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Assessment of the quality and structural integrity of a complex glycoprotein mixture following extraction from the formulated biopharmaceutical drug product

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ABSTRACT

Biological drugs represent an important and rapidly growing class of therapeutics useful in the treatment of a variety of disorders ranging from cancer to inflammation to infectious diseases. Unlike single chemical entities, the recombinant production of these drugs in living cells confers considerable structural and chemical heterogeneity to the biologically derived protein product that constitutes the active pharmaceutical ingredient (API). In mammalian based expression systems, much of this diversity is conferred through heterogeneous protein glycosylation. These post-translational modifications can have significant effects on the structure, biological function, and pharmacological properties of the API. In addition, the bulk proteins that comprise the API are further formulated through the use of multiple excipients designed to ensure product stability, solubility, and lot-to-lot consistency. Unfortunately, these matrices can interfere with commonly available analytical methods used in the thorough chemical characterization of the biological drug product. At the same time, a demonstration of the suitable extraction of the bulk drug substance in a manner and form that does not destabilize the active ingredient or introduce any structural bias with direct reference to the original drug product is both critical and necessary. Here, we use recombinant human follicle stimulating hormone (follitropin alpha for injection) from a pharmaceutical source as an example to illustrate a suitable purification strategy to effectively extract the bulk drug substance from the formulated drug product with high purity and yield. We assess the suitability of this extraction method in preserving the structural integrity and overall quality of the drug substance relative to the formulated drug product, placing a particular emphasis on glycosylation as a key product attribute. In so doing, we demonstrate that it is possible to effectively extract the active pharmaceutical ingredient from a formulated biological drug product in a manner that is consequently sufficient for its use in comparability studies.

1. Introduction

For many pharmaceutics, drug product characterization is relatively straightforward. Non-biological agents, often referred to as small-molecule drugs, are typically of low molecular size and are manufactured in chemical reactors rather than in biological systems. The structure of small-molecule drugs can be verified through established analytical testing, including monodimensional NMR and mass spectrometry. However, of increasing interest and importance are drugs of biological origin (hereafter referred to as biologics) that are manufactured by inserting genes into living cells so as to recombinantly produce a therapeutic protein of interest. Many biologics are glycoproteins, consisting both of a protein backbone and additional post-translational modifications, including, for example, phosphorylation and/or N- and O-linked glycosylation. Protein glycosylation, in particular, represents one of the most structurally complex, post-translational modifications; as such, the resultant glycoproteins are, in reality, comprised of a heterogeneous mixture of many isoforms. Because of their structural complexity and size, assessing the structure of biologics is a formidable task compared to assessing the structure of small-molecule drugs, which generally have fewer than 100 atoms. For example, using molecular weight as a measure of size, human

Abbreviations: rh-FSH, recombinant human follicle stimulating hormone; HPLC, high-performance liquid chromatography; IEX, ion-exchange chromatography; SEC, size-exclusion chromatography; SAX, strong-anion exchange chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF, isoelectricfocusing; PCD, postcolumn derivatization; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

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growth hormone is more than 150 times larger than aspirin and a monoclonal antibody is more than five times larger still than human growth hormone.

The glycosylation status of a given biologic has been shown to be important to its biological function, stability, and pharmacological properties; numerous studies have identified roles for specific monosaccharides on glycoproteins in terms of modulating biological activity. Examples include the degree of sialylation and half-life [1–4], core fucosylation and antibody-dependent cytotoxicity [5] and the presence of galactose $\alpha 1 \rightarrow 3$ galactose and immunogenicity [6–8]. In these cases, complex glycoprotein biopharmaceuticals, such as antibodies, follicle stimulating hormone (FSH), and erythropoietin as examples, consist of a range of isoforms due to the presence of different glycoforms, each with potentially different activity, stability, and/or serum half-life.

Given the complexity of biologics and the fact that the presence and amount of different isoforms dictates their biological function, characterization of the mixture becomes critical to the overall assessment of the product obtained from a given manufacturing process. Numerous techniques have been used to characterize biologics, including HPLC and CE [9–11], also coupled to mass spectrometry [12], and NMR [13], among others. One critical component of many of these studies is the requirement for API to be free of components within the drug product formulation, which may confound analysis through signal suppression, presence of background analytes, and other technical hindrances. To this end, we present here a straightforward purification strategy to effectively isolate a biologics API from its original drug product formulation, resulting in quantitative productive yields while also preserving product integrity and the overall product quality profile relative to original pharmaceutical drug product from which it was derived. Gonal-F® (generic name follitropin alpha for injection), a commercial preparation of recombinant human follicle stimulating hormone (FSH) was used as a model glycoprotein biologic to demonstrate our approach. The drug product formulation of Gonal-F is rather complex; it includes a number of excipients such as salts (e.g., sodium phosphate), osmotic regulators (e.g., sucrose) and other stabilizers (e.g., methionine and polyoxamer). In fact, on a mass basis, less than 0.1% of the bulk drug product is actually comprised of recombinant human FSH (follitropin-α). As such, quantitative extraction represents a challenging scientific problem. Furthermore, FSH exists not as a single molecular structure, but rather as multiple, charged isoforms that result from variations in the composition of the four carbohydrate chains (in particular their sialic acid content) attached to the two (α and β) inter-linked protein subunits [14–16]. These isoforms differ in their molecular weights, biological potency, elimination half-life and immunoreactivity [17–19]. Furthermore, the α and β subunits of FSH interact with one another through non-covalent interactions. Therefore, dissociation of these subunits from one another is an exquisitely sensitive readout of whether disruption of the API has occurred. Preventing the dissociation of these subunits is critical to ensure the native structure of the protein and, accordingly, an un-biased analysis.

In this study, rh-FSH was isolated from Gonal-F® using different chromatographic fractionation methods and one workflow was found optimal to meet our satisfaction. The isolated FSH was subsequently analyzed through a series of established analytical methods to assess the overall quality and structural integrity of the API following extraction. A particular emphasis was placed on protein integrity (i.e., maintenance of the α and β subunits interaction) and profiling of protein glycosylation. Based on this assessment, we conclude that rh-FSH, when carefully isolated from the drug product maintains its overall structural integrity and glycan profiles (composition) relative to the drug product from which it was derived.

2. Methods

2.1. Materials

Recombinant gonadotrophins (r-hFSH, Gonal-F®) were produced by Merck KGaA/EMD Serono (Switzerland). Reagents used in this study, unless otherwise specified, were purchased from either Sigma (St. Louis, MO) or Fluka (Bucho, Switzerland). 10% Bis–Tris and 14% Tris–Gly gels were purchased from Invitrogen. PNGase F (Glycerol Free), supplied in 50 mM NaCl, 20 mM Tris–HCl (pH 7.5) and 5 mM EDTA, was purchased from New England Biolabs.

2.2. FSH isolation by Hitrap DEAE-FF–IEX

Each Gonal-F® pen contains approximately 1.5 mL in liquid formulated solution. For this study, multiple pens of the same lot were dispensed and combined, then loaded on to a Hitrap DEAE-FF column (GE Healthcare life science, Cat# 17-5154-01) at a flow rate of 2 mL/min under isocratic condition of 100% buffer A (2 mM phosphate, pH 7.4). The baseline of the flowthrough was monitored at 230 and 280 nm until a return to baseline was observed, then the enriched FSH was eluted under a gradient of buffer B (2 mM phosphate + 500 mM NaCl) from 0 to 50% within 20 min. The eluted peak was collected and applied to an Amicon Ultra 10k MWCO (Millipore, Billirico, MA) Centrifuge device (15 mL). The centrifuge was spun for 1 h at 3000 rpm at 4 °C and washed with buffer of 5 mM phosphate containing 150 mM NaCl pH 7.4. After concentration, fractions were combined and stored at −80 °C.

2.3. FSH quality and quantity by SEC-HPLC

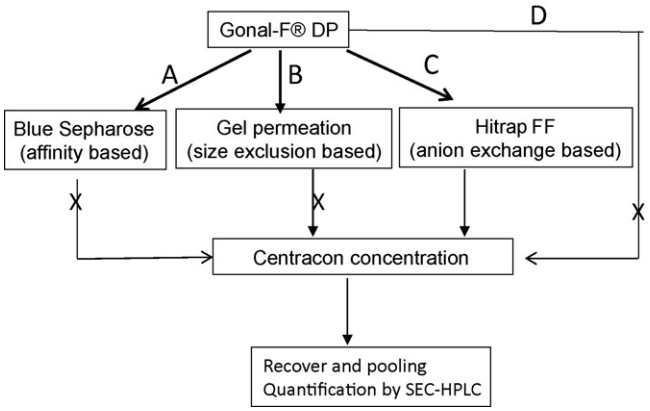
Chromatography was performed on Dionex Ultimate 3000 system with two SEC columns connected in series (Tosoh Bioscience, G3000SW and G2000SW). The sample (either Gonal-F® drug product or isolated rh-FSH) was dissolved in running buffer (5 mM sodium phosphate, 150 mM sodium chloride) at a concentration range of 0.5–1 mg/mL. 20 μL of sample solution was injected onto the LC system and eluted under isocratic conditions within 1 h at flow rate of 0.5 mL/min. The eluted peaks were monitored by UV detector (absorbance at 214, 230 and 280 nm) and refractive index detector sequentially. The isolated FSH concentration was determined, and purity, quality, and recovery were compared to Gonal-F®.

2.4. r-hFSH intact and dissociated subunit content by SDS-PAGE

NuPAGE® Novex 10% Bis–Tris Gel 1.0 mm × 10 well (Invitrogen) and Tris–Glycine Invitrogen™ 14% Tris–Glycine Gel 1.0 mm × 12 well were used to evaluate the content of intact FSH as well as dissociated subunits. Protein markers (SeeBlue® Plus Pre-stained Standard (Invitrogen)) were used on the gel to assess molecular weight. Gels were run under standard conditions (25 V, 131 min, 24 mA, power 3.0 W, room temperature).

2.5. FSH isoform distribution by IEF

Precast Novex® pH 3–7 IEF Gel 1.0 mm, 10 well was purchased from Invitrogen (Invitrogen, Cat. No. EC6645BOX) and directly used to evaluate FSH isoform pattern distribution according to the vendor manual. IEF protein Marker 3-7 (Cat. No. 39212-01) was used as a standard. The IEF gel was stained by Coomassie G-250 (Invitrogen).



Scheme 1. Schematic workflow of FSH isolation from Gonal-F®. Workflow C was found to be the optimal isolation pathway for FSH.

2.6. Intact FSH isoform profiling by high resolution IEX-HPLC

Gonal-F® (Lot# Y40B7171) and isolated rh-FSH from this lot (~6 μg FSH) were individually injected onto a SAX-HPLC Column (Sepax Tech, Proteomix SAX-NP1.7) using a Dionex ICS3000 system with two detectors: a UV–vis detector and a fluorescence detector. The flow rate was 0.1 mL/min and column was kept at 50 °C. FSH isoforms were eluted using a gradient of buffer B (10–55% in 50 min). Buffers A: 20 mM Tris/HCl, pH 8.0 and B: 20 mM Tris/HCl + 450 mM NaCl, pH 8.0. Detection was completed using UV at 214, 230, 280 nm as well as intrinsic tryptophan fluorescence emission at 320 nm (excitation at 272 nm).

2.7. Glycan preparation

Prior to digestion, Gonal-F® was concentrated by centrifugation using a Centricon filter without passing through Hitrap DEAE-FF catridge (workflow D in Scheme 1). This concentrated FSH (workflow D) was used as a reference of Gonal-F®, which represents FSH drug product. Due to the low concentration of rh-FSH present in the commercial drug product, (~44 μg/mL), it was not technically feasible to obtain directly from the commercial Gonal-F® sufficient glycan quantities, for downstream structure assessment without first concentrating the recombinant glycoprotein by ultrafiltration The isolated FSH through workflow C and workflow D, separately was used for glycan analysis after enzymatic release using PNGase F. Briefly, about 100 μg of FSH was incubated in denaturing buffer at 100 °C for 10 min in a heat block. Then sequential addition of NP-40, G7 reaction buffer and PNGase F (1 μL/20 μg of glycoprotein) was performed, followed by incubation at 37 °C for 18 h. The resulting mixture was applied to a pre-treated 3 mL Hypercarb cartridge (Thermo-Fisher Scientific) which had been pre-equilibrated with 2× 5 mL of 50% acetonitrile/0.1% TFA. After addition of the glycans, the cartridges were washed with 5 mL of water followed by 5 mL of 5% acetonitrile/0.1% TFA and eluted with 2× 2.5 mL of 50% acetonitrile/0.1%TFA. Samples were frozen and dried by lyophilization. The resulting glycan samples were stored at −20 °C prior to use.

2.8. Glycan mapping by SAX-HPLC followed by online postcolumn derivatization (PCD)

Unlabeled glycans were directly profiled by strong anion HPLC and detected using in-line postcolumn derivatization with 2-cyanoacetoamide. The mobile phases were defined as A: water; B: 2 M sodium chloride, pH 7.0. The pH was adjusted using either sodium hydroxide (1.0N) or hydrochloric acid (1.0N). A CarboPac PA-100 strong anion exchanger column was used for separation using a gradient of B from 2% to 50% over 60 min. The total flow rate was kept constant at 1 mL/min The eluent was subjected to in-line derivatization using a 3% 2-cyanoacetoamide solution and 0.6 M sodium hydroxide by mixing and then passing the mixture through a reaction heating coil set in a dry temperature-controlled bath at 125 °C, followed by a cooling coil. Derivatized glycans were subsequently monitored by fluorescence (emission at 410 nm and excitation at 346 nm).

2.9. Glycan analysis by MALDI-TOF-MS

The unlabeled glycan mixture was mixed at 1:9 with a matrix comprised of a 10 mg/mL ATT (6-azo-thiothymine) solution in anhydrous ethanol 1.0 μL, and spotted on a MALDI plate (Applied Biosystems or equivalent, Cat. V700666). The plate was predeposited and dried with 1.0 μL of Nafion. As an alternate matrix saturated DHB (2,6 dihydroxybenzoic acid) in 300 mM aqueous spermine tetrahydrochloride mixed 1:1 with the sample. 1.0 μL of each matrix:sample mixture was likewise spotted. All spotting was done in a humidity control room to minimize sample moisture. The spectra produced by each sample were examined for signs of glycan degradation as well as for the presence of significant alkali metal adducts. Analysis of acidic glycans was performed in reflectron mode selecting for negative ions. The sensitivity was evaluated by determining the lowest detectable amount of each glycan for each of the matrices tested and calculating the corresponding signal to noise ratio.

3. Results

3.1. Purification of FSH from Gonal-F®

The principal goal of this study was to effectively isolate the API (i.e., rh-FSH) of Gonal-F® in a facile and reproducible manner while achieving high yield, and the preservation of critical sample quality. Toward this end, we first examined several approaches (schematically depicted in Scheme 1). These empirical approaches sought to utilize one or more of the physico-chemical properties or the recombinant glycoprotein product, including molecular mass, charge, and hydrophobicity. In Scheme 1, workflow A was based on the affinity based fractionation of FSH using Blue Sepharose® CL-6B. This design was attempted since Blue Sepharose is commonly used chromatographic isolation procedure whereby many proteins are able to bind under conditions of relatively low ionic strength, and are subsequently eluted by raising the salt concentration or changing the mobile phase pH. We found that this approach resulted, however, in a low recovery of FSH (~30%) from the commercial drug product. Workflow B used semi-preparative SEC-HPLC fractionation and resulted in a reasonably high recovery of rh-FSH (~70%). The overall process by workflow B was relatively time consuming. However, for this reason this particular workflow was likewise not pursued any further. Workflow D was straightforward and efficient; however, the recovery (60–85%) was not consistently high. Of all the isolation procedures examined, workflow C was found to be superior to the rest inasmuch as it met our requirements of high product yield and workflow efficiency. This workflow involved online separation of excipients from the rh-FSH with concomitant concentration of the glycoprotein following multiple rounds of binding (4× 4 mL) to a Hitrap FF cartridge. Subsequent desalting of the eluted product was achieved by simply using an Amicon Ultra membrane (10k MWCO). Isolation of rh-FSH from Gonal-F® by this two-step process resulted in a mass recovery of ≥90%.

To assess reproducibility, three additional batches of Gonal-F® materials were processed using this isolation procedure (workflow

Table 1
Summary of the recovery yield from three batches of Gonal-F® purification.

Gonal-F lot#	# Pen received	# Pen processed	Recovery yield (%)
Y40B1502	166	164	90
Y40B4802	70	68	95
Y40B1667	70	68	92

C). Table 1 summarizes the results from the isolation of rh-FSH (bulk drug substance) from three lots of Gonal-F®. All three-batches' drug substance fractions were quantified by SEC-HPLC: a quantitatively reproducible yield of approximately 90% was routinely achieved. Of note is the fact that the actual amount of FSH present in Gonal-F® in comparison with the mass of excipients is low, accounting for ~0.1% of the total mass of the formulated drug product. As such, quantitative isolation for this drug product is challenging; Detection is likewise difficult given the low concentration of the recombinant protein product in such a formulation (Fig. 1A); FSH present in a single pen of Gonal-F® is below the limits of detection by UV absorbance at 280 nm. However, after online enrichment (4× 4 mL loading), the FSH-specific protein peak is clearly observed as shown in Fig. 1B. To ensure the absence of introduced bias, we extensively analyzed the attributes of the isolated drug substance and compared these to the attributes of FSH in the initial drug product formulation, using a number of analytical procedures.

3.2. Assessment of FSH integrity by SEC-HPLC

Initially, to assess the quality and purity of isolated FSH, we completed analysis by SEC-HPLC. SEC-HPLC is known to separate intact protein from degraded size variants, as well as monomers from oligomers. Additionally, SEC-HPLC has recently been used to quantify FSH for clinical use (http://www.fertilitylifelines.com/index.jsp). Additionally, the drug product vial is filled by mass [15,16]. As a result, FSH can be reliably quantified using an optimized size exclusion high-performance liquid chromatography (SEC-HPLC) method.

Fig. 2 shows a comparison of Gonal-F® and isolated FSH by SEC-HPLC analysis. The isolated FSH sample eluted as a single peak using UV detection, which has an identical elution time (12.5 min) as FSH in drug product matrix. These results suggest that the isolated FSH is the same size as it is within Gonal-F®. This does not appear to be either aggregation or degradation of the sample. Importantly, dissociation of FSH into α and β subunits, an indicator of sample instability, is not observed. Further inspection of the HPLC profile for Gonal-F® indicates that there are two other peaks present besides that of FSH. These two peaks eluted later (16 min, 26 min) than FSH. The largest peak at 26 min is identified as the low molecular weight excipients, such as sucrose, methionine, cresol as well as salts. The peak eluting at 16 min is likely to be the result of micelles formed from polysorbate in the formulation buffer. As additional demonstration of the robustness of this approach, analysis of multiple-batches of isolated FSH from Gonal-F®, indicates high reproducibility and recovery. We quantitatively analyzed the SEC-HPLC profile for concentration determination as shown in Fig. 3. Fig. 3A shows a comparison of isolated FSH to FSH within the Gonal-F® drug product. Fig. 3B shows an overlaid SEC-HPLC profiling from three independent isolations of FSH from three lots of Gonal-F®. In each case, the SEC-HPLC profiles overlay almost perfectly. Additionally, it should be noted that the SEC-HPLC resulted in single peak monitored by refractive index detection (data not shown), and recovery yields were calculated based on this mass concentration measurement (Table 1). Consistent, with the analysis of the UV profiles, we find that isolation of FSH from drug product results in near quantitative recovery. To confirm and extend the results obtained from SEC-HPLC, we examined the isolated FSH product by a variety of analytical techniques.

3.3. Comparative analysis of rh-FSH by SDS-PAGE

SDS-PAGE was used as an initial (and routinely used) analytic to directly compare the relative product attributes of isolated rh-FSH drug substance to the original drug product (Gonal-F) from which it was derived, especially in terms of subunit integrity and the apparent molecular weight distribution of the various heterogeneously glycosylated isoforms that collectively comprised the biological product. In its native state, FSH is a heterodimer comprised of non-covalently associated α and β subunits. The apparent molecular weight of native FSH (heterodimeric form) is reported as approximately 33 kDa [20,21], with ~30% of this molecular weight being comprised of carbohydrates. We sought to benchmark our studies using high resolution TOF-MS to measure the respective masses of the α and β subunits. MS analysis of the isolated rh-FSH (Gonal-F®

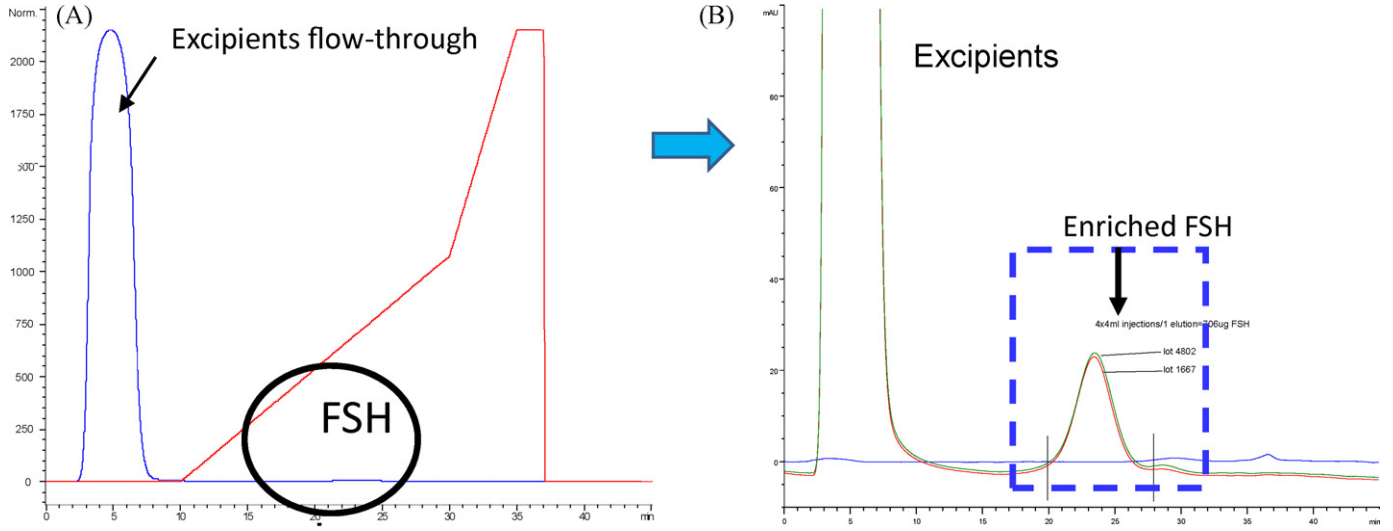


Fig. 1. Chromatographic fractionation of recombinant human FSH from drug product (Gonal-F®) by ion-exchange chromatography. FSH was fractionated on a Hitrap DEAE-FF Cartridge as described in Section 2. (A) Full-scale of the chromagogram with the gradient elution after a single load (4 mL) of Gonal-F® drug product solution. The elution gradient is shown. (B) Zoomed scale chromatogram with the same gradient elution after multiple loading (4× 4 mL) of Gonal-F® (Lot# Y40B7171) solution to increase product detection by UV absorbance.

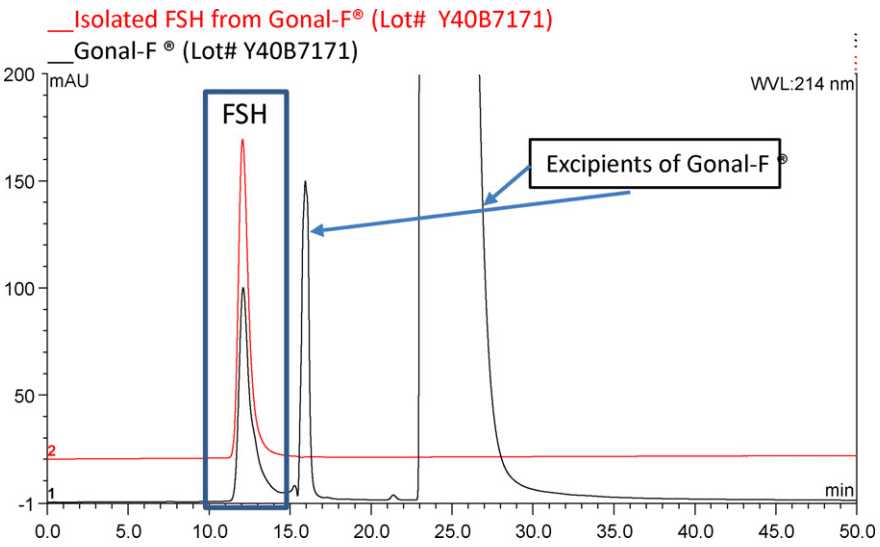


Fig. 2. Comparison of isolated FSH with Gonal-F (Lot# Y40B7171) by SEC-HPLC (zoomed scale) with detection at 214 nm. Red traces represent the isolated FSH, the black traces represent analysis of Gonal-F®. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

Lot# Y40B4802) indicated a highest abundance molecular mass of 14606.7 and 17542.8 Da for the α and β subunits, respectively (data not shown). This yielded a calculated molecular mass of 32149.5 Da for the most abundant heterodimer FSH isoform. SDS-PAGE analysis confirms the molecular mass of multiply isolated rh-FSH batches to be consistently in this range (Fig. 4A). Notably, isolated FSH remains as heterodimer, and is neither dissociated, nor present in higher molecular order oligomers (aggregates) when analyzed under native conditions. Moreover, rh-FSH analyzed by this method appears to be physically indistinguishable from the Gonal-F drug product present on the same gel. Such a first pass analysis indicates at least no gross changes in the overall structure between the drug substance and the drug product, including post-translational modifications due to glycosylation.

3.4. Intact FSH isoform pattern and distribution by IEF gel

While SEC-HPLC and SDS-PAGE methods are useful as first-pass methods to quickly demonstrate the overall integrity and purity of isolated FSH, these two techniques are clearly limited in their ability to provide more detailed structural information on glycoform diversity and, along these lines, any impact our purification of the recombinant drug product may have on glycan structure relative to the formulated drug product from which it was derived. Therefore, we chose to investigate the use of two orthogonal, higher resolution methods for the comparative analysis of intact isoforms of FSH, namely electrophoresis-based IEF and chromatography-based IEX-HPLC.

Isoelectric focusing (IEF), is a technique for separating different molecules by their electric charge differences. It is a type of zone electrophoresis, which takes advantage of the fact that a molecule's charge changes with the pH of its surroundings. Based on the amino acid composition of FSH, the appropriate IEF gel was chosen to resolve FSH isoforms in the pH 3–7 range. Fig. 5 shows a comparison of multi-lot analysis of rh-FSH by IEF gel. All lots of isolated FSH qualitatively appear to have the same number of isoforms; additionally the isoform distributions of the samples are very consistent with one another. The migration pattern for a given glycoprotein within an IEF gel is predominantly dictated by the net charge of the glycans, including sialic acid, and possibly sulfation, or phosphorylation [22,23]. Based on this comparison of the IEF results we conclude that isolation of rh-FSH from the drug product did not result in any detectable charge change in the fine structure of FSH, including the number and distribution of acidic glycan species.

3.5. Intact FSH isoform profiling by high resolution IEX-HPLC

To further analyze the distribution of isoforms, we used a high resolution ion-exchange chromatography technique to compare and quantify isoform distributions of the isolated FSH and FSH in

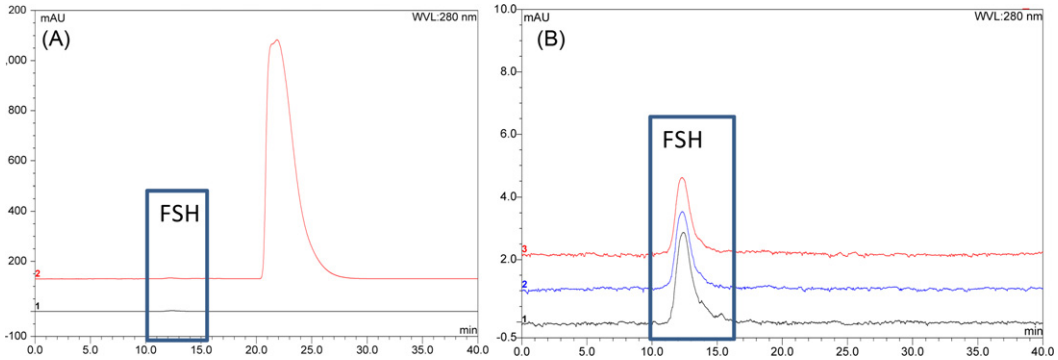


Fig. 3. (A) Comparison of one batch Gonal-F (Lot# Y40B7171) and isolated FSH by SEC-HPLC (full-scale) with detection at 280 nm. Red trace represents Gonal-F and black trace represents isolated FSH. (B) Overlaid SEC-HPLC of three batches of FSH isolation from respective lots of Gonal-F (zoomed); red trace represents Gonal-F Lot# Y40B1502, blue trace represents Lot# 40B4802, black trace represents Lot# Y40B1667. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

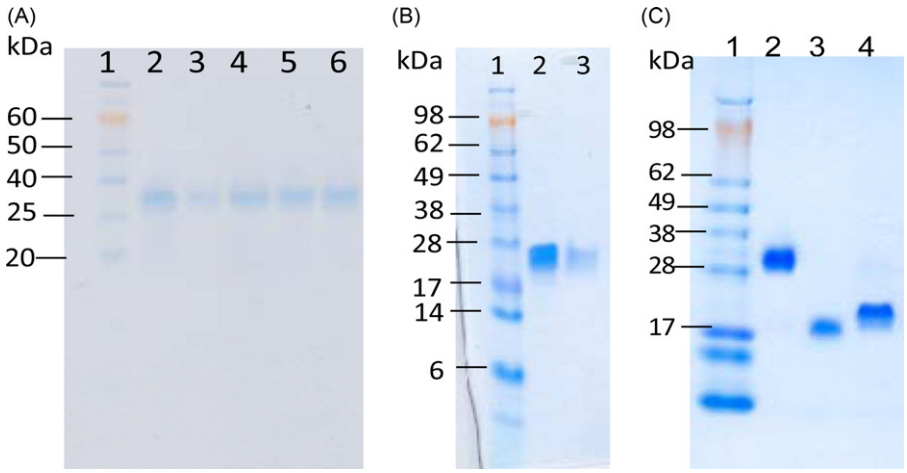


Fig. 4. Characterization of extracted recombinant FSH by non-reducing SDS-PAGE. Gels were stained by Coomassie G-250. (A) Samples were not heated prior to running on 10% Bis–Tris NuPAGE MES. Lane 1, protein marker (Invitrogen BenchMarkTM Pre-stained, Cat#10748-010); Lane 2, the isolated FSH (~1 μg) from workflow C (Gonal-F® Lot# Y40B7171); Lane 3, Gonal-F DP Lot# Y40B1502 (~0.44 μg). Lanes 4, 5, 6, the isolated FSH (~0.8 μg) from Gonal-F® Lot# Y40B1502, Y40B4802, Y40B1667, respectively. (B) Samples were heated to 80 °C for 5 min and remained as α, β subunits (non-separated) and run on 10% Bis–Tris NuPAGE. Lane 1, protein marker (Invitrogen, SeeBlue® Plus2 Pre-Stained Standard (Cat. No. LC5925)); Lane 2, isolated FSH from Gonal-F Lot# Y40B7171 (1.4 μg loaded); Lane 3, Gonal-F Lot# Y40B7171 (~0.88 μg FSH loaded). (C) Samples were run on 14% Tris–Glycine SDS-PAGE. Lane 2, the isolated FSH (~1.4 μg from Gonal-F® Lot# Y40B71710) was intact and unheated; Lane 3, purified and isolated FSH subunit-α from intact FSH (Gonal-F® Lot# Y Y40B7171); Lane 4, purified and isolated FSH subunit-β from intact FSH (Gonal-F® Lot# Y40B7171).

the context of drug product. As shown in Fig. 6, the isolated FSH and Gonal-F® were injected and analyzed by this system. Careful examination of the region bounded by retention times between 35 and 65 min indicated that there are at least 10 peaks which are eluted for both samples under identical conditions. Comparison of the results for isolated FSH and Gonal-F® indicate that the two profiles are essentially superimposable, each one being comprised of the same number of peaks. The consistency of the isoform profiles in Fig. 6 demonstrates two facts, at least. First, FSH after isolation maintains a correct higher-order structure. To confirm this observation, the dissociated α, β subunits were observed to elute at different retention times and different distribution patterns (data not shown here) under identical conditions. Therefore it is concluded that the α and β subunits within isolated FSH remain associated with the correct stoichiometry. Second, based on this analysis, there is no detectable change in the charged glycoform states and distribution of glycans. The sialic acid content is labile to changes in buffer pH, temperature, salt content, and storage conditions. No such desialylation of isolated FSH was observed as shown in Fig. 6.

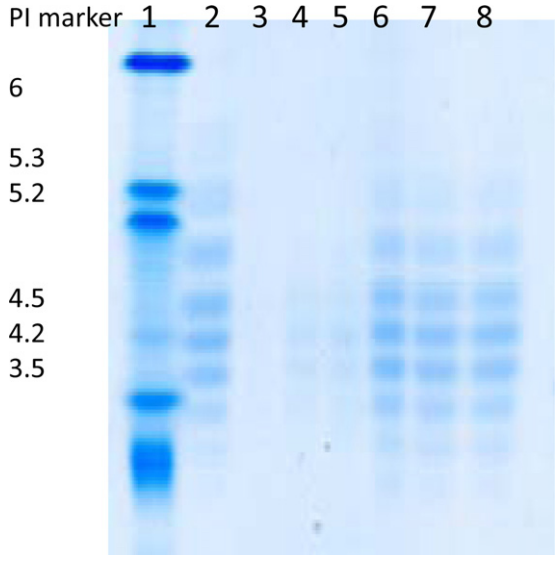


Fig. 5. Examination of isolated FSH from Gonal-F by IEF gel (pH 3–7 1.0 mm). The IEF gel was stained by Coomassie G-250. Lane 1, IEF marker; Lane 2, ~4 μg concentrated by workflow D (Gonal-F Lot# Y40B7171); Lane 3, 0.44 μg Gonal-F DP Lot# Y40B1502; Lane 4, 0.88 μg Gonal-F Lot# Y40B1502; Lane 5, 1.32 μg Gonal-F Lot# Y40B4802; Lane 6, 4 μg isolated FSH from Gona-F lot Y40B1502; Lane 7, 4 μg FSH isolated from Gona-F Lot Y40B4802; Lane 8, 4 μg FSH isolated from Gona-F lot Y40B1667.

3.6. Glycan profiling by SAX-HPLC-PCD

The SEC-HPLC and high resolution IEX-HPLC are orthogonal but complementary HPLC-based techniques to evaluate the quality and structural integrity of isolated FSH protein as compared to FSH in the context of Gonal-F®. However, neither technique explicitly looks at glycan fine structure. In case of FSH, there are a total of four N-linked glycosylation sites [24,25]. In an effort to assess the overall quality and content of the glycans present on isolated FSH, they were profiled by SAX-HPLC-PCD (Fig. 7A) and compared to the glycans obtained the directly from Gonal-F® (Fig. 7B). In this analysis, the glycans elute as a function of the number of sialic acids, which, in turn, is linked to their charge. In this technique, the groups of peaks corresponding to glycan species with different levels of sialylation are well separated without overlap. As shown, the stacked SAX-HPLC-PCD profiling for released glycans of isolated FSH are similar and consistent to FSH in the context of drug product. Quantitative analysis through integration of each peak for these two profiles also suggest that relative distribution of charged glycan clusters such as neutral, mono-, di-, tri-, and tetra- sialylated glycans is consistent between isolated FSH and FSH in the context of drug product.

3.7. Glycan profiling by MALDI-TOF-MS

To further assess the glycan structures present on FSH, MALDI-TOF-MS was utilized to evaluate relative glycan composition. MALDI-TOF-MS under linear mode is a high throughput and sensitive technique to determine glycan composition. As shown in Fig. 8, the isolated glycans were profiled by MALDI-TOF-MS. Glycan composition and proposed structures are depicted above each MALDI-TOF-MS peak. Taken together with the results from SAX analysis, we find that there is no difference in either the type or distribution of glycans between isolated FSH and FSH in the context of drug product. The interpretation of the MALDI-TOF-mass spec-

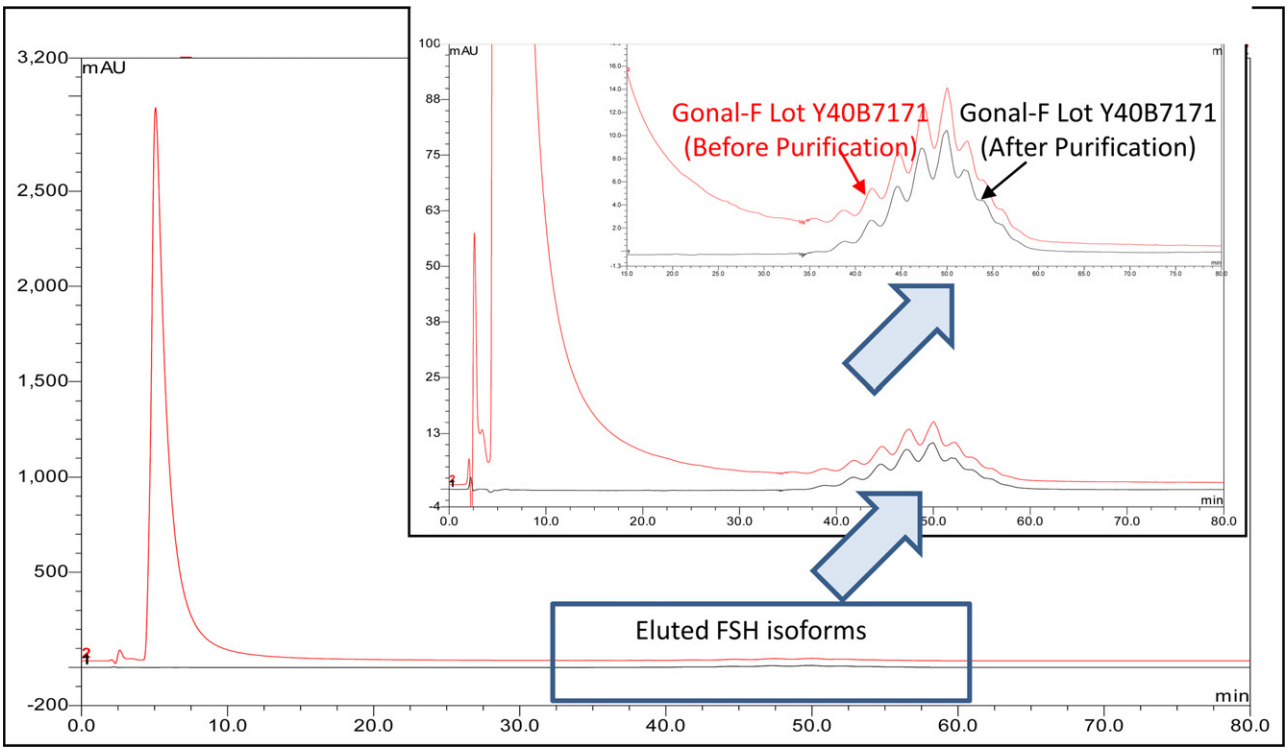


Fig. 6. Comparison of intact isoforms of the isolated FSH and Gonal-F® by IEX-HPLC. About 6 μg FSH was injected to the SAX-HPLC Column (Sepax, Proteomix SAX-NP1.7) using a Dionex ICS3000 system with two detectors: a UV–vis detector and a fluorescence detector. The flow rate was 0.1 mL/min and column was kept at ambient temperature. FSH isoforms were eluted using a gradient of buffer B (10–55% in 50 min). Buffers A: 20 mM Tris/HCl, pH 8.0 and B: 20 mM Tris/HCl + 450 mM NaCl, pH 8.25. Detection was completed using UV at 214, 230, 280 nm as well as fluorescence emission at 320 nm (excitation at 272 nm).

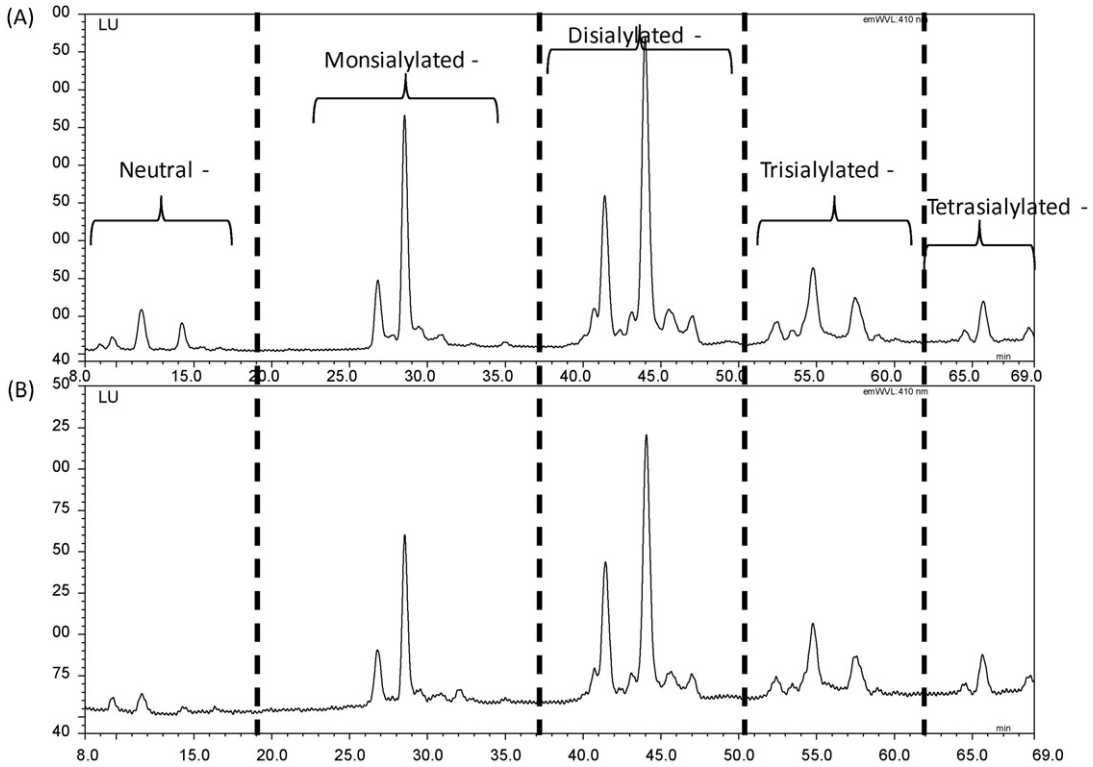


Fig. 7. Glycan profiling by SAX-HPLC online PCD (postcolumn derivatization) analysis. HPLC conditions were described in "Section 2" in detail. These two profiles were obtained from slightly different amounts of intact FSH protein. (A) Profiling of glycans obtained from the isolated FSH by workflow C (Gonal-F® Lot# Y40B7171). (B). Profiling of glycans obtained from the concentrated FSH by workflow D (Gonal-F® Lot# Y40B7171).

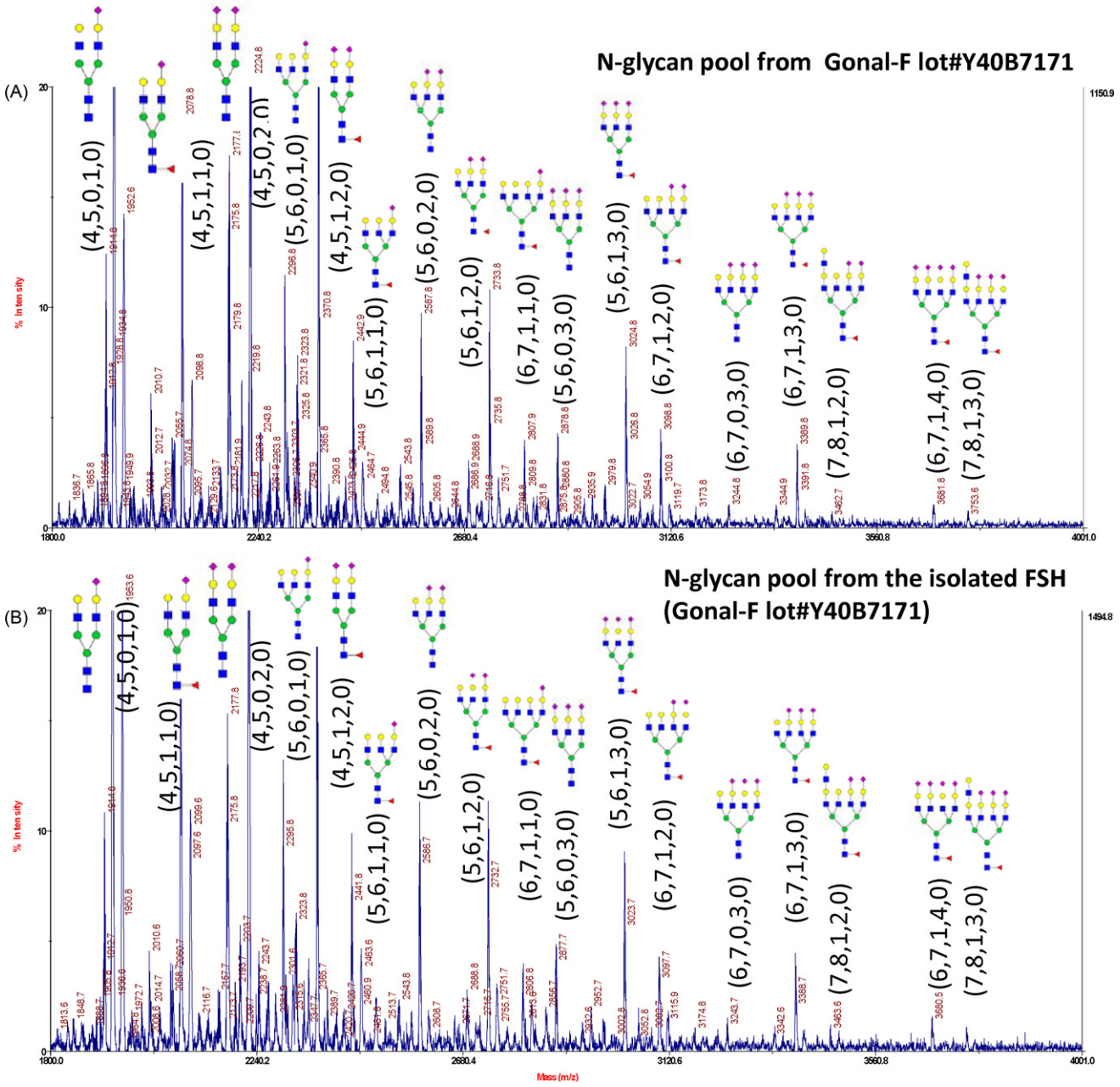


Fig. 8. Comparison of the intact glycan pool by MALDI-TOF-MS under linear negative mode. (A) Glycans were generated from concentrated Gonal-F (Lot# Y40B7171), which was through Centricon centrifugation only. (B) Glycans were generated directly from isolated FSH by workflow C (Lot# Y40B7171).

tra confirmed that the N-glycans are of complex type with di-, tri-, tetra-antennary species. Fucosylated and non-fucosylated species were detected. The charged glycans with different number of sialic acids are also detected. Although the MALDI-TOF-MS is not a quantitative technique, the distribution trend of glycans with charged numbers and antennarity is correlated with the charged glycan profiling by SAX-LC-PCD (Fig. 7). For instance, the monosialylated, disialylated glycans, i.e., biantennary species either fucosylated (4,5,1,1,0), (4,5,1,2,0), or non-fucosylated (4,5,0,1,0), (4,5,0,2,0) species are the most abundant species, as consistently observed by both SAX-HPLC-PCD (Fig. 7) and MALDI-MS (Fig. 8). There is no detected degraded forms lacking one or two hexose residues (galactose residues, loss of 162 Da), N-acetylhexosamine residues (GlcNAc, loss of 203), and/or sialic acid residue (NeuAc, loss of 291 Da). Furthermore, the glycan structures observed by this analysis are consistent with previous reports [25]. Therefore taken together, this analysis allows us to conclude that the glycans remain intact before and after isolation from Gonal-F®, confirming the development of a robust, quantitative method to isolate FSH from drug product.

4. Discussion

In this study we report an optimized isolation procedure for isolating recombinant FSH (FSH) from a commercial drug product in a manner that preserves the integrity and glycan properties of the starting material based on directed analytical methods intended to assess these attributes while also providing valuable information related to product recovery. Using FSH as our model protein, we, in fact, identified a robust, simple workflow (workflow C, Scheme 1). Recombinant glycoprotein derived from this procedure was demonstrated to be highly pure with reproducibly

demonstrated yields exceeding 90%. Notably, the approach of using immunoaffinity chromatography [26,27] was not evaluated in this study, for at least two reasons: (1) using a FSH-specific monoclonal antibody may result in antibody lot-to-lot variability; (2) typical conditions used to elute isolated products typically involves low pH, conditions which are known to affect fine structure, especially glycosylation. Gonal-F® 900 IU/1.5 mL (66 μg/1.5 mL) was chosen as a test case since it contains a large excess of excipients, such as salts, sucrose, methionine, m-cresol and polysorbate; the active ingredient FSH is usually present at less than 0.1% by mass. The difficulty of isolating heterogeneous active ingredient from biologics drug product and assessing structural integrity has been recognized and limited not only by the complexity of structural heterogeneity/diversity but also by the art of the state of analytical tools. As demonstrated by this work, we have surmounted many of these challenges and designed an approach that allows us to obtain a high recovery of the API isolated from the drug product. Also, we have put into place a set of analytical methods post-isolation that allow for a robust assessment of quality attributes of the API, which provides assurance that the isolation procedures used do not compromise the structural attributes of the sample.

In this context, a total of six methods were deployed to evaluate and assess the sameness of the isolated FSH in comparison to FSH present in Gonal-F®. SEC-HPLC and SDS-PAGE were utilized first to assess protein quality, purity, recovery and structural integrity. We find that upon using an optimized isolation procedure, FSH remains intact as a heterodimer, and is not dissociated into its subunits (Figs. 2–4); nor was there any indication of aggregation or oligomer formation. Furthermore, no significant impurities were introduced by the isolation procedure. As a result, the isolated FSH preserves its quality and higher-order structure to the same extent as it does in the context of drug product. IEF and high resolution IEX-HPLC methods (described in Sections 2.5 and 2.6) were deployed to assess the structural integrity of intact protein glycoforms. Ten bands (Fig. 5) were visualized on IEF gel, and at least 11 peaks (Fig. 6) were resolved by HR-IEX-HPLC. IEF separates intact glycoprotein isoforms based on the net charge of protein, while HR-IEX-HPLC separates intact glycoprotein isoforms based on the surface charge of protein. The consistency of intact protein glycoform distribution and profiles by IEF gel and IEX-HPLC confirmed again the quality and structural integrity were assured after the FSH isolation from its formulated drug product.

The results observed by multiple analyses (SEC-HPLC, SDS-PAGE, IEF and IEX-HPLC methods) provide substantive evidence that the quality, chemical properties, and structural integrity of the isolated FSH are preserved and unchanged by the isolation process from the original drug product presentation. Each of these methods has its own level of sensitivity and resolution. In certain respects, certain methods not only capture potential changes within the primary structure of FSH, including changes in glycosylation; a subset of these methods (e.g., SEC-HPLC and possibly IEX-HPLC) also possess the requisite sensitivity to detect global changes in product conformation. As such, these methods represent a reasonable and potentially more sensitive approach to monitor changes in product structure and integrity in comparison to the orthogonal use of an in vivo biological assay. Of particular note here, SEC-HPLC is routinely used by the manufacturer as a standard method to “fill-by-mass” (FbM) for the final product without the requirement of an in vivo bioassay [16].

The complex composition of glycans attached to glycoprotein drugs, i.e., FSH in this case, has drawn more attention due to its significant role in biological functions [14,25,28]. Furthermore, given the lability of the glycans (specifically sialic acids), it is important to ensure that the isolation procedures have not compromised the glycan structural attributes. Therefore, in the current work, after assessing the intact protein quality and structural integrity by the above four methods (SEC-HPLC, SDS-PAGE, IEF, IEX-HPLC) that were focused on assessing the overall glycoprotein attributes, two methods (SAX-HPLC-PCD) and MALDI-TOF-MS were deployed to specifically evaluate glycan charge, size and composition masses distribution. The SAX-PCD method was ion-exchange chromatography based that separates glycan species according to their charge and size, whereas the MALDI-TOF-MS method, typically utilized to profile glycan compositional masses, provides an orthogonal set of information. As demonstrated in Figs. 7 and 8, consistent data were observed across the methods. Combined together, these two methods sufficiently demonstrate glycan quality is preserved before and after isolation procedures.

In particular, for glycan profiling (Figs. 7 and 8) assessment, it should be pointed out that a direct glycan analysis of the formulated Gonal-F® (directly from the pen) was technically not feasible without first concentrating the formulated product prior to glycan release by enzymatic digestion with PNGase F. The API (FSH) concentration present in Gonal-F® is extremely low even when present in the highest dose (900 IU/1.5 mL pen = 44 μg/mL). For this reason, we view workflow D (direct concentration of the drug product) as a reasonable facsimile of the drug product inasmuch as it does not fractionate the API through any purification step or reconstitute it in an entirely different formulation. Our results indicate that FSH quality and structural integrity by workflow D were maintained during the straight forward step of product concentration. In practice, however, while workflow D preserved the product quality of the formulated drug product, the resultant yields made this a suboptimal method for recovering the API.

Taken all presented evidence together in Figs. 2–8, we conclude that the quality and structural integrity of the isolated FSH is maintained, and our work has demonstrated a feasible pathway to isolate API (i.e., complex heterogeneous biologics) from a commercially formulated drug product. While FSH presents a representative case in our current methodology, the overall approach presented here can be likely extended to other glycoprotein drugs, including monoclonal antibodies.

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References

[1] Y. Kaneko, F. Nimmerjahn, J.V. Ravetch, Analysis of glycoprotein-derived oligosaccharides in glycoproteins detected on two-dimensional gel by capillary electrophoresis using on-line concentration method, Science 313 (2006) 670–673.
[2] B.J. Scallon, S.H. Tam, S.G. McCarthy, A.N. Cai, T.S. Raju, Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality, Mol. Immunol. 44 (2007) 1524–1534.
[3] K. Bork, R. Horstkorte, W. Weidemann, Increasing the sialylation of therapeutic glycoproteins: the potential of the sialic acid biosynthetic pathway, J. Pharm. Sci. 98 (2009) 3499–3508.
[4] A. Varki, Sialic acids in human health and disease, Trends Mol. Med. 14 (2008) 351–360.
[5] T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, N. Hanai, K. Shitara, The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity, J. Biol. Chem. 278 (2003) 3466–3473.
[6] U. Abdel-Motal, S. Wang, S. Lu, K. Wigglesworth, U. Galili, Increased immunogenicity of human immunodeficiency virus gp120 engineered to express Galalpha1-3Galbeta1-4GlcNAc-R epitopes, J. Virol. 80 (2006) 6943–6951.
[7] F.J. Dor, Y.L. Tseng, J. Cheng, K. Moran, T.M. Sanderson, C.J. Lancos, A. Shimizu, K. Yamada, M. Awwad, D.H. Sachs, R.J. Hawley, H.J. Schuurman, D.K. Cooper, alpha1,3-Galactosyltransferase gene-knockout miniature swine produce natural cytotoxic anti-Gal antibodies, Transplantation 78 (2004) 15–20.
[8] R.B. Mandell, R. Flick, W.R. Staplin, L.D. Kaniewski, A.K. Carzoli, R.P. Manuszak, J. Wang, G.R. Rossi, N.N. Vahanian, C.J. Link, The alphaGal HyperAcute(R) Technol-

ogy: Enhancing Immunogenicity of Antiviral Vaccines by Exploiting the Natural alphaGal-Mediated Zoonotic Blockade, Zoonoses Public Health, 2009.
[9] S.T. Dhume, G.N. Saddic, K.R. Anumula, Monitoring glycosylation of therapeutic glycoproteins for consistency by HPLC using highly fluorescent anthranilic acid (AA) tag, Methods Mol. Biol. 446 (2008) 317–331.
[10] R.P. Oda, J.P. Landers, High-resolution glycoprotein analysis using capillary electrophoresis, Mol. Biotechnol. 5 (1996) 165–170.
[11] S. Kamoda, K. Kakehi, Capillary electrophoresis for the analysis of glycoprotein pharmaceuticals, Electrophoresis 27 (2006) 2495–2504.
[12] H. Geyer, R. Geyer, Strategies for analysis of glycoprotein glycosylation, Biochim. Biophys. Acta 1764 (2006) 1853–1869.
[13] V.M. Lo, M.G. Hahn, N. Hong, T. Ogawa, H. van Halbeek, Complete assignment of the 1H NMR spectra of phytoalexin elicitor-active oligoglucosides, Carbohydr. Res. 245 (1993) 333–345.
[14] Q.R. Fan, W.A. Hendrickson, Assembly and structural characterization of an authentic complex between human follicle stimulating hormone and a hormone-binding ectodomain of its receptor, Mol. Cell. Endocrinol. 260–262 (2007) 73–82.
[15] R. Bassett, D. Ceccarelli, C. Crisci, A.M. di Tria, M. Mancinelli, F. Martelli, D. Mendola, A. Pezzotti, Comparative analytical characterization of two commercial human follicle-stimulating hormones extracted from human urine, Curr. Med. Res. Opin. 21 (2005) 899–905.
[16] R.M. Bassett, R. Driebergen, Continued improvements in the quality and consistency of follitropin alfa, recombinant human FSH, Reprod. Biomed. Online 10 (2005) 169–177.
[17] C. Yding Andersen, L. Leonardsen, A. Ulloa-Aguirre, J. Barrios-De-Tomasi, L. Moore, A.G. Byskov, FSH-induced resumption of meiosis in mouse oocytes: effect of different isoforms, Mol. Hum. Reprod. 5 (1999) 726–731.
[18] E. Zambrano, T. Zarinan, A. Olivares, J. Barrios-de-Tomasi, A. Ulloa-Aguirre, Receptor binding activity and in vitro biological activity of the human FSH charge isoforms as disclosed by heterologous and homologous assay systems: implications for the structure-function relationship of the FSH variants, Endocrine 10 (1999) 113–121.
[19] A. Ulloa-Aguirre, C. Timossi, P. Damian-Matsumura, J.A. Dias, Role of glycosylation in function of follicle-stimulating hormone, Endocrine 11 (1999) 205–215.
[20] J.U. Baenziger, E.D. Green, Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin, Biochim. Biophys. Acta 947 (1988) 287–306.
[21] C.J. Gray, Molecular weight of human follicle stimulating hormone, Nature 216 (1967) 1112–1113.
[22] J.W. Mulders, M. Derksen, A. Swolfs, F. Maris, Prediction of the in vivo biological activity of human recombinant follicle stimulating hormone using quantitative isoelectric focusing, Biologicals 25 (1997) 269–281.
[23] P.L. Storring, R.E. Gaines Das, J.W. Mulders, M. Halder, Physicochemical methods for predicting the biological potency of recombinant follicle stimulating hormone: an international collaborative study of isoelectric focusing and capillary zone electrophoresis, Biologicals 30 (2002) 217–234.
[24] K.M. Fox, J.A. Dias, P. Van Roey, Three-dimensional structure of human follicle-stimulating hormone, Mol. Endocrinol. 15 (2001) 378–389.
[25] A. Gervais, Y.A. Hammel, S. Pelloux, P. Lepage, G. Baer, N. Carte, O. Sorokine, J.M. Strub, R. Koerner, E. Leize, A. Van Dorsselaer, Glycosylation of human recombinant gonadotrophins: characterization and batch-to-batch consistency, Glycobiology 13 (2003) 179–189.
[26] D.F. van de Wiel, P.A. van Rijn, R.H. Meloen, R.J. Moormann, High-level expression of biologically active recombinant bovine follicle stimulating hormone in a baculovirus system, J. Mol. Endocrinol. 20 (1998) 83–98.
[27] V. Borromeo, A. Amsterdam, A. Berrini, D. Gaggioli, A. Dantes, C. Secchi, Characterization of biologically active bovine pituitary FSH purified by immunoaffinity chromatography using a monoclonal antibody, Gen. Comp. Endocrinol. 139 (2004) 179–189.
[28] D.G. Mottershead, M.M. Pulkki, P. Muggalla, A. Pasternack, M. Tolonen, S. Myllymaa, O. Korchynskyi, Y. Nishi, T. Yanase, S. Lun, J.L. Juengel, M. Laitinen, O. Ritvos, Characterization of recombinant human growth differentiation factor-9 signaling in ovarian granulosa cells, Mol. Cell. Endocrinol. 283 (2008) 58–67.