Comparison of Follicle-Stimulating Hormone Glycosylation Microheterogeneity by Quantitative Negative Mode Nano-Electrospray Mass Spectrometry of Peptide-N-Glycanase-Released Oligosaccharides

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Abstract

Glycans from six highly purified hFSH preparations were released by peptide-N-glycanase digestion and analyzed by negative mode nano-ESI mass spectrometry before and after neuraminidase digestion. Pituitary glycan structures were mainly high-mannose, di-, tri-, and tetra-antennary, and their abundance largely paralleled that reported by other investigators using different approaches. For most of the FSH preparations, the differences in glycosylation appeared to be restricted to relative abundances of the major glycan families, as defined by their neutral core oligosaccharide structures. Qualitative differences between glycan populations were largely relegated to those species that were lowest in abundance. Significant qualitative differences were noted in two cases. Recombinant hGH-hFSH triantennary glycans appeared to have the third antenna exclusively on the mannose6-branch, in contrast to all pituitary and urinary hFSH triantennary glycans, in which this antenna was exclusively attached to the mannose3-branch. The hypo-glycosylated hFSH preparation isolated from purified hLH was decorated with high mannose glycans that accounted for over 40% of the total in this population. As this preparation was found to be consistently 20-fold more active in FSH receptor-binding assays, it appears that both macroheterogeneity and microheterogeneity in FSH preparations need to be taken into account.

Keywords: Follicle-stimulating hormone; Oligosaccharides; Mass spectrometry

Introduction

Human Follicle-Stimulating Hormone (FSH) is a heterodimeric member of the glycoprotein hormone subfamily of the cystine knot growth factor superfamily [1-3]. FSH consists of a common subunit that it shares with three other glycoprotein hormones, Luteinizing Hormone (LH), Chorionic Gonadotropin (CG), and Thyroid-Stimulating Hormone (TSH). A non-covalently associated FSHβ subunit confers hormone specificity. Both subunits possess two potential N-glycosylation sites. While the FSHα subunit exhibits only microheterogeneity in its glycosylation, the FSHβ subunit exhibits both macro- and micro-heterogeneity [4-8].

Macroheterogeneity in FSH glycosylation, which can be characterized by Western blotting, using FSHβ subunit-specific antibodies, has revealed four variants that differ in the number or position of their β-subunit N-glycans. FSHβ7 possesses both Asn1 and Asn7 N-glycans, FSHβ8 possesses only the Asn1 glycan, FSHβ9 possesses only the Asn7 glycan, while FSHβ10 lacks both N-glycans [9]. Since the FSHα subunit always possesses both Asn1 and Asn7 N-glycans, FSH glycoforms are identified by their FSHβ subunit variants, thus FSHβ7, FSHβ8, FSHβ9, and FSHβ10. The two most abundant human (h)FSH glycoforms are FSHβ7 and hFSHβ8, which can be detected in Western blots of pituitary extracts and urinary samples [10]. The former represents 80% of hFSH in pooled pituitary and post-menopausal urinary hFSH samples, while the latter was found to comprise 52-70% of hFSH samples isolated from pituitaries derived from autopsies of women in their twenties [8-10]. The potential physiological effect of hFSHβ7 stems from its greater apparent affinity for and more rapid association with the FSH receptor. In addition, it occupies more FSH binding sites than FSHβ8 [7]. Thus, a decrease in hFSHβ7 abundance is attended by a substantial loss of circulating FSH activity. FSH glycoforms also appear to vary in relative abundance in the circulation during the normal menstrual cycle [11].

Minor differences in electrophoretic mobilities of FSH subunit preparations probably reflect microheterogeneity in the structures of the associated N-glycan populations. For the most part, FSH microheterogeneity has been evaluated indirectly by separating differentially charged isoforms using electrophoretic techniques, followed by FSH radioimmunoassay detection and quantification [12]. Direct analysis of chromatofocusing-derived hFSH isoform glycosylation by glycopeptide mass spectrometry, did not confirm the underlying assumption that FSH isoforms possessed differing populations of N-glycans [6]. All isoforms possessed the same core set of glycans, differed by only a few glycans, and these did not exhibit the expected trend toward greater negative charge with decreasing isoelectric point [6]. Nevertheless, FSH isoform preparations have been reported to exhibit differences in biological activity associated with follicle development and granulosa cell gene expression [13-16].

FSH oligosaccharide populations are challenging to characterize because of their heterogeneity [17-19]. Advances in mass spectrometric techniques provide a means to make detailed comparisons of the glycan populations in FSH preparations using 10-30 µg samples [20-23] that are, at most, 3% of the milligram quantities of hFSH required for oligosaccharide analysis in earlier reports cited above. In this paper, we compare the N-glycan populations of oligosaccharides released from samples of pituitary, urinary, and recombinant hFSH preparations, as well as three hFSH glycoform preparations.
Methods

Hormone preparations

The highly purified human pituitary hFSH\(^{21,22}\) preparation, AF7298A, was obtained from the National Hormone and Pituitary Program. It is comprised of 70% FSH\(^{23}\) and 30% FSH\(^{24}\) and exhibits a biological activity of 8560 IU/mg. Highly purified urinary hFSH was obtained from ProSpec (East Brunswick, NJ). The biological activity of this preparation is 10,000 IU/mg. Recombinant hFSH was purified from transformed GH\(_4\) cells that express both hFSH\(^{25}\) and hFSH\(^{26}\) [24] using immunoadsorption chromatography followed by gel filtration [10]. Human pituitary hFSH glycoforms hFSH\(^{4}\) and hFSH\(^{5}\) were prepared from crude human pituitary glycoprotein extract [25], as outlined below, and hFSH\(^{21,22}\) from purified hLH preparations [7].

Pituitary hFSH glycoform purification

Pituitary FSH glycoform purification is summarized in Figure 1. A human pituitary glycoprotein preparation [25] was fractionated by Sephadex G-100 chromatography [26] followed by phenyl-Sepharose chromatography [27], FSH in fraction G-100B (Figure 1A) was recovered in a single fraction following phenyl-Sepharose chromatography (Figure 1C). This fraction was immunopurified with anti-FSH monoclonal antibody 4882 provided by SPD Development Co., Ltd., Bedford, UK (Figure 1E). As this antibody recognizes an epitope largely on the α subunit, it can also capture LH and TSH [24]. FSH in fraction G-100C was subjected to phenyl-Sepharose chromatography (Figure 1D) and the FSH-containing fractions were further separated by Sephacryl S-100 chromatography (Figure 1F). The foregoing produced three purified FSH preparations that were separated into FSH\(^{1}\) and FSH\(^{2}\) glycoform preparations by high-resolution Superdex 75 gel filtration chromatography (Figure 1G-11). Glycoforms were pooled on the basis of Western blot analysis of individual column fraction samples [24]. The glycoforms were characterized by FSH radioimmunoassay, using National Hormone and Pituitary Program polyclonal antibody AFP-005 at 1:10000 dilution [4], FSH radioligand assay, SDS-PAGE, and Western blot [7].

FSH deglycosylation

FSH samples consisting of 13-30 μg purified hormone were reduced and carboxymethylated [26]. The reaction mixtures were desalted and equilibrated with 0.2 M ammonium bicarbonate, pH 8.5, in Amicon (EMD Millipore, Billerica, MA) Ultra-4 (10,000 MW cutoff) ultrafiltration cartridges. Each FSH sample was then incubated overnight at 37°C with 2.5 μM pepti-N-glycanase F (PNGaseF) obtained from ProZyme (Hayward, CA). PNGaseF-released oligosaccharides were separated from deglycosylated FSH subunits by reverse-phase HPLC and recovered by evaporation in a Thermo Fisher Scientific (Waltham, MS) Savant™ SpeedVac as previously described [7].

Mass Spectrometry

Glycan sample preparation, purification using Nafion membranes, and nano-electrospray mass spectrometry were described in a recent publication [8]. The analytical procedures and analyses have been described previously [20-23]. The instrument employed was a Waters quadrupole-time-of-flight (Q TOF) Ultima Global instrument in negative ion mode. Glycan abundance was calculated on the basis of oligosaccharide ion intensity. Data are organized in a table based on the m/z value for the neutral core ion and relative abundance values for all charge variants resulting from sialic acid or sulfate residues are tabulated in columns to the right of the corresponding neutral core oligosaccharide composition.

Results

Mass spectrometry of intact glycans

Intact oligosaccharide mass spectra for six hFSH glycan preparations are shown in Figure 2. In each spectrum, glycan diagrams illustrate likely structures for individual ions using the Oxford Glycobiology Institute system [28,29]. Using a scale that permits fitting the mass spectra to a single page, some ion peaks separated by only 1-2 mass units appear as single peaks. Accordingly, quantitative values for glycan abundance listed in Table 1 are used for determining differences in glycan abundance. Quantitative values are affected by two major factors:

1. Glycans ionize in different states ([M-H]\(^{-}\) and [M-2H]\(^{2-}\) for acidic glycans and [M+H\(_{2}\)PO\(_{4}\)]\(^{+}\) for the neutral compounds) and the relative ionization efficiency for production of each ion, which is unknown. Thus, although the reported percentages do not accurately reflect the absolute amount of the individual glycans, the results can still be used to compare the relative amounts of the glycans between samples.

2. Some ions contain isomeric and isobaric structures and it is sometimes not possible to determine the contribution of each to the ion current. Percentages are cited to two decimal places to produce a concentration range of five orders of magnitude and do not signify an accuracy to this level.

Over 100 pituitary hFSH-derived glycans were found in 24-56 families, based on shared neutral core glycan structure (Figure 2A and Table 1). Some ambiguity remained, as the 24 neutral glycan ions represented 56 glycan structures, because more than one isobaric structure was consistent with the same m/z value. Moreover, ms/ ms experiments sometimes confirmed the presence of more than one isobaric glycan. Seventeen pituitary hFSH glycans with greater than 2% relative abundance (range 2.05-9.25%), accounted for over 77% of the total glycan population. Six of these were bi-antennary, nine were tri-antennary, and two were tetra-antennary. Ten glycans were quantitatively capped with sialic acid, leaving no free galactosyl residues exposed. The remaining glycans were missing one or more sialic acid residues.

Urinary hFSH oligosaccharides are shown in Figure 2B. Five of the fifteen >2% abundance glycan structures were bi-antennary, seven were tri-antennary and three were tetra-antennary glycan. Eight of these glycans were completely capped with two sialic acid residues. These accounted for just over 72% of the urinary FSH glycans.

The thirteen >2% abundance GH\(_{4}\)-hFSH glycans totaled a little over 68% of this oligosaccharide population (Figure 2C). Seven were bi-antennary, varying by core fucosylation, or substitution of GalNAc for Gal in the Man3-branch. Six glycans were tri-antennary, with the third antenna on the Man6-branch. All tri-antennary glycans in this sample exhibited the same branching pattern, despite the presence of both GlcNAc transferase IV isozymes in GH\(_{4}\) cells. These transferases have the potential to attach GlcNAc to the Man3-branch, but did not appear to, at least with the most abundant glycans in this preparation [24].

There were fourteen glycans derived from fully-glycosylated hFSH\(^{1}\) that exceeded 2% abundance (Figure 2D). Nine of these glycans were bi-antennary, four were tri-antennary, one was tetra-antennary, and collectively they accounted for over 47% of the glycan population. Three bi-antennary glycans were core-fucosylated and one was terminated with two sulfate residues, a modification associated with LH, rather than FSH glycans.
Fourteen glycan variants isolated from hFSH\textsuperscript{30} exceeded 2% abundance (Figure 2E). Nine were bi-antennary, five of these were disialylated, one was sulfated as well as sialylated, while two were disulfated. Five possessed core fucose, while four lacked this monosaccharide residue. There were five tri-antennary glycans and one was fully sialylated, while two were core fucosylated. There were no tetra-antennary glycans that were greater than 2% relative abundance.

A striking qualitative difference was observed in the glycosylation of hFSH\textsuperscript{30} isolated from hLH preparations [7]. All but two of the eleven most abundant glycans were oligomannose-type glycans possessing 8, 7, 6, 5, or 3 manose residues (Figure 2F). These are biosynthetic intermediates, one processed in the rough endoplasmic reticulum (GlcNAc\textsubscript{2}Man\textsubscript{5}) and the others processed in the Golgi apparatus prior to complex branch addition [30].

**Desialylated, neutral glycan ion analysis**

The number of neutral glycan ions in the intact glycan spectra ranged from only 2 in highly purified pituitary hFSH to as many as 40 in the hLH-derived glycoform preparation hFSH\textsuperscript{30} (Table 2). The number of neutral glycan cores inferred from detection of one or more corresponding acidic glycan ions ranged from 25 to 50. For all three hFSH preparations and one of the FSH glycoform preparations, hFSH\textsuperscript{14}, 80-96% of the neutral glycan ions were detected in the desialylated glycan spectra. The missing glycans were either minor species or major sulfated glycan variants. Of the 14 major neutral
glycan ions derived from pituitary hFSH that are shown in Figure 3A, 11 were also major ions in the urinary hFSH glycan spectrum (Figure 3B), and only 8 were major ions in the recombinant GH₃-hFSH glycan spectrum (Figure 3C). Termination with sulfate resulted in the absence of a major inferred neutral core glycan ion from the desialylated hFSH glycan spectrum in Figure 3D, as neuraminidase cannot remove sulfate residues. Otherwise, all 14 major glycan ions indicated in the pituitary hFSH desialylated glycan spectrum were found in the desialylated hFSH glycan spectrum. For glycoform preparations, hFSH and hFSH₂/₁₈, 3 or 4 additional neutral glycan ions were observed after desialylation than were inferred from the acidic ions in the intact oligosaccharide spectra (Figure 3E and 3F). The hFSH desialylated glycan spectrum possessed 11 of the major glycan ions associated with pituitary hFSH, while the hypo-glycosylated hFSH₂/₁₈ glycan population only possessed 6 and five were m/z 2102.7 or greater.

All core glycan structures, both inferred and directly observed in either intact or desialylated glycan spectra, are shown in Figure 4. These are organized by increasing m/z value and their presence in each hFSH preparation is indicated as follows: P = pituitary, U = urinary, G = recombinant hFSH produced by GH₃ cells, 4 = pituitary hFSH₄, 1 = pituitary hFSH₁, and H = hypo-glycosylated pituitary hFSH₂/₁₈.

**Figure 2.** Negative mode nano-ESI mass spectrometry of oligosaccharides released from reduced, carboxymethylated hFSH samples by peptide-N-glycanase F digestion. Structures of the more abundant glycans are shown for each preparation along with the monoisotopic m/z value. Because the ¹³C ion is often more abundant than the former, peaks may be labeled with m/z values that do not match values found in Table 1. A. Glycans from pituitary hFSH preparation AP7296A. B. Glycans from purified urinary hFSH. C. Glycans from recombinant GH₃-hFSH. D. Glycans from hFSH glycoform preparation. E. Glycans from hFSH glycoform preparation. F. Glycans from hypo-glycosylated hFSH₂/₁₈ preparation isolated from highly purified LH preparations. Note the expanded scale indicated by the dashed line.

**Figure 3.** Glycan structures (Figure 3B) glycosylation of these two preparations compares the outcomes of those studies with results of the present study. All agree on the absence of high mannose glycans, high abundance of complex glycans that are largely capped with sialic acid, and in one study, with the degree of sialylation. While Green and Baenziger did not find any Man₆-linked triantennary glycans, Renwick et al. reported that about a third of hFSH triantennary glycans possessed this structural feature. Our study was consistent with the former and differed from that of the latter, as we did not find Man₆-linked glycans in the pituitary hFSH glycan sample nor in any other glycan sample except recombinant GH₃-hFSH glycans (see below).

**Classic glycan class comparison with pituitary hFSH glycans**

Two other research groups have analyzed pituitary hFSH oligosaccharides using lectin affinity chromatography, exoglycosidase digestion, and other chromatographic methods [17-19]. Figure 5A compares the outcomes of those studies with results of the present study. All agree on the absence of high mannose glycans, high abundance of complex glycans that are largely capped with sialic acid, and in one study, with the degree of sialylation. While Green and Baenziger did not find any Man₆-linked triantennary glycans, Renwick et al. reported that about a third of hFSH triantennary glycans possessed this structural feature. Our study was consistent with the former and differed from that of the latter, as we did not find Man₆-linked glycans in the pituitary hFSH glycan sample nor in any other glycan sample except recombinant GH₃-hFSH glycans (see below).

Based on conventional comparisons of urinary and pituitary hFSH glycan structures (Figure 5B) glycosylation of these two preparations was very similar. The major differences resulted from fewer fucosylated and bisected glycans associated with urinary than with pituitary hFSH. Comparing the glycan structure abundance with pituitary hFSH, GH₃-hFSH possessed more bi-antennary and fewer tri-antennary glycans (Figure 5C). Sialylated glycan abundance decreased, while neutral
## Table 1. Masses and abundances of N-glycans found in pituitary, urinary and recombinant hFSH preparations compared with pituitary hFSH glycoform preparations.

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<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
<td>91,92,93</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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**Table 2: Summary of glycan ions detected by nano-electrospray mass spectrometry of six hFSH preparations.**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Pituitary hFSH</th>
<th>Urinary hFSH</th>
<th>GH, hFSH</th>
<th>hFSH&lt;sup&gt;43&lt;/sup&gt;</th>
<th>hFSH&lt;sup&gt;118&lt;/sup&gt;</th>
<th>hFSH&lt;sup&gt;148&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amt. anal. (µg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycan Ions</td>
<td>19.1</td>
<td>30.2</td>
<td>22.5</td>
<td>21.4</td>
<td>26.5</td>
<td>13.1</td>
</tr>
<tr>
<td>Neutral Cores Inferred</td>
<td>280</td>
<td>11/67</td>
<td>1133</td>
<td>30/109</td>
<td>25/108</td>
<td>40/43</td>
</tr>
<tr>
<td>Asialo-Neutral Cores</td>
<td>34</td>
<td>25</td>
<td>25</td>
<td>49</td>
<td>50</td>
<td>39</td>
</tr>
<tr>
<td>Detected</td>
<td>27</td>
<td>24</td>
<td>20</td>
<td>42</td>
<td>53</td>
<td>43</td>
</tr>
</tbody>
</table>

- a) Calculated mass of \([\text{M+H}]^+\) ion. Experimental masses were within ± 0.1 mass units.
- b) Compounds are listed in order of increasing mass of the neutral glycans. The masses for acidic glycans found in each sample are listed after the neutral structures. Compounds not found as neutral structures are not listed.
- c) H = hexose, N = GlcNAc, F = fucose.
- d) Source of FSH preparations: P = pituitary, U = urinary, G = recombinant GH cell medium, F<sup>43</sup> = pituitary hFSH<sup>43</sup>, F<sup>118</sup> = pituitary hFSH<sup>118</sup>, and F<sup>148</sup> = LH-associated hFSH<sup>148</sup>.
- e) The numbers represent the % of total identified glycans. T = trace.
- f) Structures of the neutral glycans as shown in Figure 3.
- g) Numbers in bold represent glycans whose structures have been confirmed by CID.
- h) 1 = [M+H]<sup>+</sup>, 2 = [M+H]<sup>2+</sup>, 3 = [M+H]<sup>3+</sup>.

Comparisons with hFSH glycans on the basis of common neutral core structure

The relative abundance of each glycan family (based on the percentage of glycans sharing a common core structure) for the pituitary hFSH preparation is shown in Figure 6A. Of the eleven >4% abundance glycan families, five were biantennary, five were triantennary and one was tetraantennary. Together, they comprised almost 85% of those found in pituitary hFSH. Comparing the pituitary and urinary glycan families possessing a common neutral glycan core (Figure 6B), both populations displayed similar overall patterns, as the most abundant glycan family in pituitary hFSH was also most abundant in urinary hFSH. The nine most abundant urinary hFSH glycan families consisted of four bi-antennary, four tri-antennary, and three tetra-antennary glycans. These accounted for over 83% of the urinary glycan population. Striking differences in abundance were noted for two glycan families represented by ions m/z 2467.8 and 2613.9, which were more abundant in urinary hFSH and m/z 2435.8, which was more abundant in pituitary
hFSH. The overall similarity between pituitary and urinary FSH glycan populations is consistent with the hypothesis that pituitary, urinary, and serum FSH glycosylation is the same. Thus, in contrast to unique FSH isoform patterns for FSH present in these three compartments [12], suggesting marked differences in FSH glycosylation, pituitary and urinary glycosylation is very similar. Since the only way for FSH to get from the pituitary to the urine is via the bloodstream, more readily available urinary FSH can be used to infer the glycosylation status of circulating FSH.

The eight more abundant glycan families that exhibited >4% relative abundance in recombinant GH-hFSH, accounted for just over 78% of the glycan population (Figure 6C). Six families were bi-antennary and two were tri-antennary. When comparing GH-hFSH with pituitary hFSH glycan families, the most obvious trend was smaller glycans indicated by $m/z$ 1356-2087 ions that were more abundant in the former than in the latter. Larger glycans were almost always more abundant in pituitary hFSH, the exception being the glycan family indicated by neutral glycan $m/z$ 2248.8.

There were nine FSH$^{14}$ glycan families that exceeded 4% abundance in the fully-glycosylated glycoform preparation, hFSH$^{14}$, and these represented over 59% of the total population. Five families were bi-antennary, three were tri-antennary, and one was tetra-antennary. Several glycan core structures, represented by $m/z$ 1413.5, 1819.6, 1924.6, 1965.7, 2127.7, and 2289.8, were much more abundant than their counterparts in pituitary hFSH glycans Figure 6D). All of these represented glycans that possessed at least one GalNAc residue and several charged variants of these core structures were sulfated.

The ten most abundant glycan families accounted for just over 64% of the hFSH$^{14}$ glycoform glycans. Three of the more abundant core structures possessed 1-2 GalNAc residues (Figure 6E) and included several charged variants of these core structures were sulfated. For the other hypo-glycosylated hFSH preparation, as the most abundant oligomannose glycans included no charge variants, they also represented the most abundant glycan families, comprising 63% of the glycan population (Figure 6F). As this hFSH glycoform preparation consistently exhibited a 20-fold greater apparent affinity for the rat and human FSH receptors [7], glycan structure, as well as the absence of one FSHβ glycan, may play an important role in defining FSH receptor-binding properties.
Figure 4: Diagrams showing the structures of the neutral core glycans associated with the 6 hFSH and hFSH glycoforms analyzed in this study. The m/z values for the ion associated with the neutral glycan and composition are shown with each structure. In several cases, more than one structure was possible and either could not be eliminated by MS/MS analysis or else all were shown to be present by MS/MS characterization. FSH preparations in which at least 1 glycan ion was detected that shared the same core glycan structure are indicated as follows: P = pituitary FSH, U = urinary FSH, G = recombinant GH, hFSH, 4 = hFSHβ, 1 = FSHβ, and H = hFSHβ4H from hLH preparations.
Characterization of FSH glycoforms by gel filtration

Samples of hFSH glycoform preparations isolated from the triple Superdex chromatograms shown above in Figure 1G were characterized to select the best candidates for oligosaccharide analysis. When chromatographed on a single Superdex 75 column, aliquots of three adjacent hFSH glycoform fractions produced single peaks, with progressively longer retention times, (Figure 7). Western blot analysis confirmed that fraction A possessed largely hFSH24 and fraction C possessed largely hFSH21 (Figure 7A, inset). The more highly purified sample of fully-glycosylated hFSH24 (lane 2) and that of hypo-glycosylated hFSH21 (lane 5) were deglycosylated and the released oligosaccharides analyzed by mass spectrometry, as described above.

Comparison of FSH24 and FSH21 glycoform glycan populations

Comparison of the FSH24 and FSH21 glycoform glycan populations with each other revealed a high degree of similarity in glycan structure type (Figure 8A). Both populations possessed largely complex, sialylated glycans, with few neutral and very few high mannose glycans. FSH24 possessed more tetra-antennary glycans, consistent with the larger size of this glycoform, yet also possessed more neutral glycans than FSH21. As the relative abundances of both these large and small glycan classes were in the 5-10% range, this did not seem to affect FSH size. Comparison of the glycan populations by glycan core revealed that most of the 56 glycans were common to both glycoforms. The major differences appeared to be in the patterns of relative abundance, as
The strikingly different pattern of hFSH glycan families accounted for the bulk of the oligosaccharides attached to family members [8]. A more satisfactory pattern was obtained by figure that required zooming in to evaluate relative abundance of unsatisfactory because the information related to a single glycan family was not characterized. To provide a global picture of glycan abundance across with the other five hFSH glycan preparations made extensive use of side-by-side comparisons of purified pituitary hFSH glycan families. E. Comparison of P-hFSH with pituitary hFSH glycan families. F. Comparison of hypo-glycosylated hFSH$^{21/18}$ with pituitary hFSH glycan families. The solid bars represent the hFSH glycan families while the gray bars represent the other hFSH preparation glycan families.

31 glycan families were more abundant in FSH$^3$ than in FSH$^4$, while only 15 were more abundant in FSH$^4$ than in FSH$^3$ (Figure 8B). Both hFSH glycoforms differed primarily in the relative abundance of each glycan family, rather than qualitative differences, such as those noticed in hypo-glycosylated hFSH$^{21/18}$. 

**Relative abundance of glycan families in each FSH preparation**

Side-by-side comparisons of purified pituitary hFSH glycan families with the other five hFSH glycan preparations made extensive use of bar graphs. To provide a global picture of glycan abundance across all six FSH preparations, we employed heat maps to highlight the most abundant glycans. For individual glycan variants, this was highly unsatisfactory because the information related to a single glycan family was distributed across the map by singly, doubly, and triply charged ions. Even grouping these by neutral core family produced an unsatisfactory figure that required zooming in to evaluate relative abundance of family members [8]. A more satisfactory pattern was obtained by comparing core glycan family abundance, as shown in Figure 9. Seven glycan families accounted for the bulk of the oligosaccharides attached to five of six hFSH preparations. These are represented by bi-antennary glycan ions, $m/z$ 1737.6, 1778.6, and 1883.7, tri-antennary glycan ions $m/z$ 2102.7, 2249.8, and 2305.8, and a single tetra-antennary glycan ion $m/z$ 2613.9, found in high abundance only in urinary hFSH glycan. The strikingly different pattern of hFSH$^{21/18}$ glycosylation stands out as five high mannose-type glycans, $m/z$ 1007.3, 1331.4, 1493.5, 1655.5, and 1817.6.

**Challenges quantifying FSH in glycoform preparations**

FSH concentrations in each of the glycoform fractions derived from triple Superdex 75 chromatography (Figures 1H and 11) were determined by ultra-performance, size-exclusion chromatography (Figures 10 A-D). The hFSH$^3$ preparation was shown to have a small amount of high molecular weight contaminant that was confirmed by SDS-PAGE (Figure 10E) and, therefore, excluded from the FSH concentration calculation. Coomassie Blue-stained FSH bands exhibited 63-71% the intensity as the pituitary hFSH preparation used as reference marker. Both hFSH$^3$ preparations resulted in single peaks characterized by increased retention times consistent with lower molecular weights, due to the absence of one N-glycan. Based on common $\alpha$-subunit band intensities, the hFSH glycoform samples possessed 20-70% greater $\alpha$-subunit immunoactivity than the hFSH reference preparation (Figure 10F). However, when 1 µg samples of the same preparations were subjected to Western blotting (Figure 10G), hFSH$^{21/18}$ was found to possess only 53% of the expected immunoactivity, hFSH$^3$ from fraction B only 36%, while hFSH$^4$ from fraction C possessed only 12%. An LH$\beta$ subunit-specific Western blot provided a potential rationale for the greater than anticipated $\alpha$-subunit immunoactivity, as fractions with lower FSH$^3$ immunoactivity possessed greater LH$\beta$ immunoactivity (Figure 10H). LH$\beta$ immunoactivity was undetectable in fraction B hFSH$^{21}$ and only 4% was associated with fraction C hFSH$^{21}$, which were used for glycoform glycan analysis described above. Significantly more LH was associated with fraction D FSH$^{21}$ glycoform preparation, which was not characterized.
**Figure 7:** FSH characterization by size exclusion chromatography and Western blot analysis. Samples of hFSH24, hFSH24/21, and hFSH21 were applied to a 1 x 30 cm Superdex 75 column and the chromatogram developed with 20% acetonitrile in 0.2 M ammonium bicarbonate at a flow rate of 0.4 ml/min. Retention times (min) for each FSH peak are indicated. A. Fully-glycosylated hFSH24 from Superdex 75 fraction A (Fig. 1G). Inset: Lane 1, fraction A; lane 2, fraction A'; lane 3, fraction B; lane 4, fraction B'; lane 5, fraction C; lane 6, fraction C'; lane 7, purified pituitary hFSH24/21. B. Mixture of both hFSH glycoforms. C. Hypo-glycosylated hFSH21 from fraction C, Fig. G.
Figure 8: Comparison of glycan populations derived from FSH glycoform preparations, FSH24 and FSH21. A. Comparison by glycan structure type. B. Relative abundance of all variants of glycan core families.
Figure 9: Heat map comparing relative abundances of glycan core families for all six hFSH preparations. Glycan ion m/z values are listed in increasing order and relative abundance in percentage reported for each family. Gray scale used to highlight the abundance data. Structure diagrams shown for the more abundant families.
N-glycan populations were largely variations in the relative abundance of virtually identical glycan populations. The apparent loss of core fucosylated and bisected glycans and the enrichment for partially sialylated glycans in urinary hFSH suggests it would be informative to isolate urinary FSH from several individuals and pool hFSH from several pituitary glands. Isolation of hFSH from both sources using the same procedure followed by glycan evaluation by mass spectrometry would permit identification of glycan structures favoring clearance in the liver [31-33] from those favoring kidney clearance [34]. Application of the same purification procedure would be necessary because differences in glycan abundance observed in the present study could have been due to enrichment by FSH purification procedures used by the National Hormone and Pituitary Program and ProSpec.

As all but one of the most abundant recombinant GH-hFSH glycans were the same as the most abundant glycans derived from pituitary and urinary hFSH preparations, we have expressed this recombinant hormone on a large scale and have been able to purify hFSH\textsuperscript{118} and hypo-glycosylated hFSH\textsuperscript{118} glycoform preparations for in vitro and in vivo studies [9, 24]. We did note that in GH-hFSH tri-antennary glycans the third antenna was located on the mannos3-branch rather than on the mannos3-branch, where the third antenna was found in both pituitary and urinary hFSH tri-antennary glycans. Antenna fucosylation was also more abundant in GH-hFSH glycans, although it was observed in pituitary FSH glycoform preparations, particularly hFSH\textsuperscript{118}.

The hFSH\textsuperscript{118} isolated from hLH preparations, represented the 1% FSH activity associated with most pituitary LH preparations [7], displayed striking differences in the associated glycan population, which possessed only 30% complex glycans, while over 40% were high.
mannose. As this preparation was consistently 20-fold more active than hFSH 21, the glycan population may also contribute to altered FSH activity.

When hFSH 21 was first identified, despite the glycoprotein appearance of the FSHβ band in Western blots, MALDI-TOF-MS revealed the presence of non-glycosylated FSHβ, which was confirmed by the detection of PhNCS-Asn following Edman degradation [4]. This observation led to the idea of all-or-none N-glycosylation of FSHβ, resulting in two major glycoforms. The activities of these were assumed to be dictated by the presence or absence of both FSHβ N-glycans. However, characterization of the first hypo-glycosylated hFSH glycoform preparation, which possessed an additional FSHβ band in Western blots, revealed the presence of carbohydrate in both FSHβ 21 and FSHβ 23 bands [7]. The non-glycosylated FSHβ 23 band was never seen in intact FSHβ Western blots, but was readily detected following peptide-N-glycanase digestion of denatured FSHβ samples. In FSH receptor-binding assays, the ID 50 values for hFSH 21 preparations are always less than those of hFSH 23, but the differences vary from 2-fold to 20-fold [7, 24]. Part of this seems to be the result of the challenge of accurately quantifying FSH in glycoform preparations, as shown above. Part of it could be due to the structures of the N-glycans influencing FSH receptor-binding and cellular activation, as hypo-glycosylated hFSH 23-24 isolated from hLH preparations was consistently 20-fold more active than hFSH 21 in receptor-binding assays, as mentioned above. The radically different glycan population associated with this preparation may contribute to the higher receptor-binding activity, as well as the absence of one of the FSHβ N-glycans. Indeed, KGN granulosa tumor cells responded differentially to hFSH preparations that failed to bind concanavalin A, presumably decorated with tetra-antennary, tri-antennary, and bisected bi-antennary glycans, as compared with those that bound tightly, and presumably are enriched for hybrid-type glycans [16]. Accordingly, it is necessary to take into account both macroheterogeneity, which can be assessed by Western blots, and microheterogeneity, which can be assessed by mass spectrometry techniques, such as those employed in this study.

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References


