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Article

Batch-to-batch consistency of human-derived gonadotrophin preparations compared with recombinant preparations

Claudio Wolfenson obtained his PhD in 1985 at the department of organic chemistry, University of Buenos Aires, Argentina. During the last 20 years he has been working in the pharmaceutical industry primarily with development of purification processes for glycoproteins derived from biological sources. Research interests include purification and characterization of glycoproteins and other types of glycoconjugates from biological sources.

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Abstract

Different gonadotrophin preparations derived from human urine or manufactured by recombinant technology are currently used in clinical practice for the treatment of infertility. It has been widely assumed that gonadotrophin products manufactured by recombinant technology have better batch-to-batch consistency compared with human-derived preparations and that this potentially will be shown to provide a more constant clinical response, but there is little evidence for either statement. This study compared the batch-to-batch consistency between urinary-derived and recombinant manufactured gonadotrophin preparations using standard analytical techniques, as well as a novel in-vitro follicle bioassay to evaluate the consistency of the biological response at the target organ. Oligosaccharide isoform profiling, immunoassay testing, size exclusion chromatography analysis and in-vitro bioassay testing of urinary derived gonadotrophin preparations (MENOPUR® and BRAVELLE®) confirm that these products display a high degree of batch-to-batch consistency, similar to recombinant FSH (GONAL-f®) either filled by mass or bioassay. The data also suggest that the batch-to-batch variation is independent of the manufacturing procedure (filled-by-bioassay or filled-by-mass) for the recombinant preparation (Gonal-f), but that the total FSH bioactivity delivered from a single dose preparation after reconstitution differs between the two manufacturing procedures.

Keywords: *batch-to-batch consistency, gonadotrophin, human-derived, recombinant FSH, in-vitro follicle bioassay*

Introduction

Several different drug products containing gonadotrophin hormones derived from urine of postmenopausal women are currently used in clinical practice for the treatment of infertility, such as HMG (human menopausal gonadotrophin) preparations containing a 1:1 ratio of FSH and LH bioactivity, as well as preparations containing only FSH bioactivity. From 1995, gonadotrophin products, manufactured by recombinant DNA technology, have become available. The importance of the manufacturing procedure used for gonadotrophin preparations, together with the purity and consistency of the isoform profile, has been discussed recently (Recombinant Human FSH Product Development Group, 1998; Driebergen and Baer, 2003; Gervais *et al*., 2003). Furthermore, it is widely assumed that gonadotrophin products manufactured by recombinant technology have less batch-to-batch variability and that this eventually will be shown to provide a more consistent clinical response. However, there is little evidence for either statement.

FSH, LH and human chorionic gonadotrophin (HCG) belong to the same family of complex glycoprotein hormones. These

gonadotrophins are heterodimers composed of an alpha- and a beta-subunit. While the alpha-subunit is common for these three gonadotrophins, the beta-subunits are unique, giving them their different biological characteristics. The *N*-linked carbohydrates are complex, with different numbers of antennae (from two to five) and various levels of sialylation and sulphation (Green and Baenziger, 1988a,b; Gervais *et al*., 2003). These variations in structure and type of sugar residues within a glycoprotein are commonly known as isoforms, and they can display significantly different in-vivo biological activity (Baenziger and Green, 1988; Burgon, 1996; Gervais *et al*., 2003). It is also well established that in-vivo bioactivity of these hormones is related to the plasma half-life of the isoforms. Wide and Hobson showed that the more acidic forms (higher content of sialic acid) of human FSH exhibited longer plasma half-lives and higher in-vivo bioactivity than the less acidic isoforms (Wide, 1986; Wide and Hobson, 1986; Galway *et al*., 1990; Bishop *et al*., 1995). Another aspect of the in-vivo bioactivity of different isoforms relates to differences in interaction at the hormone receptor and post-receptor effects level (for review, see Barrios-de-Timossi *et al*., 2002). Regarding isoform heterogeneity, recombinant FSH manufactured using Chinese hamster ovary (CHO) cells shows an intermediate complexity as compared with human-derived FSH. This is most likely because the CHO cells lack the enzymatic functions to construct the more complex carbohydrate structures found in the human (Horsman *et al*., 2000).

The labelled potency of gonadotrophin preparations is determined in accordance with the European and United States Pharmacopoeias using bioassays, e.g. the FSH bioactivity in the product is determined using the Steelman and Pohley assay (Steelman and Pohley, 1953). This assay measures the ovarian weight gain in rats as an endpoint and has a good predictive value for the response in humans. Bioassays are still the only way to measure the integrated physiological response of an isoform mixture of gonadotrophins on its target organ. Gonadotrophin products are normally manufactured by adding a fixed number of International Units (IU), as determined by bioassay, per vial (FbIU). Recently, the concept of filling by mass (FbM) was introduced, where the specific bioactivity is related to protein content and a fixed amount (μg) of protein is added to each vial instead (Driebergen and Baer, 2003). As previously mentioned, different isoforms can have significantly different in-vivo biological activity (Burgon *et al*., 1996; Rose *et al*., 2000; Gervais *et al*., 2003), which to a large extent is due to differences in the relative rate of clearance. For example, it has been shown that acidic isoforms can be 100- to 200-fold more bioactive *in vivo* than basic isoforms. Thus, relatively small changes in isoform profiles may significantly impact the in-vivo bioactivity of the preparation (Mulders *et al*., 1997). Therefore, even though a product has a constant protein content, there can still be a large variation in the biological activity between drug product batches when FbM.

Urinary derived gonadotrophins have been used clinically for over 40 years and their safety is well established (Westergaard *et al*., 2001; EISG, 2002). Recently, a second generation of highly purified (HP) urinary derived gonadotrophins compared with the first generation, was introduced. The increased purity is obtained by adding additional purification steps, such as anion exchange and hydrophobic interaction chromatography to remove urinary proteins without FSH and/or LH bioactivity. The significantly increased purity of the second generation gonadotrophin preparations facilitates more comprehensive characterization studies providing additional information on the composition. Whereas the action of FSH is mediated by a distinct receptor, the beta chains of LH and HCG share 82% protein sequence homology and exert their actions through the same receptor (McFarland *et al*., 1989). Urine from post-menopausal women contains both LH and HCG molecules (Armstrong *et al*., 1984; Akar *et al*., 1990; Alfthan *et al*., 1992) and consequently HMG preparations may contain both gonadotrophins. However, in-vivo bioassays cannot distinguish between LH and HCG molecules; instead, specific antibodies can be used to estimate the LH and HCG content in these preparation.

The principal aim of this study was to investigate batch-to-batch consistency of currently available highly purified urinary derived gonadotrophin preparations to allow comparisons among commercially available preparations, e.g. preparations manufactured using recombinant technology. The analytical techniques used include immunoassay; size-exclusion chromatography (SEC); isoelectric focusing (IEF); anion exchange chromatography (AEC) and a multiparametric in-vitro follicle bioassay. The in-vitro follicle bioassay was used to evaluate the response at the target organ for both the specificity and consistency for each of the preparations (Cortvrindt and Smitz, 2002).

Materials and methods

The gonadotrophin drug products GONAL-f® (recombinant), PERGONAL® (urinary) (Serono, Geneva, Switzerland) and HUMEGON® (urinary) (Organon, Oss, The Netherlands) were purchased in Europe and Argentina. MENOPUR® and BRAVELLE® were obtained directly from the manufacturer, Ferring Pharmaceuticals (Copenhagen, Denmark), and were randomly selected to contain different HMG-HP and FSH-HP drug substance batches respectively. In general, a drug product batch is a finished dosage form that contains a drug substance in association with other ingredients. A drug product or drug substance batch produced on a specified occasion should have uniform characteristics and quality, within specified limits. Drug substance batches used in Bravelle and Menopur and other reference standards were provided by Instituto Massone, Buenos Aires, Argentina. A complete list of the samples used in this study is provided in **Table 1**.

Sialic acid profiling (HPAEC-PAD)

Standard oligosaccharides and sialic acid (A0812) were obtained from fetuin purchased from Sigma (Saint Louis, Missouri, USA). Peptide *N*-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA, USA).

The *N*-linked carbohydrates were released from the FSH-HP and HMG-HP drug substances and fetuin by PNGase F digestion. The samples were denatured in 5% SDS, 10% β-mercaptoethanol, and then incubated with PNGase F (~1 mU per 20 mg sample), 10% Nonidet P-40 detergent (NP-40) in 0.5 mol/l sodium phosphate pH 7.5 for 18 h at 37°C. Subsequent to enzymatic cleavage from the protein, *N*-glycans were purified using Ultrafree McFilter (molecular weight cut-off 5000; Millipore, Bedford, MA, USA).

PNGase F released oligosaccharides were analysed by high performance anion exchange chromatography (HPAEC) using

Table 1. Sample information and performed analyses. Three subsets of samples have been used: (i) pharmaceutical drug products (DP); (ii) gonadotrophin drug substances (DS) in the pharmaceutical drug products Menopur and Bravelle; and (iii) WHO International Reference Standards.

Sample	*Labelled bioactivity content (IU)*		*Drug product batch identification number*	*Drug substance batch identification number*	*Analyses*[a]
	FSH	*LH*			
Menopur (DP)	75	75	32509	1817184003	1,2,3,5
Menopur (DP)	75	75	32307	1741175902	1,2,3,5
Menopur (DP)	75	75	34104	1872189403	1,2,3,5
Bravelle (DP)	75	–[b]	FMA010	1302132101	1,3,5
Bravelle (DP)	75	–[b]	FMA015	1386139701	1,3,5
Bravelle (DP)	75	–[b]	FMA018	1398141201	1,3,5
Gonal-f (DP)	75	–	BM210B	Not available	1,3,5
Gonal-f (DP)[c]	75	–	02L09	Not available	1,3,5
Gonal-f (DP)	75	–	G3310B	Not available	1,3,5
Gonal-f (DP)[c]	75	–	1971006C04	Not available	1,3
Gonal-f (DP)[c]	75	–	MQN657A04	Not available	1,3
Gonal-f (DP)[c]	75	–	1970512B02	Not available	1,3
Gonal-f (DP)	75	–	BM215C	Not available	1,3
Gonal-f (DP)	75	–	G3311G	Not available	1,3
Pergonal (DP)	75	75	0331206B	Not available	1,2
Humegon (DP)	75	75	43905119	Not available	1,2
HMG-HP (DS in Menopur)			–	1817184003	4
HMG-HP (DS in Menopur)			–	1741175902	4
HMG-HP (DS in Menopur)			–	1872189403	4
HMG-HP (DS in Menopur)			–	1277129201	4
HMG-HP (DS in Menopur)			–	1462147601	4
HMG-HP (DS in Menopur)			–	1580161102	4
FSH-HP (DS in Bravelle)			–	1302132101	4
FSH-HP (DS in Bravelle)			–	1386139701	4
FSH-HP (DS in Bravelle)			–	1398141201	4
FSH-HP (DS in Bravelle)			-	78881800	4
FSH-HP (DS in Bravelle)			–	1665163902	4
2nd WHO standard[d]	54	46		71/223	1,2
4th WHO standard[d]	72	70		98/704	1,2

[a]Analyses: 1 = FSH immunoassay; 2 = LH and HCG immunoassays; 3 = size-exclusion chromatography; 4 = sialic acid profiling; 5 = in-vitro follicle bioassay.
[b]Bravelle contains ≤2% of the total FSH activity.
[c]Labelled bioactivity is 75 IU FSH and the mass is 5.5 µg.
[d]International Reference Standards for human urinary FSH and LH (National Institute for Biological Standards and Control, NIBSC–WHO, Hertfordshire, UK). Menotrophin extracted from urine from post-menopausal women in the 2nd and 4th standards was originally provided by Instituto Massone Buenos Aires, Argentina and Instituto farmacologico Serono Rome, Italy respectively.
HP = highly purified.

a Dionex DX-300 (Sunnyvale, CA, USA), equipped with an amperometric pulse detector (PAD). Sialylated oligosaccharides were separated on a Carbopack PA-100 column (4 × 250 mm) with pre-column Carbopac Guard PA-100 (Dionex, Sunnyvale, CA, USA) using a 100 mmol/l sodium hydroxide solution over the whole run and sodium acetate 50 mmol/l for the first 5 min, followed by a gradient of sodium acetate from 50 to 170 mmol/l over 60 min, with a flow rate of 1 ml/min.

Immunoassay

Immunoactivities were measured by enzyme linked fluorescent assay (ELFA) using a MiniVidas automatic analyser (BioMérieux, Marcy-l'Etoile, France) with corresponding VIDAS® FSH, HCG and LH kits. Testing was performed according to the instructions provided with the kit. Each drug product vial was dissolved in 1 ml of saline solution.

Size exclusion chromatography (SEC)

Size exclusion chromatography was performed using a TSK G 2000 SW 7.5 × 600 mm column (Tosoh Bioscience, Montgomeryville, PA, USA) together with Waters 2690 separation module (Waters, Milford, MA, USA). Phosphate/sulphate pH 6.7 was used as mobile phase at a flow rate of 0.7 ml/min. The eluate was monitored at 210 nm. Each drug product vial was dissolved in 1 ml water and 80 µl was injected on to the column.

Statistical calculations for immunoassay and SEC comparison

The relative standard deviation is expressed as a percentage and is obtained by multiplying the standard deviation (per batch) by 100 and dividing this value by the average (per batch). The relative intra-product standard deviation is obtained by multiplying the standard deviation (per product using the average values per batch) by 100 and dividing this value by the average (per product).

In-vitro follicle bioassay

Mice were housed and bred following the Belgian standards for animal breeding. Animals were killed in accordance with the Belgian regulations for animal welfare. These experiments were approved by the ethics committee of the Free University of Brussels. Follicle culture assays were set up as previously described by Cortvrindt and Smitz (2002). In short, intact preantral follicles (100–130 µm) were isolated from ovaries from prepubertal mice (C57BlCa/CBA) and cultured individually in 96-well plates for 12 days up to the preovulatory stage, when the ovulatory stimulus (1.5 IU/ml HCG + 5 ng/ml recombinant human epidermal growth factor (rEGF, purchased from Boehringer Mannheim, Mannheim, Germany) was given (Cortvrindt and Smitz, 2002). The basal culture medium (CM) was composed of alpha Minimal Essential Medium (α-MEM) with 5% heat inactivated fetal calf serum (HIA FCS, both from Life Technologies, Merelbeke, Belgium) and insulin–transferrin–selenium mixture (ITS, from Sigma, Bornem, Belgium).

On days 4, 8 and 12, follicles were scored morphologically for survival and differentiation stage. Half of the medium was refreshed and conditioned medium was collected and pooled per set of 10 follicles. From each refreshment of culture medium, samples were kept frozen at –20°C for batchwise measurement of testosterone, oestradiol and progesterone. At 18 h after the ovulatory stimulus, follicles were scored for mucification of the cumulus–oocyte complex (COC). Oocytes were denuded, measured and analysed for nuclear maturity. Testosterone, 17β-oestradiol and progesterone concentrations were measured in the conditioned medium at days 8 and 12 and progesterone concentration was also determined on day 13. Antrum formation (a parameter selected for the evaluation of follicle growth and differentiation) and oocyte nuclear maturation stage (a parameter selected for the evaluation of oocyte quality) were respectively scored at days 12 and day 13 of the culture period. The rates were expressed as percentages and calculated in relation to the number of follicles that were initially put into culture.

Results

Oligosaccharide isoform profiles

The degree of sialylation between batches determined by HPAEC was used to substantiate process reproducibility and ultimately batch-to-batch consistency of the urinary-derived gonadotrophins used to manufacture Bravelle and Menopur. The relative content of mono-, di-, tri-sialylated and other oligosaccharides released from five FSH-HP (Bravelle) and six HMG-HP (Menopur) drug substance batches are shown in **Figure 1**. Approximately 90% of the oligosaccharides are di- or tri-sialylated and for either type the difference between minimum and maximum is less than 10% between batches for both FSH-HP (Bravelle) and HMG-HP (Menopur) at two separate testing occasions. However, per testing occasion the range is <5% between batches for both drug substances. The variation of the assay is illustrated by including a reference batch on all occasions.

Immunoassay

Three batches of both Menopur and Bravelle were compared with three batches of Gonal-f. The immunological FSH content in these nine batches together with the relative standard deviation (SD) per batch ($n = 5$) and per product (also referred to as intra product variation) ($n = 3$) are provided in **Table 2**. The data indicate a larger intra-product variation for Gonal-f as compared with Menopur and Bravelle. However, one Gonal-f batch (02L09) appeared to deviate from the other two Gonal-f batches, thus increasing the total average content and intra product variation. Batch 02L09 is labelled to contain 75 IU (5.5 µg), previously referred to as Gonal-f FbM, whereas the other two Gonal-f batches are only labelled with 75 IU and presumably FbIU.

The immunological FSH content of additional Gonal-f batches either FbM or FbIU was determined to confirm whether this apparent difference can be linked to the manufacturing method (**Table 3**). The average FSH value from four different batches of Gonal-f (FbIU) was normalized to 100 and the FSH content in batches of Gonal-f (FbM) were expressed as a percentage of the normalized FbIU average. The data suggest that Gonal-f

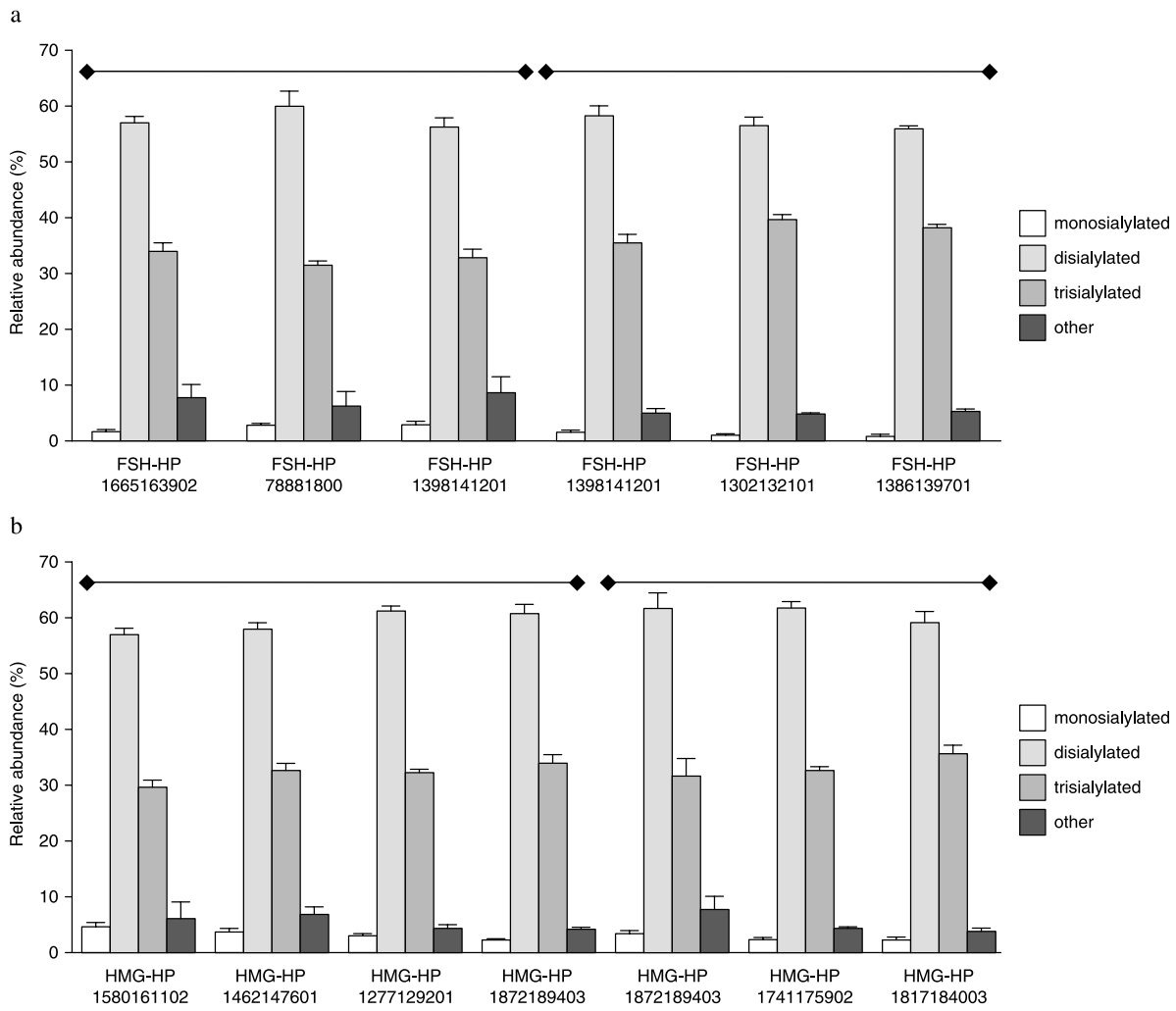

Figure 1. Relative content of mono-, di-, and tri-sialylated and other oligosaccharides released from (**a**) five FSH-HP (Bravelle) and (**b**) six HMG-HP (Menopur) drug substance batches using high performance anion exchange chromatography with amperometric pulse detector (SD displayed with error bars, n = 3–5). (♦-♦) represents different testing occasions and one FSH-HP (1398141201) or HMG-HP (1817184003) reference batch was tested on both occasions. HP = highly purified.

Table 2. FSH immunoactivity in nine gonadotrophin drug products (3 × Menopur, 3 × Bravelle and 3 × Gonal-f) all labelled to contain 75 IU of FSH bioactivity per vial.

Product	*Drug product batch no.*	*FSH immunoactivity IU/vial (relative SD, n = 5)*	*Average and relative intra-product SD (n = 3)*
Menopur	32509	74.17 (1.9)	76.74 (6.6)
Menopur	32307	73.44 (3.9)	
Menopur	34104	82.62 (1.3)	
Bravelle	FMA010	74.48 (2.8)	75.45 (5.2)
Bravelle	FMA015	79.75 (1.7)	
Bravelle	FMA018	72.12 (5.4)	
Gonal-f	BM210B	72.34 (3.5)	84.31 (13.9)
Gonal-f	02L09	95.70 (2.3)	
Gonal-f	G3310B	84.90 (7.6)	

Table 3. FSH immunoactivity in eight batches of Gonal-f, either filled by mass (FbM) or filled by bioassay (FbIU). The data have been normalized to the average of the four FbIU.

GONAL-f	*Drug product batch no.*	*FSH normalized (relative SD, n = 5)*	*Normalized average and relative intra-product SD (n = 4)*
FbM	1971006C04	123 (4.1)	125 (4.4)
FbM	MQN657A04	126 (4.0)	
FbM	02L09	132 (2.3)	
FbM	1970512B02	119 (3.4)	
FbIU	BM210B	99 (3.5)	100 (11.8)
FbIU	BM215 C	93 (4.6)	
FbIU	G3311G	91 (3.0)	
FbIU	G3310B	117 (7.6)	

(FbM) contains more FSH than Gonal-f (FbIU).

FSH, HCG and LH immunological content values are provided in **Table 4** for Menopur in comparison with other commercially available menotrophin drug products and two generations of the WHO international reference standard for urinary FSH and LH. All preparations tested contain both HCG and LH; however, the LH content in Menopur appears to be lower than other preparations. It should be noted that immunoactivity cannot be related to bioactivity, since the assays are based on different principles. Therefore, the vast majority of LH bioactivity in Menopur is provided by HCG. The intra-product variation (10.5%, calculated from data in **Table 4**) of immunological HCG content in Menopur, is of the same order of magnitude as the variation observed for FSH immunoactivity in Menopur, Bravelle and Gonal-f (**Tables 2** and **4**).

Size-exclusion chromatography (SEC)

The nine batches tested by immunoassay (**Table 2**), were further studied by SEC and the obtained area under the curve (AUC), relative SD and intra-product variation are provided in **Table 5**. Representative chromatograms for each product are shown in **Figure 2**. As expected, the obtained AUC for Menopur is higher than the values obtained for Bravelle and Gonal-f, which are comparable. This difference is to a large extent due to the 75 IU of LH bioactivity also present in Menopur.

The total intra-product variation seen by SEC is in line with that obtained by FSH immunoassay (**Table 2**). In addition, the AUC values obtained for the three Gonal-f batches show the same trend as seen for the FSH immunoassay results, i.e. AUC for the FbM batch (02L09) is higher than the other two FbIU Gonal-f batches (**Table 2**). This difference in AUC (of the main peak at retention time 20 min) is further illustrated by two representative SEC chromatograms of Gonal-f FbM and FbIU (**Figure 3**). The chromatograms in **Figure 3** also show a significant difference in the SEC profiles (retention time 25–40 min) between Gonal-f FbM and FbIU, which is most likely linked to differences in excipients (inactive ingredients such as polysorbate, lactose, etc.).

The Gonal-f batches analysed by FSH immunoassay (**Table 3**) were further studied by SEC and the obtained areas under the curve (AUC) are given in **Table 6**. The SEC data confirm the results obtained by FSH immunoassay, i.e. that Gonal-f (FbM) contains more FSH than Gonal-f (FbIU). The relative intra-product SD for both FSH immuno content and AUC (SEC) are summarized in **Table 7**.

In-vitro follicle bioassay

Three drug product batches of Menopur and Bravelle have been compared with three drug product batches of Gonal-f using a multiparametric in-vitro follicle bioassay (Cortvrindt, 2002). The bioassay was used to evaluate and compare the nine batches at two relevant doses with respect to folliculogenesis, oogenesis and steroidogenesis.

Folliculogenesis and oogenesis

Using one-way ANOVA, no inter-batch differences were observed when drug preparations were applied to the follicle cultures at concentrations of 25 and 100 mIU/ml. **Figure 4** illustrates that both the proportion of follicles that progressed to the final differentiation stage and the percentage of oocytes extruding a polar body (PB) after ovulatory stimulation, were comparable for all batches of each product at the two concentrations. Follicle survival during the whole culture period did not differ between batches of each gonadotrophin preparation (data not shown).

Steroidogenesis

No significant differences between batches with respect to steroid hormone production were observed in the in-vitro follicle bioassay, when gonadotrophin preparations were applied at 100 and 25 mIU/ml. Testosterone and oestradiol secretion (by the theca cells and the granulosa cells respectively) were comparable for the different batches per product. Due to the presence of LH bioactivity, Menopur tended to induce a higher testosterone production with higher oestradiol concentrations as a consequence, compared with the two other preparations at both concentrations (**Figure 5**). In all preparations the oestradiol secretion increased time-dependently, as measured at day 8 and

Table 4. FSH, LH and HCG immunoactivity in different HMG preparations.

Product	*Drug product batch no.*	*FSH immunoactivity IU/vial (relative SD, n = 5)*	*LH immunoactivity IU/vial (relative SD, n = 5)*	*HCG immunoactivity IU/vial (relative SD, n = 5)*
Pergonal	0331206B	58.77 (2.2)	13.49 (3.6)	3.39 (1.7)
Humegon	43905119	65.12 (1.7)	5.77 (1.0)	6.86 (1.8)
2nd WHO standard		77.72 (5.0)	7.39 (2.4)	7.22 (4.8)
2nd WHO standard		86.14 (5.3)	3.82 (1.8)	10.10 (5.1)
Menopur	32509	74.17 (1.9)	0.29 (5.2)	9.61 (2.3)
Menopur	32307	73.44 (3.9)	0.48 (1.7)	9.05 (3.3)
Menopur	34104	82.62 (1.3)	0.39 (3.1)	11.06 (1.8)

Table 5. Area under curve (AUC) obtained by size exclusion chromatography (SEC) for nine gonadotrophin drug products (3 × Menopur, 3 × Bravelle and 3 × Gonal-f).

Product	*Drug product batch no.*	*AUC (SEC) (relative SD, n = 3)*	*Average and relative intra-product SD (n = 3)*
Menopur	32509	32.4 (0.4)	32.9 (6.5)
Menopur	32307	31.0 (0.4)	
Menopur	34104	35.2 (0.3)	
Bravelle	FMA010	16.1 (0.8)	16.1 (1.9)
Bravelle	FMA015	16.4 (1.0)	
Bravelle	FMA018	15.8 (0.3)	
GONAL-f	BM210B	13.3 (2.4)	15.1 (12.5)
GONAL-f	02L09	17.1 (0.6)	
GONAL-f	G3310B	15.0 (1.0)	

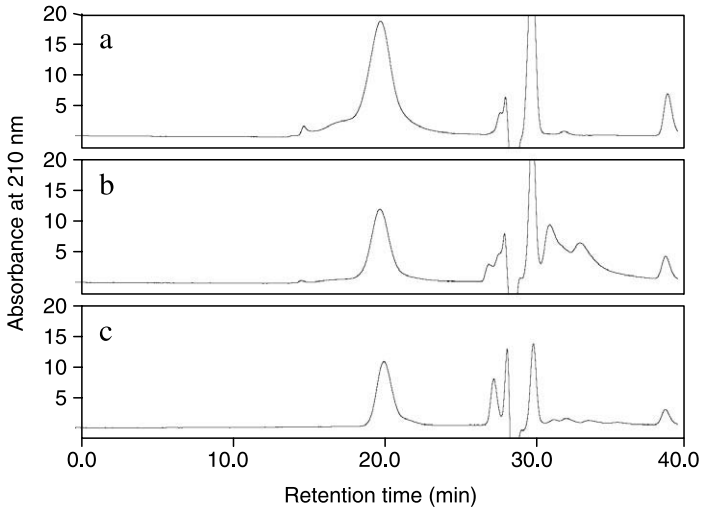

Figure 2. Representative size exclusion chromatography chromatograms of Menopur (**a**), Bravelle (**b**) and Gonal-f (filled by bioassay) (**c**).

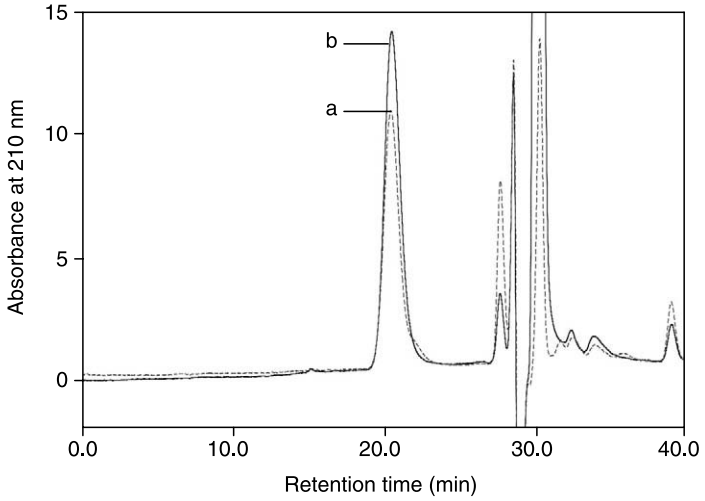

Figure 3. Representative size exclusion chromatography chromatograms of GONAL-f filled by bioassay (**a**) and Gonal-f filled by mass (**b**).

Table 6. Area under curve (AUC) (size exclusion chromatography, SEC) data in eight batches of for Gonal-f, either filled by mass (FbM) or filled by bioassay (FbIU). The data have been normalized to the average of the four FbIU results.

GONAL-f	*Drug product batch no.*	*AUC (SEC) normalized (relative SD, n = 3)*	*Normalized average and relative intra-product SD (n = 4)*
FbM	1971006C04	142 (0.1)	135 (5.2)
FbM	MQN657A04	127 (0.2)	
FbM	F02L09	140 (0.2)	
FbM	1970512B02	132 (0.3)	
FbIU	BM210B	99 (2.9)	100 (3.0)
FbIU	BM215 C	96 (2.1)	
FbIU	G3311G	101 (0.2)	
FbIU	G3310B	103 (4.6)	

Table 7. Relative intra-product SD for FSH content and area under curve (AUC) (size exclusion chromatography: SEC) in Menopur, Bravelle and Gonal-f (filled by mass: FbM) and (filled by bioassay: FbIU).

Drug product	*Relative intra-product SD*	
	FSH content	*AUC (SEC)*
Menopur	6.6	6.5
Bravelle	5.2	1.9
Gonal-f (FbM)	4.4	5.2
Gonal-f (FbIU)	11.8	3.0

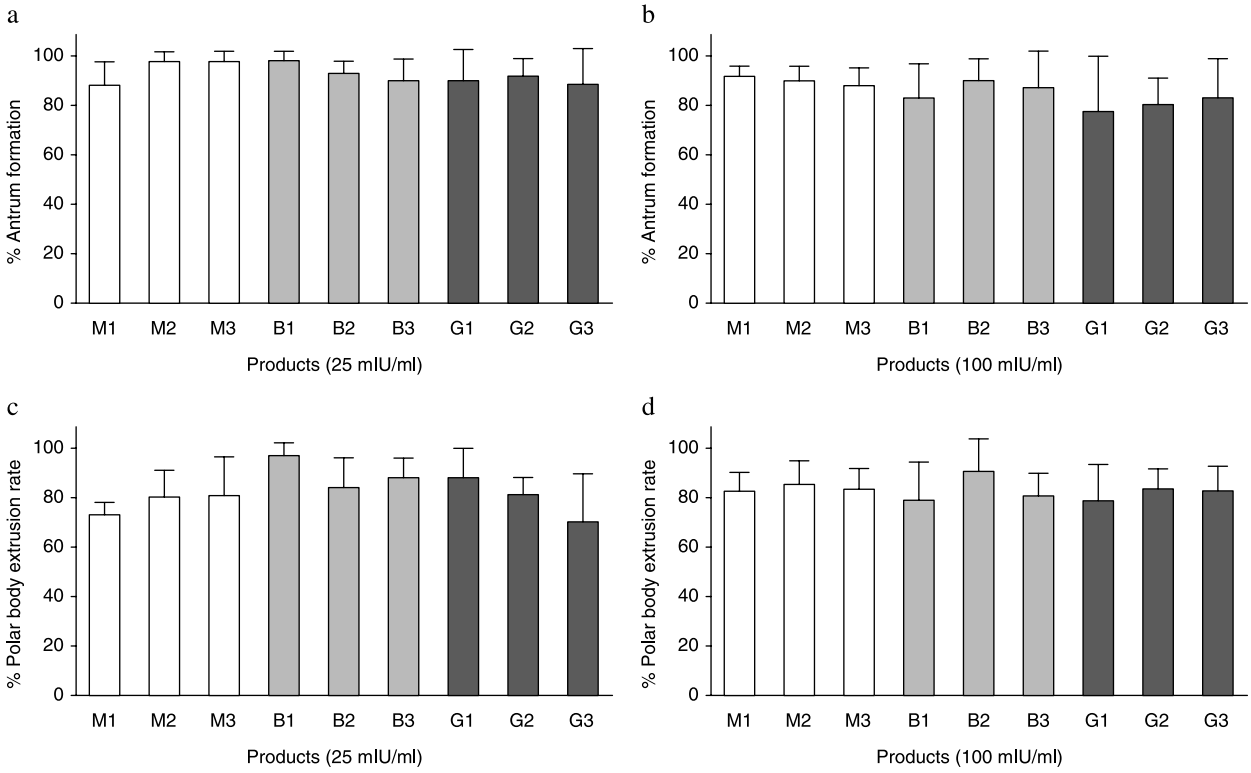

Figure 4. Inter-batch comparison of follicle differentiation (antrum formation) and oocyte quality (polar body extrusion rate) for doses of 25 mIU/ml (**a** and **c**) and of 100 mIU/ml (**b** and **d**) for the nine product batches: Menopur batch numbers 32509 (M1), 32307 (M2) and 34104 (M3); Bravelle batch numbers FMA010 (B1), FMA015 (B2) and FMA018 (B3); Gonal-f batch numbers BM210B (G1), 02L09 (G2) and G3310B (G3). Rates in percentage are represented as the mean ± SD.

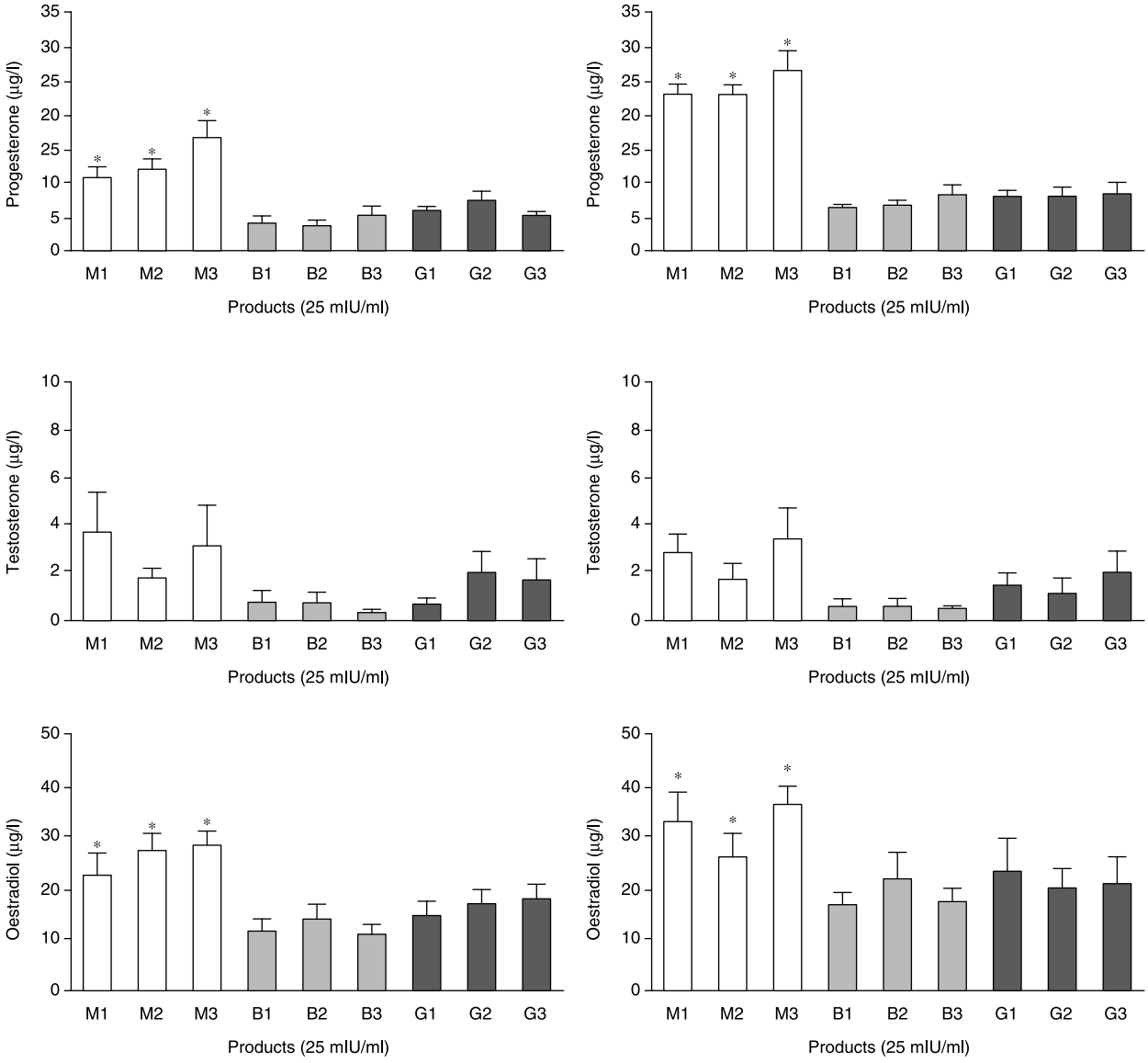

Figure 5. Progesterone, testosterone and oestradiol produced in conditioned medium on day 12 (prior to ovulation trigger). Hormone concentrations are shown for doses of 25 mIU/ml (left panel) and 100 mIU/ml (right panel). Columns represent mean concentrations (± SEM) for the nine product batches; Menopur batch numbers 32509 (M1), 32307 (M2) and 34104 (M3); Bravelle batch numbers FMA010 (B1), FMA015 (B2) and FMA018 (B3); Gonal-f batch numbers BM210B (G1), 02L09 (G2) and G3310B (G3). Columns topped by an asterisk are significantly different from those without asterisk (ANOVA: $P < 0.05$).

Table 8. Percentage of FSH with isoelectric points (pI) >4.25 and pI range for different gonadotrophin preparations.

	Gonal-f[a]	*Puregon*[a]	*Metrodin HP*[b]	*Metrodin*[b]	*Bravelle*[c]
pI >4.25 (%)	56	64	~15	~40	~100
pI range	3.5–6.1	3.2–5.7	3–5.5	–	3.5–7.0

[a]Horsman *et al*. (2000).
[b]Lambert *et al*. (1995).
[c]Internal Data Instituto Masdone (isoelectric focusing).

day 12 of the culture period (data not shown). In addition, progesterone concentrations were two to three times higher in Menopur than in Bravelle or Gonal-f cultures at both doses. For all product batches, comparison of the three steroid concentration profiles by ANOVA demonstrated batch-to-batch consistency. For all three products, the HCG ovulatory stimulus given on day 12 of culture induced a prompt luteinization, as measured by the steep progesterone increase on day 13 (data not shown).

Discussion

Bravelle and Menopur drug substances are manufactured from the same source of urine and during the purification procedure FSH and LH bioactivities are fractionated. The fraction containing FSH bioactivity is used to manufacture Bravelle, and appropriate quantities of FSH and LH bioactivity are pooled to achieve a 1:1 ratio in the manufacturing of Menopur. As previously mentioned, the highly purified HMG in Menopur contains both LH and HCG molecules, but LH molecules are preferentially lost compared with HCG during purification (internal data). The urine from post-menopausal women is collected in large quantities for the production of highly purified urofollitrophin (Bravelle) and highly purified menotrophin (Menopur). More than 100,000 individually screened donors contribute urine several times for the manufacture of each batch of drug substance. This ensures that a consistent starting material is generated, since potential individual variations are minimized by the large pool of urine used. The variation between batches is further minimized by using validated extraction and purification procedures to obtain large batches of consistent preparations. The anion exchange chromatography steps applied during the manufacturing of HMG in Menopur are not only used to increase the specific activity of the hormones, but also affect the obtained sialic acid profile of the final drug substance. When the purification process includes AEC steps the isoforms present in the final product will consistently reflect both the isoform profile of the loaded material and the specific conditions applied in the AEC step. The low variation in sialic acid content data (**Figure 1**) for Menopur and Bravelle confirms that using large quantities of urine and well-controlled manufacturing procedures result in preparations with a consistent profile between batches.

The in-vivo bioassays best define the FSH potency, since both the biological action at the target tissue and the rate of biological clearance are taken into account. It has been suggested that bioassays may be associated with a larger variation, but this to a large extent depends on the number of times the assay is repeated and the breed of animals used. The principal aim of this study was to document the batch-to-batch consistency of Menopur and Bravelle, both of which are FbIU, in comparison with Gonal-f either FbM or FbIU. FSH content and AUC (SEC) data show a comparable variation between batches for Menopur, Bravelle and Gonal-f FbM and FbIU (summarized in **Table 7**). The intra-product variation for HCG immunoactivity in Menopur is also comparable to that obtained for FSH, confirming a consistent content. The FSH and SEC data obtained for Gonal-f strongly indicate a difference in FSH content between batches FbM versus FbIU. However, the relative SD between FbIU and FbM batches is similar based on SEC, suggesting that the batch-to-batch variation is independent of the manufacturing procedure. The observed difference in FSH content between Gonal-f FbM or FbIU is probably due to (i) different compositions and/or (ii) different overages of drug substance. (The term overage describes the addition of extra drug substance to compensate for potential loss of drug substance during manufacture and/or for example during reconstitution). The most important compositional difference between Gonal-f (FbM) and (FbIU) is the presence of polysorbate in FbM product. The detergent polysorbate is known to minimize the adsorption of proteins to surfaces, and will probably affect the measured FSH concentration after reconstitution. However, the large differences observed in this study could also be linked to a potential difference in FSH overage.

The potentially higher content of FSH in Gonal-f (FbM) might induce a more prompt recruitment of oocytes by reaching the FSH threshold dose (conducive to multiple folliculogenesis) earlier. Indeed, a recent clinical study comparing Gonal-f (FbIU) and Gonal-f (FbM) indicated that a shorter duration of gonadotrophin treatment was observed in the FbM compared with the FbIU group (Balasch *et al*., 2004).

When evaluating effects of complex mixtures of FSH isoforms or FSH and LH/HCG isoforms, it makes sense to use a bioassay that permits the evaluation of integrated function on relevant end parameters. The bioassay was primarily used to mimic the in-vivo situation as used in infertility clinics (at supraphysiological concentrations) and demonstrated that small differences as detected by analysis of the physicochemical characteristics had no impact on the bioactivity of the preparations tested. Validation of the culture system from Cortvrindt and Smitz (2002) has shown in extensive preliminary studies that growth and steroid secretion patterns of the mouse ovarian follicle units are both time- and concentration dependent on FSH (Adriaens *et al*., 2004). The

cultured follicle is also sensitive to LH or HCG action during its growth and final maturation. On a purely theoretical basis, it is now well accepted that when measuring a combination of variables in a biological system (instead of a univariate approach) there are significant increases in power and efficiency of information and a decreased probability of error in hypothesis testing (Gad and Weil, 1989). The drug products Menopur, Bravelle and Gonal-f are designed to be used for superovulation where exposure of antral follicles to supra-threshold doses of FSH induce multiple follicle development. Isolated secondary mouse follicles were therefore exposed to two supra-threshold FSH concentrations: 25 and 100 mIU/ml as labelled by the manufacturer on their respective ampoules.

The clear formation of antral-like cavities in this culture system, being a robust parameter for follicle differentiation, was recorded on average in 80% of all surviving follicles with the three preparations and for the two doses. For this parameter, the lowest variability between different batches was observed for Menopur. As was previously demonstrated in this culture system, the presence of LH bioactivity had a clear effect on antral cavity formation (Cortvrindt *et al*., 1998). The triggering of the LH receptor present on thecal cells in early preantral follicles might provide additional trophic factors to granulosa cell growth. It is known that in rodent models complementary action of LH to FSH has anti-apoptotic effects via oestradiol on the earliest stages of antral follicles (Richards, 1975). Hence, LH bioactivity support provided within a certain window of concentrations might improve robustness in follicle selection by inducing expression of key enzymes in theca cells at onset of inter-cycle recruitment (Sasano *et al*., 1989; Tamura *et al*., 1992).

Extrusion of the first polar body, a marker of normal nuclear maturation, was seen on average in 80% of the oocytes. There were no significant differences between preparations or between batches. It should be pointed out that all in-vitro bioassay samples were reconstituted using PBS (phosphate-buffered saline) plus 1% BSA (bovine serum albumin). The BSA in the buffer probably reduces the importance of polysorbate as a blocking agent in the Gonal-f (FbM), thereby off-setting the observed differences in FSH content in Gonal-f (FbM) and (FbIU), observed by immunoassay and SEC (sample preparation without BSA), as discussed above. Not unexpectedly, the basal steroid production profile by cultured follicles on day 12 was different for Menopur compared with the two FSH preparations. However, there were no overall significant differences in steroid profile that could be detected in relation to batch-to-batch differences for the three preparations. The LH bioactivity in Menopur had a significant dose-dependent enhancing effect upon progesterone production, illustrating the effect upon the P450 side chain cleavage production. Menopur-stimulated follicles had a higher basal testosterone production than the FSH-alone preparations, but the dose-effect was not seen because more testosterone substrate was converted into oestradiol at the highest FSH dose. Basal oestradiol production increased dose dependently and was significantly higher in Menopur compared with the FSH preparations. This experiment shows that the follicle bioassay has a predictive value regarding the endocrine profile to be expected in human serum.

In this study, all FSH preparations used had broad FSH isoform mixtures, with Bravelle having a higher degree of less acidic isoforms as compared with Gonal-f (**Table 8**). It should be noted that FSH isoform profiles in Menopur cannot be differentiated from the LH and HCG isoforms using, e.g IEF and therefore a comparison with FSH preparations is not applicable. It has been shown by at least two laboratories that non-overlapping fractions of FSH isoforms had a different effect on follicle differentiation, induction of oocyte meiosis, fertilization and the percentage of embryos that proceed to the two-cell stage. As previously mentioned, more acidic isoforms exhibit longer plasma half-lives and higher in-vivo bioactivity than less acidic isoforms. However, the less acidic isoform fractions have been shown to be more biologically active, at the receptor level, compared with more acidic preparations (Vitt *et al*., 2001; Andersen, 2002). Furthermore, several research groups have shown that it is conceivable that different isoforms exert different functions on the gonad (Dahl *et al*., 1988; Timossi *et al*., 1998a,b, 2000; Vitt, 2001; Andersen, 2002; Barrios-de-Timossi, 2002). Isoform populations might exert physiological functions eventually through variant gonadotrophin receptor populations (Sairam *et al*., 1997; Babu *et al*., 2000). However, the implications of different isoform profiles upon embryo quality need to be further evaluated, as recently discussed by Andersen *et al*. (2004).

Oligosaccharide isoform profiling, immunoassay testing, SEC analysis and in-vitro bioassay testing of Menopur and Bravelle confirm that these products display a high degree of batch-to-batch consistency, similar to recombinant FSH (Gonal-f) either FbM or FbIU. It also appears that the variation in protein content seen in Gonal-f is independent of the two manufacturing procedures (FbIU or FbM).

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