Introduction

Follicle stimulating hormone (FSH) is a glycoprotein produced and secreted by the anterior pituitary gland. It exists not as a single molecular structure, but rather as multiple, charged isoforms that result from variations in the composition of the four carbohydrate chains (in particular their sialic acid content) attached to the two (alpha and beta) inter-linked protein subunits. These isoforms differ in their molecular weights, biological potency, elimination half-life and immunoreactivity. Sialic acid content is the main determinant of half-life – the greater the sialic acid content, the higher the acidity of the isoform and the longer the biological half-life. The amount and relative composition of FSH secreted, as well as its bioactivity, are influenced by the hormonal milieu.

FSH plays a key physiological role in both males (spermatogenesis) and females (regulation of follicular growth). Over the past 40 years, injectable forms of FSH have established a leading role in the management of male and female infertility. In women, FSH is used in...
the treatment of ovulatory disorders and as part of controlled ovarian stimulation (COS) regimens for assisted reproductive technologies (ART). In men, it is most commonly used to treat hypogonadotropic hypogonadism.

Prior to the advent of recombinant DNA technology, all therapeutic FSH preparations were urinary-derived. The rather crude manufacturing methods used during the mid-to-late 20th century required the collection of vast quantities of urine and led to FSH preparations with varying degrees of protein contamination (although the most recent urinary FSH product – highly purified urinary FSH – contains > 95% pure FSH). Nevertheless, given the highly variable composition of urinary gonadotrophins, there were (and continue to be) significant batch-to-batch inconsistencies in terms of contamination as well as FSH isoform profile. As a result, the quantification of FSH presented a serious problem, given that its expression by mass was meaningless. In order to quantify FSH content and standardise proprietary preparations, therefore, it was necessary to use an in vivo bioassay. Bioassays make use of internationally accepted standards (provided by the WHO) that enable samples of unknown biopotency to be estimated. The test results on the bulk preparations allow filling of FSH vials/ampoules according to the desired FSH bioactivity measured in international units (IU). This activity is confirmed by a final bioassay on the finished product just prior to its release; the result has to be within a specification range of 80–125% of the labelled potency.

Although a number of FSH bioassays have been developed, the one required by regulatory agencies is the classic Steelman–Pohley in vivo assay1. This is based on the fact that immature female rats (21–22 days old), pretreated with human chorionic gonadotrophin (hCG), are sensitive to exogenous FSH and that there is a linear relationship between administered FSH and ovarian weight. FSH is injected subcutaneously once daily for 3 days with an autopsy being performed after 72 h. One of the advantages of this assay is that it takes FSH clearance into account.

The manufacture of FSH using recombinant DNA technology now ensures a constant supply of the most biochemically pure FSH preparation (r-hFSH, > 10 000 IU FSH/mg protein) with high batch-to-batch consistency in isoform profile and glycan species distribution. The most significant advantage of this isoform and glycan species consistency is that it permits FSH to be reliably quantified by mass. This article considers the rationale and methodology behind this development and the advantages of non-bioassay-based quantification methods.

Limitations of the Steelman–Pohley FSH bioassay

Although the Steelman–Pohley bioassay has proved to be a robust, specific in vivo assay for FSH, and remains the mainstay of pharmacopoeial 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: the assay’s limited precision, its requirement for the sacrifice of large numbers of animals and its cumbersome data generation and interpretation procedures, which require highly controlled conditions and standardised procedures.

The bioassay is widely acknowledged to have only limited precision – its coefficient of variation (CV) in a single determination is 10–20%, implying that the acceptable range of FSH activity in proprietary preparations is highly variable. A vial that nominally contains 75 IU of FSH on the basis of an initial assay meets the 80–125% (i.e. 60–94 IU) fiducial limits. However, in reality the vial may contain anything from 48 IU to 117 IU FSH1. Furthermore, because biological systems are not entirely predictable, their use in the context of bioassays leads to variable responses between tests and between test laboratories. Bioassays therefore require highly standardised conditions. In order to reduce variability to approximately 10%, and to obtain a sufficient number of valid tests to calculate the final test result, multiple replicates, multiple standard points and multiple independent tests are required. Consequently, it is very common for 150–200 immature rats to be sacrificed to obtain a single final test result per sample. Given that, on an industrial scale, several hundreds of samples are submitted for testing each year, it is not unusual to find that tens of thousands of animals are being killed annually. These days, there is increasing ethical and political pressure to reduce the use of laboratory animals.

Overall, therefore, the Steelman–Pohley bioassay is cumbersome, time-consuming, and lacks precision. There is a clear need for a more precise, and more ethically acceptable, physico-chemical alternative to measure the potency of r-hFSH preparations.

Alternative physico-chemical methodology

Size exclusion high performance liquid chromatography (SE-HPLC) represents an alternative physico-chemical method by which Serono – the manufacturers of
Gonal-F* (r-hFSH, follitropin-alfa) – are now able to quantify r-hFSH protein in both drug substance and final product with high reliability. This technique has far greater precision than the Steelman–Pohley in vivo bioassay (%CV 1–2% vs %CV 10%) and supports an intended ‘target mass’ ± 10% specification range. This represents a significant improvement in product quality and batch-to-batch consistency compared with the current protein content range of ±23% for Gonal-F 75 IU vials/ampoules, filled by IU and released by bioassay against the 80–125% biopotency specification. The SE-HPLC method used is very robust and provides highly consistent analytical results at multiple laboratories, as shown in Table 1. However, this optimised SE-HPLC method is only viable because:

- The drug substance manufacturing process produces r-hFSH protein with a consistent physico-chemical quality
- The compounding, filling and finishing process maintains the integrity of the r-hFSH molecule, ensuring consistent biological quality
- A reliable conversion factor has been determined to calculate the equivalent of IU in µg.

**Table 1. Analysis of protein content – results (µg/vial) obtained at four different quality control laboratories on a 6 µg r-hFSH drug product control sample using a size exclusion high performance liquid chromatography (SE-HPLC) method optimised for the r-hFSH drug product fill-by-mass process (Serono, data on file)**

<table>
<thead>
<tr>
<th>Site</th>
<th>Mean (µg)</th>
<th>SD</th>
<th>%CV</th>
<th>N</th>
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<tr>
<td></td>
<td>5.95</td>
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<td>1.4</td>
<td>56</td>
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</tr>
<tr>
<td></td>
<td>5.98</td>
<td>0.06</td>
<td>1.0</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>0.09</td>
<td>1.5</td>
<td>18</td>
</tr>
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Human pituitary FSH can be separated into at least 20 isoform fractions with the endocrine milieu strongly influencing the overall isoform profile. For this reason, urinary gonadotrophins have a highly variable composition and a highly variable level of bioactivity, necessitating the quantification of FSH by bioassay. By contrast, the manufacturing processes used to produce Gonal-F lead to a product with a highly consistent glycosylation profile resulting in a consistent isoform pattern and bioactivity. Glycan mapping and isoelectric focusing (IEF) have both shown that Gonal-F has a high physico-chemical consistency that is reflected in a consistent specific bioactivity. Analysis of the vial filling and finishing operations (including lyophilisation) has confirmed the consistent biological integrity of the r-hFSH molecule (Serono, data on file).

**Glycan Mapping**

Glycan mapping produces a ‘fingerprint’ of the glycan (polysaccharide) species of r-hFSH and an estimation of the degree of sialylation. The glycan species are separated by charge – a function of their sialic acid content – and the results are expressed as the relative percentage of neutral, mono-sialylated, di-sialylated, tri-sialylated and tetra-sialylated glycan species and as a hypothetical charge number ‘Z’ calculated from the different proportions of the different species. The Z number is a very precise estimation of the degree of sialylation and has been assessed with a CV of just 2%.

Evaluation of the batch data on a year-by-year production basis (1998–2000) shows that the glycoform distribution has been highly consistent over the years (Figure 1), reflecting the high consistency of the production process.

**Figure 1. Glycan mapping – glycan species distribution comparison of control sample ((a), left) (51 independent values) and 121 batches of r-hFSH (Gonal-F) drug substance ((b), right) produced in 1998 (n = 60), 1999 (n = 41) and 2000 (n = 20).**

(Serono, data on file)

*Gonal-F is a registered trade name of Serono S.A., Switzerland
molecular profile of the Gonal-F product (Serono, data on file).

Iso-electric Focusing (IEF)

The IEF of Gonal-F is performed in acrylamide gels across a pH range of 3.5–7.0. The reference standard produces seven major bands – one band above pI 5.20; three bands between pI 5.20 and 4.55 and three bands below pI 4.55. The isoform distribution consistency of the commercial manufacturing process over the years has been confirmed by evaluating the relative intensity of each of the major and minor bands on the IEF gel, measured by its integrated optical intensity (IOD) (Figure 2). The distribution of the main bands is very similar to that of the reference standard; the mean %IOD obtained for each of the major bands is within 2–4% of the mean %IOD of the same band of the standard (Serono, data on file).

Specific Activity Data

Specific activity is a ratio of Gonal-F bioactivity (measured using the Steelman–Pohley assay) and protein content (measured by SE-HPLC) and expressed in IU/mg protein. Detailed statistical analysis has been performed on specific activity data for 100 Gonal-F batches of drug substance manufactured in 1997, 1998 and part of 1999 from nine different bioreactor runs. These demonstrated that the specific activity of Gonal-F was normally distributed, stable, and that there was no bioreactor run effect. At an average specific activity of 13 745 IU/mg, a target mass of 5.5 µg was judged to be equivalent to 75 IU. Detailed evaluation of the drug substance production data from 1998 to 2000 confirmed, within process and analytical variability, the well-controlled behaviour and consistency of the process (Figure 3) and supported this conversion factor (Serono, data on file).

The highly consistent physico-chemical and biological properties of the product now permit FSH quantification by SE-HPLC and vials/ampoules to be reliably filled by mass (FbM) rather than by specific bioactivity – this product is referred to as Gonal-F FbM.

Figure 2. Isoelectric focusing – isoform distribution comparison of reference standard (top) (26 independent values) and 63 batches of r-hFSH (Gonal-F) drug substance produced in 1999 (n = 41) and 2000 (n = 22). (Serono, data on file)

Regulatory implications of Gonal-F FbM

Gonal-F FbM represents a significant technological advance over current FSH products and scientific data supporting its development and use have been submitted to both the FDA and the EMEA. During the registration process of Gonal-F, both agencies asked Serono to consider developing the FbM process in order to improve batch-to-batch consistency further. This
request follows a general regulatory trend (and in fact a commitment of the European Pharmacopoeia Commission) to reduce animal usage, wherever possible, in testing of pharmaceuticals. Regulatory agencies increasingly require manufacturers of biopharmaceutical products to replace in vivo and/or in vitro bioassay procedures by more sophisticated physico-chemical analytical methods providing more adequate pharmaceutical control. Recombinant human growth hormone and recombinant human insulin are examples of protein products that pharmacopeial monographs require to be tested for potency by physico-chemical methods. Indeed, the rapid scientific progress in biopharmaceutical analysis has made state-of-the-art, highly computerised analytical methodology available for quality control purposes.

Serono is committed to the use of SE-HPLC methodology to test Gonal-F FbM (and other gonadotrophins) for potency. This choice required, as explained before, that the r-hFSH product produced by Serono had to be thoroughly characterised to understand in detail the physico-chemical properties of the product. In addition, the manufacturing process had to be optimised and thoroughly validated to demonstrate that a highly consistent r-hFSH product is obtained, from batch to batch, under routine manufacturing conditions. Lastly, the SE-HPLC method had to be optimised, and validated in agreement with International Conference on Harmonisation (ICH) guidelines, to be able to support a 90–110% protein content specification range.

Clinical implications of Gonal-F FbM

In a clinical study, four batches of Gonal-F were each filled by bioassay, as well as by mass. When comparing the clinical response of these batches, those patients receiving the filled-by-mass preparation had a more consistent clinical response in terms of oocytes retrieved. Greater control of the dose of FSH delivered is therefore associated with an improved consistency of clinical response. Initial clinical experience in women undergoing COS for ART indicates that Gonal-F FbM is more effective than standard filled-by-bioassay Gonal-F in terms of the quantity and quality of oocytes and embryos, number of days of stimulation and number of doses required. Gonal-F FbM is, of course, also at least as well tolerated as the standard Gonal-F product. Further studies using the novel FbM product are ongoing.

Conclusions

Extensive analyses have shown that Serono’s r-hFSH (Gonal-F) has extremely high physico-chemical and biological consistency, which is reflected in its status as the first FbM r-hFSH. As a result, Gonal-F can now be characterised by non-bioassay methodology; Serono uses an optimised SE-HPLC technique for quantification of r-hFSH protein in both the drug substance and final product. Not only is this method far more precise than the Steelman–Pohley in vivo bioassay, representing a significant improvement in product quality and batch-to-batch consistency, but it also reduces the necessity to sacrifice tens of thousands of laboratory animals. In prescribing Gonal-F FbM, clinicians can be assured that they are using a product manufactured to the highest standards of quality and consistency that delivers a guaranteed dose. In conclusion, therefore, in view of the well-known drawbacks of in vivo bioassay, methods such as SE-HPLC are likely to gain increasing popularity for quality testing and regulatory purposes, provided that the manufacturing process is well controlled, fully validated and results in a protein product of highly consistent physico-chemical properties.
References


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