitate oxidation of the PEG-rhGH conjugate. Fig. 3 shows a chromatographic overlay of a PEG-rhGH sample that was force-oxidized prior to PEGylation (top) and an untreated PEG-rhGH conjugate (bottom). The mass obtained for T2 in the PEGylated rhGH was 979.50 Da. This agrees with the theoretical mass for T2 of 979.71 Da based on previously reported data. The mass obtained for the peak labeled as 'Oxidized T2' was 995.63 Da, which agrees with the theoretical mass for oxidized T2 of 995.50 Da. Both chromatograms were generated with the integrated system. Of note is the increase in the OxT2 peak in the top chromatogram. Resolution of the force-degraded peptide indicates that the integrated system is also stability indicating for oxidation.

3.3. Quantitation of methionine oxidation and free N-terminus

Because the drug product is described as PEGylated only at the N-terminus, it is necessary to monitor the amount of free N-terminus present in the drug product as a key quality marker. Free N-terminus can be quantified based on a comparison of relative peak areas (since T1 is the unPEGylated free N-terminus) according to the following equation:

$$\%\text{Free N} = \frac{\text{PA}_{\text{peg}}}{\text{PA}_{\text{hgh}}} \times 100\%$$

where PA_{peg} is the peak area of T1 in the PEG-rhGH sample and PA_{hgh} is the peak area of T1 in the rhGH sample. Fig. 4 illustrates how this quantitation can be performed. This figure shows a chromatographic overlay of an rhGH (unPEGylated growth hormone standard; top) sample analyzed with the integrated system, a PEG-rhGH conjugate sample analyzed via the integrated system (middle) and the same PEG-rhGH conjugate sample analyzed via batch digestion (bottom). The mass obtained for T1 in the unPEGylated rhGH was 930.77 Da. This agrees with the reported theoretical mass for T1 of 930.54 Da. It is clear from this figure that the integrated system is able to resolve the unPEGylated T1 fragment. Trace levels of the T1 peptide appear in PEG-rhGH samples analyzed via batch digestion and by the integrated system at similar levels. Quantitation of these peaks compared to the hGH standard using the above formula resulted in values of 4.3% T1 for the batch digestion analysis and 2.9% T1 for the online analysis.

Quantitation of methionine oxidation in the T2 fragment can also be calculated and expressed as a relative peak area according to the following formula:

$$\%\text{Ox} = \frac{\text{PAox}}{\text{PAT2}} \times 100\%$$

where PAox is the peak area of the OxT2 peak and PAT2 is the peak area of the parent T2 peak. Fig. 4 shows that this information can be obtained from the same chromatogram. Area quantitation of the OxT2 yielded values of 1.8% oxidation for the batch digestion analysis and 1.0% oxidation for the integrated system. The online system appears to have a slight bias relative to the batch digestion analysis. This could indicate either a reduced digestion efficiency of the integrated system or a fractionation of the sample that is delivered to the second HPLC system.

3.4. Evaluation of method precision of the online and offline procedures

One critical method attribute for routine use in a pharmaceutical environment is method precision. Method precision was evaluated for both the integrated system and the batch digestion analysis. Batch digestion precision was evaluated by preparing twelve separate trypsin digestions of the same PEG-rhGH sample. These samples were then each analyzed by the RP-HPLC procedure. Online precision was evaluated by performing twelve separate injections of the same PEG-rhGH sample. The peak area %RSD for the representative tryptic peaks (OxT2, T2, T1, T11 and T12) was then calculated. The %RSD ($n = 12$) for the T2, OxT2, T1, T11 and T12 via batch digestion analysis was 4.1%, 13.2%, 12.9%, 3.0% and 3.6%, respectively. While the %RSD for the same peaks via the integrated approach was 1.7%, 4.9%, 6.4%, 2.2% and 1.5%, respectively. The method precision of the integrated system was better than that of the batch digestion procedure. Presumably, this is a consequence of the inherently less precise manual sample preparation wherein analyst error is a significant component. Conversely, the temporal and volumetric precision of the integrated system is inherently higher.

4. Conclusions

This manuscript shows that currently available, commercial IMER technology can be an effective alternative to more laborious batch digestion approaches to peptide mapping. Sample digestion times can be reduced from 16 h to less than 5 min, which represents almost a 200-fold increase in throughput. Despite the dramatic reduction in digestion time, the integrity of the peptide map was preserved. Evidence of partially digested peaks were present in the online system, however, these peaks did not interfere with the ability of the integrated system to positively confirm protein identity, quantitate methionine oxidation or assay for free N-terminus of a PEGylated rhGH conjugate.

Overall, the sensitivity of the batch digestion method was moderately better than the integrated system. This could be due to increased digestion efficiency in the batch digestion method and therefore improved sensitivity in assessing oxidation (1.8% via batch analysis vs. 1.0% online) and free N-terminus (4.3% via batch analysis vs. 2.9% online). This may also indicate that further optimization of the sample transfer to the second HPLC system is required. Evidence was not presented to offer a definitive explanation either way. Loss in sensitivity is offset not only by a dramatic decrease in digestion time but also an increase in method precision. In comparing representative peaks across the peptide map, area %RSD is better for the online system in every case. This can be attributed to the removal of the manual sample handling steps that are needed to complete the batch digestion. The integration of the digestion and analysis into a single, online step improves the overall precision of the method and greatly increases the throughput of the analytical laboratory.

References

[1] V.M. Ingram, Nature (London) 178 (1956) 792.
[2] K. Moon, E. Smith, J. Biol. Chem. 248 (1973) 3082.
[3] A. Huberman, M.B. Aguilar, J. Chromatogr. 443 (1988) 337.
[4] G. Pohl, M. Kallstrom, N. Bergsdorf, P. Wallen, H. Jornvall, Biochemistry 23 (1984) 3701.
[5] R.L. Garnick, N. Solli, P.A. Papa, Anal. Chem. 60 (1988) 2546.
[6] K.F. Medzihradszky, Methods Enzymol. 405 (2005) 50.
[7] G. Massolini, E. Calleri, J. Sep. Sci. 28 (2005) 7.

[8] N. Markoglou, I.W. Wainer, in: I.D. Wilson (Ed.), Bioanalytical Separations, Elsevier, Amsterdam, 2003, p. 25.
[9] J. Krenkova, F. Foret, Electrophoresis 25 (2004) 3550.
[10] F. Svec, Electrophoresis 27 (2006) 947.
[11] J. Ma, L. Zhang, W. Zhang, Y. Zhang, J. Sep. Sci. 30 (2007) 3050.
[12] J. Krenkova, F. Svec, J. Sep. Sci. 32 (2009) 706.
[13] D.S. Peterson, T. Rohr, F. Svec, J.M. Fréchet, J. Prot. Res. 1 (2002) 563.
[14] J. Krenkova, N. Lacher, F. Svec, Anal. Chem. 81 (2009) 2004.
[15] P. Gellerfors, G. Eketorp, K. Fholenhag, B. Pavlu, S. Johannsson, L. Fryklund, J. Pharm. Biomed. Anal. (1989) 173.
[16] F.M. Veronese, Biomaterials 22 (2001) 405.
[17] P. McPhie, Methods Enzymol. 22 (1971) 23.