Published in final edited form as:

J Glycomics Lipidomics. 2015 ; 5(1): . doi:10.4172/2153-0637.1000129.

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

George R. Bousfield¹, Vladimir Y. Butnev¹, William K. White¹, Aaron Smalter Hall², and David J. Harvey³

¹Department of Biological Sciences, Wichita State University, Wichita, KS 67260

²Molecular Graphics and Modeling Laboratory, University of Kansas, Lawrence, KS 66045

³Department of Biochemistry, University of Oxford, Oxford, OX1 3QU, UK

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 GH₃-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 than hFSH²⁴ 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 α subunit that it shares with three other glycoprotein hormones, luteinizing hormone (LH), chorionic gonadotropin (CG), and thyroid-stimulating hormone (TSH). A non-covalently associated FSH β subunit confers hormone specificity. Both subunits possess

two potential N-glycosylation sites. While the FSH α subunit exhibits only microheterogeneity in its glycosylation, the FSH β subunit exhibits both macro- and microheterogeneity [4-8].

Macroheterogeneity in FSH glycosylation, which can be characterized by Western blotting using FSH subunit-specific antibodies, has revealed four variants that differ in the number or position of their β -subunit N-glycans. FSH β^{24} possesses both Asn⁷ and Asn²⁴ N-glycans, FSH β^{21} possesses only the Asn⁷ glycan, FSH β^{18} possesses only the Asn²⁴ glycan, while FSH β^{15} lacks both N-glycans [9]. Since the FSH α subunit always possesses both Asn⁵² and Asn⁷⁸ N-glycans, FSH glycoforms are identified by their FSH β subunit variants, thus FSH²⁴, FSH²¹, FSH¹⁸, and FSH¹⁵. The two most abundant human (h)FSH glycoforms are hFSH²⁴ and hFSH²¹, 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 hFSH²¹ stems from its greater apparent affinity for and more rapid association with the FSH receptor. In addition, it occupies more FSH binding sites than FSH²⁴ [7]. Thus, a decrease in hFSH²¹ 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 μ g 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^{24/21} preparation, AFP7298A, was obtained from the National Hormone and Pituitary Program. It is comprised of 70% FSH²⁴ and 30% FSH²¹ 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 GH₃ cells that express both hFSH²⁴ and hFSH²¹ [24] using immunoaffinity chromatography followed by gel filtration [10]. Human pituitary hFSH glycoforms hFSH²⁴ and hFSH²¹ were prepared from crude human pituitary glycoprotein extract [25], as outlined below, and hFSH^{21/18} from purified hLH preparations [7].

Pituitary hFSH glycoform purification

Pituitary FSH glycoform purification is summarized in Fig. 1. A human pituitary glycoprotein preparation [25] was fractionated by Sephadex G-100 chromatography [26] and FSH immunoactivity was found in three fractions (Fig. 1A). Each FSH-positive G-100 fraction was subjected to phenyl-Sepharose chromatography [27]. FSH in fraction G-100A was distributed across five phenyl-Sepharose column fractions (Fig. 1B). We were unable to purify these fractions further. FSH in fraction G-100B was recovered in a single phenyl-Sepharose fraction (Fig. 1C). FSH in fraction G-100C was recovered from 3 adjacent phenyl-Sepharose fractions (Fig. 1D). FSH from the chromatogram in Fig. 1C was immunopurified with anti-FSH monoclonal antibody 4882 provided by SPD Development Co., Ltd., Bedford, UK (Fig. 1E). As this antibody recognizes an epitope largely on the α subunit, it also captures LH and TSH [24]. The FSH-containing, phenyl-Sepharose fractions from the chromatogram in Fig. 1D were further separated by Sephacryl S-100 chromatography (Fig. 1F). Immunopurified FSH from Fig. 1E was separated into FSH²⁴ and FSH²¹ fractions by gel filtration using three Superdex 75 columns connected in series (Fig. 1G), as previously described [24]. Triple Superdex 75 chromatography was also applied to two FSH-positive S-100 fractions, S-100B and S-100C (Figs. 1H & 1I, respectively). Glycoforms in each Superdex 75 chromatogram were pooled on the basis of Western blot analysis of individual column fractions [24]. Some glycoform preparations 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 μ g 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 mU peptide-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) SavantTM 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 Fig. 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 $[M+H_3PO_4]^-$ 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 (Fig. 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 tetraantennary. 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 Fig. 2B. Five of the fifteen >2% abundance glycan structures were bi-antennary, seven were tri-antennary and three were tetra-antennary glycans. 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 GH₃-hFSH glycans totaled a little over 68% of this oligosaccharide population (Fig. 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 isoforms in GH₃ 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 hFSH²⁴ that exceeded 2% abundance (Fig. 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 biantennary 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 hFSH²¹ exceeded 2% abundance (Fig. 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 only 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^{21/18} 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 mannose residues (Fig. 2F). These are biosynthetic intermediates, one processed in the rough endoplasmic reticulum (GlcNAc₂Man₈) 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^{21/18} (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, hFSH²⁴, 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, shown in Fig. 3A, 11 were also major ions in the urinary hFSH glycan spectrum (Fig. 3B), and only 8 were major ions in the recombinant GH₃-hFSH glycans spectrum (Fig. 3C). Termination with sulfate resulted in the absence of a major inferred neutral core glycan ion from the desialylated hFSH²⁴ glycoform glycan spectrum in Fig. 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 hFSH²⁴ glycan spectrum. For glycoform preparations, hFSH²¹ and hFSH^{21/18}, 3 or 4 additional neutral glycans were observed after desialylation than were inferred from the acidic ions in the intact oligosaccharide spectra (Figs 3E & 3F). The hFSH²¹ desialylated

glycan spectrum possessed 11 of the major glycan ions associated with pituitary hFSH, while the hypo-glycosylated hFSH^{21/18} 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 Fig. 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 hFSH²⁴, 1 = pituitary hFSH²¹, and H = hypo-glycosylated pituitary hFSH^{21/18}.

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]. Fig. 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 (Fig. 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 biantennary and fewer tri-antennary glycans (Fig 5C). Sialylated glycan abundance decreased, while neutral glycan abundance increased. Both core-fucosylation and the presence of a bisecting GlcNAc residue were more frequent in GH₃-hFSH than in pituitary hFSH. An additional feature of GH₃-hFSH oligosaccharide structures was antenna-fucosylation, which was rare in pituitary hFSH preparations. In an earlier study, higher fucose content in glycoform hydrolysates was associated with PNGaseF resistance [7], suggesting the novel presence of α 1-3-linked core fucose, however, the absence of ms/ms evidence for two core-linked fucose residues indicate this is unlikely. The largest structural differences between FSH²⁴ and pituitary hFSH were the presence of more neutral, sulfated, and GalNAc-containing glycans derived from the former than from the latter (Fig. 5D). For hFSH²¹, there was a reduction in tetra-antennary and sialylated glycans as compared with pituitary hFSH, but an increase in sulfated and GalNAc-possessing glycans (Fig. 5E). While complex glycans were present in the other hypo-glycosylated glycoform preparation, hFSH^{21/18}, they represented only about 30% of the total, in contrast to >90% complex glycans in all other hFSH preparations (Fig. 5F). Neutral glycans represented over 70% of the glycan population.

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 Fig. 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 (Fig. 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 tetraantennary 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 GH₃-hFSH, accounted for just over 78% of the glycan population (Fig. 6C). Six families were bi-antennary and two were tri-antennary. When comparing GH₃-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 FSH²⁴ glycan families that exceeded 4% abundance in the fully-glycosylated glycoform preparation, hFSH²⁴, and these represented over 59% of the total population. Five families were bi-antennary, three were tri-antennary, and one was tetraantennary. 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 (Fig. 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 hFSH²¹ glycoform glycans. Three of the more abundant core structures possessed 1-2 GalNAc residues (Fig. 6E) and included sulfated variants. 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 (Fig. 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 FSH β glycan, may play an important role in defining FSH receptor-binding properties.

Characterization of FSH glycoforms by gel filtration

Samples of hFSH glycoform preparations isolated from the triple Superdex chromatograms shown above in Fig. 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, (Fig. 7). Western blot analysis confirmed that fraction A possessed largely hFSH²⁴ and fraction C possessed largely hFSH²¹ (Fig. 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 (Fig. 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 glycans 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 31 glycan families were more abundant in FSH²¹ than in FSH²⁴, while only 15 were more abundant in FSH²⁴ than in FSH²¹ (Fig. 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 glycans with the other five hFSH glycan preparations made extensive use of bar graphs. To provide a global picture of glycan abundance across all six hFSH 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 Fig. 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 glycans. 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 (see above Figs. 1H & 1I) were determined by ultra-performance, size-exclusion chromatography (Figs. 10A-D). The hFSH²⁴ preparation was shown to have a small amount of high molecular weight contaminant that was confirmed by SDS-PAGE (Fig. 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 hFSH²¹ 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 α -subunit band intensities, the hFSH glycoform samples possessed 20-70% greater α -subunit immunoactivity than the hFSH reference preparation (Fig. 10F). However, when 1 μ g samples of the same preparations were subjected to Western blotting (Fig. 10G), hFSH²⁴ was found to possess only 53% of the expected immunoactivity, hFSH²¹ from fraction B only 36%, while hFSH²¹ from fraction C possessed only 12%. An LH β subunit-specific Western blot provided a potential rationale for the greater than anticipated α -subunit immunoactivity, as fractions with lower FSH β immunoactivity possessed greater LH β immunoactivity (Fig. 10H). LH β immunoactivity was undetectable in fraction B hFSH²⁴ and only 4% was associated with fraction C hFSH²¹, which were used for glycoform glycan analysis described above. Significantly more LH was associated with fraction D FSH²¹ glycoform preparation, which was not characterized.

Based on FSH content determined by size exclusion chromatography, samples of each glycoform were serially diluted and FSH immunoactivity measured by FSH radioimmunoassay. As can be seen in Fig. 11A, the ID₅₀ values were very similar to each other and no significant difference was found when these values were compared with Prism (Table 3). All glycoform preparations were 3- to 4-fold more active than the pituitary hFSH reference preparation.

Using the Western blot-derived FSH β immunoactivity values to correct the amounts of FSH added to a radioligand assay, both hFSH²¹ preparations exhibited greater than 10-fold higher apparent affinity for the FSH receptor than hFSH²⁴ (Fig. 11B and Table 3). However, LH contamination rendered the hFSH²¹ preparations unsuitable for assessment of biological activity, as some assays employed whole follicles and other assays involved whole ovaries, which would respond synergistically to the mixture of FSH and LH activities. Well after these purifications were performed, glycoform-selective monoclonal antibodies were identified that will improve the quality of future hFSH glycoform preparations.

Discussion

Detailed analysis of glycan structures associated with 10-30 μ g samples of FSH that previously required milligram quantities to obtain [17-19] permitted characterization of a low-abundance hFSH glycoform, two more abundant glycoforms, two highly purified hFSH preparations, and a recombinant hFSH preparation that was a candidate for large-scale expression. For pituitary and urinary hFSH preparations obtained from different suppliers, the differences in 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 GH₃-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 hFSH²⁴ and hypo-glycosylated hFSH^{21/18} glycoform preparations for *in vitro* and *in vivo* studies [9, 24]. We did note that in GH₃-hFSH tri-antennary glycans the third antenna was located on the mannose6-branch rather than on the mannose3-branch, where the third antenna was found in both pituitary and urinary hFSH tri-antennary glycans. Antenna fucosylation was also more abundant in GH₃-hFSH glycans, although it was observed in pituitary FSH glycoform preparations, particularly hFSH²⁴.

The hFSH^{21/18} 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 hFSH²⁴, the glycan population may also contribute to altered FSH activity.

When hFSH²¹ was first identified, despite the glycoprotein appearance of the FSH β band in Western blots, MALDI-TOF-MS revealed the presence of non-glycosylated FSH β , 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 FSH β , resulting in two major glycoforms. The activities of these were assumed to be dictated by the presence or absence of both FSH β N-glycans. However, characterization of the first hypo-glycosylated hFSH glycoform preparation, which possessed an additional FSH β band in Western blots, revealed the presence of carbohydrate in both FSH β ²¹ and FSH β ¹⁸ bands [7]. The non-glycosylated FSH β ¹⁵ band was never seen in intact FSH β Western blots, but was readily detected following peptide-N-glycanase digestion of denatured FSH β samples. In FSH receptor-binding assays, the ID₅₀ values for hFSH²¹ preparations are always less than those of hFSH²⁴, 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 hypoglycosylated hFSH^{21/18} isolated from hLH preparations was consistently 20-fold more active than hFSH²⁴ 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 FSH β 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.

Acknowledgements

We thank Ms. Alice Hwang for her assistance in editing tables and figures. This work was supported by National Institutes of Health grants P01 AG-029531, G20 RR-031092, and P20 GM-103418.

References

1. Bousfield, GR.; Jia, L.; Ward, DN. Gonadotropins: chemistry and biosynthesis. In: Neill, JD., editor. Knobil and Neill: Physiology of Reproduction. 3rd ed. Vol. 1. Elsevier; San Diego: 2006. p. 1581-1634.
2. Hearn MTW, Gomme PT. Molecular architecture and biorecognition processes of the cystine knot protein superfamily: part I. The glycoprotein hormones. *J. Mol. Recognit.* 2000; 13:223–278. [PubMed: 10992290]
3. Fox KM, Dias JA, Van Roey P. Three-dimensional structure of human follicle-stimulating hormone. *Mol. Endocrinol.* 2001; 15:378–389. [PubMed: 11222739]
4. Walton WJ, Nguyen VT, Butnev VY, Singh V, Moore WT, et al. Characterization of human follicle-stimulating hormone isoforms reveals a nonglycosylated β -subunit in addition to the conventional glycosylated β -subunit. *J. Clin. Endocrinol. Metab.* 2001; 86:3675–3685. [PubMed: 11502795]
5. Dalpathado DS, Irungu JA, Go EP, Butnev VY, Norton K, et al. Comparative glycomics of the glycoprotein hormone follicle-stimulating hormone (FSH): glycopeptide analysis of isolates from two mammalian species. *Biochemistry.* 2006; 45:8665–8673. [PubMed: 16834341]
6. Bousfield GR, Butnev VY, Bidart JM, Dalpathado D, Irungu J, et al. Chromatofocusing fails to separate hFSH isoforms on the basis of glycan structure. *Biochemistry.* 2008; 47:1708–1720. [PubMed: 18197704]
7. Bousfield GR, Butnev VY, Butnev VY, Hiromasa Y, Harvey DJ, et al. Hypoglycosylated human follicle-stimulating hormone (hFSH21/18) is much more active in vitro than fully-glycosylated hFSH (hFSH24). *Mol. Cell. Endocrinol.* 2014; 382:989–997. [PubMed: 24291635]
8. Bousfield GR, Butnev VY, Rueda-Santos MA, Brown A, Smalter Hall A, et al. Macro and micro heterogeneity in pituitary and urinary follicle-stimulating hormone glycosylation. *J. Glycomics Lipidomics.* 2014; 4:125.
9. Davis JS, Kumar TR, May JV, Bousfield GR. Naturally occurring follicle-stimulating hormone glycosylation variants. *J. Glycomics Lipidomics.* 2014; 4:e117. [PubMed: 25893134]
10. Bousfield GR, Butnev VY, Walton WJ, Nguyen VT, Singh V, et al. All or none N-glycosylation in primate follicle-stimulating hormone β subunits. *Molec. Cell. Endocrinol.* 2007; 260:262–40.
11. Wide L, Eriksson K. Dynamic changes in glycosylation and glycan composition of serum FSH and LH during natural ovarian stimulation. *Ups. J. Med. Sci.* 2013; 118:153–164. [PubMed: 23527482]
12. Ulloa-Aguirre A, Midgley AR Jr, Beitins IZ, Padmanbhan V. Follicle-stimulating isohormones: characterization and physiological relevance. *Endocr. Rev.* 1995; 16:765–787. [PubMed: 8747835]
13. Andersen CY, Leonardsen L, Ulloa-Aguirre A, Barrios-de-Tomasi J, Moore L, et al. FSH-induced resumption of meiosis in mouse oocytes: effect of different isoforms. *Mol. Hum. Reprod.* 1999; 5:726–731. [PubMed: 10421799]
14. Barrios-de-Tomasi J, Nayudu PL, Brehm R, Heistermann M, Zariñán T, et al. Effects of human pituitary FSH isoforms on mouse follicles in vitro. *Reprod Biomed Online.* 2006; 12:428–441. [PubMed: 16740215]

15. Loreti N, Ambao V, Andreone L, Trigo R, Bussmann U, et al. Effect of sialylation and complexity of FSH oligosaccharides on inhibin production by granulosa cells. *Reproduction*. 2013; 142:127–135. [PubMed: 23166369]
16. Loreti N, Fresno C, Barrera D, Andreone L, Albarran SL, et al. The glycan structure in recombinant human FSH affects endocrine activity and global gene expression in human granulosa cells. *Mol. Cell. Endocrinol*. 2013; 366:68–80. [PubMed: 23261981]
17. Renwick AGC, Mizuochi T, Kochibe N, Kobata A. The asparagine-linked sugar chains of human follicle-stimulating hormone. *J. Biochem*. 1987; 101:1209–1221. [PubMed: 3115970]
18. Green ED, Baenziger JU. Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin: I. Structural elucidation of the sulfated and sialylated oligosaccharides on bovine, ovine and human pituitary glycoprotein hormones. *J. Biol. Chem*. 1988; 263:25–35. [PubMed: 3121609]
19. Green ED, Baenziger JU. Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin II. Distributions of sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *J. Biol. Chem*. 1988; 263:36–44. [PubMed: 3121612]
20. Harvey DJ. Fragmentation of negative ions from carbohydrates: part 3. Fragmentation of hybrid and complex N-linked glycans. *J. Am. Soc. Mass Spectrom*. 2005; 16:647–659. [PubMed: 15862766]
21. Harvey DJ. Fragmentation of negative ions from carbohydrates: part 2. Fragmentation of high-mannose N-linked glycans. *J. Am. Soc. Mass Spectrom*. 2005; 16:631–646. [PubMed: 15862765]
22. Harvey DJ. Fragmentation of negative ions from carbohydrates: part 1. Use of nitrate and other anionic adducts for the production of negative ion electrospray spectra from N-linked carbohydrates. *J. Am. Soc. Mass Spectrom*. 2005; 16:622–630. [PubMed: 15862764]
23. Harvey DJ, Royle L, Radcliffe CM, Rudd PM, Dwek RA. Structural and quantitative analysis of N-linked glycans by matrix-assisted laser desorption/ionization and negative ion nanospray mass spectrometry. *Anal. Biochem*. 2008; 376:44–60. [PubMed: 18294950]
24. Butnev VY, Butnev VY, May JV, Shuai B, Tran P, et al. Production, purification, and characterization of recombinant hFSH glycoforms for functional studies. *Mol. Cell. Endocrinol*. 2015; 405:41–52.
25. Hartree AS. Purification of human pituitary glycoprotein hormones. *Meth. Enzymol*. 1975; 37:380–389. [PubMed: 1128252]
26. Bousfield GR, Ward DN. Purification of lutropin and follitropin in high yield from horse pituitary glands. *J. Biol. Chem*. 1984; 259:1911–1921. [PubMed: 6420415]
27. Chapman GE, Renwick AGC, Livesey JH. The isolation of human pituitary hormones from frozen glands. *J. Clin. Endocrinol. Metab*. 1981; 53:1008–1013. [PubMed: 6793612]
28. Harvey DJ, Merry GH, Royle L, Campbell MP, Dwek RA, et al. Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds. *Proteomics*. 2009; 9:3796–3801. [PubMed: 19670245]
29. Harvey DJ, Merry AH, Royle L, Campbell MP, Rudd PM. Symbol nomenclature for representing glycan structures: Extension to cover different carbohydrate types. *Proteomics*. 2011; 11:4291–4295. [PubMed: 21954138]
30. Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem*. 1985; 54:631–634. [PubMed: 3896128]
31. Hudgin RL, Pricer WE Jr, Ashwell G, Stockert RJ, Morell AG. The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. *J. Biol. Chem*. 1974; 249:5536–5543. [PubMed: 4370480]
32. Fiete D, Srivastava V, Hindsgaul O, Baenziger JU. A hepatic reticuloendothelial cell receptor specific for SO₄-4GalNAc β 1,4GlcNAc β 1,2Man α that mediates rapid clearance of lutropin. *Cell*. 1991; 67:1103–1110. [PubMed: 1662117]
33. Park EI, Mazella SM, Baenziger JU. Rapid clearance of sialylated glycoproteins by the asialoglycoprotein receptor. *J. Biol. Chem*. 2003; 278:4597–4602. [PubMed: 12464602]
34. Emmanouel DS, Stavropoulos T, Katz AI. Role of the kidney in metabolism of gonadotropins in rats. *Am. J. Physiol*. 1984; 247:E786–E792. [PubMed: 6439050]

Figure 1.

Isolation of hFSH glycoform preparations, FSH²⁴ and FSH²¹. **A.** Sephacryl G-100 chromatography of 1502 mg soluble human pituitary glycoprotein fraction on a 5 × 200 cm column equilibrated in 0.126 M ammonium bicarbonate at a flow rate of 150 mL/h. FSH was found in fractions A-C by RIA (dotted squares). **B.** A 111 mg sample of G-100A was chromatographed on a 5 × 15 cm phenyl-Sepharose column. The chromatogram was developed with: 1 M ammonium sulfate in 0.05 M sodium phosphate buffer, pH 7.0, followed by 0.2 M ammonium sulfate in 0.05 M sodium phosphate buffer, pH 7.0, 0.05 M sodium phosphate buffer, pH 7.0, and 40% ethanol/water. FSH emerged in fractions A, B, and D (inset bar graph). **C.** A 155 mg sample of G-100B was applied to the phenyl-Sepharose column and the chromatogram developed as in Fig. 1B. FSH largely emerged in Fraction B1 (inset bar graph). **D.** A 349 mg sample of G100-C was applied to the phenyl-Sepharose column. FSH was recovered in fractions A2, B1 and B2 (inset bar graph). **E.** FSH in fraction B1 from panel C was immunopurified using anti-hFSH monoclonal antibody 4882. **F.** A 51 mg sample of fractions A2, B1, and B2 from panel D was applied to a 2.5 × 200 cm Sephacryl S-100 and the chromatogram developed with 0.126 M ammonium bicarbonate buffer at a flow rate of 45 mL/h. FSH was found in fractions B and C (upper inset). **G-I.** Triple-Superdex 75 gel filtration of purified hFSH. Three 1 × 30 cm Superdex 75 columns were connected in series and equilibrated with 0.2 M ammonium bicarbonate containing 20% acetonitrile. The chromatogram was developed with the same buffer at a flow rate of 0.3 mL/min. Individual fractions were collected and samples of each fraction associated with the FSH peak analyzed by FSHβ (panel G inset) or FSHβ and FSHα-specific Western blots (panels H and I, insets).

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 ^{13}C 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 AFP7298A. **B.** Glycans from purified urinary hFSH. **C.** Glycans from recombinant GH₃-hFSH. **D.** Glycans from hFSH²⁴ glycoform preparation. **E.** Glycans from hFSH²¹ glycoform preparation. **F.** Glycans from hypo-glycosylated hFSH^{21/18} preparation isolated from highly purified hLH preparations. Note the expanded scale indicated by the dashed line.

Figure 3.

Neuraminidase-digested oligosaccharides analyzed by nano-ESI mass spectrometry. Aliquots of samples analyzed in Fig. 2 were digested with *Arthrobacter ureafaciens* sialidase prior to analysis. Structures of the more abundant neutral core glycans (Table 1) are shown in each panel. **A.** Desialylated glycans from purified pituitary hFSH. **B.** Desialylated glycans from purified urinary hFSH. **C.** Desialylated from recombinant GH₃-hFSH. **D.** Desialylated glycans from hFSH²⁴. **E.** Desialylated glycans from hFSH²¹. **F.** Desialylated glycans from hypo glycosylated hFSH^{21/18}. Note the expanded scale indicated by the dashed line.

Figure 4.

Diagrams showing the structures of the neutral core glycans associated with the 6 hFSH and hFSH glycoform preparations analyzed in this study. The m/z value 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 GH₃-hFSH, 4 = hFSH²⁴, 1 = FSH²¹, and H = hFSH^{21/18} from hLH preparations.

Figure 5.

Comparison of glycan abundance by structure type. These comparisons were made because previous FSH glycan structure determinations were summarized using this type of grouping. In each case pituitary hFSH glycan relative abundance is compared with the other FSH preparations in this study as well as two published hFSH glycan studies. **A.** Comparison of pituitary hFSH AFP-7298 with pituitary hFSH [17] and pituitary hFSH [18, 19]. **B.** Comparison of pituitary and urinary hFSH glycans by structure type. **C.** Comparison of pituitary hFSH glycans with those from recombinant GH₃-hFSH. **D.** Comparison of pituitary hFSH and hFSH²⁴ glycans. **E.** Comparison of pituitary hFSH and hFSH²¹ glycans. **F.** Comparison of pituitary hFSH and hFSH^{21/18} glycans.

Figure 6.

Comparison of glycan abundance by neutral core structure. The abundances of all variants sharing a common core were summed and plotted. Comparisons were made between highly purified pituitary hFSH glycans and those of the other five preparations analyzed in this study. **A.** Purified pituitary hFSH glycans, showing the glycan families accounting for at least 4% of the total. **B.** Comparison of urinary with pituitary hFSH glycans. The structures in this and subsequent panels represent urinary glycans, as pituitary glycans were shown in panel A, above. **C.** Comparison of recombinant GH₃-hFSH with pituitary hFSH glycans. **D.** Comparison of hFSH²⁴ with pituitary hFSH glycans. **E.** Comparison of hFSH²¹ with pituitary hFSH glycans. **F.** Comparison of hypo-glycosylated hFSH^{21/18} with pituitary hFSH glycans. The solid bars represent the hFSH glycan families while the gray bars represent the other hFSH preparation glycans.

Figure 7.

FSH characterization by size exclusion chromatography and Western blot analysis. Samples of hFSH²⁴, hFSH^{24/21}, and hFSH²¹ were applied to a 1 × 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 hFSH^{24/21}. **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.

Figure 10.

Size exclusion chromatography and Western blot characterization of hFSH. **A-D.** Samples of hFSH were subjected to UPLC-SEC using a 0.46×15 cm Waters BEH200 SEC column equilibrated with 20% acetonitrile in 0.2 M ammonium bicarbonate at a flow rate of 0.4 ml/min. **A.** 0.5 µg GonalF recombinant hFSH; **B.** 1.5 µg pituitary hFSH²⁴; **C.** 1 µg pituitary hFSH²¹; **D.** 20 µg pituitary hFSH²¹. **E-H.** Electrophoretic characterization. **E.** SDS-PAGE with Coomassie Blue staining. Lane 1, BioRad broad MW pre-stained standards; 2, 10 µg pituitary hFSH AFP-7298A; lane 3, 10 µg pituitary hFSH²⁴ from panel B; lane 4, 10 µg hFSH²⁴ from panel C; lane 5, 10 µg pituitary hFSH²¹; lane 6, 10 µg pituitary hFSH²¹ from panel D. **F.** Common α-subunit Western blot probed with monoclonal antibody HT13 diluted 1:4000. **G.** FSHβ Western blot probed with monoclonal antibody RFSH20 diluted 1:10,000. **H.** LHβ Western blot probed with anti-eLHβ antibody VB-I-118 diluted 1:1000. Lane 1, 1 µg pituitary hFSH AFP-7298A (missing in panel H); lane 2, 1 µg hLH; lane 3, 1 µg pituitary hFSH²⁴ from panel B; lane 4, 1 µg pituitary hFSH²¹ from panel C; lane 5, 1 µg pituitary hFSH²¹; lane 6, 1 µg pituitary hFSH²¹ from panel D.

Figure 11.

FSH radioimmunoassay and radioligand assays. **A.** FSH radioimmunoassay using ^{125}I -hFSH tracer and FSH dimer-specific polyclonal antibody AFP005, diluted 1:10,000. FSH glycoform concentrations based on SEC peak areas. **B.** FSH radioligand receptor assay employing ^{125}I -eFSH tracer and rat testis receptors. FSH glycoform concentrations corrected for Western blot results (Fig. 10G).

Table 1

Masses and abundances of N-glycans found in pituitary, urinary and recombinant hFSH preparations compared with pituitary hFSH glycoform preparations.

Neutral compounds										Acidic compounds										
<i>m/z</i> ^{<i>a,b</i>}	Comp. ^{<i>c</i>}				Sample ^{<i>d,e,f</i>}					Structure ^{<i>e</i>}	<i>m/z</i>	Acid groups ^{<i>g</i>}		Ion ^{<i>h</i>}	Sample ^{<i>d,e,f</i>}					
	H	N	F	P	U	G	F ²⁴	F ²¹	F ^{21/18}			Si	SO ₃ H		P	U	G	F ²⁴	F ²¹	F ^{21/18}
1007.3	3	2	0	0.11	-	-	0.37	0.64	5.10	1	-	-	-	-	-	-	-	-	-	
1153.4	3	2	1	0.18	-	-	0.47	0.54	4.82	2	-	-	-	-	-	-	-	-	-	
1169.3	4	2	0	-	-	-	0.17	-	0.74	3	-	-	-	-	-	-	-	-	-	
1210.4	3	3	0	-	-	-	0.12	0.15	1.95	4,5	-	-	-	-	-	-	-	-	-	
1331.4	5	2	0	-	-	-	0.52	0.29	11.67	6	-	-	-	-	-	-	-	-	-	
1356.5	3	3	1	-	-	0.91	0.29	-	2.41	7,8	-	-	-	-	-	-	-	-	-	
1372.5	4	3	0	-	-	-	0.49	0.47	0.96	9,10	1565.5	1	0	1	0.76	0.23	-	0.58	1.33	1.74
1413.5	3	4	0	-	-	-	0.33	0.28	1.69	11,12	1395.4	0	1	1	-	-	-	1.56	1.04	1.13
											1606.6	1	0	1	0.21	-	-	0.46	0.66	0.77
1493.5	6	2	0	-	-	-	0.11	0.12	11.20	13	-	-	-	-	-	-	-	-	-	-
1518.5	4	3	1	-	1.10	-	0.34	0.26	0.95	14,15	1711.6	1	0	1	0.62	0.17	-	0.38	0.74	0.97
1559.5	3	4	1	-	-	-	0.07	0.12	2.84	16,17	1541.5	0	1	1	-	-	-	0.44	0.74	0.37
1575.5	4	4	0	-	0.11	0.54	0.22	0.22	0.64	18,19,20	1557.5	0	1	1	-	-	-	0.03	0.85	-
											1768.6	1	0	1	-	-	0.66	0.30	0.46	0.54
1616.6	3	5	0	-	-	2.48	0.10	-	0.85	21	1598.5	0	1	1	-	-	-	0.27	0.29	0.41
											1809.7	1	0	1	-	-	0.11	0.12	0.15	0.32
1655.5	7	2	0	-	-	-	0.09	0.10	7.31	22,23,24	-	-	-	-	-	-	-	-	-	-
1721.6	4	4	1	-	-	0.90	0.26	0.30	0.78	25,26	1703.5	0	1	1	-	-	-	0.11	0.14	-
											1914.7	1	0	1	0.26	0.09	-	0.20	0.27	0.47
											1004.8	1	1	2	0.25	0.41	-	0.32	0.29	-
											1110.4	2	0	2	9.25	8.82	10.61	4.36	4.09	1.86
1737.6	5	4	0	-	0.20	0.28	0.83	0.10	0.30	27	1719.5	0	1	1	-	-	-	0.27	0.34	-
											1930.7	1	0	1	0.35	1.56	0.88	1.85	0.63	0.32

Neutral compounds													Acidic compounds												
$m/z^{a,b}$	Comp. ^c			Sample ^{d,e,f}						Structure ^e	m/z	Acid groups ^g		Ion ^h	Sample ^{d,e,f}										
	H	N	F	P	U	G	F ²⁴	F ²¹	F ^{21/18}			Si	SO ₃ H		P	U	G	F ²⁴	F ²¹	F ^{21/18}					
1762.6	3	5	1	-	-	-	-	-		28,29,30	1744.6	0	1	1	1	-	0.12	0.57	0.13	0.14	-				
											919.8	0	2	2	2	-	-	-	0.22	0.26	0.13				
											1025.3	1	1	1	2	2.05-	0.82	-	2.81	3.97	2.34				
1778.6	4	5	0	-	0.12	2.27	0.65	0.24	0.59	31,32,33	1130.9	2	0	2	2	6.57	3.97	8.06	3.78	6.19	2.86				
											1760.6	0	1	1	1	0.18	0.16	0.67	1.30	0.78	0.33				
											1971.7	1	0	1	1	0.48	0.67	2.47	1.50	1.07	0.83				
1817.6	8	2	0	-	-	-	0.08	0.13	11.50	34	2262.8	2	0	1	1	0.05	0.09	-	0.08	0.11	-				
											-	-	-	-	-	-	-	-	-	-	-				
											940.3	0	2	2	2	0.23	0.06	-	2.80	2.15	1.14				
1819.6	3	6	0	-	-	-	-	-	-	35,36	1045.8	1	1	1	2	0.29	0.14	-	0.42	0.49	0.28				
											1801.6	0	1	1	1	-	-	-	0.04	-	-				
											2012.7	1	-	1	1	-	-	-	-	-	0.08				
											1077.8	1	1	1	2	-	0.39	-	0.34	0.29	-				
1883.6	5	4	1	-	0.22	-	0.54	0.10	0.38	37,38	1183.4	2	0	2	2	7.66	8.61	12.96	2.99	3.77	1.16				
											1865.6	0	1	1	1	-	-	-	0.10	0.02	-				
											2076.7	1	0	1	1	0.34	1.35	1.80	1.03	0.46	0.24				
											2367.8	2	0	1	1	0.05	0.14	-	0.12	0.06	-				
											992.8	0	2	2	2	-	-	-	T	-	-				
											1098.4	1	1	1	2	1.08	0.45	-	3.27	4.94	1.59				
1924.6	4	5	1	-	0.06	2.18	0.34	0.13	0.38	39,40,41 42,43,44	1203.9	2	0	2	2	0.62	0.47	2.44	0.93	1.24					
											1906.6	0	1	1	1	T	0.08	-	1.05	0.63	0.21				
											2117.8	1	0	1	1	T	0.18	2.39	-	0.28	0.29				
											2197.7	1	1	1	1	-	-	-	-	0.12	-				
											1211.9	2	0	2	2	4.44	4.03	4.90	1.79	2.90	0.74				
1940.7	5	5	0	-	0.11	0.49	0.35	0.07	0.26	45	2133.8	1	0	1	1	0.19	0.67	1.44	0.60	0.35	0.25				
											2424.9	2	0	1	1	0.04	0.07	0.30	-	-	-				

Neutral compounds										Acidic compounds											
$m/z,a,b$	Comp. ^c			Sample ^{d,e,f}						Structure ^e	m/z	Acid groups ^g		Ion ^h	Sample ^{d,e,f}						
	H	N	F	P	U	G	F ²⁴	F ²¹	F ^{21/18}			Si	SO ₃ H		P	U	G	F ²⁴	F ²¹	F ^{21/18}	
											1013.3	0	2	2	0.16	-	-	5.46	7.23	1.14	
1965.7	3	6	1	-	-	-	-	-	0.27	46,47	1053.3	0	3	2	-	-	-	0.15	0.13	-	
											1118.9	1	1	2	-	-	-	0.46	0.49	-	
											1947.7	0	1	1	-	-	-	0.15	0.11	0.09	
1979.6	9	2	0	-	-	-	-	4.85	-		-	-	-	-	-	-	-	-			
1981.7	4	6	0	-	-	-	-	-	-	49	1963.7	0	1	1	-	-	0.03	-	-		
											2174.8	1	0	1	-	-	-	-	-	0.09	
2029.7	5	4	2	-	-	-	-	0.03	0.04	50,51	1256.4	2	0	2	0.28	-	-	0.32	0.18	-	
											2222.8	1	0	1	-	-	0.37	-	-	-	
											2052.7	0	1	1	-	-	-	0.23	0.34	0.18	
2070.7	4	5	2	-	-	0.87	0.09	0.05	0.12		52,53	1179.4	1	1	2	-	-	0.10	0.07	-	
2086.7	5	5	1	-	0.12	0.66	0.11	0.07	0.22	54,55,56	1285.0	2	0	2	4.72	2.75	4.51	2.03	3.27	0.94	
											2279.8	1	0	1	0.16	0.37	1.16	0.50	0.25	0.13	
											1292.9	2	0	2	4.44	8.15	3.01	5.27	2.69	0.29	
2102.7	6	5	0	-	0.05	-	0.22	-	0.08		57,58	1438.5	3	0	2	6.48-	5.08	4.36	1.78	2.72	-
										2295.8		1	0	1	0.03	0.36	-	0.58	0.10	0.03	
										2586.8		2	0	1	T	0.07	-	0.05	-	-	-
2111.7	3	6	2	-	-	-	-	-	-	59		2093.7	0	1	1	-	-	0.16	0.16	-	
										60,61	1199.9	1	1	2	-	-	-	0.11	0.24	-	
											1313.5	2	0	2	-	-	-	2.58	-	-	-
											1458.9	3	0	2	-	-	-	0.49	-	-	-
2127.7	4	6	1	-	-	-	-	0.09	2117.7		1	0	1	0.50	-	-	-	-	-	-	
									2109.7	0	1	1	-	-	-	0.04	0.02	-	-		
									2117.7	1	0	1	-	-	-	0.40	-	-	-		
										2320.8	1	0	1	-	-	-	-	-	0.02		
										2336.8	1	0	1	-	-	-	0.30	-	-	-	

Neutral compounds											Acidic compounds										
$m/z^{a,b}$	Comp. ^c			Sample ^{d,e,f}						Structure ^e	m/z	Acid groups ^g		Ion ^h	Sample ^{d,e,f}						
	H	N	F	P	U	G	F ²⁴	F ²¹	F ^{21/18}			Si	SO ₃ H		P	U	G	F ²⁴	F ²¹	F ^{21/18}	
2143.7											1207.9	1	1	2	0.87	0.53		1.51	0.99	-	
											1313.5	2	0	2	3.12	1.68	2.15	-	3.18	0.49	
	2143.7	5	6	0	-	-	0.14	0.02	0.12	62,63,64 65,66,67 68	1353.4	2	1	2	0.84	-	-	0.85	0.77	-	
											1459.0	3	0	2	1.42	0.60	-	-	1.28	-	
											2125.7	0	1	1	-	-	-	0.17	0.05	-	
											2336.8	1	0	1	0.05	0.08	-	0.12	0.03		
2184.7	4	7	0	-	-	-	-	-	-		1122.8	0	2	2	-	-	-	0.35	0.17	-	
										1228.4	1	1	2	-	-	-	0.63	-	-		
											-	-	-	-	-	-	-	-	-		
2232.8	5	5	2	-	-	0.50	-	0.05		69,70											
											1260.4	1	1	2	0.33	0.35	-	0.39	0.16	-	
2248.8	6	5	1	-	0.05	-	0.16	-	0.07	71,72,73 74	1366.0	2	0	2	4.39	6.52	4.33	4.52	2.59	0.37	
											1406.0	2	1	2	--	0.29	-	0.06	0.04	-	
											1511.5	3	0	2	4.45	3.06	6.23	1.55	1.86	-	
											2441.9	1	0	1	0.03	0.28	-	0.36	0.08	0.03	
											1175.2	0	2	2	-	-	-	0.17	0.23	-	
2289.8	5	6	1	-	-	0.03	-	0.06	75,76,77 78	1280.9	1	1	2	-	0.36	-	1.80	1.90	-		
										1386.5	2	0	2	0.75	0.47	1.93	0.74	0.86	-		
										1426.4	2	1	2	-	-	-	0.81	1.17	-		
										1532.0	3	0	2	-	-	-	0.05	0.25	-		
										2271.7	0	1	1	-	-	-	0.16	0.06	-		
										2482.9	1	0	1	0.01	0.04	-	0.08	0.04	0.02		
2305.8	6	6	0	-	0.03	-	0.12	-	0.08	79,80	1394.5	2	0	2	3.76	4.74	1.43	2.80	1.79	-	
											1540.0	3	0	2	5.48	2.61	1.06	0.84	1.32	-	
											2498.9	1	0	1	0.02	0.16	-	0.22	0.05	0.02	
											1195.9	0	2	2	-	-	-	0.41	0.39	-	
2330.8	4	7	1	-	-	-	-	-	-		81	1305.1	1	1	2	-	-	-	0.22	0.22	-
										1228.4		1	1	2	-	-	-	-	-	0.27	-

Neutral compounds											Acidic compounds										
$m/z^{a,b}$	Comp. ^c			Sample ^{d,e,f}						Structure ^e	m/z	Acid groups ^g		Ion ^h	Sample ^{d,e,f}						
	H	N	F	P	U	G	F ²⁴	F ²¹	F ^{21/18}			Si	SO ₃ H		P	U	G	F ²⁴	F ²¹	F ^{21/18}	
2394.8	6	5	2	-	-	-	-	-	-	-	82	1439.0	2	0	2	-	-	1.72	-	-	-
												2587.9	1	0	1	0.01	-	-	0.03	-	-
2451.9	6	6	1	-	-	-	-	0.10	-	0.05	83,84,85	1467.5	2	0	2	2.81	2.47	1.90	2.40	2.48	0.27
												1613.0	3	0	2	2.51	0.98	0.84	0.70	1.84	-
												2644.9	1	0	1	0.01	0.07	-	0.12	0.04	0.01
												1475.5	2	0	2	0.50	5.10	-	1.64	0.91	-
2467.8	7	6	0	-	-	-	-	0.03	-	-	86,87	1621.1	3	0	2	1.18	1.05	0.78	1.18	1.07	-
												1766.6	4	0	2	0.57	0.23	-	0.29	0.37	-
												2660.9	1	0	1	-	-	-	0.06	-	-
												1390.5	1	1	2	-	-	-	0.39	0.36	-
2508.9	6	7	0	-	-	-	-	-	-	-	88,89	1496.0	2	0	2	-	-	-	0.47	0.69	-
												1536.0	2	1	2	0.18	-	-	0.40	0.44	-
												1641.6	3	0	2	0.31	-	-	0.16	0.65	-
2549.9	5	8	0	-	-	-	-	-	-	-	90	1305.4	0	2	2	-	-	-	0.18	0.14	-
												1548.5	2	0	2	1.30	8.44	-	2.35	0.92	-
												1588.5	2	1	2	-	0.16	-	0.05	-	-
2613.9	7	6	1	-	-	-	-	-	-	0.02	91,92,93	1694.1	3	0	2	2.72	2.84	1.31	1.29	0.95	-
												1839.6	4	0	2	2.57	0.76	0.51	0.26	0.30	-
												1985.2	5	0	2	0.23	-	-	-	-	-
												2807.0	1	0	1	-	-	-	0.04	0.01	-
2654.9	6	7	1	-	-	-	-	-	-	-	-	1463.5	1	1	2	-	-	-	0.55	0.59	-
												1569.1	2	0	2	0.23	-	0.59	0.09	0.11	-
												1609.0	2	1	2	-	-	-	0.41	0.42	-
												1577.1	2	0	2	0.37	0.07	-	0.09	0.18	-
2670.9	7	7	0	-	-	-	-	-	-	-	94,95	1722.6	3	0	2	0.71	0.36	0.71	0.10	0.24	-
												1868.2	4	0	2	0.27	-	-	-	0.07	-
2817.0	7	7	1	-	-	-	-	-	-	-	96	1650.1	2	0	2	0.58	0.73	-	0.79	0.69	-

Neutral compounds										Acidic compounds											
<i>m/z</i> ^{a,b}	Comp. ^c			Sample ^{d,e,f}						Structure ^e	<i>m/z</i>	Acid groups ^g		Ion ^h	Sample ^{d,e,f}						
	H	N	F	P	U	G	F ²⁴	F ²¹	F ^{21/18}			Si	SO ₃ H		P	U	G	F ²⁴	F ²¹	F ^{21/18}	
												3	0	2	1.06	0.50	-	0.28	0.59	-	
												1941.2	4	0	2	0.81	-	-	0.14	-	
2833.0	8	7	0	-	-	-	-	-	-	97	1658.1	2	0	2	0.21	0.09	-	0.12	-	-	
											1803.6	3	0	2	0.21	0.13	-	0.15	0.19	-	-
											1949.2	4	0	2	0.11	-	-	-	-	-	-
2979.1	8	7	1	-	-	-	-	-	98	1731.1	2	0	2	0.20	0.30	-	0.21	0.16	-	-	
										1876.7	3	0	2	0.24	0.33	-	0.14	0.14	-	-	
										2022.2	4	0	2	0.58	0.29	-	-	0.14	-	-	
										2167.7	5	0	2	0.10	-	-	-	-	-	-	
3020.0	7	8	1	-	-	-	-	-	-	-	1791.6	2	1	2	-	-	-	0.08	-	-	
3036.0	8	8	0	-	-	-	-	-	-	99	1839.6	2	2	2	-	-	-	-	0.30	-	
3182.1	8	8	1	-	-	-	-	-	-	-	1832.7	2	0	2	0.12	0.05	-	0.09	-	-	
											1978.2	3	0	2	0.21	-	-	-	-	-	-
3262.1	8	8	1	-	-	-	-	-	-	100	1832.7	2	0	2	-	-	-	-	0.07	-	
3344.1	9	8	1	-	-	-	-	-	-	-	2059.2	3	0	2	0.08	0.14	-	-	-	-	
											2204.8	4	0	2	0.06	-	-	-	-	-	-

^{a)} Calculated mass of [M+H₂PO₄]⁻ 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 GH₃ cell medium, F²⁴ = pituitary hFSH²⁴, F²¹ = pituitary hFSH²¹, and F^{21/18} = hLH-associated hFSH^{21/18}.

^{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)} Si - Neu5Ac (sialic acid), SO₃H = sulfate.

$$-2^2[\text{2H-M}] = 2^2 \cdot -[\text{H-M}] = 1^{(4)}$$

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

Summary of glycan ions detected by nano-electrospray mass spectrometry of six hFSH preparations.

Preparation	Pituitary hFSH	Urinary hFSH	GH ₃ hFSH	hFSH ²⁴	hFSH ²¹	hFSH ^{21/18}
Amt. anal. (µg)	19.1	30.2	22.5	21.4	26.5	13.1
Glycan Ions ^I	2/80	11/67	11/33	30/109	25/108	40/43
Neutral Cores Inferred	34	25	25	49	50	39
Asialo-Neutral Cores Detected	27	24	20	42	53	43

^I Neutral / acidic glycan ions detected in negative mode nano-electrospray MS.

Table 3

RIA and RLA results from Figs. 11A and 11B, respectively.

Preparation	RIA ID ₅₀ (ng) ^a	RIA Potency (%)	RLA ID ₅₀ (ng) ^b	RLA Potency (%)
hFSH (AFP7298A)	1.15	100	383.2	100
Fraction A hFSH ²⁴	0.26	442	268.7	143
Fraction B hFSH ²¹	0.37	311	19.4	1975
Fraction C hFSH ²¹	0.39	295	8.6	4456

^aDetermined by SEC chromatography (Figs. 10B-D).

^bDetermined by Western blotting (Fig. 10G).