A Novel Cell Based Approach for Potency Determination of Recombinant Human Follicle Stimulating Hormone

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Abstract: Sialic acid plays a very important role in the in vivo biological activity of Follicle Stimulating Hormone (FSH). The in vitro assays for some proteins are able to pick up variation in the efficacy of the molecule and therefore closely reflect the in vivo function. However, not always in vitro assays can replace the in vivo assays. This often leaves animal-based testing as the best option for looking into potency. The biopotency of human FSH is determined on the basis of in vivo (Steelman-Pohley) bioassay that is ovarian weight augmentation assay. The present work focuses on an in vitro receptor-binding assay in which binding of FSH to its receptors (expressed in CHO cells) gives a dose and/or activity dependent production of Cyclic adenosine monophosphate (cAMP) quantified by a luciferase assay system. This in vitro assay is used to detect subtle changes in the sialic-acid, which alters the binding of FSH to its receptor, thereby affecting luminescence intensity. The resulting signal is quantified in terms of in vitro specific activity using a reference standard whose specific activity is known. This study shows an inverse linear correlation between the in vitro and in vivo activity indicating that the in vitro cell-based activity assay can allow substitution of in vivo biological potency assay that rely on the use of animals.

Abbreviations: Drug Substance (DS) • Drug Product (DP) • Chinese hamster ovary (CHO) • National Institute for Biological Standards and Control (NIBSC) • hour (h) • Standard Deviation (SD) • International Unit (IU) • International System of Units (SI)

Keywords: Isohormones • Recombinant Glycoprotein Hormone • Bioactivity • Sialylation

INTRODUCTION

Follicle Stimulating Hormone (FSH) is responsible for reproductive function, key regulator in developmental process the ovarian follicle and spermatogenesis. FSH has been widely used in the treatment of both female and male infertility [1, 2]. FSH is structurally heterodimer and composed of two non-covalently linked subunits which contain several heterogeneous oligosaccharide molecules which play a vital role in the In vivo as well as in vitro biological activity of the hormone. FSH binding to its receptor on gonadal cells increases intracellular levels of cAMP [3] by activating adenylyl cyclase [4].

The biological activity of therapeutic protein is very crucial for quality control aspect. For that purpose animal models are mostly used. The example includes the weight gain assay in hypophysectomized rats for growth hormone (GH), Reticulocyte count in Polycythaemic mice for Erythropoietin (EPO), the increase in seminal vesicle weight in immature rats for luteinizing hormone (LH) and chorionic gonadotropin (CG), and the rat hypocalcaemia bioassay for calcitonin and rat ovarian weight augmentation test (FSH).

All Biological products should be fully characterized as per the guidelines of the International Conference on Harmonization [5] that includes physico-chemical, biological and immunological properties of the molecules.
The potency of FSH is determined in the rat ovarian weight augmentation test (Steelman-Pohley In vivo assay) [6].

Although the Steelman-Pohley bioassay has proved to be a robust, specific In vivo assay for FSH and remains the mainstay of pharmacopeial monographs for the statutory determination of the FSH potency of therapeutic preparations, it has a number of important limitations. The most important of these are: (technically) the assay's limited precision and non-robustness, (Ethical) its requirement for the sacrifice of large numbers of animals, (Commercial concern) High cost and its cumbersome data generation and interpretation procedures, which require highly controlled conditions and standardized procedures. There is a need of 150-200 immature rats to be sacrificed to obtain single final test results per sample which led to thousands of animals being killed annually. Furthermore, animal testing is widely discouraged when alternative solutions exist [7, 8].

And considering the ethical and political pressure on the use of laboratory animals there is clearly a need for an alternative assay with greater precision and accuracy. There are two alternative to replace the animal based bioassay, that includes the In vitro cell based bioassay approach and a physicochemical method. The In vitro bioassay alternative bears less risk when compared to the physicochemical assay in terms of potency determination and also ensure the accepted quality of the "biological" analyte (rHuFSH) which cannot be characterized adequately by chemical and/or physical means alone. On the other hand physicochemical assay can only be used for potency determination when there are no expected changes to an established process. The development of more complex biological therapies is continuing to increase the dependency on Biological assays rather than on physicochemical assay. However, for biological reference preparation the replacement of In vivo bioassay with physicochemical assays cannot be possible because the chemical reference substance assigned in SI (mg) not units (IU). On other side the replacement of In vivo bioassays so difficult to replace with physicochemical alternatives because the physicochemical characteristics of glycoproteins are highly heterogeneous, they do not contain a single active entity, a mixture of active forms is conceptually difficult for an HPLC potency assay and the multi-factorial nature of an In vivo response as receptor binding and half-life determine the total activity. And for glycoprotein like rHuFSH the requirements of ICH Q6B were not met, ‘…..Where the battery of physico-chemical tests have been shown to adequately characterize the molecule, a physico-chemical assay alone may be employed’ [5]. Because there have been cases where differences between products were identified by bioassay but not by physicochemical tests due to a lack of understanding of the possibility of certain modifications and again biological activity depends on the integrity of certain features of a molecular structure, including higher-order structure[9]. The way forward is not to eliminate the In vivo bioassay, but to replace it with an In vitro alternative.

Although there is a drive to replace In vivo bioassays with non-animal based assays that is In vitro cell based assay, this is not yet feasible in the case of rHuFSH as the two do not correlate. The major reason for this disparity is the role of the liver which expresses AGPR (asialoglycoprotein receptor) which clear inadequately sialylated glycoprotein faster from the circulation[10].

Correct glycosylation is essential to the In vivo activity of rHuFSH [11]. It is believed that the carbohydrate component and the degree of sialylation of glycoproteins are responsible for transmitting different physiologic signals to the target cells. The degree of sialylation of a recombinant glycosylated protein is often affected by the conditions used in expressing the recombinant protein and therefore the heterogeneity of sialic acid (SA) content is dictated by culture conditions. Also purification (process) condition can influence the levels of sialic acid content of purified glycoprotein. Sialylation imparts a negative charge on the glycoprotein molecule giving rise to many different isoforms. All these isoforms have different In vivo half-lives and consequently different pharmacodynamic behavior. The heterogeneity in the degree of sialylation is assayed by the In vivo bioassay in animal models.

The present study offers a reliable and high throughput In vitro (Reporter Gene) bioassay for FSH with higher accuracy and precision as compared to the conventional animal based In vivo assay. The work illustrates the relevant correlation with In vivo biological activity by generating stressed variants considered likely to arise from the manufacturing process. With a well established manufacture history one can replace the animal based assay with cell based In vitro bioassay.
MATERIALS AND METHODS

Reagents: IEF reagents Tris-buffered saline (TBS) buffer (10X), Tris-buffered saline (TBS) buffer (1X, 100mL), Tris-glycine buffer for Electro-transfer, Tris-glycine buffer (10X, 100mL), Blocking buffer (5% skim power non fat in 1x TBS), Primary antibody solution (1:250) (Serotec, cat#: MCA 338), Mouse anti human FSH intact (Serotec, cat#: MCA 337), Secondary antibody solution Goat anti mouse ALP Conjugate (1:250), Substrate solution (BCIP/NBT is used as the substrate solution which is available as a ‘ready to use’ solution).

SEC-HPLC reagents HPLC (Agilent 1100/1200 Series) column (Brand name: Phenomenex Bio-Sep-SEC-S 2000, CAT# 00 H-2145-k0).

Cell culture reagents MAM PF-2 (Bioconcept, Catalog No. 10-2P24-I), Fluoric F-68 (Bioconcept, Catalog No. 5-7F02-H), Phenol Red (Sigma, Catalog No. P3532 or any cell culture tested), Dialyzed FBS (Invitrogen, Catalog No. 26400-044), Gentamicin (Ranbaxy), L-Glutamine (Sigma, Catalog No.G7513 or HyClone, Catalog No. SH30034), Luciferase assay system (Promega, Catalog # E1501).

Desialylation of rHuFSH: The first aim of this experiment was to generate a range of increasingly desialylated rHuFSH samples. These samples are then used for comparison of results between In vivo and In vitro assays. The rHuFSH was dissolved in buffer A (Provide in Kit Prozyme GK80110) to give final concentration 2.18 mg/ml. This solution was then vortex and equal volume of different aliquots (90 μl, 196 μg/90μl) were incubated for different time point (such as 0, 12, 24, 36 and 48 hrs) with Sialidase A (Provide in Kit Prozyme GK80110) in a water bath at 37° C and removed respectively from water bath at different time as shown above. After removing from water bath the samples were immediately kept at-20° C to stop/arrest the reaction of Sialidase A.

CHO-hFSHR-Luc In vitro bioassay: CHOSI FSHR CRELuc B3-Cells (Eugenics Biotechnologies, Switzerland) were grown in Growth media (supplemented with MAM PF-2975mL/1000mL, L-Glutamine (100X) 10mL/1000mL, Pluronic F-68 10 mL/1000mL, Phenol Red Solution 5 mL/1000mL, Gentamicin ml/1000mL) and seeded in a white 96 well half area Assay Plates (50,000 cell/well). After require a time period for incubation at 37°C different concentration (range of 10.5-0.02 mlU/mL) of test component was added and incubated for ~4 hrs (12). At the end of FSH incubation, cells were lysed (use 5X lysis buffer), incubated for 15 min at room temperature before reading the plate in a luminometer (Turner BioSystems USA). After these add 50 μl of lularase assay reagent which provided in the Luciferase assay system (Promega, Catalog # E1501). Specific activity was calculated using PLA software.

SEC-HPLC: In order to investigate the content of rHuFSH (μg/ml) the size exclusion chromatography has been used. SEC-HPLC was carried out with the Agilent 1100/1200 series on Phenomenex Bio-Sep-SEC-S 2000 (Diameter 7.8 mm, Length 300 mm, particle size of 5 micron and pore size of 125 Å). The mobile phase was sodium phosphate buffer (pH 6.7; 0.1 M), with a flow rate of 1.0 ml/min.

Isoelectrofocusing: The isoelectrofocusing (IEF) was carried out using Dry IEF Phast gels (Dry IEF PhastGel™, Amersham Biosciences, Catalog # 17-067-01) having pH range 3 to 6. The gel was run loading 50 μg of the chronologically desialylated samples for each lane. The run was performed in a PHAST System for 2000 V at 2.5 mA, 200V at 2.5 mA, 2000V at 2.5 the entire run would take approximately 35 min. After completion of the run gel transfer in to transfer buffer. Place a PVDF membrane, cut to the size of the gel (activated with methanol and equilibrated in the transfer buffer for 5 min), on the gel. Connect the electrodes with the power pack and set current 400mA, 100V and 45 minute. Keep the membrane to blocking solution for 1 hour. Decant the blocking solution after 1 hr and add the primary antibody on the membrane incubate for 1hr at room temperature. Wash the membrane thrice (5 min each wash) with 1X TBS. Add Secondary antibody and incubate for 1hr at room temperature. Decant the secondary antibody and wash same as mention above. Incubate the membrane with the substrate solution till 7-8 isoforms of Gonal-F is visible. Decant the substrate and wash the membrane with water to stop further reaction. Scence the membrane, dry and preserve it [13].
HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection): HPAEC is an improved chromatographic technique developed to separate carbohydrates coupled with pulsed amperometric detection (PAD) which permits direct quantification of nonderivatized carbohydrates at low Pico-mole level. The oligosaccharides moiety (Sialic acid) of rHuoFSH was studied by carbohydrate profiling. The released sialic acid was quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on CarboPac PA10 column (Dionex). Sialic acid was detected by pulsed amperometric detection.

Steelman–Pohley FSH bioassay: rHuoFSH activity was determined by the rat ovarian weight gain method of Steelman and Pohley [5] and the assay was conducted in accordance with the national protection laws on animal welfare. A group of Wister immature female rats (age 21±1 days) is injected subcutaneously with hCG and then injected with the international standard of FSH once daily for 3 days; a second group of animals is injected with the same amount of hCG and then with the samples 0.5, 1 and 3 IU (with different level of Desialylation) to be tested once daily for 3 days. An autopsy is performed 72 hr after the first injection, at which time the ovaries are dissected and weighed. Statistical analysis of the assay data was carried out according to Finney, by the parallel line method (3X3), using PLB 2.0 software (Stegmann System-beratung, Rodgau, Germany)

Method Equivalence: In order to replace a bioassay, one should have to show a strong body of data that strongly correlates product activity between existing bioassay (In vivo) and intended replacement (In vitro) is necessary. The proposed In vitro cell based reporter gene bioassay can be used to assess the potency of lot release sample, stability samples, Stressed samples, Product variants, simulated potency (spiked) samples. Equivalency approach (with log potency values) at 90 % CI for difference between means was applied for establishing the equivalence of the two methods. This was carried out for potency determination of 89 different batches of rHuoFSH that includes 9 different batches of DS, 20 different batches of DP and 60 Stability samples (DS and DP) using both the methods.

Acceptance criteria to establish equivalence:
- The In vivo bioassay and In vitro bioassay method for the testing of mean specific activities of rHuoFSH should result in a 90% CI limit at 0.1% of Difference between Means (as defined above) to be within 80-125% (14) to be considered as Equivalent.
- The residual plot of D value (Potency obtained by In vivo bioassay-Potency obtained by In vitro bioassay, Log10 transformed for each sample tested) and percentage recovery against the average potency value should show a random distribution around a mean value of D. The range of D+2SD and D-2SD of these D values should contain 95% of all the estimated D values.

Selection of time points is based on scientific understanding of equivalent potency estimation by the In vitro bioassay compared to the In vivo bioassay without affecting the end result on quality of the DS, DP and stability samples.

RESULTS

Desialylation of rHuoFSH: The first aim of this experiment was to generate a range of increasingly desialylated rHuoFSH samples. These are then used for comparisons of results between In vivo and In vitro assays. Figure. 1 shows the gradual increase in the basic isoforms of rHuoFSH which correlates with the length of incubation with sialidase. As rHuoFSH loses negatively charged sialic acids (i.e. becomes more positive) it migrates towards the negative electrode (cathode). rHuoFSH sample ‘0’ h had all the isoforms of normally sialylated FSH sample. The longer the samples were incubated with sialidase, the lesser their acidic isoforms. A 48 h incubation in sialidase resulted in a sample having the highest basic isoforms.

SEC-HPLC: Size Exclusion Chromatography (SEC) is a method which separates molecules based on their hydrodynamic volume. Molecules pass through the column and, depending on their sizes, elute through a stationary phase at different rates. The SEC was used for quantifying amount of FSH.

The follicle stimulating hormone is a heterodimer molecule containing one alpha and one beta subunit. Herein, the objective of the study is to quantify total rHuoFSH present in the protein. The SEC method dose
Fig. 1: Isoelectric focusing gel with anti-FSH antibody of rHuFSH treated with sialidase for increasing lengths of time. IEF analysis over the pH range 3 to 6 was performed on a thin-layer 6% polyacrylamide gel in the presence of urea and the protein was visualized by immunoblottting. Lane 1 represents standard Gonal-F and Lane 2 to 6 represent the rHuFSH treated with sialidase for increasing lengths of time (t=0, t=12, t=24, t=36, t=48).

Table 1: Content determination of rHuFSH treated with sialidase by SEC-HPLC

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>rHuFSH Conc. (mg/ml)</th>
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<tbody>
<tr>
<td>0</td>
<td>2.72</td>
</tr>
<tr>
<td>12</td>
<td>2.80</td>
</tr>
<tr>
<td>24</td>
<td>2.60</td>
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<tr>
<td>36</td>
<td>2.52</td>
</tr>
<tr>
<td>48</td>
<td>2.33</td>
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not resolves the alpha and beta subunits; rather they elute intact as along with aggregates and free subunits in single peaks. Table 1 represent the content determination of rHuFSH treated with sialidase for increasing lengths of time.

CHO-hFSHR-Luc In vitro and Steelman–Pohley In vivo bioassay: The In vitro and In vivo activities of the rHuFSH treated with sialidase for increasing lengths of time in the CHO-hFSHR-Luc In vitro bioassay and Steelman-Pohley In vivo assay are shown in Figure 2. The gradual increase in the basic isoforms (‘48’ h sample) of rHuFSH which correlating with the length of

Fig. 2: Specific Activity of sialidase enzyme treated samples with control in the CHO-hFSHR-Luc In-Vitro bioassay and Steelman-Pohley In-Vivo Bioassay. There is inverse relationship found between sialic acid content and receptor-binding activity. In-Vivo activity decrease with the sialic acid content of the glycohormone indicating the amount of sialic acid containing carbohydrate chain regulates clearance while the In-Vitro bio activity increase due to the highest affinity for the FSH Receptor (FSHR).
incubation in sialidase A. Sialic acid containing carbohydrate can affect receptor binding activity of rhUFSH. As rhUFSH loses negatively charged sialic acids (i.e. becomes more positive) it shows higher Specific Activity in the CHO-hFSHR-Luc In vitro bioassay (t=12 and t=48 h samples). Whereas the less acidic rhUFSH were less potent in the Steelman-Pohley In vivo assay (i.e. t=48 hr compared to t=12 hr) (Table 2 and Figure 2). These results indicate that sialic acid containing carbohydrates were required for maintenance of In vivo activity and the In vivo activity loss were proportional to the amount of sialic acid loss from the rhUFSH with increasing lengths of time (Table 2). Figure 3 shows the rat ovary weight in mg in the Steelman-Pohley In vivo assay which was found to be significantly less while moving from ‘12’ hrs to ‘48’ hrs
disialidase enzyme treated samples. This result confirms the direct relationship between sialic acid moles/mole of FSH, circulating half-life and In vivo biological activity, but an inverse relationship with receptor affinity In vitro.

Moles of sialic Acid/Moles of FSH by Dionex: Figure 4A represents the comparison of different chromatograms (Dionex) of rhUFSH treated with sialidase for increasing lengths of time, while Figure 4B represents the moles of sialic acid/mole of FSH in the sialidase treated samples based on the retention time of respective sialidase treated sample window (t=12, t=24, t=36 and t=48 hours). This result shows, the time of sialidase enzyme incubation with sample is increased the moles of sialic acid/moles of FSH are also increased. Overall we can say that there is a direct relationship between moles of sialic acid/mole of FSH and In vivo activity. These results indicated that as increasing lengths of time with sialidase enzyme the sample released more sialic acid and became less acid thereby having less In vivo activity and more receptor binding activity In vitro.

Fig. 4 A: The Figure represents the comparison of different chromatogram (Dionex) of rhUFSH treated with sialidase for increasing lengths of time. Sialic acid moles/mole of FSH profile analysis of recombinant human follicle stimulating hormone (rHuFSH). Sialic acid released from 2.18mg/ml rHuFSH was analyzed by high performance anion exchange chromatography (HPAEC-PAD). Windows for t=0, t=12, t=36, t=48 hrs peaks were presented.
Fig. 4B: The Figure represents the moles of sialic acid/moles of FSH in the sialidase treated samples represent the longer the sample were incubated with sialidase, the higher their released sialic acid content.

Table 3: Analysis of statistical validity for equivalence

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity (Log10)</th>
<th>Specific Activity (Log10)</th>
<th>Mean D</th>
<th>-0.004</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-vivo bioassay</td>
<td>4.117</td>
<td>4.121</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum of Squares of Errors (SSE)</td>
<td>0.169</td>
<td>0.307</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Assays (N)</td>
<td>89</td>
<td>89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degrees of freedom (df)</td>
<td>176</td>
<td>176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower acceptance limit</td>
<td>80.0</td>
<td>80.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper acceptance limit</td>
<td>125.0</td>
<td>125.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower 90% Confidence Interval (CI) of DM</td>
<td>96.2</td>
<td>96.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper 90% Confidence Interval (CI) of DM</td>
<td>102.1</td>
<td>102.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>176</td>
<td>176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum of Squares of Errors (SSE)</td>
<td>80.0</td>
<td>80.0</td>
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<tr>
<td>EQUIVALENCE</td>
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Negative correlation of In vitro and In vivo bioassay was found in Table 2 indicating that the loss of sialic acid from the glycan structure correlates with the loss of In vivo biological activity. This was supported in the present study by the decrease in the rate of ovarian weight in comparison with those isoforms which have an intact glycan structure or more acidic isoforms as determine by Steelman-Pohley assay. While less acid isoforms (i.e. t=48 hr sample) retain full receptor binding and showed higher Specific activity. The apparent paradox, that high sialic acid moles/mole of FSH with lower receptor-binding activity have increased In vivo activity, is explained by the counteracting effects of sialic acid containing carbohydrate on clearance. These results indicate that sialic acid containing carbohydrate was required for maintenance of In vivo activity and the In vivo activity loss was proportional to the length of sialidase treatment. These results indicate that high amount of free sialic acid (beyond those found in t=0 hrs sample), could decrease the In vivo activity and that there was a direct relationship between the number of sialic acid moles/mole of FSH and rHuFSH In vivo activity. The number of sialic acid moles/mole FSH increased, receptor binding (In vitro) bioactivity also increased in a linear-log relationship.

Method Equivalence: Both the methods were found to be equivalent. The residual plot a show random distribution around the mean and thus rules out any bias. Analysis of the statistical validity of equivalence [14] is shown in Table 1. The observed range of 90% confidence interval of the difference in means was 89.9 to 100.1 (Table 3). From the analysis it has been concluded that the both methods are equivalent for potency recovery (with a precision of <6% of 90% Confidence Interval of Difference between Mean Potency Values) and the In vivo assay can be replaced selectively with that of the in-vitro alternative. The observed range of 90% confidence interval of the difference in means was 96.2 to 102.1 (Table 3). The residual plot shows a random distribution around mean and thus rules out any bias. Moreover 95% of the residuals as expected lies within ± 2 SD (Standard Deviation) (Figure 5).
Fig. 5: Residual plot of difference between means of potency of the methods against average percentage potency recovery for each sample tested.

DISCUSSION

Proposed Change and objective of this study: Proposed changed and objective of this study is to switch over from animal based In vivo bioassay to reporter gene cell proliferation In vitro bioassay for determination of potency of rHuFSH. Objective of this study is to provide justification and analysis of risk associated with this change control has been categorized in three categories that include 1) regulatory perspectives, 2) technical and quality perspectives. 3) Logistics, administrative and commercial perspectives. Justification from regulatory perspective: It has been interpreted that, on ethical ground regulatory authorities encourage minimizing the laboratory animal during analysis [7] European pharmacopoeia 6.0 (introduction part; page no VI). EDQM also putting stress on establishing an alternative method to Replace, Reduce and Refine the use of animals in quality control of biological (3R concept, Proceeding of the international symposium, Strasbourg, 7-8 November 2002). Proposed In vitro bioassay method is based on the use of recombinant human FSH receptor cotransfected into Chinese hamster ovary cells with a CAMP-responsive Luciferase gene. The recombinant cell gives dose dependant induction of Relative Luciferase Unit (RLU) and that is directly propositional to the binding of FSH molecules to its receptor. There is the provision of alternative method provided suitability of the method for the intended purpose, for this method qualification and method validation activity has been carried out. Ability of the proposed method for unequivocal decision, for this method has been carried out and both methods found to be comparable (detail regarding comparability exercise has been provided in Method).

Justification from technical and quality perspective: FSH is a glycosylated protein having two noncovalently linked, heterogeneous α- and β-subunits, α is subunit composed of 92 amino acids having two carbohydrates moieties-linked to Asn-52 and Asn-78 and the β-subunits contain 111 amino acids carries two carbohydrate moieties-linked to Asn-7 and Asn-24. Therefore, the glycoprotein is highly heterogeneous in terms of charge, molecular weight and biological activity [15-19]. This heterogeneity leads to 20 different isoform with varying degree of glycosylation [20] forming a range of isoforms with differences in charge, bioactivity and t½ half-lives [16, 21]. The more sialylated molecules possess a longer circulation half-life as compared to the desialylated molecules which, are eliminated faster by the liver’s asialoglycoprotein receptors (ASGPR) that binds galactose residue that are exposed once the terminal sialic acid have been removed from the glycan chain there by decreasing In vivo bioactivity [22-25]. It has been also demonstrated that inadequately sialylated FSH and FSH variants having a higher amount of Asn-linked galactose and mannose oligosaccharide are cleared faster from circulation of rat [22]. rHuFSH upon desialylation of the oligosaccharide gives basic isoforms which have highest In vitro bioactivity the difference accounted for decrease sialic acid content and less carbohydrate related stearic hindrance [22].
It indicates that there is a direct correlation between degree of sialylation and In vivo activity and inverse relationship in the case of the In vitro bioassay. There are several complementary methods during either manufacturing of rHuFSH DS or in final DS e.g. Capillary zone electrophoresis (CZE), IEF, Sialic acid estimation by Dionex etc. If results of these analyses are in accordance with predefined acceptance criteria then it can be technically concluded that during In vitro analysis activity obtained is a function of FSH molecule itself, not the degree of desialylation. The bio-pharmaceutical manufacture of rHuFSH ensues in mammalian cells, as mammalian cells have ability to add sugar chain in the protein by a process called glycosylation (rather than bacteria, which lack there necessary enzyme). The degree of sialylation of a recombinant glycosylated protein is often affected by the conditions used in expressing the recombinant protein and therefore the heterogeneity of sialic acid content is dictated by culture conditions. As this protein glycosylation process is not so tightly regulated it leads to batch to batch variation and which ultimately affect on biological properties of the glychormone. Attempts to replace the animal tests with any other alternative test that includes Physico-chemical assay like IEF, SE-HPLC and In vitro cell based bioassay. During development of In vitro method, the special rHuFSH samples were generated forcefully in order to find the capability of the method to detect such samples. The lists of samples were generated forcefully in order to find the capability of the method to detect such samples. And sample responded well to such samples that includes thermally aggregated samples (data not shown), Chronologically removal of sialic acid from the FSH [30-32] results in the loss of its In vivo biological activity due to faster removal from circulation by the hepatic ASGR (asialoglycoprotein receptors) [21]. The results reported here show that less acidic (t= 48 h) and intact (t=0 h) isoforms/glycomhormone induced dose-dependent response with significant difference in the biological activities. The less acidic isoforms group shows a higher In vitro biopotency than the acidic isoforms and these differences were found due to the effect of sialic acid on the FSH-FSHR interaction. It is believed that the protein and carbohydrate components of FSH are responsible for transmitting different physiologic signals to target cells (in the present study modified CHO cells). Less acidic isoforms increased the receptor binding activities of the hormone, as assessed by CHO-hFSHR-Luc In vitro bioassay. These results corroborate with the finding of Manjunath P. [34] who has shown that the less acidic isoforms are potent and specific antagonists of the intact hormone in In vitro bioassays. The inverse correlation between In vitro and In vivo bioactivities of rHuFSH treated with sialidase confirms the importance of the Sialic acid as regulatory determinants of In vivo potency and metabolism. These results demonstrated an inverse relationship between the sialic acid number (moles/mole FSH) and receptor binding activity and therefore In vivo bioactivity. It was clearly demonstrated that there are consistent and reproducible relationship between assay response and different type of sample that is equivalent or not inferior in comparison with In vivo bioassay. As per one of the presentation by Dr. Adrian Bristow (Head, technology development, NIBSC) for batch release, physicochemical characterization of glycan structure in combination with In vitro bioassay seems to offer adequate control on potency. Why In vitro cell based reporter gene bioassay should implement? Among this alternative method the measurement of the rHuFSH by isoforms distribution by IEF [26, 27] cannot be used to assess the rHuFSH of the final pharmaceutical form (solution for injection). While replacement of animal study with physico-chemical method like SE-HPLC [28] cannot be possible as the former cannot assign unit IU i.e. "biological effect" rather than "mass unit" (SI) to the recombinant protein. So the physicochemical analysis is not immediately aperients to determine the potency for rHuFSH and other glycosylated proteins [29] However the assessment of the rHuFSH by In vitro bio-assays were able to pick up variation in the efficaciy of the molecule and therefore closely reflect the In vivo function. As the cell-based reporter gene assay is based on the use of recombinant CHO cells expressing the FSH receptor as well as a reporter gene construct and is highly specific for the FSH mediated gene signaling so in other word the cell based bioassay is more physiologically relevant to the clinical endpoint. The Potency determination of drug substance, a drug product and stability sample by In vitro bioassay shows an acceptable precision and accuracy. Justification from logistic and administrative perspective: Proposed In vitro cell based potency assay is advantageous for company who doesn’t have their animal house or those company who are dependant on CRO (clinical research organization) for their routine batch release and stability study sample analysis because in proposed In vitro cell based assay is carried by QC (quality Control) department which is more feasible to evaluate and
investigate any OOT (out of trend) and OOS (Out of Specification) results, which is difficult in case of contract laboratories. In case of outsourcing the company needs to send the sample to the contract laboratory by courier service and because of not maintaining the proper cold chain the sample quality may get deteriorate during transportation. For commercial perspective also the cost of Animal study (In vivo) is too high compare to In vitro assay. For quality control department it is critical for timely release of batches in order to fulfill market commitment. There is a huge dependency on contract laboratories for release of the batch. Many a times due to several factors e.g. unavailability of animals or other resources this is not possible. After implementation of this In vitro cell based potency assay this issue shall be resolved. Bicassay are required for complex medicinal products such as glycosylated therapeutic proteins which are depend on quantitative biological methods such as bicassay to express their potency.

CONCLUSION

Based on the excellent (inverse linear) correlation between In vitro and In vivo bioactivity assay as shown in the present study, it is clear that In vitro receptor-binding assay using CHO-hFSHR-Luc assay can substitute for animal based-ovarian weight gain assay. The former can therefore be used to determine the potency of the FSH for different manufacturing lots and therefore can be used for batch release of drug substance and drug product. The In vitro assay is sensitive and high throughput, making it easier and reliable to study variations in the manufacturing process. The study confirms that both the methods are equivalent in terms of potency recovery and thus the In vitro bioassay can be used wherever suitable. The sample size (n=89) and its quality of this study allows the replacement of the In vivo assay. It is recommended that few commercial batches should be analyzed by both the methods as per the availability, for potency determination and statistical validity tests similar to the one described here be done. This will ensure the complete replacement of the monograph based In vivo bioassay with the In vitro bioassay.

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