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Potential of hydrophilic interaction chromatography for the analytical characterization of protein biopharmaceuticals

Aurélie Periat[a], Szabolcs Fekete[a], Alessandra Cusumano[a], Jean-Luc Veuthey[a], Alain Beck[b], Matthew Lauber[c], Davy Guillarme[a,*]

[a] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland
[b] Center of Immunology Pierre Fabre, 5 Avenue Napoléon III, BP 60497, Saint-Julien-en-Genevois, 74160, France
[c] Waters Corporation, 34 Maple Street, Milford, MA 01757-3696, United States

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ABSTRACT

A new stationary phase based on wide-pore hybrid silica bonded with amide ligand has been used to explore the utility of HILIC for the analytical characterization of protein biopharmaceuticals. Various, highly-relevant samples were tested, including different insulins, interferon α-2b and trastuzumab. This work shows that HILIC can be successfully employed for the analysis of therapeutic proteins and mAbs, using mobile phase compositions comprised of between 65 and 80% ACN and 0.1% TFA. In terms of elution order and selectivity, these HILIC separations have proven to be highly orthogonal to RPLC, while the kinetic performance remains comparable. In the case of characterizing trastuzumab, HILIC was uniquely able to resolve several important glycoforms at the middle-up level of analysis (fragments of 25–100 kDa). Such a separation of glycoforms has been elusive by other separation mechanisms, such as RPLC and IEX. Besides showing orthogonality to RPLC and improved separations of glycoforms, HILIC offers several additional benefits for biopharmaceutical characterization: i) an inherent compatibility with MS, ii) a reduced requirement for very high mobile phase temperatures that are otherwise needed in RPLC to limit undesirably strong adsorption to the surface of the stationary phase, and iii) the possibility to couple several columns in series to improve resolving power, thanks to comparatively low mobile phase viscosity.

1. Introduction

The importance of biotechnology products has been ever increasing, and it is expected that the majority of new drug approvals will be biologics [1,2]. Therapeutic proteins are more complex than small molecules due to their high molecular weights, numerous possible conformations, post-translational modifications and microheterogeneity [3]. The heterogeneity of biopharmaceuticals is a consequence of their expression from living organisms and the fact that modifications can readily occur during production, extraction, purification, formulation and storage [4]. These changes can affect the efficacy and safety of biopharmaceuticals. Therefore, it is important that they be properly characterized. For this purpose, a variety of orthogonal analytical techniques can be used, including liquid chromatography, gel and capillary electrophoresis, mass spectrometry (MS), and spectroscopy [5].

Liquid chromatography is an indispensable tool for intact protein analysis and different modes can be employed, such as reversed-phase liquid chromatography (RPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and hydrophilic interaction chromatography (HILIC) [6,7]. SEC, IEX and HIC are historical techniques, well established in the biopharmaceutical industry for intact proteins analysis, though they suffer from a difficult hyphenation to MS. For this reason, RPLC in combination with UV, fluorescence and, above all, MS is more and more widely used today to assess protein batch purity and highlight any protein degradation or misfolds [8–10]. In the RPLC of large proteins, an analyst often encounters adsorption, carryover, multiple peak formation and low chromatographic performance due to slow diffusion and secondary interactions with stationary phase. Nonetheless, it has been shown that RPLC of large proteins can be enhanced through the use of elevated mobile phase temperature (in the range 60–90 °C), addition of ion-pairing agents to the mobile phase (e.g. TFA), use of columns packed with wide-pore particles of 300–1000 Å pore sizes, and/or use of silica-based

* Corresponding author.
E-mail address: davy.guillarme@unige.ch (D. Guillarme).

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stationary phases with reduced silanol amount and/or acidity [2].

HILIC is a variant of normal phase chromatography in which the stationary phase is polar and the mobile phase consists of water and 60–95% of an aprotic, miscible organic solvent, usually acetonitrile (ACN). The highly organic mobile phase is particularly well suited to ESI–MS detection, leading to substantial improvements in sensitivity for a large variety of compounds [11,12]. The high organic content of the mobile phase also generates relatively low back pressures, allowing the use of high flow rates or long columns packed with small particles. The retention mechanism of HILIC is based mainly on hydrophilic partitioning of polar compounds between a water-enriched layer formed at the surface of the stationary phase and the highly organic mobile phase. Depending on the nature of both the stationary phase and the mobile phases, as well as the physico-chemical properties of the analytes, additional interaction mechanisms can occur, including hydrogen bonding, dipole-dipole interactions, and ionic interactions [13–15]. HILIC is nowadays largely employed for the analysis of polar and/or ionizable small compounds, peptides, and glycans [16–18]. However, it has not yet been applied for the analysis of intact proteins, except for the characterization of lipophilic membrane proteins, which could bind irreversibly to a stationary phase under RPLC conditions [18–21].

In this first study of its kind, a new wide-pore, amide-bonded stationary phase has been used to investigate the applicability of HILIC to the characterization of intact protein biopharmaceuticals. Compelling separations have been obtained for a set of highly relevant biopharmaceuticals, including insulins, interferon α-2b, and trastuzumab. Interestingly, it has been found that the performance of these novel HILIC separations nicely complements the capabilities of conventional separation mechanisms, such as RPLC and IEX.

2. Experimental part

2.1. Reagents and analytes

Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile (ACN) and methanol (MeOH) were ULC–MS grade and purchased from Biosolve (Valkenswaald, Netherlands). Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane (Tris) and 1 M sodium hydroxide (NaOH) solution were purchased from Sigma–Aldrich (Buchs, Switzerland).

Human insulin (Insuman rapid 100 UI/mL), insulin glargine (Lantus 100 UI/mL) and insulin glulisine (Apidra 100 UI/mL) were obtained from Sanofi Aventis (Paris, France). Insulin lispro (Humalog 100 UI) and insulin aspart (Novorapid 100 UI/mL) were purchased from Eli Lilly (Indianapolis, USA) and Novo Nordisk Pharma AG (Küsnacht, Switzerland), respectively. Bovine insulin was purchased from Sigma-Aldrich (Buchs, Switzerland). The sequences of human, bovine and synthetic insulins are provided in Table 1.

Dithiothreitol (DTT), hydrogen peroxide 30% and methionine were purchased from Sigma-Aldrich. IdeS enzyme (FabRICATOR™) and EndoS2 enzyme (GlycINATOR™) were purchased from Genovis Inc. (Lund, Sweden). Recombinant interferon α-2b was purchased from Merck (Darmstadt, Germany). Trastuzumab was kindly provided by Pierre Fabre laboratories (Saint-Julien en Genevois, France).

2.2. Instrumentation and columns

RPLC and HILIC analyses of insulins were performed on a Waters ACQUITY UPLC H-Class system (Milford, MA, USA). This system included a quaternary solvent delivery pump, an autosampler, and a column oven. The autosampler included a flow through needle (FTN) injection system with a 15 μL needle. The system was equipped with a UV-DAD detector set at 214 nm. The dwell volume was experimentally measured as 375 μL. Data acquisition, data handling and instrument control were performed with Empower 3 (Waters).

The RPLC and HILIC analyses of interferon α-2b and trastuzumab samples were performed on a Waters ACQUITY UPLC I-Class system equipped with a binary solvent delivery pump, an autosampler, a UV-DAD and a fluorescence detector (FL) set at λ_{ex} = 280 nm and λ_{em} = 360 nm. The system included a flow through needle (FTN) injection system with a 15 μL needle. The dwell volume was experimentally measured as 110 μL. Data acquisition, data handling and instrument control were performed with Empower 2 (Waters).

IEX measurements were performed on a Waters ACQUITY UPLC™ system equipped with a binary solvent delivery pump, an autosampler and fluorescence detector (FL). The Waters ACQUITY system included a 5 μL sample loop and a 2 μL FL flow-cell. The loop was directly connected to the injection switching valve. The measured dwell volume was around 100 μL. Fluorescence detection was carried out at λ_{ex} = 280 nm and λ_{em} = 360 nm. The acquisition rate and time constant were fixed at 10 Hz and 50 ms, respectively. Data acquisition and instrument control were performed by Empower Pro 3 Software (Waters).

Used in this study were ACQUITY UPLC Glycoprotein BEH Amide 300 Å 1.7 μm (2.1 mm id × 150 mm,) and ACQUITY UPLC Peptide BEH C18 300 Å 1.7 μm (2.1 mm id × 150 mm,) columns, both obtained from Waters (Milford, MA, USA). The IEX columns were YMC BioPro SP-F (4.6 mm i.d. 100 mm, 5 μm) strong cation exchanger purchased from Stacroma (Reinach, Switzerland).

2.3. Analysis of insulins

In HILIC and RPLC, the mobile phase was 0.1% (v/v) TFA in water (A) and 0.1% (v/v) TFA in ACN (B). The optimized conditions included an isocratic elution at 80% B and 31% B in HILIC and RPLC modes, respectively. The flow rates were set at 0.5 mL/min and 0.3 mL/min in HILIC and RPLC, respectively. The column temperature was set at 50 °C in both modes. All insulins were diluted at 100 μg/mL in a 1:1 (v/v) mixture of water/ACN with 0.1% FA for HILIC or in water for RPLC analysis, respectively. All experiments with insulins were performed in duplicate, and the chromatograms were identical between the two successive runs.

Human insulin is composed of 51 amino acid residues. It is a dimer of A- and B-chains, linked together by disulfide bridges. Bovine insulin differs from human insulin in only three amino acid residues: the threonine at position A8 and isoleucine at position A10 in the human insulin sequence are replaced by two valine residues, while the C-terminal asparagine of the B-chain of human insulin is replaced by alanine. Insulin Lispro is a fast acting insulin analogue in which the penultimate lysine and proline residues on the C-terminal end of the B-chain are reversed. Insulin glulisine is a rapid-acting insulin analogue that differs from human insulin in that the amino acid asparagine at position B3 is replaced by lysine and the lysine in position B29 is replaced by glutamic acid. Insulin aspart is also a fast-acting insulin analog where a single amino acid has been exchanged (proline in position B28 replaced by aspartic acid). Insulin Glargine is a long-acting basal insulin analog. It has a substitution of glycine for asparagine at position A21 and has two arginine residues added to the C-terminal position of B-chain, shifting the isoelectric point from 5.4 to 6.7 and limiting deamidation of asparagine.