MRM based Signature Peptide Quantitation Method for Follicle Stimulating Hormone (FSH) in Human serum

SCIEX QTRAP® 6500 LC-MS/MS System

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Introduction

Follicle stimulating hormone (FSH), a 35.5 kDa gonadotrophic glycoprotein hormone secreted from the anterior pituitary gland plays a key role in regulation of follicular growth in females and spermatogenesis in males[1-4]. Quantification of FSH holds clinical relevance especially in controlled ovarian stimulation regimens for assisted reproductive technologies and hypogonadotrophic hypogonadism. Traditionally quantification of FSH is done using an ELISA based assay or size exclusion UV based high-performance liquid chromatography. ELISA based commercial kits are available in the market but come with varying linear dynamic ranges and sensitivities. Human FSH is a heterodimer of glycosylated α and β subunits present in various isoforms that differ in molecular weight, biological potency, half-life of elimination and immunoreactivity. Quantification of FSH in plasma or serum is challenging due to its presence in low abundance, owing to its site specific action. The present work reports the development of sensitive, reproducible signature peptide quantification workflow with a wide linear dynamic range in serum using the SCIEX 6500 QTRAP system.

Key challenges of FSH Quantitation

- **Poor Sensitivity** – Achieving a high level of sensitivity is difficult due to its low concentration, the presence of isoforms and its short half-life.
- **Lack of reproducibility and repeatability** –Low reproducibility and repeatability for FSH at low levels due to high interference in serum.
- **High level of background** - Extensive sample preparation is required due to high level of background interference.

Unique features of FSH Bioanalytical Quantification in Human Plasma

- **High Selectivity:** This bioanalytical method for FSH in plasma achieves better selectivity using a unique signature peptide, a simple 1 step sample cleanup and a selective chromatographic method.
- **Accuracy and precision:** The accuracy and precision of the signature peptide from plasma QC samples was between 95% and 100% nominal and 10-12% CV meeting the validation requirement for regulated bio-analytical labs.

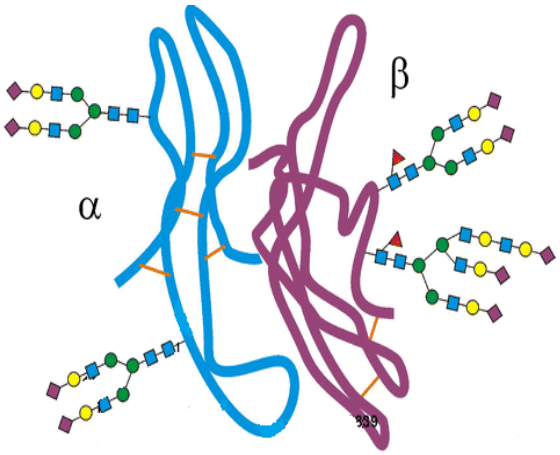

Figure 1: Structure of Follicle Stimulating Hormone (FSH)

SCIEX QTRAP® 6500 System

Materials and Method

Urofollitrophin (FSH) Injection (SITRODIN) B.P. 75/150 IU that was used as the reference standard.

Chromatographic Conditions:

Gradient profile for signature peptide identification from FSH protein digests

Gradient condition were chosen for maximum resolution of the peptides from digested protein using a Shimadzu Nexera LC system with a Phenomenex Luna RP C18, 100 x 2.1 mm, 3.5µm column at 40°C. Peptides were chromatographically separated in 35 minutes with an organic gradient of 5-35% acetonitrile (0.1% formic acid) at a flow rate of 300 µL/min. Ten microliters of digested FSH was injected.

Gradient profile for signature peptide quantitation (MRM)

LC System: Shimadzu Nexera with 30AC auto sampler

Column: Acquity CSH C18, 100 mm × 2.1 mm, 1.7 µ

Column Temp: 45 °C

Injection Volume: 30 µL

Flow Rate: 0.300 mL/min

Mobile phases:(A) 0.1% v/v Formic Acid in Water

(B) 0.1% v/v Formic Acid in Acetonitrile

Time(min)	%B
0.01	5
1.00	5
6.00	10
12.0	10
13.0	50
15.0	50
16.0	80
18.0	80
18.1	5
20.0	5

Table 1: Optimized gradient run for the quantitation of FSH in MRM mode

Sample Preparation

Solid Phase Extraction Various SPE cartridges from different vendors were evaluated to remove the interference from FSH spiked digested matrix during the method development. Based on the recovery experiments, Cleanert PCX, 30cc mixed mode strong cation cartridges from Bonna-Agela were used for final method development and validation. Detail procedure for digestion and sample cleanup were given in table 2 and 3.

Mass Spectrometry Conditions:

SCIEX QTRAP 6500® LC-/MS/MS system with Ion Drive™ Turbo V source was used to set up the peptide selection and MRM experiments. A QTRAP based non-targeted IDA workflow was used to identify a surrogate peptide from a tryptic digest of FSH (Figure 2 & 3). Sequence confirmation of a unique peptide was confirmed using ProteinPilot™ 5.0 software. Skyline software was also used to confirm the precursor of the signature peptide and its fragments. The MRM list obtained from Skyline software was exported to Analyst® 1.6.2 software for acquisition. Source and compound related parameters (table 4) were optimized to achieve highest sensitivity. Surrogated peptide "*ELVYETVR*" m/z 504.7^{2+} **>** 667.2 was identified as the most intense and stable sequence for quantitation.

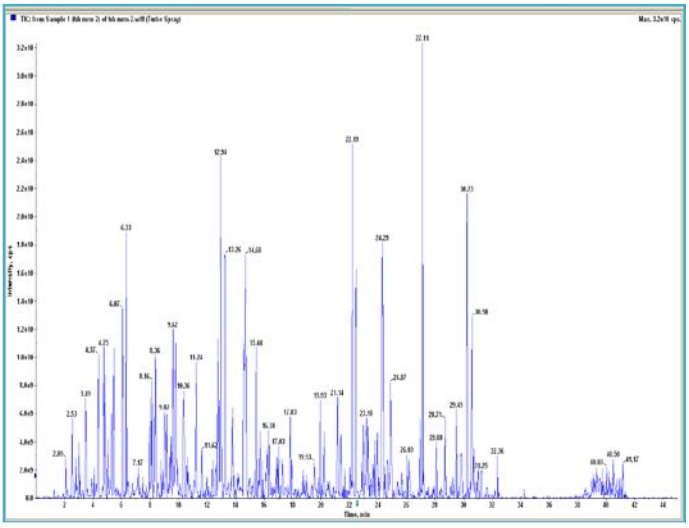

Figure 2: Chromatogram of tryptic digest of FSH using Information dependent acquisition (IDA)

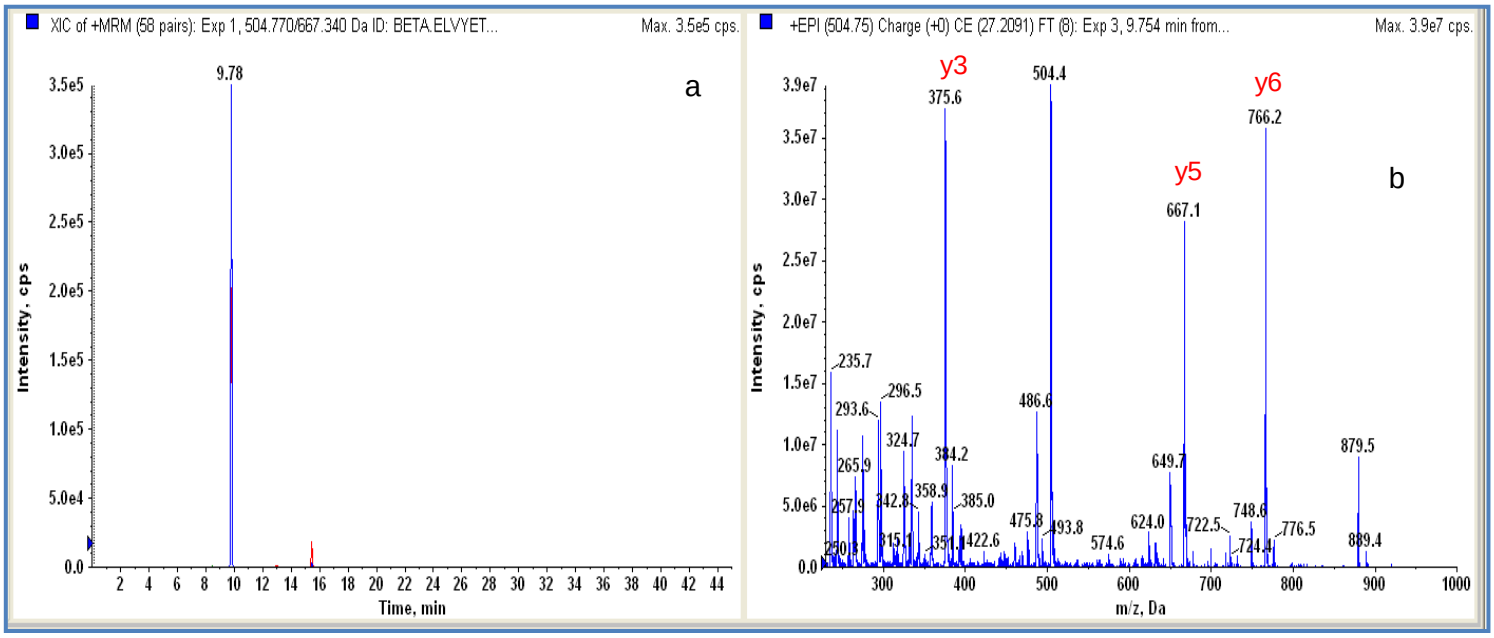

Figure 3: Extracted ion chromatogram (a) of precursor ion m/z 504. 7 with product ion (b) using QTRAP ® technology.

Optimised Procedure for FSH digestion	
Alkylation and Reduction	• 100µl serum was spiked with known concentrations of follicle stimulating hormone (FSH)
	• Volume made up to 2ml with water then 10µl of 100 mM dithiothreitol (DTT) was added
	• Add 50µl TFE (Trifluoroethanol)
	• Heat at 60^0 C for one hour
	• Add 10µl iodoacetamide (100 mM) in dark for one hour at Room Temperature
	• Add 10µl of 100mM DTT and mix for one hour at room temperature
Digestion	• Add 100µl of 200mM ammonium bicarbonate to maintain the at pH 7.5 for Trypsin
	• Add Trypsin concentration in ratio of 1:50 and incubate at 37^0 C overnight

Table 2: Optimized procedure for FSH digestion

Optimised Clean up procedure for Peptide enrichment after digestion	
Equilibration Loading and Elution	• PCX was equilibrated with 1 ml • 100mM Ammonium formate (pH 3.0)
	• Added 500µl of 100mM Ammomium Acetate (pH 3.0) to liquid digest to adjust to pH 3.0
	• Loaded the digested FSH sample into PCX and centrifuged at 500 rpm for 2 min
	• Washed PCX with 1 ml equilibration buffer Ammomium Acetate (pH 3.0) twice at 500 rpm for 2 min
	• Elution of the enriched peptide was done with 500µl of 80 % ACN and 20% 100mM Ammomium Acetate (pH 6.0)
Reconstitution for MS	• N_2 drying then reconstitution was done in 300µl of 10% ACN with 0.1% formic acid

Table 3: Cleanup and Enrichment procedure for signature peptide

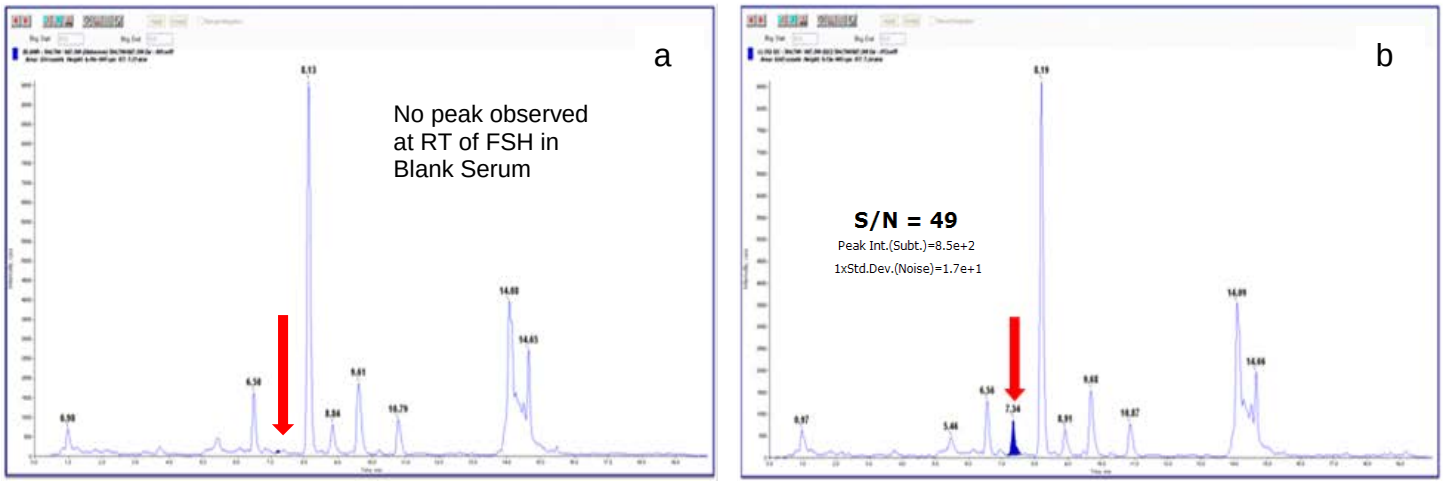

Figure 4: Chromatograms of blank plasma (a) and (b) signal to noise of LOQ sample

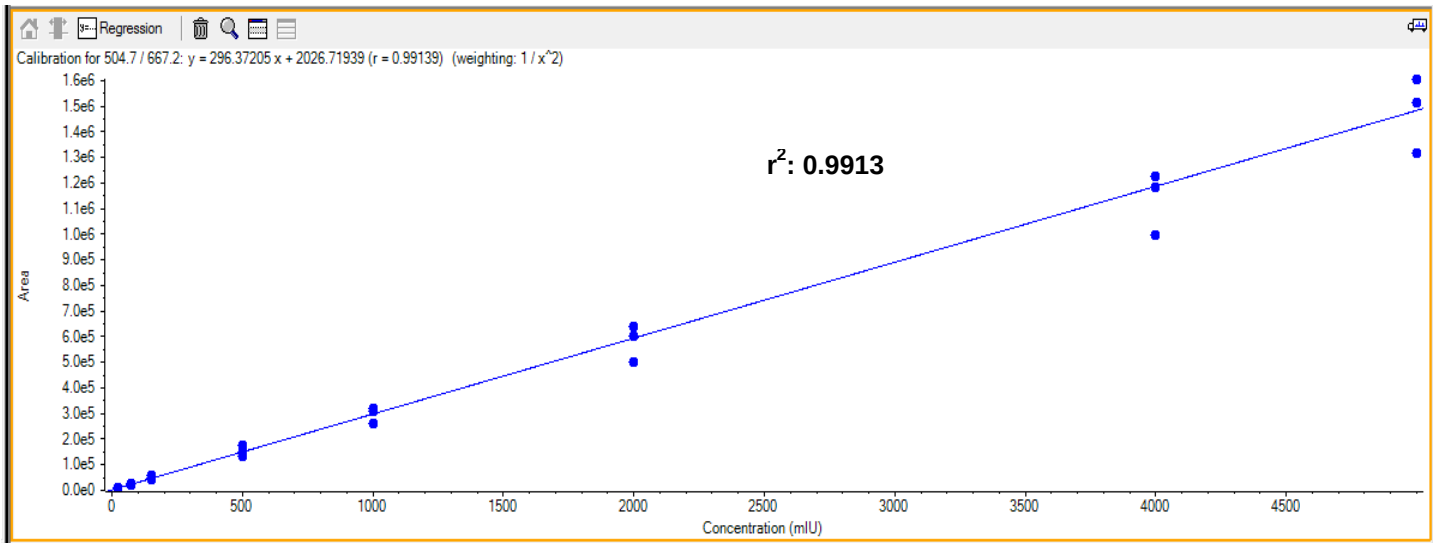

Figure 5: Calibration curve showing linearity range from 25 - 5000mIU/mL.

Data processing for peptide identification and MRM quantification

Peptide identification data was acquired using an IDA workflow method on the QTRAP® 6500 system. MRM Pilot and Skyline software was used to identify the correct precursor and its fragment ions for quantification.

Quantification data was processed using Multiquant Software™. The calibration curves were analyzed using a linear fit with $1/x^2$ weighting.

Source Parameters		Compound Parameters	
CUR	30	DP	50
IS	5500	EP	10
TEM	550	CE	28
GS1	50	CXP	05
GS2	60		
CAD	10		

Table 4: Optimized source and compound related parameters for MRM detection.

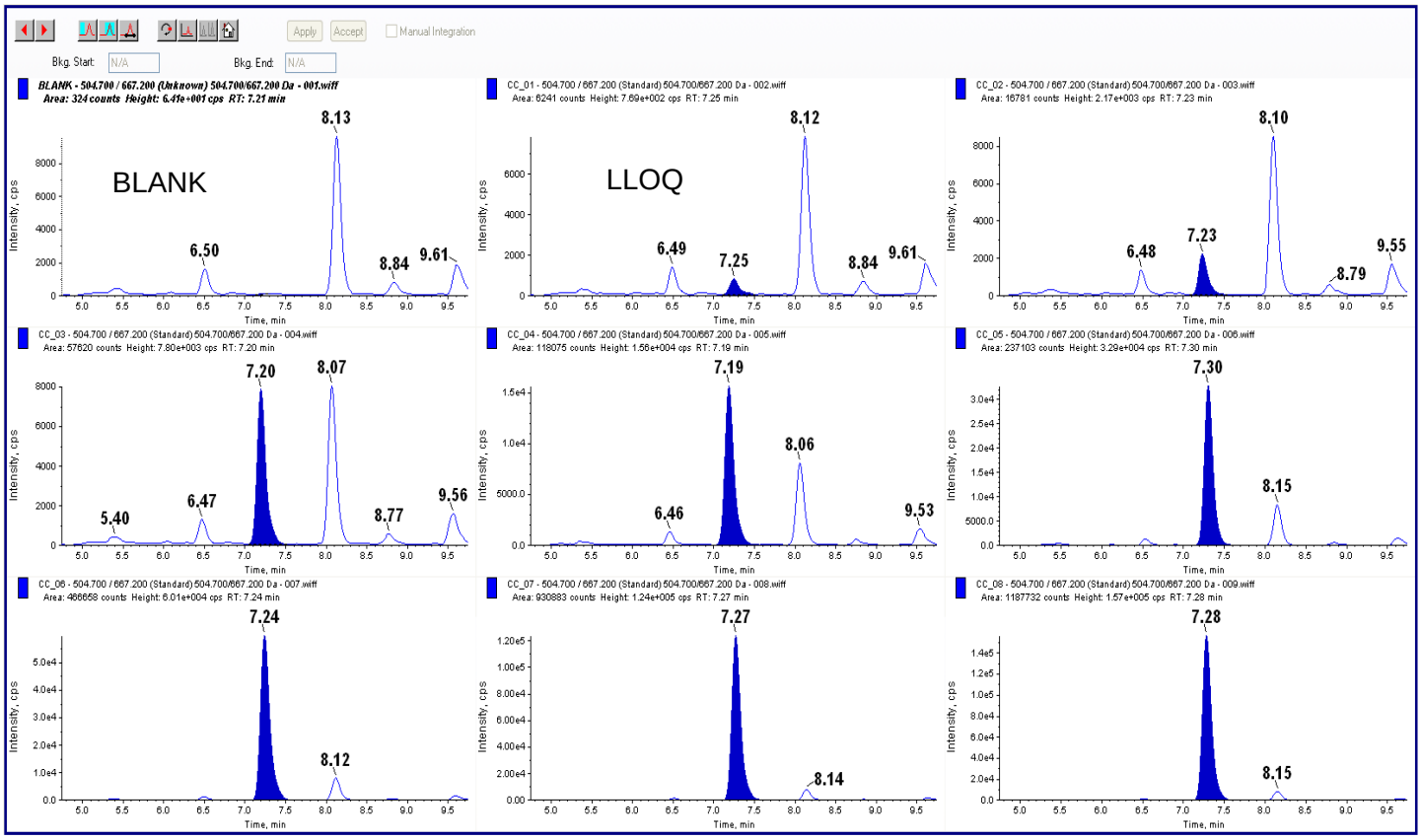

Figure 6: Chromatograms of FSH in serum ranging from 25mIU-5000mIU/mL.

Results and Discussion

The QTRAP® 6500 System was operated using Information Dependent Acquisition (IDA) mode with two looped experiments including EMS (Enhanced Mass Scan) and EPI (Enhance Product Ion) scans. IDA is a very useful tool for identification and conformation of sequence from digested protein. IDA workflow is capable of combining two or more different scan modes in the same analytical run in which the first scan is defined as a survey scan (EMS) and the second run as data dependent scan (EPI). A doubly charged ion with *m/z* 504.7^{2+} was obtained from the IDA run along with the major product ion *m/z* 667.2. Based on the precursor and product ion the *ELVYETVR* sequence was derived using ProteinPilot software and the 504.7/667.2 MRM transition was used for quantitation. Digestion and SPE based cleanup procedure for peptide enrichment were optimized to obtain maximum recovery from serum. Representative chromatograms for blank and spiked serum samples are given in Figure 6. We achieved a lower limit of quantitation (LLOQ) of 25mIU/mL in serum with excellent signal to noise ratio of 49 shown in figure 4(b).

The calibration curve in serum extracted samples showed excellent linearity with r value of >0.99 for the linear range between 25-5000mIU/mL (Fig. 5). Reproducibility of the assay was assessed by multiple replicate injections of quality control samples (Three Precision Accuracy (PA) batches, n=18) at LLOQ QC, LQC, MQC and HQC concentration levels.

Three precision accuracy batches were processed and data is shown in Table 5. The precision of all the three batches was within the acceptance criteria of %CV ±20% at LLOQ level and ±15% at other levels.

Extracted sample data from PA Batch 03 was used to calculate recovery against an aqueous digest of FSH. The average recovery of the signature peptide was 88% across the three QC concentrations.

Sample	LLOQ QC	LQC	MQC	HQC
Nominal Concentration (mIU/mL)	**25.1**	**75**	**2000**	**4000**
PA 01	28.1	78.7	2032.4	4091.0
	27.1	85.3	2107.3	4165.3
	29.8	84.6	2188.9	4294.6
	28.5	88.1	2150.2	4249.0
	28.9	76.5	2105.1	4151.6
	28.3	82.0	2047.6	4103.8
PA 02	22.7	65.2	1674.2	3281.0
	22.1	61.1	1743.5	3347.2
	20.0	62.0	1675.7	3401.7
	24.0	71.3	1733.3	3401.0
	25.5	67.3	1912.1	3677.6
	24.7	71.1	1820.8	3980.9
PA 03	34.4	96.4	2328.7	4569.0
	34.0	84.4	2319.1	5307.7
	27.9	85.1	2423.4	4151.9
	29.2	89.4	2178.8	4314.7
	26.3	74.1	2119.4	4198.4
	29.0	85.0	2269.1	4434.4
Mean	**27.3**	**78.2**	**2046.08**	**4062.3**
N	**18**	**18**	**18**	**18**
C.V. (%)	**12.5**	**12.4**	**12.5**	**10.7**
% Nominal	**98.8**	**99.6**	**97.7**	**95.5**

Table 5: Statistics of three precision accuracy batches

Conclusion

- The SCIEX QTRAP® 6500 system with IonDrive™ technology gives high sensitivity for high throughput bioanalytical peptide quantitation.
- A non targeted IDA method (EMS to EPI) was performed to identify a unique signature peptide from tryptic digested FSH.
- *"ELVYETVR"* is the doubly charged signature peptide ($504.7^{2+} > 667.2$) selected for MRM quantification.
- The digestion and peptide enrichment workflow was optimized from the generic procedure in order to achieve maximum sensitivity.
- Cleanert PCX (strong cation exchange) was used for extraction of peptides and optimisation was done to obtain 80-90% peptide recovery.
- The linear dynamic range was 25 - 5000mIU/ml of FSH spiked in serum. The signal to noise ratio at the LLOQ was 49.

References

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