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Coupling of on-column trypsin digestion–peptide mapping and principal component analysis for stability and biosimilarity assessment of recombinant human growth hormone

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ABSTRACT

Peptide mapping (PM) is a vital technique in biopharmaceutical industry. The fingerprint obtained helps to qualitatively confirm host stability as well as verify primary structure, purity and integrity of the target protein. Yet, in-solution digestion followed by tandem mass spectrometry is not suitable as a routine quality control test. It is time consuming and requires sophisticated, expensive instruments and highly skilled operators. In an attempt to enhance the fuctionality of PM and extract multi-dimentional data about various critical quality attributes and comparability of biosimilars, coupling of PM generated using immobilized trypsin followed by HPLC-UV to principal component analysis (PCA) is proposed. Recombinant human growth hormone (rhGH); was selected as a model biopharmaceutical since it is available in the market from different manufacturers and its PM is a well-established pharmacopoeial test. Samples of different rhGH biosimilars as well as degraded samples: deamidated and oxidized were subjected to trypsin digestion followed by RP-HPLC-UV analysis. PCA of the entire chromatograms of test and reference samples was then conducted. Comparison of the scores of samples and investigation of the loadings plots clearly indicated the applicability of PM-PCA for: i) identity testing, ii) biosimilarity assessment and iii) stability evaluation. Hotelling's T^2 and Q statistics were employed at 95% confidence level to measure the variation and to test the conformance of each sample to the PCA model, respectively. Coupling of PM to PCA provided a novel tool to identify peptide fragments responsible for variation between the test and reference samples as well as evaluation of the extent and relative significance of this variability. Transformation of conventional PM that is largely based on subjective visual comparison into an objective statiscally-guided analysis framework should provide a simple and economic tool to help both manufacturers and regulatory authorities in quality and biosimilarity assessment of biopharmaceuticals.

1. Introduction

Recombinant protein biopharmaceuticals comprise the fastest growing sector in the pharmaceutical market [1]. On the other hand, quality and biosimilarity assessment of biopharmaceuticals is challenging due to the inherent variability and complexity of production processes and protein structure [2,3]. Minor variation in the primary structure and pattern of post-translational modifications (PTMs) could impose profound effects on the safety and efficacy of such molecules [4–6]. Susceptibility of biopharmaceuticals to minor changes in formulation and storage conditions might result in a diverse range of degradation products due to the complexity of possible degradation pathways [7,8]. Thus, the development of validated orthogonal testing protocols has been the gold standard approach in order to overcome such challenges [9–14].

Peptide mapping (PM) is one of the very powerful identity tests for biopharmaceuticals. Its power lies in the flexibility of choices from various cleavage agents to the separation and detection techniques. In each case, a fingerprint is obtained that is as unique as the human fingerprint and enables monitoring of several critical quality attributes. In literature, PM was used to determine the primary structure [15–17]; including the positions of disulfide bonds [18,19], provide information on the N- and C-terminal peptides [20] as well as detection of single point mutations, which would cause single or multiple amino acid

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substitutions [21]. Additional information regarding PTMs can also be deduced, such as the position of glycan moiety [22,23] and amino acid isomerization [24]. Applications of PM in the assessment of the stability of biopharmaceuticals include also the detection of deamidation and oxidation of amino acid residues [6,25]. Consequently, this technique is particularly important in the quality control of biotechnology protein products. Validated PM is used routinely to: i) compare recombinant protein structure to that of its human counterpart or reference material, ii) evaluate batch-to-batch consistency, iii) demonstrate the genetic stability of the production system [26,27] and iv) biosimilarity [28–31]. Developing a biosimilar product is not a trivial task and requires proving that the new product is 'biosimilar' to the innovator's biologic as per ICH guidelines [8,32].

Coupling of PM to mass spectrophotometric detection is known as peptide mass fingerprinting (PMF). Although it has huge applications in bioinformatics and proteomics [33,34], it is not a cost effective technique when it comes to routine quality control analysis. Moreover, the highly sophisticated data produced by mass spectrometry is hard to deal with in quality control labs.

The usefulness of PM generated using HPLC-UV in routine work is limited to well-characterized small molecular weight biopharmaceuticals. On the other hand, chemometrics was first presented by researchers in analytical and physical organic chemistry in the late 1960s. It involves multivariate data analysis following the use of high-throughput techniques utilizing the revolution in computer science [35]. Application of chemometric techniques allows pattern recognition, detection of possible outliers, and detection of similarities/differences between samples [36–38]. With principal component analysis (PCA), samples are described by principal components which are linear combinations of the original variables, selected to account for the more systematic variation. Then the relation between the samples can be visualized by low-dimensional score plots, distinguishing possible subgroups and detecting outliers [39]. In literature, PCA is the primary technique used to recognize small changes in the chromatographic data which cause significant variance among the samples and find latent structures in the analyzed data [37,40]. Chemometrics was applied to the quality control of small molecule biopharmaceuticals; such as granulocyte colony stimulating factor (G-CSF) and large ones; such as monoclonal antibodies (mAb) to find a measure for the similarity of different protein variants during product development stages [41,42]. Moreover, the possibility of using chemometrics for the assessment of process comparability in biomanufacturing processes has been discussed in several reports representing a promising field of applications especially with the growing acceptability of the concepts of quality by design (QbD) and process analytical technology (PAT) among the regulatory authorities and industrial companies [40,43].

In this work, we aim to develop a reliable and objective comparability framework for stability and biosimilarity assessment of a model biopharmaceutical product (rhGH) using the complex data originating from PM. Immobilized enzymatic digestion and HPLC-UV analysis were employed in order to develop a cost-effective analytical tool. PCA supported with Hotteling's T^2 and Q statistics were employed to enhance reliability to detect minor differences between the studied products. The applicability of the optimized model was demonstrated using a set of commercially available biosimilars as well as deamidated and oxidized samples prepared using three oxidizing agents of different selectivity.

2. Experimental

2.1. Materials

Reference standard of rhGH was kindly supplied by the National Organization for Research and Control of Biologicals (Egypt). The standard used was in the form of lyophilized powder, labelled to contain 1.30 mg/vial. Biosimilars of rhGH from different manufacturers were obtained from local market. The used products were labelled to contain: 3.3 mg/mL (liquid formulation) or 1.6 mg Somatropin (lyophilized powder) in mannitol, histidine, poloxamer 188 and phenol. Commercial names and batch numbers of biosimilar products were not disclosed for confidentiality purposes. All other chemicals were of HPLC grade or higher and were purchased from Sigma (USA). Ultra-pure water was obtained using a MilliQ UF-Plus system (Millipore, Germany) with a resistivity of at least 18.2 MΩ cm at 25 °C and TOC value below 5 ppb.

2.2. Instruments

On-column trypsin digestion was carried out using a vacuum manifold and Bond Elut solid phase extraction (SPE) columns, 1 mL with two Frits (Agilent Technologies, Germany). All chromatographic separations were performed using an Agilent 1260 HPLC series with photodiode array detector, temperature-controlled autosampler and column compartment. Instrument control and data analysis were achieved using Chemstation software (Agilent Technologies, Germany).

2.3. Preparation of degraded rhGH samples

2.3.1. Deamidation

Deamidation was performed by incubation of rhGH samples (2.00 mg/mL) in 30 mM ammonium bicarbonate for 24 h at 37 °C.

2.3.2. Oxidation

Samples of rhGH (2.00 mg/mL) were prepared in phosphate buffer (pH 7.2) and incubated away from light with either i) 0.01 M Cu (II) in the presence of 2 mM ascorbic acid, ii) 50 μM Fe (II) in the presence of 50 μM EDTA and 5 mM ascorbic acid or iii) H_2O_2 at a ratio of 1:50 protein/oxidant at room temperature for 24 h. After incubation, the reaction was stopped by the addition of 12% trichloroacetic acid. The precipitated protein was collected by centrifugation (3000 rpm, 20 min) and re-dissolved in 100 mM Ammonium bicarbonate buffer pH 8.5. The pH was adjusted to 7.5 using 1 M HCl before the addition of trypsin.

2.3.3. Chromatographic separation of degraded samples

RP-HPLC was performed using Grace Vydac C4 column (4.6 × 250 mm, 5 μm). Separations were performed according to the Ph. Eur. [44]. Briefly, the mobile phase composition was 0.05 M Tris-HCl (pH 7.5):1-propanol (71:29 v/v) and the run time was 40 min with isocratic elution at a flow rate of 0.5 mL/min. The injection volume was 20 μL and UV detection was carried out at 220 nm. The temperature of the column compartment and sample tray were maintained at 45 °C and 5 °C, respectively.

2.4. Preparation of tryptic digests

Three replicates of both in-solution and on-column digestion experiments were performed and the resulting peptide maps were compared to demonstrate the consistency of digestion process under the proposed conditions.

2.4.1. In-Solution digestion

Conventional digestion was performed as outlined in the Ph. Eur. [44]. In brief, a solution of rhGH was prepared in 0.05 M Tris buffer solution pH 7.5 to obtain a solution containing 2.0 mg/mL. Dimethylated trypsin from porcine pancreas was reconstituted in 1 mM HCl to a concentration of 1.00 mg/mL then stored at −20 °C. Trypsin was added to the rhGH solution in a ratio of 1:66 enzyme/protein and incubated at 37 °C for 4 h with shaking. The reaction was stopped by freezing at −80 °C until the chromatographic analysis. No additional denaturing, reduction and alkylation steps were carried out as described in the pharmacopoeia procedure [44].