HUMAN FOLLICLE-STIMULATING HORMONE GLYCOFORM ABUNDANCE DURING THE NORMAL MENSTRUAL CYCLE IN WOMEN

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HUMAN FOLLICLE-STIMULATING HORMONE GLYCOFORM ABUNDANCE DURING THE NORMAL MENSTRUAL CYCLE IN WOMEN

The following faculty have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biological Sciences.

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ABSTRACT

Human FSH exists as two major glycoforms, tetra-glycosylated and di-glycosylated hFSH. Tetra-glycosylated FSH possesses both α and β subunit carbohydrates while di-glycosylated hFSH possesses only α subunit carbohydrates. Western blotting differentiated the glycosylated, 24 kDa hFSHβ band from the non-glycosylated 21kDa FSHβ band. As part of the laboratory-wide effort to define glycoform abundance in individual humans, FSH was isolated from pituitaries. The findings from this assay were combined with previous data from individual human pituitaries showing the highest abundance of di-glycosylated hFSH in the youngest individuals, intermediate variation in perimenopausal individuals, and low abundance in post menopausal women.
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LIST OF ABBREVIATIONS

$^{125}\text{i}$............................................... A Radioactive Isotope of Iodine

$^\circ\text{C}$............................................... Degree Celsius

$\mu\text{g}$............................................... Microgram

$\mu\text{l}$............................................... Microliter

Asn............................................... Asparagine

BSA............................................... Bovine Serum Albumin

CG............................................... Chorionic Gonadotropin

CHO............................................... Chinese Hamster Ovary

cpm............................................... Counts per Minute

FSH............................................... Follicle-Stimulating Hormone

g............................................... Gravity

Gal............................................... Galactose

GlcNAc........................................... N-acetylglucosamine

GnRH............................................... Gonadotropin-Releasing Hormone

h............................................... Human

HPLC............................................... High-Performance Liquid Chromatography

l............................................... Liter

LH............................................... Luteinizing Hormone

MAb............................................... Monoclonal Antibodies

MCR............................................... Metabolic Clearance Rate
LIST OF ABBREVIATIONS (Cont.)

mg………………………………………… Milligram
ml………………………………………… Milliliter
MWM……………………………………… Molecular Weight Marker
ng………………………………………… Nanogram
pl………………………………………… Isoelectric Point
PVDF…………………………………… Polyvinylidene Fluoride
RIA……………………………………….. Radioimmunoassay
SA………………………………………… Sialic Acid, N-Acetyl-Neuraminic Acid
SDS-PAGE…………………………… Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TSH………………………………………. Thyroid-Stimulating Hormone
vol………………………………………. Volume
WBB…………………………………… Western Blotting Buffer
INTRODUCTION

Follicle-stimulating hormone (FSH), one of the hormones produced by the anterior pituitary gland, is a member of the glycoprotein hormone family, which also includes thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and chorionic gonadotropin (CG). FSH plays a key role in the regulation and maintenance of gametogenesis. In females, FSH regulates many ovarian processes associated with the female cycle including: follicular development, ovulation (Arslan et al. 2003, Ulloa-Aguirre et al. 1995), prevention of follicular atresia, aromatase induction, granulosa cell proliferation, LH receptor induction, and induction of its complementary receptor (West et al. 2002). FSH does not exist as a single molecular form because populations of glycans are found at each of four N-glycosylation sites (Bousfield GR et al. 2008a, Dalpathado et al. 2006). Consequently, FSH preparations can be fractionated into isoforms by zone electrophoresis, isoelectric focusing, and chromatofocusing, techniques that are assumed to exploit the variation in the number of negative charges conferred by all four glycans. Under changing endocrine conditions associated with the female reproductive cycle, corresponding alterations in relative abundance of FSH isoforms are observed that suggest gonadal feedback may regulate FSH glycosylation. However, high resolution glycopeptide mass spectrometry has revealed considerable overlap in the glycans associated with purified hFSH isoforms. Our laboratory identified a novel form of hFSH, which lacked both β subunit N-glycans, which we define as a di-glycosylated FSH glycoform because the glycosylation difference is known rather than assumed (Walton et al. 2001). Chromatofocusing failed to separate this glycoform from the presumably more negatively charged tetra-glycosylated hFSH, which possesses all
4 glycans (Bousfield GR et al. 2008a). An age-related difference in abundance of di-glycosylated hFSH form has been observed during analysis of individual human pituitary glands. This project addresses menstrual cycle-associated changes in FSH glycoform abundance.

**FSH structure and function**

Like all other members of the glycoprotein hormone family, FSH is composed of two non-covalently linked subunits, alpha (α) and beta (β) (Garcia-Campayo and Boime 2001). The α-subunit is common for all glycoprotein hormones. The human α subunit contains 92 amino acid residues and is encoded by a single gene (Einstein et al. 2001, Ma et al. 2004, Mann et al. 2003, Manna et al. 2002, Nguyen et al. 2003, Takahashi et al. 1998). The hCG and hFSH crystal structures define five regions of the α-subunit primary structure: an N-terminal end, three cystine knot loops (αL1, αL2, and αL3), and a C-terminal region (Combarnous 1992). The α subunit has two N-linked glycosylation sites, Asn^{52} and Asn^{78}. Oligosaccharides attached to Asn^{52} play a primary role in signal transduction initiated by LH and FSH receptors (Bousfield GR et al. 2004). Asn^{78} oligosaccharides are involved in α-subunit folding because the proximal N-acetylglucosamine (GlcNAc) residues interact with side chains of residues in both αL1 and αL3 loops (Lapthorn et al. 1994, Wu et al. 1994). The β subunits are usually encoded by a single gene (hFSHβ, hTSHβ and hLHβ). These subunits are called hormone-specific because when combined with an α subunit from any other hormone, the identity of the β subunit confers hormonal specificity. The hFSHβ subunit contains 111 amino acid residues (Darling et al. 2001, Perlman et al. 2003). FSHβ has two N-linked glycosylation sites, Asn^{7} and Asn^{24} (D’Antonio et al. 1999, Rose et al. 2000). Both
FSHβ glycosylation sites are located in the βL1 loop (Bousfield GR et al. 2004, Combranous 1992). The hFSH crystal structure defines three cystine knot loops, βL1, βL2, and βL3, along with a seatbelt loop that embraces the αL2 loop in the heterodimer (Fox et al. 2001). Amino terminal residues 1-2 are missing from 80% of the hFSHβ molecules, which has been suggested to result from alternative cleavage by signal peptidase (Walton et al. 2001).

Glycans in FSH are major structural components comprising in excess of 30% of the gonadotropin mass (Ulloa-Aguirre et al. 2003). The Asn (N)-linked carbohydrate side chains present in the glycoprotein hormones can be classified into three categories: 1) high mannose-type, 2) complex-type, and 3) hybrid-type (Hearn and Gomme 2000). High mannose-type glycans posses 4-9 mannose residues and two core N-acetylglucosamine (GlcNAc) residues, while the complex-type possess a common pentasaccharide core with structural variations occurring at the two non-reducing terminal α-mannose residues. These structural variations typically consist of a variable number of NeuAc-Gal-GlcNAc tri-saccharides, although SO₄-GalNAc-GlcNAc is also common in pituitary gonadotropins. Human FSH is unusual in that sulfated glycans are relatively rare as compared with FSH derived from other mammalian species (Dalpathado et al. 2006, Green E and Baenziger 1988a, b). FSH complex-type glycans include di-, tri- or tetra-antennary branched oligosaccharides which are terminated in galactose or sialic acid (Green E et al. 1985, Hearn and Gomme 2000). Hybrid-type glycans possess a combination of the features derived from both the high mannose and complex subtypes. Like the complex subtype, the hybrid subtype contains a common pentasaccharide core with a 1-3 linked α-mannose residue containing one or more
NeuAc-Gal-GlcNAc trisaccharides or a single SO$_4$-GalNAc-GlcNAc branch. The 1-6 linked α-mannose residue is linked to a variable number of mannose residues derived from incomplete processing of the high mannose precursor of all N-glycans. Oligosaccharides play a key role in determining a number of structural and biosynthetic properties, including α/β subunit assembly, intracellular sorting, metabolic clearance (Dias et al. 1998, Ulloa-Aguirre et al. 1995, Ulloa-Aguirre et al. 1999, Ulloa-Aguirre et al. 2003) receptor-binding affinity and capability of gonadotropins to activate the receptor and efficiently trigger intracellular signaling (Manna et al. 2002, Sairam 1989, Zariñan et al. 2001).

Carbohydrate chain composition and structure among glycoprotein hormones are highly variable, a feature that depends on the genetic background of the cell in which the hormones are synthesized (Furuhashi and Suganuma 2003, Ulloa-Aguirre et al. 2003). Oligosaccharides on the α-subunit, in particular those attached to Asn$_{52}$, are required for signal transduction, intracellular stability, and association with the β-subunit (Rose et al. 2000) Those on the β-subunit are required for β-subunit folding and for correct disulfide bond formation (Rose et al. 2000). Experiments conducted with recombinant hFSH have demonstrated that prevention of carbohydrate addition at Asn$_{52}$ reduced estradiol production 4-fold (Bishop et al. 1994). However, elimination of either glycosylation site from the β-subunit resulted in variable effects, as did elimination of both β-subunit carbohydrate attachment sites. In one study, recombinant di-glycosylated hFSH exhibited an increase in estradiol production by rat Sertoli cells (Bishop et al. 1994). In another study, there was a 2-fold decrease in estradiol production by rat granulosa cell cultures (Flack et al. 1994). Naturally occurring di-glycosylated hFSH was
25-fold more active than tetra-glycosylated hFSH in a rat testis FSH receptor-binding assay, and 88-fold more active than tetra-glycosylated hFSH in the rat granulosa cell bioassay (Bousfield GR et al. 2008b).

Glycosylation of the glycoprotein hormones is important for circulatory survival and may dictate the clearance pathway (Fox et al. 2001, Ulloa-Aguirre et al. 1995, Ulloa-Aguirre et al. 1999). Bishop et al. (Bishop et al. 1995) determined that recombinant hFSH mutants lacking both β subunit oligosaccharides were cleared from circulation 10 times faster than wild-type recombinant hFSH, while removal of the βAsn7 glycosylation site resulted in a 5-fold increase in MCR as compared with the wild type recombinant hFSH. Elimination of βAsn24 glycosylation, alone, produced less than a 3-fold increase in MCR, demonstrating that the MCR of recombinant hFSH was determined by both of the oligosaccharides present on the β-subunit. On the other hand, loss of either one or both α subunit oligosaccharides did not significantly change MCR’s in this study, consistent with studies involving TSH that indicated the β subunit glycan, rather than α subunit glycans determined clearance rates of this related hormone (Weintraub et al. 1995).

Changes in overall charge of the FSH molecule due to variations on sialic acid content have been shown to be important factors for the survival of human FSH in the circulation (Suttajit et al. 1971, Vitt et al. 1998). Less acidic human pituitary FSH isoforms, as well as similar recombinant FSH preparations, have been found to have a reduced circulatory half-life and a lower in vivo bioactivity than acidic isoforms, as measured by pharmacokinetic tests and the ovarian weight augmentation test in immature mice, respectively (Ulloa-Aguirre et al. 1995, Vitt et al. 1998). As a result, one
of the major factors that control FSH action in vivo is the relative rate of clearance via liver or kidney. Liver clearance via the asialo-glycoprotein receptor has been proposed for nearly 40 years as a mechanism for glycoprotein clearance following peripheral neuraminidase treatment (Morell et al. 1971). When tested experimentally, however, the lectin-mediated clearance mechanism did not appear to be involved in hCG clearance, as saturating the system with asialo-fetuin had no effect on intact hCG clearance, while significantly slowing clearance of asialo-hCG (Lefort et al. 1984). As hFSH glycans are almost exclusively sialylated, the foregoing results suggest that this mechanism is unlikely to eliminate hFSH. However, α2-6-linked sialylated glycans have been reported to be cleared by the asialo-glycoprotein receptor (Park EI et al. 2005) because the sialic acid in this position does not prevent galactose binding (Fig. 1). As human FSH possesses both α2-3 and α2-6 linked sialic acid residues, while hCG glycans possess only α2-3 linked sialic acid residues, the liver mechanism may be involved in FSH clearance. In this case it may be the ratio or location of α2–6 linked sialic acid residues that determines the site of removal. Recovery of FSH from human postmenopausal urine on an industrial scale suggests that another important mechanism for elimination of FSH is through glomerular filtration, and this has been confirmed by one study involving renal clearance of FSH (Perlman et al. 2003).
Figure 1. Comparison of sialic acid linkages $\alpha(2\text{--}3)$ and $\alpha(2\text{--}6)$ and interaction of the Gal residue to the potential binding site for the asialo-glycoprotein receptor (ASGPR) carbohydrate recognition domain.

The first step in kidney clearance is ultrafiltration in the glomerulus, which is influenced primarily by molecular size (<30Å) and secondarily by charge and molecular shape. Proteins having diameters of ~30Å are rapidly cleared if positively charged and more slowly cleared if negatively charged (Maack et al. 1992). In general, ultrafiltration membranes are calibrated with spherical proteins and their ultrafiltration properties vary according to size. On the other hand, when non-spherical proteins are subjected to
ultrafiltration, they do not follow patterns established with spherical proteins and this is particularly true for glycoproteins. The structure of hFSH is ellipsoid rather than spherical (Fox et al. 2001). The α subunit oligosaccharides emerge along the long axis of the molecule. In consequence, the presence or absence of these oligosaccharides probably does not affect filtration rate because when hFSH encounters a renal capillary pore in a sideways orientation, the 75Å protein itself may be too long to enter. In contrast, the β subunit oligosaccharides project from the side of the hormone, making the narrow, 30-35Å, diameter of the hormone bigger, as glycans can extend up to 30Å (Rudd et al. 1999). These oligosaccharides, therefore, may retard ultrafiltration of hFSH in the kidneys, thereby prolonging circulatory survival (Walton et al. 2001).

**FSH Isoforms**

Gonadotropins exist in multiple molecular forms that are assumed to represent differences in N-glycosylation. These variant forms are called isoforms and have been found to vary in number and relative abundance among different species and in various biological fluids. The overall pattern of gonadotropin polymorphism is also variable according to the particular endocrine status. On a molecular basis, changes in isoform charge are associated with variable sialic acid and sulfate content indicating underlying differences in glycan structure (Papandreou et al. 1993). Several studies revealed that the anterior pituitary gland contains a spectrum of FSH isoforms with different isoelectric properties, bioactivities and circulating half lives (D'Antonio et al. 1999, Ulloa-Aguirre et al. 1995, Ulloa-Aguirre et al. 1999). The varying abundance of sialylated side chains theoretically allows the FSH isoforms to be distinguished on the basis of their different isoelectric points (pI). The FSH isoform profile is influenced by its source, and by the
gender and age of the donor (Ulloa-Aguirre et al. 1995, Vitt et al. 1998). Isoform profile changes during the female menstrual cycle are influenced by the endocrine status as well. For example, rising concentrations of estrogens during the follicular phase are correlated with an increase in the amount of the less acidic FSH isoform fractions (Ulloa-Aguirre et al. 1995, Vitt et al. 1998). More acidic isoforms stimulate the follicular maturation process at a slower rate, while the less acidic isoforms appear to provide a short, but potent stimulus, necessary for the stimulation of ovulation (D'Antonio et al. 1999). An overall change in FSH isoform pattern has been observed in the middle of the menstrual cycle according to Wide et al. (Wide and Bakos 1993) in response to a GnRH challenge during puberty. In women, more acidic forms of FSH appear after the menopause, the longer half-life of which may contribute to the increased peripheral serum FSH concentrations associated with this stage of life.

The first FSH isoforms were detected by gel filtration, and sialic acid content was deemed responsible for the two major forms detected (Peckham WD and Knobil 1976a). Subsequent studies employed zone electrophoresis, isoelectric focusing, and chromatofocusing (Ulloa-Aguirre et al. 2003). Chromatofocusing received widespread use because it could separate FSH isoforms in larger tissue and physiological fluid samples, thereby enabling human studies. Again, sialic acid content reportedly influenced isoform patterns. This was consistent with structural studies reviewed above that revealed glycans could impart 0-3 negative charges at each of four N-glycosylation sites. Moreover, the first study that characterized purified hFSH isoform preparations indicated a correlation between low isoelectric point and high sialic acid content, although subsequent anion exchange chromatography revealed heterogeneity in each
“isolectric” fraction (Stanton et al. 1992). Two major hFSH isoforms were identified in previous studies from this laboratory that differed by an all-or-none pattern of FSHβ N-glycosylation (Walton et al. 2001). Both di- and tetra-glycosylated hFSH glycoforms were observed in most hFSH isoform fractions obtained by chromatofocusing (Bousfield GR et al. 2008a). Glycopeptide mass spectrometry indicated that glycans associated with hFSH isoforms exhibited considerable overlap, thus calling into question the assumption that separation on the basis of overall charge selected differentially glycosylated hFSH forms (Bousfield GR et al. 2008a).

**FSH isoform clearance**

Isoforms of FSH, apparently differing by charge variations, have been shown to exhibit different metabolic clearance rates. Investigators have shown that the MCR for less acidic hFSH isoforms is faster (Anobile et al. 1998) while more acidic isoforms exhibit a longer plasma half life (Bishop et al. 1995, Ulloa-Aguirre et al. 1992). The impact that the varying degrees in sialylation of circulating FSH may have on its net plasma half-life is exemplified by the study from Zambrano et al. (Zambrano et al. 1995), which showed that the increased release of less sialylated isoforms occurring during the preovulatory phase of the human menstrual cycle correlated with a significant reduction in the plasma half-life of FSH.

**Hypothalamic-Pituitary-Gonadal-Axis**

The hypothalamo-pituitary-gonadal-axis mediates one of the most important biological functions of life: the ability to reproduce. After a brief period of activity at during the first year after birth, hypothalamic secretion of GnRH remains quiescent throughout juvenile life, before reactivation at the onset of puberty (Jayasena et al.
The resulting episodic release of gonadotropins permits follicle development to reach completion, enables ovulation, and transforms the post-ovulation follicle into a corpus luteum. During the menstrual cycle, steroidal and non-steroidal substances produced by developing follicles, the dominant follicle, and the corpus luteum mediate the effects of the ovaries on the hypothalamic-pituitary-axis, causing changes in GnRH pulses and gonadotrope responsiveness to GnRH stimulation. As FSH isoform abundance changes during the menstrual cycle, these ovarian factors presumably influence FSH glycosylation.

**FSH variation during the menstrual cycle**

In the female, FSH is necessary for the selection and growth of ovarian follicles and for the production of estrogen from androgen substrate provided by LH-stimulated theca cells. The biological activity of FSH is the result of a complex combination of processes: release from the pituitary, survival in the circulation, transport to the site of action, binding to the receptor, and activation of signal transduction pathways. These processes may be modified by other factors that may affect, for example, FSH release (pulse frequency or amplitude), or FSH receptor desensitization (Rose et al. 2000, West et al. 2002).

Circulating levels of FSH vary significantly across the human menstrual cycle. They are relatively elevated during the luteal-follicular transition and early follicular phase, then decline roughly 4-fold during the late follicular phase, but rise 10-fold at the time of the pre-ovulatory gonadotropin surge before decreasing and remaining low during most of the luteal phase (Christin-Maitre et al. 1996). These changes reflect the negative feedback regulation of FSH by estrogen and inhibin B as the dominant follicle
undergoes final maturation leading up ovulation and conversion to a corpus luteum (Hohmann et al. 2001). Inhibin B may act as a paracrine factor inhibiting the growth of neighboring follicles, thus participating in the mechanism of follicular selection.

Gonadotropin secretion is under control of hypothalamic GnRH and pituitary peptides, such as activin and follistatin. Feedback from the gonads, such as inhibins, and sex steroids (progesterone and estradiol) inhibit or modulate the secretory signals (Christin-Maitre et al. 2003). At least 20-30 isoforms of both FSH and LH circulate in blood during the menstrual cycle. More basic isoforms of both hormones appear in serum at midcycle than in the follicular or luteal phases (Wide and Bakos 1993). Due to the demise of the corpus luteum, as indicated by the decrease in progesterone, estradiol and inhibin biosynthesis, FSH serum concentrations start to increase during the late luteal phase (Hohmann et al. 2001).

**Menstrual Cycle**

The human menstrual cycle lasts 21-35 days and is divided into three parts, the follicular phase, ovulation, and the luteal phase. The follicular phase lasts 7-17 days and is characterized by the final development of the dominant follicle, which produces progressively increasing amounts of estradiol. Ovulation occurs when the follicle wall ruptures and the cumulus-oocyte complex is released in a process lasting 15 min (Matzuk and Lamb 2008, Nature News 2008). The remaining follicular tissue collapses and differentiates into a solid body called the corpus luteum. In most species, this tissue secretes only progesterone, however, in humans estradiol is also secreted. This probably results from the fact that complete intermixing of granulosa and theca cells does not occur as in other species. In humans, the corpus luteum consists of fragments
of theca-basement membrane-granulosa tissue, the functional unit that is responsible for estradiol synthesis during the follicular phase (Baerwald et al. 2005). FSH begins to rise in the late luteal phase following regression of the corpus luteum and consequent decrease in progesterone, estradiol and inhibin biosynthesis. (Hohmann et al. 2001, Klein et al. 2002, Miro and Aspinall 2005, Park SJ et al. 2002). This event is often defined as luteal-follicular transition.

**Follicular phase**

FSH typically reaches a maximum in the early follicular phase and begins to decline just about 1 day before peak inhibin B levels are achieved, suggesting that inhibin B may be the closest regulator of FSH in this phase (Klein et al. 2002, Welt et al. 2003). FSH concentrations are elevated for the first 5 days of the follicular phase, then progressively decrease during the mid to late follicular phase as a result of negative feedback by inhibin B and estradiol (Arslan et al. 2003, Hohmann et al. 2001). The follicular phase can be divided into two distinct stages according to circulating estrogen concentrations: early follicular phase from day 1 (the first day of menstruation) to the beginning of the progressive rise in estrogen, and late follicular phase from the day after start of the estrogen rise to the day of the pre-ovulatory gonadotropin peak. During the early follicular phase, small antral follicles recruited in the preceding cycle, begin to grow, yet exhibit very low steroidogenic activity (Park SJ et al. 2002). Elevated FSH at the beginning of the follicular phase supports development of this new cohort of follicles. The progressive 4-fold decrease in circulating hFSH is insufficient to support development of all selected follicles. One by one they become atretic as a single dominant follicle emerges (Klein et al. 2002). The second stage of the follicular phase is
characterized by the production of estrogens (Miro et al. 2004). LH stimulates theca cells to produce androstenedione, the precursor for estradiol, while FSH stimulates aromatase synthesis in granulosa cells. This enzyme converts androgens to estradiol. During the late follicular phase, the dominant follicle produces progressively more and more estradiol. When a threshold concentration (~250 pg/ml) is exceeded, estradiol exerts a stimulatory effect on the hypothalamo-pituitary axis and triggers the preovulatory gonadotropin surge that stimulates ovulation and recruits the small antral follicles for the next cycle (Klein et al. 2002).

Ovulation

Ovulation is triggered by a 10-fold rise in circulating gonadotropins. While LH is traditionally considered to be the ovulatory hormone, FSH concentrations also increase, as GnRH stimulates release of both gonadotropins (Padmanabhan et al. 1997, Padmanabhan et al. 2003). The LH surge activates oocyte maturation and resumption of meiosis as well as inducing ovulation. The FSH surge recruits a new cohort of follicles for the next cycle. Studies involving FSH isoforms suggest that FSH stimulates oocyte maturation and that less acidic isoforms are more effective in this activity than more acidic isoforms (Barrios-De-Tomasi et al. 2002). Ovulation results from localized, uninhibited activity of matrix metalloproteases that destroy granulosa cells, the basement membrane, theca cells, and the ovarian epithelium over a period of 10 hr following the peak in LH secretion (Chabbert Buffet et al. 1998). Both theca and granulosa cells respond to LH because FSH induces dominant follicle granulosa cells to express LH receptors (Barrios-De-Tomasi et al. 2002). When the stigma ruptures, follicular fluid leaks out accompanied by the cumulus-oocyte complex. The emergence
of a human oocyte was inadvertently captured on film recently and the process required 15 min to complete (Matzuk and Lamb 2008, Nature News 2008). The remainder of the follicle collapses and differentiates into the corpus luteum. A reduction in the length of the follicular phase is one of the early known effects of reproductive aging. Since this reduction occurs in parallel to an elevation in the initial FSH levels, it has been hypothesized that acceleration in follicular development occurs due to the higher FSH levels (Miro and Aspinall 2005). A reduction in follicular phase length could, in principle, affect both the early and late stages. However, if FSH is involved in the reduction, because levels are higher during the early follicular phase, this stage should be more affected than the late follicular phase (Park SJ et al. 2002).

**Luteal Phase**

During the luteal phase, the corpus luteum inhibits follicular development by secreting the hormones progesterone, estradiol and inhibin A (Miro and Aspinall 2005). These are known to suppress FSH production, and peripheral FSH concentrations are depressed throughout most of the luteal phase. The luteal-follicular transition rise in FSH might well be related to a drop in the production of these suppressive hormones (Miro and Aspinall 2005).

**Luteal-follicular transition**

Clear changes in the activity of gonadotropin-releasing hormone pulse generator occur during the luteal-follicular transition, with an abrupt increase on day 11 after the LH peak (Miro and Aspinall 2005). The first hormonal event in the cycle is the rise in FSH level. This event occurs late in the luteal phase and is often defined as the luteal-follicular transition (Miro and Aspinall 2005). The luteal-follicular transition is
characterized by decreasing plasma levels of estrogen, progesterone, and inhibin A, with simultaneous increases in FSH and LH levels. LH and GnRH pulse frequencies increase from 1 pulse every 3-4 h during the luteal phase to 1 pulse/h at the mid cycle LH surge (McCartney et al. 2002).
PURPOSE OF THIS STUDY

A highly significant difference in pituitary FSH glycoform abundance was observed between 3 women in their 20’s expressing predominantly di-glycosylated hFSH and 3 postmenopausal women in their 70’s and 80’s who expressed predominantly tetra-glycosylated hFSH. A single pituitary from an elderly woman receiving estrogen-replacement therapy expressed mainly di-glycosylated hFSH, suggesting estrogen could reverse the shift from high to low di-glycosylated hFSH abundance. This raised the possibility that changes in circulating steroids during the menstrual cycle could alter FSH glycoform abundance. Comparison of postmenopausal pituitary and urinary FSH glycoform abundance suggested the limited sample size could be overcome by analyzing hFSH in urine. Additional data indicated about 1 µg hFSH could be recovered from first void urine samples provided by a single postmenopausal individual. The purpose of the present study was to determine the feasibility of measuring glycoform abundance in urine samples provided by young, cycling women. If this was possible, then glycoform abundance during the menstrual cycle would be measured in order to determine if it varied under changing feedback from the ovary.

A possible title: FSH Glycoform Changes During the Reproductive Cycle in Women.
MATERIALS AND METHODS

Pituitary hFSH Isolation

Each pituitary was homogenized in 3-ml of 0.02 M sodium phosphate buffer, pH 7.0, along with 100 µl general-purpose protease inhibitors using a Teflon-glass homogenizer. Homogenization was repeated twice with 3-ml aliquots of phosphate buffer. Pooled extracts were centrifuged at 20,000 x g in a Sorvall RC-26 centrifuge and the clear supernatant stored at -20°C in a 15-ml polypropylene culture tube (Bousfield GR et al. 2007). Pituitary extract was recycled 3 times through a 2-ml anti-hFSHβ monoclonal antibody column. The column was washed with 0.1 M NaPO₄, pH 7.0, containing 0.3 M NaCl for 20 min, or until the UV absorbance returned to baseline. Bound protein was eluted with 0.2 M glycine-HCl, pH 2.7, containing 0.5 M NaCl. The effluent was collected in an Amicon Ultra-4 centrifugal ultrafiltration cartridge containing 400 µl 1 M Tris-HCl, pH 9.5. The volume was reduced to <200 µl by 20 min centrifugation at 3500 rpm at 4°C in the HL-6000 rotor of a Sorvall RC-2B Plus centrifuge. Retentates were dried in a Savant Speed Vac. Dried, antibody-retained samples were dissolved in 50 µl 0.2 M ammonium bicarbonate containing 20% acetonitrile and applied to a Superdex 75 gel filtration column equilibrated with 0.2 M ammonium bicarbonate / 20% acetonitrile at a flow rate of 0.4 ml/min. The chromatogram was developed under the same conditions. Six fractions were collected by hand in 1.5 ml vials and dried using a Speed Vac. The samples were redissolved in 300 µl aliquots of Milli-Q water and dried twice to remove residual ammonium bicarbonate. Portions of the hFSH fraction were analyzed by Western blot analysis, and
the relative densities of the 21 and 24 kDa bands used to determine the relative abundance of di- and tetra-glycosylated hFSH, respectively.

**Tandem-column pituitary hFSH glycoform abundance analysis**

Pituitary hFSH was chromatographed using two 10 X 300 mm Superdex 75 columns connected in tandem. Otherwise, chromatographic conditions were as described above except absorbance at 280 or 210 nm were recorded in separate experiments. Aliquots estimated to contain 1 µg hFSH were analyzed 3 times at each wavelength. Relative abundance was determined and compared with Western blot-determined abundance.

**Urinary hFSH isolation**

**Participants**

Four normal women who had regular (24-30 day) menstrual cycles and were not using steroid contraceptives were recruited. They provided urine samples each day during a complete menstrual cycle. Volunteers were classified in either one of two groups, a young cycle group (WSU-2, WSU-4, WSU-5 and WSU-6), and a perimenopausal group (WSU-3).

**Ethics**

All volunteers gave written, informed consent before entering the study. Subjects were enrolled in the department office and assigned a subject number (WSU#) to maintain confidentiality. All urine containers were labeled only with subject number, cycle day, and date.
Urine Samples

All subjects in the study were provided with instructions for urine collection and storage at the time of enrollment. Daily, early morning, first void urine samples were collected in a plastic container, one for each day of the cycle, without any additions and stored in a refrigerator.

Affinity purification with Affi-gel blue beads

The pH of each urine sample was adjusted to 8.6 using sodium hydroxide (NaOH). Next, 2 ml of Affi-gel blue beads were added to each sample and stirred for 10 minutes. The urine containing suspended Affi-gel beads was transferred to a 1 L Sorvall centrifuge bottle and the beads recovered by 5-minute centrifugation at 500 rpm in the Sorvall RC-3B centrifuge. The supernatant was decanted back into the original urine container and the beads transferred to a 50 ml polypropylene culture tube. The beads were concentrated with a 5 min centrifugation. The supernatant was decanted and the beads transferred to a 20 ml Bio-Rad Econo-Column. The beads were washed with 20 ml phosphate buffered saline, pH 8.6, (washing buffer), the wash was collected in a 50 ml centrifuge tube, and then the beads were eluted with 15 ml 0.1 M sodium phosphate buffer, pH 7.4, containing 2 M NaCl. The effluent was collected in an Amicon Ultra-15 (10,000 MW cutoff) and concentrated to 1 ml by centrifugation at 4°C for 30 minutes at 2000 rpm. The concentrated bound fraction was stored at -20°C until immunoaffinity chromatography.

Urinary Protein Ethanol Precipitation
All procedures were performed at 4°C. Urine volume was measured for each specimen and a 4 ml sample was removed for FSH and LH measurements by RIA and stored in the freezer. The remaining sample was mixed with 4 volumes of 200-proof ethanol in a 2 L beaker. A separatory funnel was used to deliver ethanol at a rate of 100 ml/min, with constant stirring until all the ethanol was delivered. After that, the sample was stirred for 1 h. Then the stir bar was removed and the sample allowed to stand overnight in order to complete protein precipitation. The following day, 2/3 of the supernatant was decanted and the remainder swirled to suspend the protein precipitate. The suspension was transferred to a 1 L Sorvall centrifuge bottle and centrifuged at 4°C for 20 min at 3000 rpm in a Sorvall RC-3B+ centrifuge to separate the protein from the ethanol. After that, the supernatant was removed and the pellet extracted four times with 10 ml 0.1 M sodium phosphate buffer, pH 7.0, containing 0.3 M sodium chloride. The extracts were combined in a 50 ml Sorvall polypropylene centrifuge tube, and centrifuged for 20 min at 12,000 rpm in a Sorvall (Thermo Electron Corp., Asheville, NC) RC-26 refrigerated high-speed centrifuge at 4°C. The supernatant was transferred to a 50 ml tissue culture tube and stored at -20°C until immunoaffinity chromatography.

**Immuonoaffinity chromatography**

The 50 ml tube containing urinary proteins was thawed and placed in an ice bath. If precipitate formed during storage, the sample was filtered before immunoaffinity chromatography was performed. The affinity column was composed of an anti-hFSHβ monoclonal antibody, designated 46.3H6.B7, coupled to 2-ml of GE Healthcare agarose (Walton et al. 2001). The column was equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl for 20 min. After that, the inlet tubing from the affinity
column was placed in the bottom of the tube containing urinary protein and the outlet tubing placed at the top of the same tube. A peristaltic pump recycled the sample through the affinity column twice at a flow rate of 1 ml/min. The column was then washed with the same buffer for 20 min, or until the 280 nm absorbance returned to baseline. The bound fraction was eluted with 0.1 M glycine-HCl, pH 2.7, containing 0.5 M NaCl, and collected in an Amicon Ultra-4 (10,000 MW cutoff) centrifugal ultrafiltration cartridge containing 400 μl 1 M Tris-HCl, pH 9.5. The fraction was mixed by inversion 3 times and centrifuged 20 min at 3000 rpm in the H6000 rotor of a Sorvall RC-3B+ centrifuge to reduce the volume to less than 200 μl. The retentate was transferred to a 0.5 ml microfuge tube, the Amicon Ultra-4 chamber washed 2 times with 100 μl Milli-Q water to recover residual bound fraction, and dried using a Savant Speed Vac. Dry samples were stored at -4°C until gel filtration chromatography.

**Single-column gel filtration–Western blot urinary hFSH analysis**

Antibody-bound hFSH was applied to a 10 X 300 mm Superdex column equilibrated with 0.2 M ammonium bicarbonate containing 20% acetonitrile at a flow rate of 0.4 ml/min. Fractions were collected by hand based on retention times determined for pituitary hFSH, dried in a Speed Vac, and dried two additional times following addition of 300 μl water. The hFSH fraction was dissolved in 20 μl SDS sample buffer, boiled, and a 15 μl aliquot analyzed by Western blotting.

**Glycoform abundance determined by tandem column gel filtration**

Antibody-retained samples were dissolved in 50 μl 0.2 M ammonium bicarbonate containing 20% acetonitrile and applied to a pair of 3.2 X 300 mm Superdex 75 gel filtration columns linked in series and equilibrated with 0.2 M ammonium bicarbonate /
20% acetonitrile at a flow rate of 0.03 ml/min. The chromatogram was developed under the same conditions. Six fractions were collected by hand in 1.5 ml vials and dried using a Speed Vac. The samples were redissolved in 300 μl aliquots of Milli-Q water and dried twice to remove residual ammonium bicarbonate. Di-glycosylated and tetra-glycosylated hFSH were identified by retention times as compared with pituitary glycoforms and confirmed by immunoassay.

SDS-PAGE

SDS-PAGE was performed using a BioRad Protean III Mini gel apparatus. Samples were electrophoresed on 15% polyacrylamide gels at constant voltage of 200 V, using the discontinuous buffer system of Laemmli (Cleveland et al. 1977). BioRad broad range prestained molecular weight marker proteins used in SDS-PAGE consisted of a set of proteins with molecular weights of 10kD, 15kD, 25kD, 37kD, 50kD, 75kD, 100kD, 150kD, and 250kD (Walton et al. 2001). These provided visual confirmation of protein transfer to PVDF.

Western Blot

Following SDS-PAGE, proteins were transferred to Immbilon-P PVDF membranes at 4°C for 2 h using a BioRad Protean II Mini gel apparatus with a constant voltage of 100 V. The transfer buffer was composed of 25 mM Tris-HCl, pH 7.4, 190 mM glycine, and 20% methanol. The membrane was air-dried and the dried membranes probed with an anti-hFSHβ-specific antibody. All incubations were carried out at 25°C. Membranes were incubated with either monoclonal antibody (MAb) RFSH20 (diluted 1:5000) in 15 ml of Western Blotting Buffer (WBB) or anti-FSHβ peptide monoclonal antibody P03 (diluted 1:1000). WBB was composed of 150 mM sodium chloride, 1 mM
EDTA, 50 mM Tris-HCl, and 0.05% Tween 20, containing 5% milk. Incubations with the primary antibody were carried out for 1 hour. Membranes were then washed three times with 100 ml of distilled water for 5 minutes each, followed by one 100 ml wash in WBB for 1 minute. Secondary antibody (anti-IgG peroxidase conjugate) specific for mouse IgG was added to 15 ml of 5% milk in WBB, incubated with the PVDF membrane for 30 minutes, and washed as described above. Chemiluminescence was developed using the GE Healthcare ECL-Plus reagents, and measured in a BioRad VersaDoc 4000 using the Quantity One application.

**Human LH Radioimmunoassay**

In order to establish the timing of the mid-cycle LH surge, the concentration of hLH was determined on each day of the menstrual cycle using a specific hLH RIA. Uniform logarithmic concentrations of cold hormone were prepared in RLA buffer composed of: 0.1 M Tris HCl, pH 7.4, containing 0.02% sodium azide and 0.1% RIA grade bovine serum albumin (BSA). Uniform logarithmic dilutions of cold hormone in 100 µl RLA buffer were added to 12 X 75 mm polypropylene tubes containing 100 µl RLA buffer. The dosage ranges were selected to generate a semi logarithmic dose-response plots that were used for determination of hLH concentrations on each day of the menstrual cycle. Tracer and antibody were diluted in phosphate buffer: 0.05 M sodium phosphate, pH 7.4, containing 0.5% sodium azide and 0.5% RIA grade BSA (Sigma, St Louis MO). The tracer used was $^{125}$I-hLH (AFP-4305A). Constant amounts of antibody (AFP-0870124 diluted 1:30,000), cold hormone and tracer (~30.000 cpm/0.1ml) were added to duplicate polypropylene tubes, vortexed and incubated for 18 hours at 4°C. Blank control tubes containing phosphate buffer and tracer, but no cold
hormone were included to measure specific binding. Tubes with excess hLH (1 µg) served as nonspecific binding tubes. Following incubation, tubes were first transferred to an ice bath. Next, 400 µl decomplemented calf serum diluted 1:3 with phosphate buffer was added to each tube in the ice bath. Then, 1 ml of 20% polyethyleneglycol 800 diluted in phosphate buffer was added to each tube. The tubes were vortexed 30 seconds and incubated for 10 minutes in the ice bath. After incubation, the tubes were centrifuged for 50 minutes at 3500 rpm using a Sorvall RC-3B+. The supernatant was aspirated and pellets were counted in a Packard Cobra II AutoGamma counter.

**hFSH Radioimmunoassay**

In order to establish the concentration of urinary hFSH during the menstrual cycle, a specific hFSH RIA was utilized. Uniform logarithmic concentrations of cold hormone were prepared in RLA buffer composed of: 0.1 M Tris HCl, pH 7.4, containing 0.02% sodium azide and 0.1% RIA grade bovine serum albumin (BSA). Uniform logarithmic dilutions of cold hormone in 100 µl RLA buffer were added to 12 X 75 mm polypropylene tubes containing 100 µl RLA buffer. The dosage ranges were selected to generate a semi logarithmic dose-response plots that were used for determination of hFSH concentrations. Tracer and antibody were diluted in phosphate buffer: 0.05 M sodium phosphate, pH 7.4, containing 0.5% sodium azide and 0.5% RIA grade BSA (Sigma, St Louis MO). The tracer used was $^{125}$I-tetraglycosylated hFSH. Constant amounts of antibody, cold hormone and tracer (~30,000 cpm) were added to duplicate polypropylene tubes, vortexed and incubated for 18 hours at 4°C. Blank control tubes containing phosphate buffer and tracer, but no cold hormone were included to measure specific binding. Tubes with excess hLH (1µg) measured nonspecific binding. Following
incubation, tubes were first transferred to an ice bath. Next, 400 µl decomplemented calf serum diluted 1:3 with phosphate buffer was added to each tube in the ice bath. Then 1 ml of 20% polyethylene glycol 800 diluted in phosphate buffer was added to each tube. The tubes were vortexed 30 seconds and incubated for 10 minutes in the ice bath. After incubation, the tubes were centrifuged for 50 minutes at 3500 rpm using the RC-3B+ centrifuge. The supernatant was aspirated and pellets were counted in a Cobra II AutoGamma counter.

**ELISA**

A non-radioactive alternative assay for FSH measurements, developed by Dr. Alan Brown, was also used in this study. Briefly, borate saline buffer (BSB), 0.01 M borate buffer, pH 8.2, containing 0.14 M NaCl was used as diluent and plate washing solution throughout. Falcon 3910 96-well polystyrene round-bottomed plates (Becton Dickinson Labware, Oxnard, CA,) were coated with the desired antigen/antibody by applying it in 100 µl of BSB in the range of 10-100 ng/well and incubating for variable times ranging from 3 hrs to overnight. Following antigen/antibody adherence, plates were washed twice with BSB and wells then filled with 1% bovine serum albumin (Sigma Aldrich, St. Louis, MO, A-7888) in BSB (BSB-BSA), covered, and stored at 4° C until use. Just prior to use, the plates were washed twice with BSB and 25 µl of BSB-BSA was added to all wells to keep them moist.

FSH was captured using the monoclonal INN-hFSH-117 (Berger et al. 1988, Madersbacher et al. 1993) as capture antibody. Wells were coated with 100 ng antibody/well for 3 hours at 25° C. The wells were washed twice with BSB and 25 µl of 1% BSA in BSB were added to each well. FSH preparations for testing as well as
controls (0.215 ng, 0.464 ng, 1 ng, 2.15 ng, 4.64 ng and 10 ng) were delivered to wells in 100 µl 1% BSA in BSB and allowed to bind for 3 hours at 25 °C or overnight at 4 °C.

Following this binding step, plates were washed six times with 0.005% Tween 20 (Sigma Aldrich, St. Louis, MO, P-1379) in BSB. Monoclonal antibody INN-hFSH-6 with specificity for hFSHβ epitope β3, which was present in both diglycosylated and tetraglycosylated hFSH, was conjugated with biotin for the purpose of detection and amplification of monoclonal antibody binding to FSH. INN-hFSH-6-Biotin was delivered to micro-wells at 10 ng/well in 100 µl BSA in BSB incubated for 3 hours at 25 °C or overnight at 4 °C. Following another series of six washes with 0.005% Tween 20. Rabbit anti-Biotin (Bethyl Laboratories, INC, Montgomery, TX, #A150-109A) diluted 1:4000 in 100 µl 1% BSA in BSB was applied to wells for 3 hours at 25°C or overnight at 4°C. This was followed by six washes with 0.005% Tween 20. HRP-goat anti-rabbit IgG, diluted 1:5000, in 100 µl 1% BSA in BSB was applied to wells for 3 hours at 25°C or overnight at 4°C. Plates received a final series of 6 washes with 0.005% Tween 20, prior addition of substrate.

Micro plates were developed for enzyme mediated substrate conversion using 100 µl of 2 mM soluble dye indicator 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (Sigma-Aldrich, St. Louis, MO, catalog number #A1888) and 2.5 mM hydrogen peroxide (H₂O₂) substrate in 0.1 M acetate, 0.05 sodium phosphate buffer, pH 5.0. Color conversion was read after 20 and 40 minutes at 405 nm using an ELx800 optical plate reader (Bio-Tek Instruments, Winooski, VT). Optical Density (OD) readings for all wells on plates were corrected for an average empty plate
background (0.057). Averages of triplicate OD readings for individual samples were also corrected by subtracting the triplicate average background associated with wells receiving all the reagents except the experimentally tested variable. Graphical representations of data were rendered using KaleidaGraph software (Synergy Software, Reading, PA).
RESULTS

As part of the laboratory-wide effort to define glycoform abundance in individual humans, FSH was isolated from 8 pituitary glands (Table 1). A series of 1 µg samples were analyzed by Western blotting in triplicate. Representative results are shown in Fig. 2. Relative abundance of di-glycosylated hFSH was determined by comparing the relative density of the 21 and 24 kDa bands (Table 1). Di-glycosylated hFSH abundance ranged from 6.8-45.3% in 5 women and 56.4-31.9% in two men. The coefficients of variance ranged from 39.24% in males to 53.85% in females. The findings from this study were combined with previous data from individual human pituitaries. Based on uterine histology, the stage of the menstrual cycle was determined for 4 individuals (Fig. 3). Di-glycosylated hFSH abundance was low in the mid-follicular stage individual and high in the late follicular stage individual. An early luteal phase exhibited low abundance in di-glycosylated hFSH, while a higher abundance was associated with a mid-luteal phase individual. Plotting di-glycosylated hFSH abundance against age revealed the highest abundance of di-glycosylated hFSH in the youngest individuals, variable, but overall intermediate abundance in perimenopausal individuals, and low abundance in postmenopausal women (Fig. 4).
Figure 2. Western blot analysis of hFSH isolated from individual human pituitary glands. FSHβ subunit was detected with FSHβ-specific monoclonal antibody, RFSH20, in all four analyses. Pre-stained BioRad Precision proteins were used as MW markers.
A 1 µg sample of hFSH was used as standard in all four blots. Sample identities are shown above the blot. All samples represent 1 µg of hFSH.
Table 1. Relative abundance of diglycosylated hFSH in individual human pituitary glands. The relative abundance represents percentage of the total densities of the 21 and 24 kDa bands.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>Age</th>
<th>% Di-glycosylated FSH (35.4 ± 15.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAR-II-31-3</td>
<td>Unknown</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td>MAR-II-31-4</td>
<td>Female</td>
<td>45.3</td>
<td></td>
</tr>
<tr>
<td>MAR-II-31-6</td>
<td>Female</td>
<td>41.4</td>
<td></td>
</tr>
<tr>
<td>MAR-II-31-7</td>
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<td></td>
</tr>
<tr>
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<td>Female</td>
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<td></td>
</tr>
<tr>
<td>MAR-II-31-10</td>
<td>Male</td>
<td>56.4</td>
<td></td>
</tr>
<tr>
<td>MAR-II-31-11</td>
<td>Male</td>
<td>31.9</td>
<td></td>
</tr>
</tbody>
</table>

N.D Not detected
_* Unidentified pituitary from the National Hormone and Pituitary Program
Figure 3. Pituitary FSH glycoform abundance during the human menstrual cycle.

Each bar represents di-glycosylated hFSH abundance in a single pituitary gland. The uterine histology for these individuals identified the stage of the menstrual cycle. Because these glands were obtained from existing pathology samples, this information was available for only these 4 individuals.
Figure 4. Age-related changes in pituitary FSH glycoform abundance. Each point represents the mean di-glycosylated hFSH abundance for an individual human pituitary gland. Data from Table 1 as well as data obtained by other members of the laboratory are included.

Comparison of pituitary FSH glycoform abundance determined by Western blot and gel filtration

Human FSH was isolated from three pituitary glands, designated MAR-II-304, MAR-III-1 and MAR-III-2. Using 5.3 µg of GonalF as a reference, the amount of hFSH recovered from each gland was, 28.8, 2.2, and 15.5 µg, respectively. A 10 µg sample of
hFSH recovered from either MAR-II-304 or MAR-III-2 was subjected to tandem-column, Superdex 75 gel filtration chromatography (see chromatogram inset, Fig. 5). The retention time of first peak corresponded to that of purified tetraglycosylated hFSH, while that of the second peak corresponded to diglycosylated hFSH. Diglycosylated hFSH accounted for 30.8% of the hFSH isolated from MAR-II-304 and 24.5% of the hFSH isolated from MAR-III-2. Western blot analysis of the glycoform peaks revealed that the putative tetra-glycosylated hFSH peaks possessed both 24 and 21 kDa subunit bands; however, more than 1 µg was analyzed in the blot. The putative di-glycosylated hFSH peaks provided approximately 1 µg samples of hFSH and only the 21 kDa bands were detected (Fig. 6).
Figure 5. Human FSH glycoforms isolated from individual human pituitary glands.

Human FSH was recovered from individual pituitaries by immunopurification followed by Superdex 75 gel filtration using a single, 10 X 300 mm column, as described under Methods. The solid bars show the portion of the chromatogram pooled to obtain hFSH. FSH glycoforms were separated by tandem-column gel filtration using two, 10 X 300 mm Superdex 75 columns. **A.** FSH isolation from pituitary MAR-II-304. **Inset:** 10 µg MAR-II-304 FSH subjected to tandem-column chromatography. The retention time of first peak corresponded to that of tetrarlycosylated hFSH, while the second peak corresponded to that of diglycosylated hFSH. **B.** FSH isolation from pituitary MAR-III-1. **C.** FSH isolation from pituitary MAR-III-2. **Inset:** 10 µg MAR-III-2 FSH subjected to tandem-column chromatography. The first peak’s retention time corresponded to that of tetrarlycosylated hFSH, while the second peak corresponded to diglycosylated hFSH.

Figure 6. FSH glycoform abundance in individual pituitary hFSH preparations.

Samples derived from the inset chromatograms in Figs. 5A and 5C estimated to contain ≈1 µg hFSH, based on a preliminary Western blot, were subjected to SDS-PAGE under reducing conditions and transferred to PVDF membranes as described under Methods.
FSHβ immunoactivity was detected with FSHβ-specific monoclonal antibody, RFSH20, diluted 1:5000. The prestained markers in lane 1 were visualized by epiillumination of the PVDF membrane, while immunoreactive hFSHβ in lanes 2-8 was detected by chemiluminescence. Lane 1, BioRad Precision, pre-stained MW marker proteins; lane 2, 1 µg hFSH (AFP-4161B); lane 3, 800 ng MAR-II-304; lane 4, 1 µg MAR-II-304A; lane 5, 500 ng MAR-II-304B; lane 6, 750 ng MAR-III-2; lane 7, 500 ng MAR-III-2A; lane 8, 500 ng MAR-III-2B.

To establish whether or not the relative abundance estimates for di- and tetraglycosylated hFSH determined by either gel filtration and Western blot analysis were comparable, 5 µg aliquots of pituitary hFSH preparation, MAR-II-31.11 were evaluated with both methods (Fig. 7). Western blotting indicated a diglycosylated hFSH abundance of 36% ± 1.57%. Gel filtration experiments performed in triplicate and monitored at either 210 or 280 nm both provided estimates of 36.4% di-glycosylated hFSH in samples of the same preparation. One-way analysis of variance indicated no significant difference in the diglycosylated hFSH abundance. These results validated earlier studies in which FSH glycoform abundance was determined by Western blot analysis and provided an alternative method for estimating glycoform abundance.
Figure 7. FSH glycoform abundance determined by Western blotting and by gel filtration chromatography. A. Single-column isolation of hFSH immunopurified from pituitary MAR-II-31.11. The closed bar shows the portion of the chromatogram pooled to obtain the hFSH fraction. The inset shows a Western blot of the hFSH fraction.
Six aliquots of hFSH derived from the pituitary, MAR-II-31.11, were subjected to Superdex 75 gel filtration. UV absorbance was monitored at either 210 or 280 nm. Relative abundance of glycoforms was determined by comparing chromatogram peak heights or Western blot band densities for the same sample. The values represent the means of triplicate determinations. B. Representative chromatogram monitoring absorbance at 210 nm. C. Representative chromatogram monitoring absorbance at 280 nm.

**Comparison of urinary hFSH recovery following Affi-Gel Blue affinity adsorption or ethanol precipitation**

Urine samples from volunteers WSU-2 and -4, were used to compare the performance of Affi-Gel Blue affinity adsorption with ethanol precipitation for the recovery of urinary hFSH. Affi-Gel Blue beads were used to capture proteins in the set of samples represented in Fig. 8. Following immunopurification, the samples were chromatographed using a Superdex 75 column and two fractions, the first corresponding to the retention time for pituitary hFSH and the second corresponding to the retention time for hFSHβ, were collected, protein recovered by evaporation, and probed with hFSHβ monoclonal antibody PO3 (Figs. 8A and 8B). Both blots revealed the hFSHβ immunoactivity migrating with an apparent molecular weight of 10 and 12 kDa. The pituitary hFSHβ immunoreactivity migrated at 21-24 kDa. While pituitary hFSH blots were largely devoid of evidence of proteolysis, it was clear that the hFSHβ in these urinary hFSH preparations had been nicked by protease, possibly at the protease-sensitive site Tyr38-Lys39 (Shome B et al. 1988). The PVDF membranes were stripped and reprobed with hFSHα monoclonal antibody HT13 (Figs. 8C and 8D). The same
bands reappeared at 10 and 12 kDa. Because the patterns were identical to those in panels A and B, we concluded that P03 was only partially stripped from the membrane using the mild conditions necessary to avoid removing FSH subunits, and the α subunit was below the detection limit of HT13.

Figure 8. Urinary FSH affinity purification with Affi-Gel Blue beads. Western blot analysis of hFSH isolated from individual urine samples (WSU-2). A-B. Probed with
FSHβ-specific monoclonal antibody, PO3, diluted 1:1000. C-D. Membranes stripped, then reprobed with FSHα-specific monoclonal antibody, HT13, diluted 1:1000. Cruz Marker™ from Santa Cruz Biotechnology was used as MW marker that was detectable by chemiluminescence. A 1 µg sample of pituitary hFSH was used as standard in each blot. Sample identities shown above each blot indicate menstrual cycle day and the putative hFSH-containing fraction, which varied from chromatogram to chromatogram. A. Putative hFSH heterodimer fractions. B. Putative hFSHβ fractions. C. Putative hFSH heterodimer fractions. C. Putative hFSHβ fractions.

To compare the efficiency of FSH recovery using Affi-Gel Blue adsorption with FSH recovery following ethanol precipitation both methods were performed on a set of samples obtained from subject, WSU-4, (Fig. 9) FSHβ immunoactivity was isolated by immunopurification, and FSH related fractions isolated by Superdex 75 chromatography. Three fractions corresponding to aggregated hFSH, hFSH heterodimer, and hFSHβ were collected based on retention times for the corresponding fractions derived from pituitary glands. Proteins were recovered from each fraction by evaporation, blotted and probed with hFSHβ specific polyclonal antibody W556. This antibody was employed because the previous experiment expended the last of the monoclonal antibody P03. The Affi-Gel Blue fractions possessed only the nicked forms of hFSH and band intensities were lower than those in the ethanol-precipitated fractions. The ethanol-precipitated aggregated fractions (day 3 F3 and day 5 F4), possessed hFSHβ immunoactivity at 50, 24, 21, 21, and 10 kDa. The 10 and 12 kDa
bands suggested partial proteolysis, since the 24 kDa band was still present. The 50 kDa bands represented aggregated hFSH. In the putative hFSH heterodimer fractions (day 3 F.4 and day 5 F.5) only the 10 and 12 kDa bands were present, indicating quantitative proteolysis of the β subunit despite its association with the α subunit. FSHβ fragment bands at 10 and 12 kDa were the only products associated with these fractions. Partial nicking has been associated with individual pituitary gland hFSH purifications. We concluded that ethanol precipitation provided better recovery of intact hFSH than AffiGel Blue adsorption. However, the problem with proteolysis still remained. The next method taken into consideration as a source of proteolytic enzymes was the gel filtration column.

Figure 9. FSH purification following either Affi-Gel Blue adsorption or ethanol precipitation of urine samples. Western blot analysis of hFSH isolated from individual urine samples (WSU-4). FSHβ subunit was detected with FSHβ peptide-specific polyclonal antibody, W556, diluted 1:1000. A. Affi-Gel Blue-bound hFSH. Lane MWM
Bio Rad, Precision Plus, prestained high MW markers detected by epi-illumination; lane hFSH, 200 ng pituitary hFSH; lane Day 2 F3, cycle day 2 FSH heterodimer fraction; lane Day 2 F4, cycle day 2 free-hFSHβ fraction; lane Day 4 F4, cycle day 4, hFSH heterodimer fraction; lane Day 4 F5, cycle day 4 free-hFSHβ fraction; lane Day 6 F4, cycle day 6, hFSH heterodimer fraction; lane Day 6 F5, cycle day 6 free-hFSHβ fraction.

B Ethanol precipitated hFSH. Lane MWM Bio Rad, Precision Plus, prestained high MW markers detected by epi-illumination; lane hFSH, 100 ng pituitary hFSH; lane Day 3 F3, cycle day 3 FSH aggregate fraction; lane Day 3 F4, cycle day 3 FSH heterodimer fraction; lane Day 3 F5, cycle day 3 free-hFSHβ fraction; lane Day 5 F4, cycle day 5 FSH aggregate fraction; lane Day 5 F5, cycle day 5, hFSH heterodimer fraction; lane Day 5 F6, cycle day 5 free-hFSHβ fraction.

**Urinary glycoform abundance confounded by proteolysis**

The Superdex 75 gel filtration column was considered to be the possible source of protease due to bacterial contamination, because this column had been used for hFSH gel filtration of recombinant hFSHβ derived from *Eschericia coli* inclusion bodies. It was incorrectly assumed that no bacteria should survive in 20% acetonitrile, and the data from previous blots contradicted that assumption. Proteolysis appeared eliminated when the column was purged with 1 ml of 6 M GuHCl immediately before subjecting 2 µg samples of purified hFSH (Fig. 10).
Figure 10. Gel filtration after pre-treating a Superdex 75 column with 1 ml of 6 M GuHCl. Western blot analysis of 3 samples of pituitary hFSH following gel filtration over the guanidine-HCl purged column. FSHβ subunit was detected with FSHβ peptide-specific polyclonal antibody, W556, diluted 1:1000. Lane 1, MWM Bio Rad, Precision Plus, prestained high MW markers detected by epi-illumination; lane 2, 500 ng hFSH; lane 3, 250 ng hFSH; lane 4, 125 ng hFSH.

Glycoform abundance determined by tandem-column Superdex 75 chromatography

While, urinary hFSH can be detected using 10 X 300 mm columns when the samples are obtained from postmenopausal urine, as first void urine samples provide
about 1 µg samples, cycling women produce only 10% postmenopausal levels of FSH and the amount provided in urine is undetectable at 210 nm. The availability of 3.2 X 300 mm Superdex 75 columns offered a potential 10-fold increase in sensitivity and coupling two columns in series was found to separate di- and tetracygosylated hFSH glycoforms. We analyzed several urinary hFSH samples to see if glycoform abundance could be assessed by directly applying the immunoaffinity-purified urinary FSH to two 3.2 X 300 mm Superdex 75 columns coupled in series. As can be seen in Fig. 11, hFSH glycoform peaks emerged with the same retention times as pituitary hFSH glycoforms. As a result, we applied this analytical procedure to daily urine samples.
Figure 11. FSH glycoform separation by tandem Superdex 75 gel filtration chromatography. Samples of immunopurified urinary hFSH were applied to two 3.2 X 300 mm Superdex 75 columns linked in series, and equilibrated with 0.2 M ammonium bicarbonate containing 20% acetonitrile at a flow rate of 0.03 ml/min. A reference
chromatogram showing the tetra- and diglycosylated hFSH glycoforms was overlaid on each panel. A. WSU-5, cycle day 8, follicular phase. B. WSU-5, cycle day 9, follicular phase. C. WSU-4 cycle day 16, phase unknown. D. WSU-4 cycle day 17.

Glycoform abundance in regularly cycling young women assessed by tandem Superdex 75 chromatography

Urine samples were collected from individuals between the ages of 21 and 30, who reported regular menstrual cycles and who were not using steroid contraceptives. Individual urine samples were subjected to ethanol precipitation followed by immunoaffinity chromatography. FSH glycoform abundance was determined by tandem Superdex 75 gel filtration chromatography using two 3.2 X 300 cm columns, as shown above. Single cycles were analyzed for 2 individuals, while two cycles were examined for one subject, WSU-5.

Glycoform abundance during the first of two cycles provided by 27 year-old subject, WSU-5, was measured in 15 of 27 samples. Samples for days 3 and 4 were not provided by the subject, chromatograms for days 12, 13 and 21 were affected by technical issues involving the HPLC system, chromatograms for days 10, 17, and 20 could not be integrated in the same manner as the others due to low peak heights, and FSH recoveries were below the limit of detection in the remaining days. The ovulatory gonadotropin surge occurred on day 13. Diglycosylated FSH abundance was low on days 1-7, rose on day 8, progressively decreased on days 9-12, and then bounced around for the rest of the cycle. No obvious pattern emerged (Fig. 12).
Figure 12. Di-glycosylated hFSH abundance during a 27–day cycle. The subject WSU-5 and this was the first of two cycles analyzed. Each bar represents the relative abundance of di-glycosylated hFSH recovered from urine on each day of the cycle.

Glycoform abundance during the second WSU-5 cycle was measured in 12 of 22 samples. The sample for day 10 was not provided by the subject, the day 14 chromatogram was affected by technical issues involving the HPLC system, chromatograms for days 1, 2, 8, 11, and 13 could not be integrated in the same manner as the others due to low peak heights, and FSH recoveries were below the limit of detection in the remaining days lacking data. Diglycosylated hFSH abundance was 50% for the first 11 days. It rose to a maximum of 95% on day 13 and remained high until day 16, the last day FSH was measured in this sample set. According to LH RIA, the preovulatory surge occurred on day 16 (Fig.13).
Figure 13. Di-glycosylated hFSH abundance during a 22–day cycle. This was the second cycle analyzed in subject WSU-5. Each bar represents the relative abundance of di-glycosylated hFSH recovered from urine on each day of the cycle.

Glycoform abundance for a 25 year-old subject, **WSU-6**, was measured in 12 of 19 samples using tandem Superdex 75 gel filtration. Chromatograms for days 6 through 11 could not be integrated in the same manner as the others due to a large peak that emerged immediately after the diglycosylated hFSH peak. FSH recoveries were below the limit of detection in the remaining days showing no data. LH RIA indicated that the pre-ovulatory gonadotropin surge occurred on day 16. The pattern of diglycosylated hFSH abundance showed a progressive rise until day 12, followed by a progressive decline until day 19 (Fig.14).
Figure 14. Di-glycosylated hFSH abundance during a 19–day cycle. The subject was WSU-6. Each bar represents the relative abundance of di-glycosylated hFSH recovered from urine on each day of the cycle.

Glycoform abundance during a Perimenopausal Cycle

Glycoform abundance for a 43 year-old subject, WSU-3, was measured in 18 of 24 samples using tandem Superdex 75 gel filtration. The day 3 chromatogram could not be integrated in the same manner as the others due to low peak heights, chromatograms for days 8, 9,13,14,15, 16 and 20 included a large peak that emerged right before the tetra-glycosylated hFSH peak, and FSH recoveries were below the limit of detection in the remaining days showing no data. LH RIA indicated that the gonadotropin surge occurred on day 10. Di-glycosylated hFSH was elevated at the
beginning of the cycle and displayed a progressive decline over the next 20 days (Fig.15).

![Graph showing % Diglycosylated hFSH](image)

**Figure 15. Di-glycosylated hFSH abundance during a 24–day cycle.** The subject was WSU-3. Each bar represents the relative abundance of di-glycosylated hFSH recovered from urine on each day of the cycle.

**Confirmatory Western Blot**

WSU-6, day 13 fractions harvested from the gel filtration chromatogram were analyzed by Western blot using the FSHβ specific polyclonal antibody W556 (Fig.16). Simultaneously, the fractions eluted between 100-120 minutes from samples obtained from subjects, WSU-5-2 (days 17 and 19) and WSU-6 (days 6, 7, 9 and 10), were analyzed under the same conditions to confirm the separation of hFSH glycoforms (Fig.17). hFSH immunoactivity was only detected in 4 out of 6 fractions for WSU-6 day
13 as expected. Fraction 2 revealed only the 24 kDa hFSHβ band indicating only tetraglycosylated hFSH was present. Fraction 3 also possessed only the 24 kDa band. Fraction 4 possessed both the 21 and 24 kDa bands and exhibited the highest level of immunological activity. Fraction 5 possessed only the 21 kDa hFSHβ band, however, the yield was so low that a longer exposure was necessary to detect it. Fraction 6 did not contain hFSH nor did any of the other fractions collected after 100 minutes. In contrast, chromatograms lacking obvious hFSH glycoform peaks lacked detectable hFSHβ immunoactivity (Fig. 17).

Figure 16. Analysis of putative FSH glycoform fractions. A. Western blot of WSU-6 Day 13 fractions obtained by gel filtration. The blot was probed with the FSHβ peptide-specific polyclonal antibody W556 diluted 1:1000. Inset. Longer exposure of sample lanes shown in the boxed area. B. Tandem Superdex 75 gel filtration chromatogram.
Figure 17. Western blot of WSU 5-2 (day 17 and 19) and WSU 6 (day 6, 7, 9 and 10) from fractions obtained by gel filtration with an elution time between 100-120 minutes. Sample identities are shown above the blot. The blot was probed with the FSHβ specific polyclonal antibody W556. Insets at the bottom of the figure show corresponding tandem Superdex 75 chromatograms. Di-glycosylated and tetra-glycosylated hFSH were eluted at 79-80 and 89-90 min, respectively (see arrows).
FSH RIA

FSH was measured in raw urine samples, concentrated urine samples, and unbound fractions after immunopurification sample by radioimmunoassay. Only a single cycle from WSU-5 was evaluated by this assay (Table 2). The assay was not sensitive enough to detect hFSH in most urine samples, but was able to detect it in concentrated urine samples. Following immunopurification, hFSH remained detectable in most unbound fractions except those derived from urine samples with low hFSH concentrations. Results from these samples assayed demonstrate that less than 50% FSH was captured during immunopurification. That was evident from the concentration of FSH in the unbound fractions.

Table 2. Concentrations of human FSH (ng/0.1 ml) in urine samples (WSU-5-1) provided by a young woman on selected days of one menstrual cycle. Values, except where indicated, represent means of duplicate determinations.

<table>
<thead>
<tr>
<th>Day of cycle</th>
<th>Concentrated urine 3ml→0.1ml for assay*</th>
<th>Non-concentrated urine 0.1ml for assay</th>
<th>Unbound fraction from affinity column 0.1ml for assay</th>
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<tbody>
<tr>
<td>8</td>
<td>0.55</td>
<td>0.16</td>
<td>0.21</td>
</tr>
<tr>
<td>9</td>
<td>0.88</td>
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<td>0.06</td>
</tr>
<tr>
<td>13</td>
<td>6.76</td>
<td>0.35</td>
<td>1.03</td>
</tr>
<tr>
<td>15</td>
<td>1.9</td>
<td>N.S</td>
<td>0.21</td>
</tr>
<tr>
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<td>0.53</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>25</td>
<td>0.56</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.S=no sample, N.D=not detectable level, *=single determination in one concentrated sample
**LH RIA**

**Table 3.** Urinary human LH concentrations (ng/0.1 ml) during four menstrual cycles. Values represent means of duplicate determinations. Bold values represent the day of the LH surge.

<table>
<thead>
<tr>
<th>Day of Cycle</th>
<th>WSU-3</th>
<th>WSU-5-1</th>
<th>WSU-5-2</th>
<th>WSU-6</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.77</td>
</tr>
<tr>
<td>2</td>
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<td>-</td>
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<td>0.57</td>
</tr>
<tr>
<td>3</td>
<td>0.51</td>
<td>-</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>0.53</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>-</td>
<td>0.4</td>
<td>0.41</td>
</tr>
<tr>
<td>6</td>
<td>0.36</td>
<td>-</td>
<td>-</td>
<td>0.28</td>
</tr>
<tr>
<td>7</td>
<td>0.33</td>
<td>-</td>
<td>-</td>
<td>0.32</td>
</tr>
<tr>
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<td>0.16</td>
<td>0.49</td>
<td>0.53</td>
<td>0.54</td>
</tr>
<tr>
<td>9</td>
<td>0.28</td>
<td>0.34</td>
<td>0.82</td>
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</tr>
<tr>
<td>10</td>
<td>1.48</td>
<td>0.75</td>
<td>0.54</td>
<td>-</td>
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<tr>
<td>11</td>
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<td>0.6</td>
<td>0.29</td>
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<tr>
<td>12</td>
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</tr>
<tr>
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</tr>
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<td>20</td>
<td>-</td>
<td>0.48</td>
<td>0.51</td>
<td>-</td>
</tr>
<tr>
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<td>N.D</td>
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<td>0.27</td>
<td>-</td>
</tr>
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<td>0.15</td>
<td>-</td>
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<tr>
<td>27</td>
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<td>0.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

N.D= not detected
hFSH abundance determined by ELISA

Samples consisting of 10% of the fractions recovered from the following daily urinary hFSH samples, WSU-5-1, days 1, 2, 15 and 27; WSU-5-2, days 7 and 14; WSU-3, days 1, 4 and 10, were tested in triplicate for the presence of hFSH. The ELISA was performed as described under Methods. Unfortunately, FSH was detected in only 7 out of 68 fractions tested and those limited data are listed in Table 4. The corresponding chromatograms are shown in Fig. 17. When comparing the data obtained from this assay and the data from the gel filtration chromatograms these were similar. For the other samples FSH was present but a concentration lower of the limit of detection, established with purified pituitary hFSH standards, as 0.215 ng.
Table 4. hFSH detected by ELISA. Seven out of 68 samples tested had a detectable amount of hFSH. Fraction 2 corresponded to tetra-glycosylated hFSH while fraction 3 corresponded to di-glycosylated hFSH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day</th>
<th>Fraction</th>
<th>ELISA 10%</th>
<th>Total FSH Recovered</th>
<th>Chromatogram</th>
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</thead>
<tbody>
<tr>
<td>WSU-5-1</td>
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<td>1</td>
<td>0.280</td>
<td>6.743</td>
<td>N.D</td>
</tr>
<tr>
<td>Tetrargosylated</td>
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<td>2</td>
<td>4.493</td>
<td>62.957</td>
<td>69.18</td>
</tr>
<tr>
<td>Diglycosylated</td>
<td>3</td>
<td>3</td>
<td>1.746</td>
<td>25.903</td>
<td>30.82</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>0.225</td>
<td>2.333</td>
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<tr>
<td>Total FSH Recovered</td>
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<td></td>
<td>9.119</td>
<td>91.193</td>
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<table>
<thead>
<tr>
<th>Sample</th>
<th>Day</th>
<th>Fraction</th>
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<tr>
<td>Total FSH Recovered</td>
<td>2</td>
<td></td>
<td>9.119</td>
<td>91.193</td>
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</tr>
</tbody>
</table>
Figure 18. Tandem Superdex 75 chromatograms for samples that tested positive for hFSH by dimer-specific ELISA. The solid bars show the portions of the chromatogram pooled to obtain each fraction. Only 10% of the total protein recovered in each fraction was tested, and the results shown in Table 4. Fraction 2 in each chromatogram corresponded to tetrabulysylated hFSH and Fraction 4 corresponded to diglycosylated hFSH.
DISCUSSION

Our laboratory has been actively developing methods for measuring FSH glycoform abundance in humans. The initial approach utilized for this project was FSH immunoaffinity purification followed by FSHβ-specific Western blot analysis. This approach was originally applied to serum specimens, however, hFSH was not detected and considerable cross-reactivity with human IgG was encountered (Singh and Bousfield, unpublished data). Control studies involving pooled human pituitary extracts revealed two hFSHβ immunoreactive bands at 21 and 24 kDa instead of the single band anticipated (Nguyen and Bousfield, unpublished). Continued investigation lead to the discovery of a non-glycosylated hFSHβ subunit that appeared as a 21 kDa band in Western blots (Walton et al. 2001). The absence of glycans attached to β-Asn residues 7 and 24 was confirmed by MALDI-TOF-MS and automated Edman degradation (Walton et al. 2001). Subsequently, a similar approach was applied to individual human pituitary glands, which consisted of extraction and immunopurification, followed by quantitation by Western blotting. The results of these studies provided the initial evidence that suggested physiological variance in hFSH glycoform abundance (Bousfield GR et al. 2007). However, glycoform abundance was more variable in non-cycling postmenopausal women than in young cycling women. This apparent contradiction was resolved when immunopurified hFSH was fractionated by gel filtration to separate differentially glycosylated hFSHβ subunit from the hFSH heterodimer. As summarized in Fig. 4, glycoform variation increased in pituitaries derived from young women and was largely eliminated in postmenopausal pituitaries. When three batches of Pergonal, a
urinary gonadotropin preparation, were fractionated by gel filtration, no free hFSHβ was detected; suggesting that immunopurification alone would be sufficient to assess glycoform abundance in ethanol precipitated urinary proteins (Bousfield GR et al. 2006). However, analysis of hFSH in individual urine samples from an adult male and a postmenopausal female revealed the presence of free hFSHβ, necessitating the use of gel filtration (Bousfield, unpublished data). An alternative purification procedure was proposed by Dr. Elliott Bedows, University of Nebraska Medical Center, Omaha, NE, that consisted topology-directed ligand affinity purification of FSH using Affi-Gel Blue chromatography followed by reverse-phase HPLC at pH 7.6. While the affinity resin captured hFSH, as shown above, reverse-phase HPLC dissociated hFSH into its constituent subunits (Bousfield, unpublished data). Normally this would not be a problem, as this study was interested in the abundance of 21 and 24 kDa bands, however, reverse-phase HPLC also separated the bulk of the 24 kDa hFSHβ from a mixture of 24 and 21 kDa hFSHβ over several milliliters of HPLC effluent, thereby complicating quantitation (Butnev and Bousfield, unpublished data). Accordingly, in the present study, we attempted to capture FSH from urine samples using Affi-Gel Blue resin, thereby eliminating the overnight ethanol precipitation step. After several experiments the use of this method was abandoned because it appeared to fail to maintain the integrity of the hFSHβ subunit (Fig. 8). As a result, the ethanol precipitation procedure was reevaluated and some intact hFSHβ was obtained, albeit in the aggregated hFSH fraction. Because some urine samples were stored at 4°C for several days prior to gel filtration, immediate precipitation of several fresh urine samples was
evaluated. Most of the hFSHβ immunoactivity continued to migrate as 10 and 12 kDa bands, indicating proteolysis was not occurring in the urine. Other students who employed AffiGel Blue affinity chromatography as a prelude to immunopurification of recombinant hFSH reported reduced recoveries as compared with direct immunoaffinity purification from conditioned medium. More detailed evaluation of the data for this discussion indicated that the only intact hFSHβ recovered by ethanol precipitation was associated with the aggregated FSH fraction. All the hFSHβ associated with the dimer fraction was apparently nicked. While the greater integrity rationale may have been flawed, improved yield still recommended ethanol precipitation over AffiGel Blue adsorption. In any case, the eventual cause of the nicked subunit was determined to be bacterial protease activity in the Superdex columns, which could be eliminated by injection of a bolus of 6 M GuHCl prior to FSH fractionation. Bacterial proteolysis of oLHα subunit preparations ultimately proved responsible for an apparent acid-labile cleavage reported during subunit isolation by counter current distribution (Liu et al. 1977). It may be responsible for the hFSH βL2 loop nick reported during reevaluation of the hFSHβ subunit sequence (Shome B et al. 1988) as well as the urinary hFSHβ fragments encountered in the present study.

Most studies involving hFSHβ Western blots in this laboratory have employed the monoclonal antibody RFSH20 as the primary antibody. The epitope for this antibody is believed to reside near residues 67-72, in the βL3 loop (Robert 1995). When hFSHβ fragments resulting from proteolytic nicks were encountered in studies with this antibody, a single 10 kDa band was typically encountered (Singh and Bousfield,
unpublished). This observation was consistent with RFSH20 recognizing only the non-glycosylated C-terminal peptide, as most FSHβ nicks occur in βL2 in hFSH (Shome B et al. 1988) and in the same loop during protelytic nicking of eFSH preparations (Bousfield, unpublished data), whereas, the epitope is located in βL3. Because detection falls off rapidly below 1 µg hFSH, the antibody was unsuitable for urinary hFSH studies, which typically involve 100 ng samples. P03 is an anti-peptide monoclonal antibody raised against a synthetic peptide consisting of hFSHβ residues 28-50 (Robert 1995), which encompasses βL2 (polyclonal antibody W556 was raised against a similar peptide). The βL2 protease-sensitive region falls in the middle of the peptide sequence used to generate both antibodies. The urinary hFSHβ immunoactivity migrated as a pair of 10 and 12 kDa bands. Initially, we overlooked this because protein migration at the bottom of the gel is somewhat erratic. However, the repeated appearance of both bands suggested that the P03 epitope might be located in the N-terminal half of the 28-50 peptide and the 10 and 12 kDa immunoreactive bands represent the hFSHβ N-terminal peptide and glycopeptide, respectively. Supporting evidence for this hypothesis was provided by hFSHβ expression studies in bacteria that largely generated a 12 kDa band. When compared on the same blot, the urinary hFSH β subunit fragment and the putative recombinant hFSHβ preparations exhibited identical electrophoretic mobilities (Thao Tran, MS thesis, WSU). Automated Edman degradation of the recombinant hFSHβ preparation yielded a low-yield sequence consistent with the predicted N-terminus. The low yield resulted from the retention of the N-terminal formyl-Met residue, which did not react with the Edman reagent. The actual sequence represented a small
amount of the sample lacking the terminal f-Met. However, this could not be confirmed by mass spectrometry because neither the protein nor peptides derived from it ionized. The complications arising from protease cleavage during isolation contributed to the decision to use tandem Superdex 75 chromatography to measure glycoform abundance. As both intact and nicked hFSH molecules would be eluted in the same peak, other sources of proteolysis that might be encountered while processing over a hundred samples would not interfere with chromatographic quantitation.

**Validation of Western blotting glycoform abundance estimates.**

A frustrating reality associated with the study of FSH glycosylation isoforms has been the inability to isolate FSH variants for structure-function studies. Increased hFSH variant size was observed following gonadectomy of rhesus monkeys using Sephadex G-100 chromatography (Peckham WD and Knobil 1976b). However significant overlap between the high and low molecular weight forms prevented isolation of these two variants. Methods based on charge variation revealed the existence of many more FSH variants and these approaches dominated the field until recently, when our laboratory demonstrated that FSH isoforms isolated by chromatofocusing did not differ in glycoform abundance and possessed similar glycan populations at at least two N-glycosylation sites, Asn\(^{52}\) and Asn\(^{24}\) (Bousfield GR et al. 2007).

**Cycle-related changes in FSH glycoform abundance in pituitaries.**

Relative abundance of di-glycosylated hFSH isolated from pituitaries appeared to vary in a cycle-specific manner. Because these glands were obtained from existing pathology samples, the uterine histology of only four cycling females was available, providing limited data that, nevertheless, covered two thirds of the menstrual cycle.
Relative abundance of diglycosylated hFSH varied from a low of 16% in the mid-follicular stage pituitary to a high of 72% in the late follicular stage gland. This was followed by a decrease to 26% in the early luteal phase pituitary and an increase to 46% in the mid-luteal phase pituitary (Fig. 3). Most of these data were consistent with the hypothesis that estrogen feedback from the ovary selectively inhibits hFSH\(\beta\) glycosylation, as di-glycosylated hFSH reached its greatest abundance during the late follicular period, when estrogen concentrations in the blood are high. Very early studies of FSH glycoforms in rhesus and rat females implicated estrogen in altering the relative size of the FSH molecule (Bogdanove et al. 1974, Peckham WD and Knobil 1976a, Peckham WD and Knobil 1976b, Peckham WD et al. 1973). The early luteal decrease in di-glycosylated hFSH abundance was consistent with the postovulatory fall in circulating estrogen. However, the mid-luteal phase increase was inconsistent. Although circulating estrogen concentrations rise during the luteal phase, elevated circulating progesterone should antagonize its actions. Because of the very limited pituitary glycoform abundance data, the possibility of random glycoform variation could not be ruled out and led to the current effort to analyze FSH glycoform abundance in urine samples.

**Cycle related changes in glycoform abundance in urine.**

Preliminary pituitary hFSH glycoform abundance data suggested cycle-related changes associated with circulating estrogen concentrations. Preliminary analysis of follicular phase and probably luteal phase glycoform abundance by tandem-column gel filtration supported the idea that differences would be apparent between the follicular and luteal phases of the cycle. However, when three sets of daily glycoform abundance
measures were compared, the most striking feature was day-to-day variation appeared to obscure any clear physiologically related patterns in glycoform abundance. A general trend in the 25 and 27 year old women was low abundance in di-glycosylated hFSH that increased to a maximum 3-5 days before the LH surge. In contrast, diglcosylated hFSH abundance was higher early in the cycle, then declined until the day of the LH surge, when it rose again, but then dropped and remained low through the luteal phase. While there is no direct correlation between less acidic isoforms and di-glycosylated hFSH (Bousfield GR et al. 2008a), it is interesting to note that less acidic isoforms are low in abundance early in the menstrual cycle and become more abundant during the middle of the cycle (Ulloa-Aguirre et al. 1995).

**Technical issues**

The initial immunoaffinity purifications of hFSH were attended by quantitative hFSHβ nicking by proteolysis such that only a 10 kDa band was observed in Western blots using three monoclonal antibodies, while partial proteolysis was associated with 46.3H6.B7, the monoclonal antibody used in the present study (Singh and Bousfield, unpublished). Addition of protease inhibitors at the time of homogenization and placing the pituitary extract in an ice bucket during immunoaffinity chromatography eliminated proteolysis during individual human pituitary hFSH isolations. The proteolysis problem reemerged when urinary hFSH samples began to be analyzed. At first, urinary proteases were thought to be responsible because some of the urine samples were stored for several days at 4°C. However, when urine samples were immediately processed quantitative proteolysis remained evident. After proteolysis during affinity chromatography had been eliminated as the source of hFSH degradation, we
considered bacterial contamination of the Superdex 75 column could be responsible. This had been a problem when ammonium bicarbonate buffer lacking acetonitrile was used in both conventional and high performance liquid chromatography [(Ward et al. 1989) and unpublished data from our laboratory]. We had incorrectly assumed that no bacteria should survive 20% acetonitrile. Proteolysis was eliminated when the column was purged with 1 ml of 6 M guanidine-HCl immediately before hFSH isolation.

Another technical issue was encountered later while collecting daily urine samples. All subjects were instructed to collect the first urine of the day, but we forgot to mention that it was also necessary to collect any urine voided during the night so that the entire 8 hr period was sampled. While most urine samples were consistent in volume day after day, others were not. Because a first void sample representing the entire sleep period possesses just enough hFSH for Western blot analysis, partial samples fail to provide enough hFSH. During the late luteal phase, when hFSH secretion is highly suppressed 24 hour collections may be necessary to provide enough hFSH for analysis.

**hFSH glycoform recovery after affinity chromatography.**

The first set of chromatograms derived from urine samples provided by subject WSU-5.1 (Fig.12) presented well defined peaks that corresponded with hFSH glycoform peaks, and from which 10% samples provided measurable amounts of hFSH by ELISA. The last set of chromatograms derived from urine samples provided by subject WSU-6 (Fig.15) presented barely detectable peaks and hFSH was undetectable by ELISA. Several reasons could be responsible for these results, including partial urine collection or loss of capacity of the affinity column. While the former were indicated by reduced
urine volumes, the latter appeared to be the cause in the course of another student’s work. After all the assays for this thesis were completed, Sridhar Enuganti, a prospective graduate student, assessed the recovery of hFSH from 5.37 µg samples available in GonalF vials. The 2-ml 46.3H6.B7 anti-hFSHβ affinity column used for the present study recovered only 11% of the recombinant hFSH in a single vial. A 1-ml anti-hFSHβ column prepared around the same time as the 2-ml column was found to recover 38% of the hFSH. Toan Nguyen, an undergraduate student in the lab, assessed recently prepared anti-hFSHβ affinity columns and reported recoveries ranging from 37% to 68% using the same protocol. The 2-ml column had been capable of recovering 15.5-28.8 µg hFSH from individual pituitaries, although the efficiency of FSH harvest remains unknown because RIA’s were not available when these purifications were performed. These results emphasize the importance of following each step of hFSH purification by radioimmunoassay.

**hFSH abundance determined by ELISA**

The estimates of % glycoform abundance from WSU 5-1 Days 1 and 2 chromatograms were fairly close from the ratios obtained by ELISA. Day 1 urine sample possessed 66.8% tetracygosylated FSH as determined chromatographically, while 66.6% was estimated by ELISA. For diglycosylated hFSH 33.24% was estimated chromatographically, while 25.89% was the value obtained by ELISA. Day 2 presented 69.18% tetracygosylated FSH from chromatogram stimatized while 69.04% was obtained by ELISA, and 30.82% Diglycosylated FSH from chromatogram stimatized while 28.40% was obtained by ELISA. The FSH ELISA was able to detect as little as 0.215 ng FSH. We can say that in the samples in which FSH was not detected (61/68), the hormone
was probably below the limit of detection of the assay, due to low recovery by immunoaffinity chromatography.

**Future directions**

Optimization and standardization of the procedure could significantly improve the recovery and quantification of hFSH glycoforms. Fully detailed collection instructions in writing must be provided to each participant so that the total nighttime urine production is consistently captured. Alternatively, 24-hr specimens should be collected. Specimens should be collected in containers that can hold up to 1L, when first morning urine is needed because the volumes ranged from 150 to 650 ml. Obviously, larger containers are necessary for 24-hour urine collections. Ethanol precipitation must be done the same day the sample is collected to avoid freezing-thawing steps that might affect the integrity of the hFSH. Otherwise, a thawing protocol must be established to treat the samples under the same conditions. Immuno purification can be done the following day as well as the gel filtration step. To complete this procedure, for a single sample, three to four days may be necessary.
SUMMARY

This research has provided evidence that changes in hFSH glycoform abundance occur during the normal menstrual cycle. However, day-to-day variability and technical issues interfering with daily glycoform abundance measurements prevented consistent patterns from emerging. Nevertheless, within individual variation of hFSH glycoforms was observed. Better analytical procedures in the future may reveal patterns of glycoform abundance driven by endocrine changes associated with the menstrual cycle.

One of the most important results obtained from this work was the close correspondence between glycoform abundance measured by Western blots performed with monoclonal antibody RFSH20 and tandem-column Superdex 75 gel filtration. This finding established that glycoform abundance measurements made with this antibody did not merely reflect the characteristics of a single antibody, but instead represented the true abundance of both glycoforms that was confirmed by an independent method.
LIST OF REFERENCES


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