Human Reproduction vol.14 no.5 pp.1160–1167, 1999

Biological characterization of recombinant human follicle stimulating hormone isoforms

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It has been established that follicle stimulating hormone (FSH) circulates in the bloodstream as a heterogeneous population of molecules. Individual FSH isoforms, while displaying identical amino acid sequences, differ in their extent of post-translational modification. As a result of these variations, the FSH isoforms exhibit differences in overall charge, degree of sialic acid or sulphate incorporation, receptor binding affinity and plasma half-life. Taking advantage of the fact that these forms can be separated from each other on the basis of their charge, we have evaluated in rats the metabolic clearance rates of the acidic [with an isoelectric point (pI) ⩽ 4.8] and the less acidic (pI > 4.8) isoforms of recombinant human FSH (rhFSH) obtained after chromatofocusing. The less acidic isoform group was found to have a faster clearance from the circulation in rats as compared with the acidic isoform group. This finding is in agreement with the lower bioactivity *in vivo* (as determined by the Steelman–Pohley assay) of the less acidic isoform group, compared with the acidic one. The mass spectra of the two groups of isoforms showed a difference in the sialic acid content thus highlighting the importance of these residues on the in-vivo activity of FSH. Conversely, when the two groups of isoforms were tested *in vitro* by using the Y1 human FSH receptor (Y1 hFSHR) assay and a reporter gene assay, no significant differences in the biological activities between these preparations were detected when test concentrations were based on mass.

Key words: biopotency/FSH/Gonal-F/isoforms/metabolic clearance rates

Introduction

The glycoprotein hormone, follicle stimulating hormone (FSH), is essential for reproductive function since it stimulates the development of the ovarian follicle and spermatogenesis. While FSH has been widely used in the treatment of female infertility, the use of exogenous FSH also offers considerable potential for the treatment of male infertility (Bishop *et al*., 1995). FSH consists of two dissimilar, non-covalently linked polypeptide subunits (Rathnam and Saxena, 1975). The protein sequence of the α-subunit is common to all the pituitary glycoprotein hormones [FSH, luteinizing hormone (LH), thyroid stimulating hormone (TSH)] and human chorionic gonadotrophin (HCG) while the β-subunit confers each hormone with its biological specificity. The human FSH α-subunit contains 92 amino acids with asparagine-linked carbohydrate side-chains attached at position 52 and 78 whereas the β-subunit contains 111 amino acids with *N*-linked carbohydrate at asparagine residues 7 and 24 (Rathnam and Saxena, 1975).

Several studies have shown that the anterior pituitary contains a spectrum of FSH isoforms with different isoelectric properties, bioactivities and in particular, circulating half-lives due to the micro-heterogeneity of the carbohydrate side chains (Chappel *et al*., 1983). In the serum of prepubertal children, hypogonadal patients and in women during the follicular phase, heavily sialylated, acidic isoforms with long half-lives *in vivo* and low in-vitro biological potency are predominant. In contrast, during puberty, gonadotrophin-releasing hormone (GnRH) therapy and the mid-cycle gonadotrophin surge in women, the less sialylated, less acidic isoforms, with short in-vivo half-lives and high in-vitro biological activity are found (Chappel *et al*., 1983; Padmanabhan *et al*., 1988; Wide and Albertsson-Wikland, 1990). Based on the above considerations, it has been assumed that the more acidic isoforms stimulate the follicular maturation process in a longer, but less intense way, while the less acidic isoforms appear to provide a short but potent stimulus, necessary for the induction of puberty or ovulation (Wide and Albertsson-Wikland, 1990; Padmanabham *et al*., 1992).

Since terminal sialylation of the carbohydrate structures has been reported to be a leading determinant of the plasma half-life of FSH (Morell *et al*., 1971; Vaitukaitis and Ross, 1971), the present study was undertaken to evaluate the metabolic clearance rates (MCR) in female rats of both the acidic [having an isoelectric point (pI) ⩽ 4.8] and less acidic (with a pI > 4.8) rhFSH isoforms, obtained after separation by chromatofocusing. The pI cut-off was fixed at 4.8 taking into account the disappearance curves of the FSH isoforms extracted from human pituitary injected in rats (Ulloa-Aguirre *et al*., 1992). The two isoform-enriched fractions were also evaluated for their activity *in vitro* (by the Y1 hFSHR bioassay and a reporter gene assay) and *in vivo* (rat Steelman–Pohley assay).

Materials and methods

Reagents

Ham's F-10 medium, α-minimal essential medium (MEM), geneticin G-418 sulphate, fetal bovine and horse sera were supplied by Gibco BRL (Life Technologies, Paisley, UK). L-glutamine and trypsin–

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EDTA were purchased from Hyclone (Cramlington, UK). rhFSH and progesterone were assayed using an immunoradiometric assay (IRMA) (FSH MAIAclone assay, Biochem Immunosystems, Coinsins, Switzerland) according to the manufacturer's instructions. All other reagents were of analytical grade, supplied by Merck (Darmstadt, Germany).

rhFSH material

The following test compounds were used throughout the study: rhFSH acidic isoforms [ISO $\leqslant$ 4.8, 0.89 mg/ml by optical density (OD), E1% = 6.98, 9537 IU/ml by in-vivo assay, 7371 IU/ml by immunoassay]; rhFSH less acidic isoforms (ISO $>$ 4.8, 0.71 mg/ml by OD, E1% = 6.98, 2065 IU/ml by in-vivo assay, 6485 IU/ml by immunoassay).

Separation of rhFSH isoforms

Bulk rhFSH was produced at Laboratoires Serono SA (Aubonne, Switzerland) by a conventional biotechnology process from Chinese hamster ovary (CHO) cells genetically engineered to contain the human genes for the α and β-subunits of FSH (Chappel *et al*., 1992). These selected recombinant cells synthesize the subunits of FSH and secrete a glycosylated bioactive dimeric FSH. The rhFSH contained within the harvested culture medium was purified by immunochromatography using a specific anti-FSH monoclonal antibody and a series of other purification steps to yield an extremely pure product. The process to obtain acidic (having pI $\leqslant$ 4.8) and less acidic (having pI $>$ 4.8) rhFSH isoforms from the rhFSH bulk material consisted of a chromatofocusing step followed by an ultrafiltration step for the ampholine removal. The rhFSH, diluted with one volume of 25 mM piperazine hydrochloride at pH 6.2, was loaded on a glass column (26×270 mm, i.d. ×l) packed with Polybuffer exchange 94 (Pharmacia). Wash and elution by resin titration were performed with ampholine pI 7–4 (Polybuffer PBE 74) diluted 1:10 in purified water and corrected to pH 4 with 1 M HCl; rhFSH isoforms having a pI $>$ 4.8 and $<$ 4.8 were collected. The residual ampholines were removed from each of the two fractions by ultrafiltration over a 10 kDa cut-off membrane (YM10, Amicon) under nitrogen pressure.

Isoelectrofocusing

The isoelectrofocusing (IEF) was carried out using IEF gels (Novex, San Diego, CA, USA) having a pH range 3–7 in order to compare the ISO $>$ 4.8 and ISO $\leqslant$ 4.8 fractions. The gel was run loading 50 μg of the acidic and less acidic isoforms for each lane. The run was performed in a Xcell II (Novex) for 1 h at 100 V, 1 h at 200 V, then the voltage was increased to 500 V for 30 min. After completion of the run, the gel was immersed in the fixing solution (14.6 g/l sulphosalicylic acid and 114.8 g/l trichloracetic acid) in order to fix the proteins and to remove the ampholytes. The gel was placed in the staining solution containing 0.025% (w/v) of Coomassie Brilliant Blue (Biorad) in 10% acetic acid. The staining step was carried out for 30 min, then the gel was de-stained in 10% acetic acid until the desired transparency had been achieved. All fixing, staining and destaining were performed under gentle shaking. The gel was scanned and the image was processed using the Phoretix 1D full software system (version 2.01) in order to evaluate fraction composition and pI of each band.

Electrospray/mass spectrometry (ES/MS) analysis of rhFSH isoforms

In order to investigate the molecular weight distribution of the isoforms, ES/MS of the two samples was performed by using a mass spectrometer VG Quattro II triple quadrupole (Micromass Limited, Manchester, UK). The ES/MS analytical process separates rhFSH into its constituent subunits, even if performed by direct introduction into the ES source, due both to the high temperature of the source and to the acidic pH of the mobile phase to be used to allow protonation of the species. Because of this, a preliminary on-line liquid chromatography step was deemed appropriate (although subunit separation was also obtained in this case) to enhance the response of the molecules. The two subunits, however, behaved differently during ES/MS, with the α-subunit giving an interpretable spectrum, and the β-subunit yielding a confused Gaussian distribution of multiple charge ions, which prevented transformation of the spectrum into a series of defined molecular weight species. If sialic acid is removed (e.g. by neuraminidase treatment), then the β-subunit also gives an interpretable spectrum; however, since it was expected that much of the difference between the isoform preparations was due to sialic acid content, this treatment was not considered meaningful and, therefore, only α-subunit spectra were obtained.

Immunoassay for quantification of rhFSH isoforms

A commercially available IRMA was used to measure the immunoreactivity of the two rhFSH isoform-enriched fractions, spiked into the assay medium and tested within the same assay run, and to quantify FSH concentrations in rat plasma. The MAIAclone assay, which incorporates two high-affinity antibodies into an IRMA system to attain higher sensitivity and specificity, does not recognize rat FSH and shows negligible cross-reactivity with human LH, human TSH and HCG.

Animals

Sprague–Dawley Crl:CD BR adult female rats (125–150 g), obtained from Charles River Italia (Calco, Lecco, Italy) and Hsd: Sprague–Dawley immature female rats (42–52 g), purchased from Harlan–Nossan Italy (Correzzana, Milano, Italy), were used in this study. Before the experiments, the animals were allowed to acclimatize under controlled environmental conditions and artificial lighting in a circadian cycle of 12 h light: 12 h dark regime; the period of light was 07:00–19:00.

Determination of the metabolic clearance rates of rhFSH

To determine the MCRs of the rhFSH isoforms, conscious female rats (five animals/time point) were injected into the caudal vein at time 0 with a bolus dose of rhFSH (7 μg/kg based on immunoactive titres). Blood samples (~800 μl) were taken from the abdominal vein 5 min before and 5, 15, 30 min and 2, 8, 24 h after the test drug injection. Plasma was collected by centrifugation and stored at –20°C until assayed for FSH content by the IRMA within the same assay session. The computerized pharmacokinetic evaluation of plasma concentration data was performed by PCNONLIN software using the two-compartment model. Curve fitting, performed on the mean values at each sampling time, were built-in with iteratively reweighted least square procedure, using the weighting function $Wi = 1/Ci^2$ where Ci is the calculated concentration.

Steelman–Pohley in-vivo assay

The increase in ovary weights due to the test compounds was determined using the Steelman–Pohley assay (Steelman and Pohley, 1953). The two rhFSH preparations were tested at seven different doses (10 animals/dose) based on IU; the doses were 0.26–4.13 μg/rat for ISO $>$ 4.8 and from 0.07–1.12 μg/rat for the ISO $\leqslant$ 4.8. Data were evaluated by GraphPad Prism (version 2.01) software.

Y1 hFSHR in-vitro bioassay

The Y1 hFSHR in-vitro assay was employed in order to determine the in-vitro bioactivities of the rhFSH isoforms. The characteristic of

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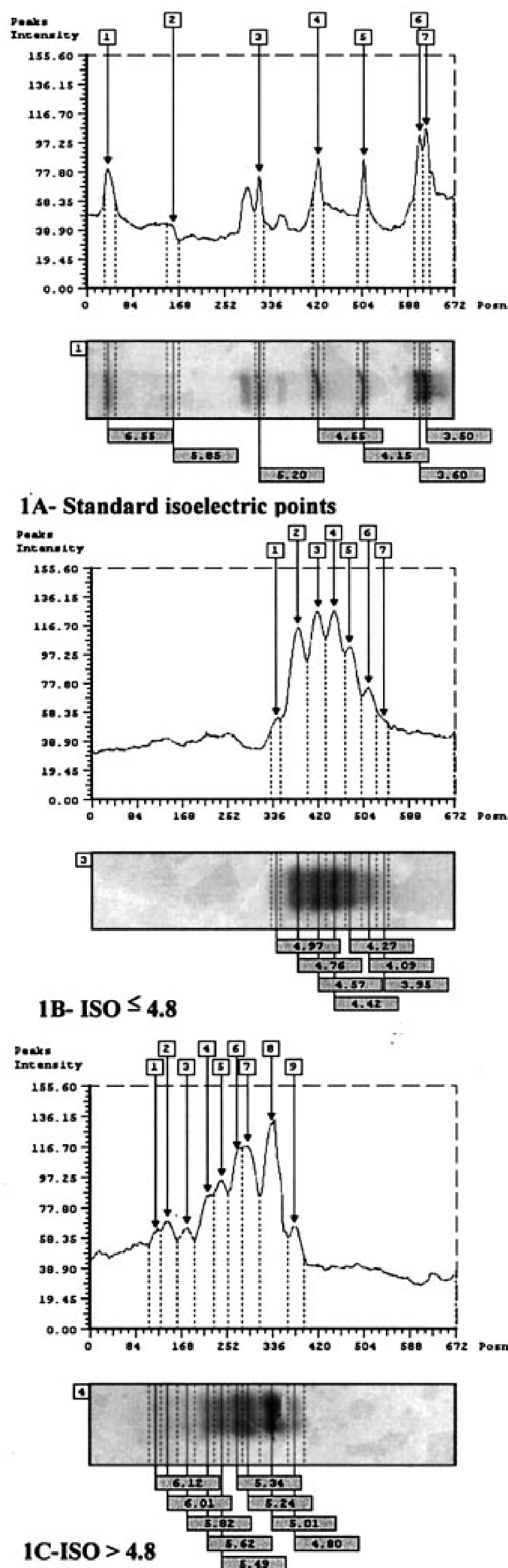
this cell line (mouse adrenal tumour transfected with human FSH receptor) has been previously described (Kelton *et al*., 1992). Briefly, Y1 human FSH receptor cells were routinely grown in Ham’s F10 culture medium supplemented with 15% horse serum, 2.5% fetal bovine serum (FBS), 80 μg/ml geneticin G-418 sulphate, 1% L-glutamine and subcultured in 96-well plates (20 000 cells/well). After 72 h, the growth medium was replaced by a serum-free medium (assay medium) before being incubated for 21 h with the test substances. At the end of FSH induction, the supernatant of each well was collected and measured for progesterone concentration by IRMA. Whole dose–response curves, on a mass basis, were generated for ISO > 4.8 and ISO ⩽ 4.8 in three independent experiments. A curve-fitting program (GraphPad Prism) was used to fit the dose–response data and to calculate EC_{50} values. The same working cell bank, horse and fetal bovine serum batches were used throughout the study.



CHO-hFSHR-Luc in-vitro bioassay

The 4Q13/Luc is a CHO DUKX cell line, prepared at Ares Advances Technologies (Randolph, MA, USA) expressing human FSH receptor which had been selected for dihydrofolate reductase (DHFR) resistance. This cell line had been subsequently transfected with 50 μg TF5/53 luciferase (Luc) vector and co-transfected with 10 μg Nα containing the *Neo* gene for G-418 resistance and, therefore, selection of transfectants. CHO–hFSHR–Luc cells were grown in α-MEM without ribonucleosides and deoxyribonucleosides (supplemented with 10% dialysed FBS, 600 μg/ml geneticin G-418 sulphate, 0.02 μM methotrexate and 1% L-glutamine) and subcultured in 96-well plates (40 000 cells/well). After incubation overnight, the growth medium was replaced by the medium containing different concentrations of the test compounds and incubated for ~5 h. At the end of FSH induction, cells were lysed, incubated for 15 min at room temperature before reading the plate in a luminometer (Dynatech, Denkedorf, Germany) after the addition of 150 μl of luciferase assay reagent (Promega Corporation, Madison, WI, USA). EC_{50} values were calculated by using the GraphPad Prism software.

Results

Isoelectrofocusing

After isoelectrofocusing, the gel was scanned to perform the image analysis so as to detect each band and measure area, position and pI. This was achieved by comparison of the bands of interest with a standard lane consisting of different proteins with known pI (Figure 1A). From the gel and the analysed data it was observed that two overlapping bands were present both in the acidic and less acidic isoform enriched fractions (Figure 1B, bands 1, 2 compared with Figure 1C, bands 8, 9). The band having pI = 4.97 (Figure 1B, band 1) in the acidic isoform fraction appeared to be a small contaminant obtained during separation procedures as it appeared more strongly in the less acidic isoform fraction (Figure 1C, band 8, pI = 5.01). Conversely, the band having pI = 4.76 (Figure 1B, band 2) appeared more marked in the acidic isoform fraction than in the less acidic one (Figure 1C, band 9, pI = 4.80). As a consequence, the acidic isoform group was named ISO ⩽ 4.8 and the less acidic group ISO > 4.8.

Figure 1. (**A**) Computer analysis run by the Phoretix software of Coomassie Brilliant Blue stained isoelectrofocusing gel. The isoelectric point of each band present in (**B**) the acidic (ISO ⩽ 4.8) and (**C**) less acidic isoforms (ISO > 4.8) were determined and compared. Standard isoelectric points (Pharmacia) (see **A**) were used as reference.

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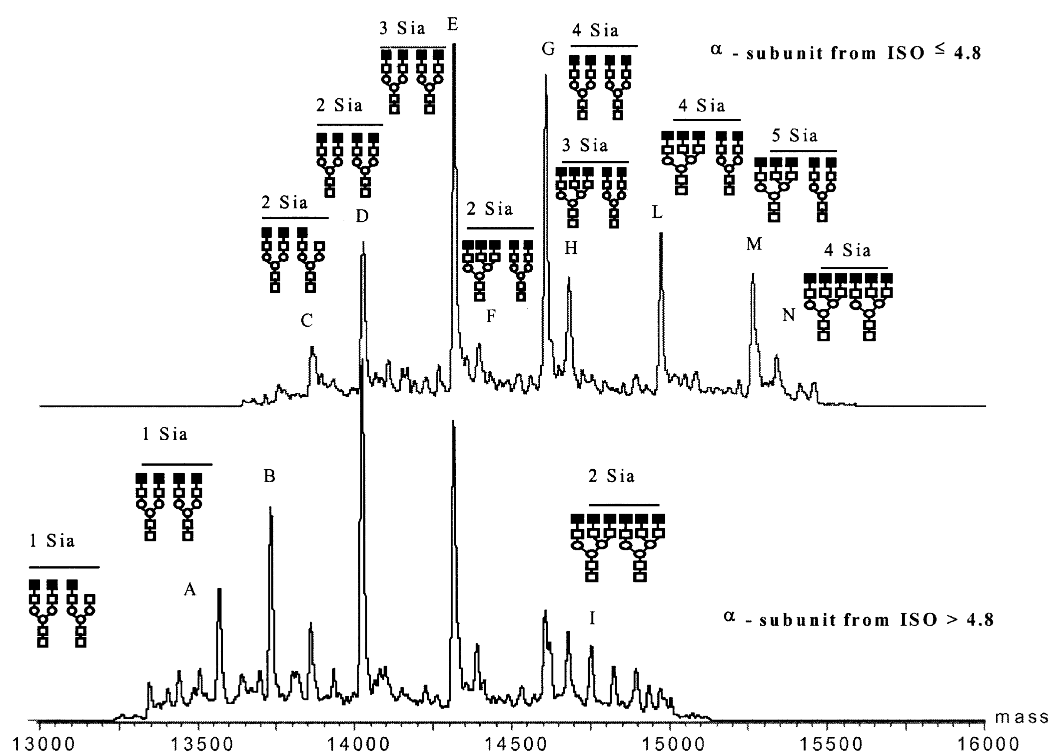

Figure 2. Overlay of electrospray/mass spectrometry (ES/MS) spectra of α-subunits from acidic (ISO ⩽ 4.8) and less acidic (ISO > 4.8) isoforms. □ = HexNAc (*N*-acetyl glucosamine); ○ = Hex (mannose); ■ = Hex (galactose).

Table I. ES-MS identification of α-subunit from acidic (ISO ⩽4.8) and less acidic (ISO >4.8) isoform preparations. The percentages reported in the table were obtained by measurement of the relative intensity of each species and are an indication of the composition of α-subunit isoforms

Species	Theoretical molecular weight	Attribution	ISO ⩽ 4.8 (%)	ISO > 4.8 (%)
A	13571.96	P + 2 Biant + 1 Sia – 1 Hex	ND	9.1
B	13734.07	P + 2 Biant + 1 Sia	ND	14.7
C	13863.19	P + 2 Biant − 1 Hex + 2 Sia	4.1	6.8
D	14025.32	P + 2 Biant + 2 Sia	11.6	25.0
E	14316.57	P + 2 Biant + 3 Sia	24.3	20.2
F	14390.65	P + 1 Biant + 1 Triant + 2 Sia	4.5	5.8
G	14607.82	P + 2 Biant + 4 Sia	22.2	7.5
H	14681.90	P + 1 Biant + 1 Triant + 3 Sia	8.6	5.8
I	14755.98	P + 2 Triant + 2 Sia	NQ	5.1
L	14973.15	P + 1 Biant + 1 Triant + 4 Sia	11.7	NQ
M	15264.40	P + 1 Biant + 1 Triant + 5 Sia	9.3	ND
N	15338.40	P + 2 Triant + 4 Sia	3.6	ND

P = r-hFSH α-subunit protein moiety (amino acid sequence residues 1–92); Biant = biantennary complex type oligosaccharide; Triant = triantennary complex type oligosaccharide; Sia = sialic acid; Hex = hexose (galactose); ND = not detected; NQ = not quantified.

Electrospray/mass spectrometry analysis of rhFSH isoforms

In Figure 2, an overlay of the ES/MS spectra of the α-subunit from the two rhFSH preparations is reported. The major difference observed between the glycoform distribution of the α-subunit from the two preparations is the shift of the less acidic isoforms towards the lower molecular weight range. This shift was essentially due to the lower amount of sialic acid in the ISO > 4.8. In fact, whereas the major glycoforms of the α-subunit from the acidic isoform preparation were the protein carrying two biantennary oligosaccharides carrying three or four sialic acid groups respectively, in the ISO > 4.8 the major species carried only two or at most three sialic acid groups.

Moreover, the highly sialylated species L and M, corresponding to the protein carrying one biantennary and one triantennary structure with an overall number of sialic acids of 4 and 5, were in the ISO > 4.8 completely absent (species M) or were only at the value of the background noise (species L).

On the other hand, the least sialylated species found in the ISO > 4.8, i.e. species A and B, were absent in the ISO ⩽ 4.8. The latter of these corresponded with the protein carrying two biantennary structures, which in the case of

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Table II. Main pharmacokinetic parameters calculated from the mean plasma concentrations in conscious female rats after a single i.v. injection of 7 μg/kg of acidic (ISO ≤ 4.8) and less acidic (ISO > 4.8) r-hFSH isoforms

	ISO ≤ 4.8	ISO > 4.8
$T_{\frac{1}{2}}$ λ 1 (distribution) (h)	0.9	0.4
$T_{\frac{1}{2}}$ λ z (terminal) (h)	6.0	5.6
AUC (0–∞) (h ng/ml)	128.5	41.2
AUC (0–∞)/dose	18.4	5.9
Vc (ml/kg)	109.1	165.4
CL total (ml/h/kg)	54.4	170.0

Vc = volume of central compartment; CL = clearance.

species A lacked one hexose, with an overall number of sialic acid equal to 1.

Table I shows the percentage of each glycoform in the α-subunit from the two preparations, confirming that the main difference between the two groups of isoforms is the extent of sialylation.

Immunoassay for quantification of rhFSH isoforms

It has been reported that the carbohydrate moieties might influence the quantification of FSH by immunoassay (Storring, 1992; Burgon *et al*., 1993) and, therefore, an immunoassay used to measure the concentration of FSH glycoforms may show different recognition for the various glycoforms of the hormone in a sample (Lambert *et al*., 1995; Zambrano *et al*., 1996). However, when serial dilutions of ISO ≤ 4.8 and ISO > 4.8 were tested by the MAIAclone assay no differences were detected between the two forms of rhFSH (data not shown), thus indicating the suitability of this assay to quantify the presence of rhFSH isoforms without bias of one isoform over another.

Determination of the MCR of rhFSH

The pharmacokinetic (PK) evaluation of the mean plasma concentrations (n = 5, pooled data) of the acidic (ISO ≤ 4.8) and the less acidic isoforms (ISO > 4.8) administered singly to rats by i.v. route at the same dose (7 μg/kg) showed a difference in the PK profile between these isoforms. For the less acidic isoforms (ISO > 4.8), the mean plasma concentrations at all sampling times were lower than those of the acidic isoforms (ISO ≤ 4.8) (Table II). Compared with the acidic isoforms, the less acidic isoforms showed a decrease in the AUC (0–∞) value (41.2 h ng/ml compared with 128.5 h ng/ml) and therefore, a corresponding increase in the total clearance (170 ml/h/kg compared with 54.4 ml/h/kg). The plasma concentrations showed a more rapid decrease immediately after the injection of the less acidic isoforms ($t_{\frac{1}{2}}$lλ = 0.4 h in comparison to 0.9 h with the acidic isoforms), while the terminal elimination phase appeared to be quite similar to that of the acidic isoforms (Table II and Figure 3). This result, however, should be considered with caution since at 24 h from treatment (last sampling time), plasma concentrations lower than the quantification limit, set at zero for curve fitting and averaged, were found in three out of five rats.

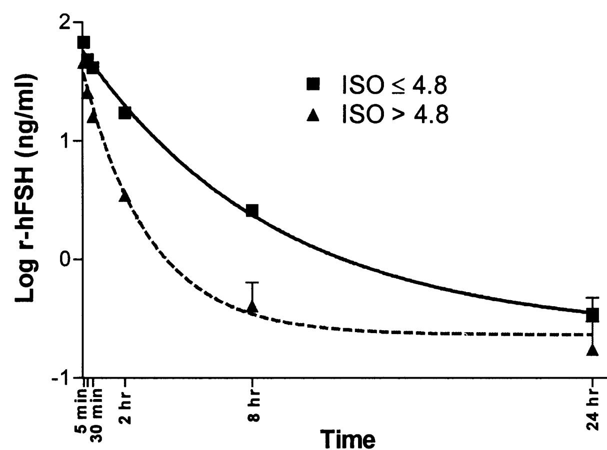

Figure 3. Plasma concentrations of immunoreactive follicle stimulating hormone (FSH) following a single i.v. injection of 7 μg/kg of ISO ≤ 4.8 and ISO > 4.8. Each time point represents the mean of five animals ± SEM.

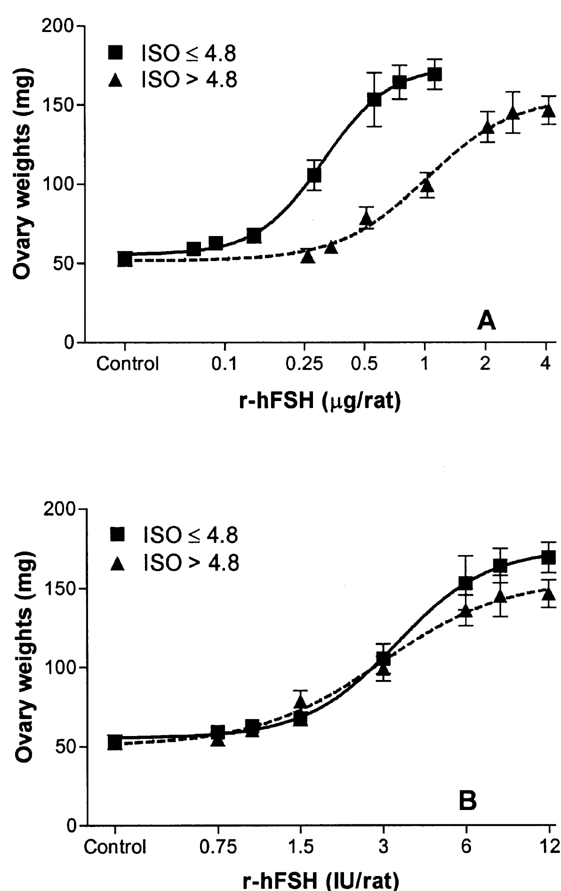

Figure 4. Dose–response curves of the ISO > 4.8 and ISO ≤ 4.8 generated in the Steelman–Pohley in-vivo assay. Doses are given in (**A**) mass and in (**B**) IU. Data represent mean response of 10 animals ± SEM.

Steelman–Pohley in-vivo assay

The effects on the rat ovary weight increase of the ISO > 4.8 and ISO ≤ 4.8 when administered on a mass basis, are reported in Figure 4A. The less acidic isoform group (with an ED_{50} = 0.9 μg/rat) was found to be significantly less potent, when compared with the acidic isoform group

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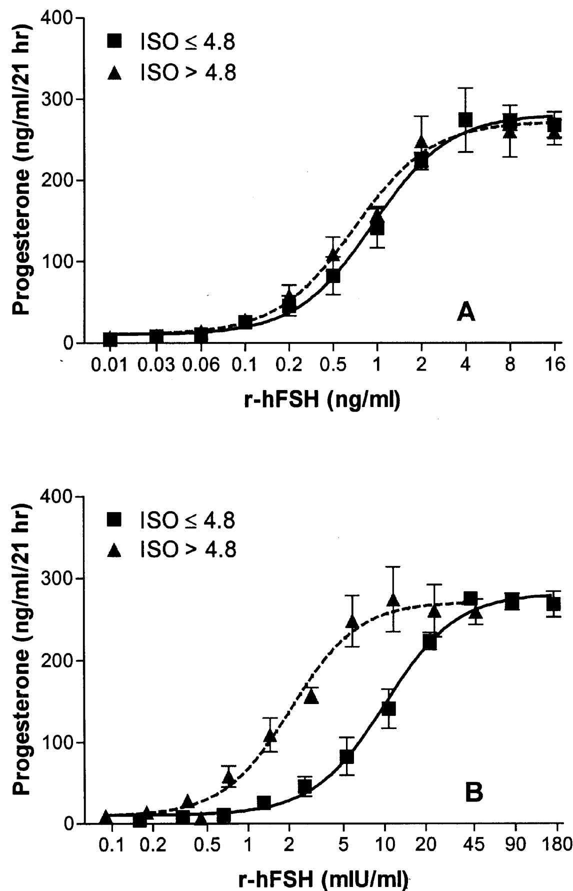

Figure 5. Dose–response curves of the ISO > 4.8 and ISO ⩽ 4.8 generated in the Y1 hFSHR in-vitro bioassay. Concentrations are given in (**A**) mass and in (**B**) IU. Data represent the average of mean responses control, subtracted from three independent experiments ± SEM.

(ED_{50} = 0.3 μg/rat) with the linear parts of the curves being parallel. As expected, when the doses were expressed in terms of IU, no differences were observed between the two preparations (ED_{50} =3 IU/rat) (Figure 4B).

Y1 hFSHR in-vitro bioassay

The in-vitro activities of the two separated groups of rhFSH isoforms in the Y1 hFSHR assay are shown in Figures 5A and 5B (average of three independent experiments). Both the rhFSH (ISO > 4.8 and ISO ⩽ 4.8) preparations were able to induce a significant dose-dependent progesterone production with no statistically significant difference between them when testing was performed based on their mass (Figure 5A). By contrast, the ISO > 4.8 was found to be significantly more potent when concentrations were expressed in IU with an EC_{50} of 2 mIU/ml, compared with 10 mIU/ml of the ISO ⩽ 4.8 (Figure 5B). A comparable pattern of results was found in the CHO–hFSHR–Luc assay (data not shown). ISO > 4.8 and ISO ⩽ 4.8 were found to induce a similar dose-dependent response when concentrations were expressed on a mass basis, the EC_{50} being 0.14 and 0.16 ng/ml, respectively. However, when concentrations were expressed in IU, the ISO > 4.8 was found to be more potent with an EC_{50} of 0.30 mIU/ml while the EC_{50} for the ISO ⩽ 4.8 was 1.4 mIU/ml (data not shown).

Discussion

The pituitary-derived gonadotrophin FSH is a hormone involved in the control of reproductive function in both the female and the male. It is a two-subunit glycoprotein which has been shown to be secreted by the pituitary as a microheterogeneous population in terms of charge, molecular weight and in-vitro and in-vivo bioactivities with the secretion of polymorphic FSH being linked to particular physiological and endocrine events (Peckham *et al*., 1973; Chappel *et al*., 1983; Padmanabhan *et al*., 1988; Wide and Albertsson-Wikland, 1990; Stanton *et al*., 1995). Up to 20 different FSH molecules with varying degree of glycosylation can be purified (Stanton *et al*., 1992) forming a spectrum of isoforms with differences in charge, bioactivity and elimination half-lives (Chappel *et al*., 1983; Ulloa-Aguirre *et al*., 1988). The more glycosylated molecules possess a longer half-life as compared to the desialylated molecules which, probably because of increased plasma clearance, have a significantly decreased *in vivo* bioactivity (Galway *et al*., 1990; Cerpa-Poljak *et al*., 1993). The sialic acid content, and particularly the number of exposed terminal galactose residues of a glycoprotein, determines the clearance rate from plasma through a mechanism that involves hepatocyte receptors for these galactose-terminal complex molecules (Ashwell and Harford, 1992; Blum and Gupta, 1985).

In the present study we employed two enriched-groups of rhFSH isoforms that were different in the degree of sialylation, with the acidic isoform preparation (ISO ⩽ 4.8) having many more sialic acid residues than the less acidic isoform preparation (ISO > 4.8), as detected by the ES/MS spectra. Similar results were obtained using another CHO-derived hFSH preparation (Puregon, NV, Organon, Oss, The Netherlands), and by employing an enzymatic method it was shown that rhFSH isoforms with a low pI have a relatively high content of sialic acid (de Leeuw *et al*., 1996). Moreover, it was also shown on the same preparation that oligosaccharides linked to the β-chain of FSH are more heavily sialylated and branched than the oligosaccharides linked to the α-chain. However, our finding of a different sialic acid content in the two isoforms obtained by the ES/MS analysis of the α-chain only might suggest that the analysis of this latter subunit alone is predictive of the relationship between pI value and sialic acid content.

The findings reported in the present study are in agreement with the previously mentioned literature data in that the less acidic isoform-enriched fraction showed a faster clearance from the general circulation in rats as compared to the acidic isoform fraction. A similar correlation between a decrease in pI and metabolic clearance rates is in good agreement with previously reported data which showed that rat acidic FSH isoforms possessed a longer circulatory half-life than the less acidic ones (Blum and Gupta, 1985). Similarly, using FSH isoforms, separated by chromatofocusing from the human anterior pituitary gland, acidic FSH isoforms with a pI < 4.5 were found to remain in the rat blood circulation for longer times than the isoforms with pI of 5.9–4.7 (Ulloa-Aguirre *et al*., 1992). A similar observation has been made with other rhFSH-derived isoforms (Puregon) injected in Beagle dogs:

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acidic isoforms displayed a long plasma residence time, whereas a short plasma residence time was observed for the less acidic isoforms (de Leeuw *et al*., 1996). Despite their faster elimination from blood, the less acidic isoforms of FSH were still able to induce a significant biological response even if only a small quantity gets to the target cells (Zambrano *et al*., 1996). This was supported in the present study by the increase, although to a lesser extent, compared with the more acidic isoforms, in the rat ovary weight as determined by the Steelman–Pohley assay and in their ability to induce ovulation in prepubertal rats (unpublished observation). Additionally, the same observation has also been made for the desialylated HCG which was shown to be capable of stimulating a full testicular response in monkey over 6 h although was eliminated from the bloodstream within 15 min (Liu *et al*., 1989).

In the present study, we have also evaluated the two rhFSH isoform-enriched groups for their capability to induce a response at the post-receptor level using the Y1 hFSHR assay and a reporter gene assay. The results of these experiments showed that both the isoform groups induced significant dose-dependent responses with no significant differences in the biological activities (between the two groups of isoforms) in both these in-vitro systems when test concentrations were based on their molecular mass. The finding of a similar in-vitro bioactivity of hFSH isoforms with low and high contents in sialic acid could indicate not only that sialic acid is not critical for receptor binding (Galway *et al*., 1990) but also that other carbohydrate moieties linked to the protein core could be essential for the FSH binding to its own receptor (Dufau *et al*., 1971; Channing *et al*., 1978; Ulloa-Aguirre *et al*., 1988). This is also supported by data obtained using rhFSH produced in CHO cells deficient either in the glycosylation enzyme *N*-acetylglucosamine transferase-I or in mutant cells defective in sialic acid transport into the Golgi body (Galway *et al*., 1990). These variant rhFSH molecules were found to exhibit a shift to a less acidic pI than the wild-type hFSH; however, these molecules were shown to retain full receptor binding and in-vitro bioactivity but exhibited minimal in-vivo activity with a shortened half-life (Galway *et al*., 1990). On the other hand, the expression of human FSH α and β cDNA in the 293-human embryonal kidney cell line, which resulted in the production of molecules with a greater number of less acidic isoforms than that of pituitary FSH standard, has also been reported (Flack *et al*., 1994). It is noteworthy that these less acidic human FSH isoforms were shown to possess a higher steroidogenic activity *in vitro* per immunological unit than more acidic FSH pituitary standards. Recently, the in-vitro bioactivity of two urinary-derived FSH batches with a very low specific activity (50 IU FSH/mg protein) has been tested in the granulosa cell bioassay (Ulloa-Aguirre *et al*., 1997) with the finding that the batch showing a less acidic profile was found to be more potent than the batch exhibiting a predominantly high-acidic charge distribution profile.

In the present study, in the two in-vitro test systems, when concentrations were based on international units, the less acidic isoform group exhibited a significantly higher in-vitro biopotency than the acidic isoforms whereas the in-vivo potency of the former group, expressed as mass, was lower than that of the latter, because of its lower specific activity. In fact, the specific activities of the acidic and less acidic isoform-enriched fractions were found to be 10 716 and 2908 IU/mg respectively (as determined by the Steelman–Pohley in-vivo bioassay). Therefore, the less acidic isoform fraction appears to have higher activity in international units because of the compensation in terms of higher quantities used. The lack of correlation between in-vitro and in-vivo bioactivities of rhFSH isoforms confirms the importance of the oligosaccharides as regulatory determinants of in-vivo metabolism (Galway *et al*., 1990; Lambert *et al*., 1995).

During the early phase of a new ovulatory cycle, the FSH signal to the ovary is mediated through long-lived acidic isoforms, the majority of which are present when pH is <4.8 (Padmanabhan *et al*., 1988); this probably allows the stimulation of a number of follicles towards the maturational process. As this cohort of follicles begins to develop, the pituitary secretes short-lived less acidic FSH isoforms in the pH range of 4.8–6.0 (Padmanabhan *et al*., 1988) so that only the largest follicles continue to grow, whereas the smaller follicles suffer atresia and can no longer participate in the follicular wave. Moreover, the particular FSH isoforms secreted during the mid-cycle period of the menstrual cycle might contribute to events associated with ovulation since the rise in FSH serves not only to release the oocyte from follicular attachments and stimulate deposition of a hyaluronic acid matrix (Wide and Bakos, 1993) but also to stimulate the production of plasminogen activator, which converts the plasminogen to the proteolytic enzyme, plasmin.

Although the presently available FSH preparations satisfactorily stimulate ovarian response, there is a general expectation that the availability of separated rhFSH isoforms would allow a more physiological treatment of patients by mimicking the natural follicle maturation process. This would also apply to in-vitro fertilization where treatment could start with very long acting FSH isoforms resulting in the recruitment of a large crop of follicles but may then be continued with short-lived, less acidic isoforms which are believed to be the most likely to induce the final maturation and growth of the follicles. It could also be argued that the use of a more physiological regimen based on differential treatment with FSH isoforms could result in the stimulation of a smaller number but of more 'naturally' matured oocytes of higher quality which may have a higher probability of developing into better quality embryos and lead to a higher take home baby rate (Lambert *et al*., 1998).

Obviously, the physiological relevance of the results of our study and the possibilities mentioned above remain open for speculation. Testing the separated rhFSH isoforms now available will help to clarify their role in modulating the different stages of the menstrual cycle influencing steroidogenesis and the follicle recruitment process.

Acknowledgements

The authors would like to thank E.De Luca for the ES/MS analysis, G.Paradisi for the separation of the rhFSH isoforms, I.Marcucci for outstanding technical skills and F.Antonetti and the Biological Control

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lab for the excellent support to this project. The authors wish to express their gratitude to G.Zanolo for kindly performing the computerized pharmacokinetic evaluation of blood concentration data and to A.Eshkol, E.Loumaye, A.Ythier and M.Dreano who critically reviewed the manuscript. Istituto di Ricerca Cesare Serono SpA is part of the Ares Serono Group.

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Received on April 24, 1998; accepted on December 18, 1998

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