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Structure–function relationship of recombinant follicle stimulating hormone (Puregon®)

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After separation by means of preparative isoelectrofocusing, the isohormones of a Chinese hamster ovary (CHO)-derived recombinant follicle stimulating hormone (rFSH, Puregon®) were characterized with respect to structural and functional features. A carbohydrate analysis revealed that rFSH isohormones with a low isoelectric point (pI) have a high sialic acid/galactose ratio and are rich in tri- and tetra-antennary *N*-linked carbohydrate chains in comparison with the high pI isohormones. The relative basic isohormones exhibit receptor binding activity and intrinsic bioactivity 2–3-fold higher than the relative acidic isohormones. However, due to their lower clearance rate these acidic isohormones displayed a 20-fold higher in-vivo bioactivity in the rat. A comparison of the isohormone profile of rFSH and urinary FSH (Metrodin®) revealed that rFSH contains about 2-fold more basic isohormones with pI ≥4.7 and 2-fold less acidic isohormones with pI <4.1. In-vitro studies showed that the receptor binding affinity and intrinsic bioactivity of both FSH preparations are similar. Also the in-vivo efficacy and the pharmacokinetic behaviour of rFSH and urinary FSH in the rat were similar, which is not surprising since both preparations were compared in terms of in-vivo bioactivity calibrated in the rat Steelman–Pohley assay. However, in dogs the bioavailability of rFSH was lower than that of urinary FSH, which is in agreement with the higher percentage of relative basic isohormones in rFSH. This suggests that the pharmacokinetic behaviour of FSH in rats and dogs is different, which is supported by the much longer elimination half-life of rFSH and urinary FSH in dogs (27.9 and 30.4 h respectively) compared with rats (11.4 and 10.4 h respectively) for rFSH and urinary FSH respectively. The observed differences in pharmacokinetic behaviour in dogs and rats indicate that the rat Steelman–Pohley assay might not be a valid model for the prediction of the FSH bioactivity in species other than rat.

Key words: biological properties/isohormones/structure/recombinant FSH (Org 32489)

Introduction

Follicle stimulating hormone (FSH) is a 34 kDa glycoprotein hormone consisting of a non-covalently linked heterodimer of two polypeptide chains, the α- and β-subunit (Baenziger and Green, 1988). Like other glycoprotein hormones, FSH displays a high degree of structural heterogeneity due to differences in the amount and/or composition of the carbohydrate residues, in particular sialic acid. FSH is produced and secreted by the anterior lobe of the pituitary and plays a pivotal role in the regulation and maintenance of essential reproductive processes such as gametogenesis, follicular development and ovulation (Catt and Pierce, 1986). FSH exerts its biological effect via binding to specific membrane receptors on Sertoli cells and granulosa cells and subsequent activation of intracellular second messenger systems, such as adenylate cyclase, resulting in the synthesis and/or secretion of various factors essential for target cell differentiation, gamete maturation and gonadal steroid production (Hsueh *et al.*, 1984; Sharp, 1990).

In cases of absent or deficient FSH production and for stimulating multiple follicular development in ovulatory women undergoing assisted reproduction technologies such as in-vitro fertilization (IVF), patients are treated with exogenous gonadotrophin preparations. Current commercially-available FSH/luteinizing hormone (LH) preparations are isolated from the urine of post-menopausal women. This source implies a number of disadvantages (Out *et al.*, 1996), such as low purity. Obviously, through the application of recombinant DNA technology, it is possible to overcome these limitations. A stable transfected cell line producing recombinant FSH (rFSH, Puregon; N.V.Organon, Oss, The Netherlands) has been established by transfection of Chinese hamster ovary (CHO) cells with a plasmid containing the two subunit genes encoding human FSH (Van Wezenbeek *et al.*, 1990; Olijve *et al.*, 1996). Recombinant FSH, the active substance of the pharmaceutical product Puregon, has a high biochemical purity (≥99%) and a high specific biological activity (~10 000 IU/mg). At the protein level the amino acid composition and amino acid sequence match those of natural human FSH (Olijve *et al.*, 1996). Carbohydrate analysis has revealed that the carbohydrate chains of rFSH are composed of mannose, fucose, *N*-acetylglucosamine (GlcNAc), galactose and sialic acid (NeuAc). However, in comparison with natural FSH, bisecting GlcNAc residues are absent and a lower percentage of α 1–6 linked fucose residues and a small amount of repeating *N*-acetyllactos-

amine units in tri- and tetrasaccharide carbohydrate chains are found (Hård *et al.*, 1990). Furthermore, the carbohydrate chains of the recombinant product contain exclusively α 2–3 linked sialic acid, whereas in the natural product α 2–6 linked sialic acid also occurs.

In this paper some structural and functional aspects of rFSH will be discussed in comparison with FSH of natural sources, e.g. urinary human FSH (Metrodin®; Serono, Aubonne, Switzerland).

In this respect it is important to realize that gonadotrophins can be quantified with four essentially different types of assays, i.e. immunoassays, receptor binding assays, in-vitro bioassays and in-vivo bioassays, all having their own specific merits. These assays measure four different fundamental characteristics of gonadotrophin molecules: (i) immunoassays measure a structural feature; it is generally believed that this type of assay provides a 'relative' measure for the amount of hormone (Chappel, 1990); (ii) receptor-binding assays provide information on the proper conformation of a gonadotrophin molecule for receptor binding; (iii) in contrast to the two previous assays, in-vitro bioassays measure a functional aspect of gonadotrophins, namely their intrinsic biological activity in terms of second messenger activation; and finally (iv) in-vivo bioassays measure the overall bioactivity of gonadotrophins. This is determined by the number of molecules, their pharmacokinetic behaviour, receptor binding activity and intrinsic bioactivity.

To relate the different aspects summarized above, the term B/I ratio has been defined. This expresses the ratio between the in-vitro or in-vivo bioactivity and the 'quantity' of immunoreactive hormone. Obviously, since different molecular properties are measured in each assay, the B/I ratios of gonadotrophin preparations do not necessarily equal one.

Characterization of rFSH isohormones

FSH, like the other gonadotrophins LH and human chorionic gonadotrophin (HCG), exists in different molecular forms (isohormones), which differ in their oligosaccharide structures, in particular the degree of terminal sialylation (Ulloa-Aguirre *et al.*, 1988; Dahl and Stone, 1992). The α- and β-subunit contain both mono- or multi-antennary *N*-linked carbohydrate chains. The degree of sialylation resulting in basic–acidic charge differences provide the basis for differences in receptor binding activity, biological activity and metabolic clearance rate (Ulloa-Aguirre *et al.*, 1988). Multiple forms of gonadotrophins have been isolated and characterized from anterior pituitary glands, serum and urine of several species including man (Wide, 1982, 1985; Reader *et al.*, 1983; Chappel *et al.*, 1984; Ulloa-Aguirre *et al.*, 1985, Chappel, 1995). Relatively acidic FSH isohormones, which are more heavily sialylated, exhibit lower receptor binding affinity and in-vitro biological activities than more basic isohormones. However, due to their lower clearance rate these more acidic forms have greater in-vivo biological activities (Ulloa-Aguirre *et al.*, 1988). Thus, the isohormone composition of FSH preparations is likely to have important biological consequences.

Therefore, the isohormones of rFSH were separated and characterized with respect to carbohydrate structure, in-vitro and in-vivo bioactivity and pharmacokinetic behaviour in female Beagle dogs.

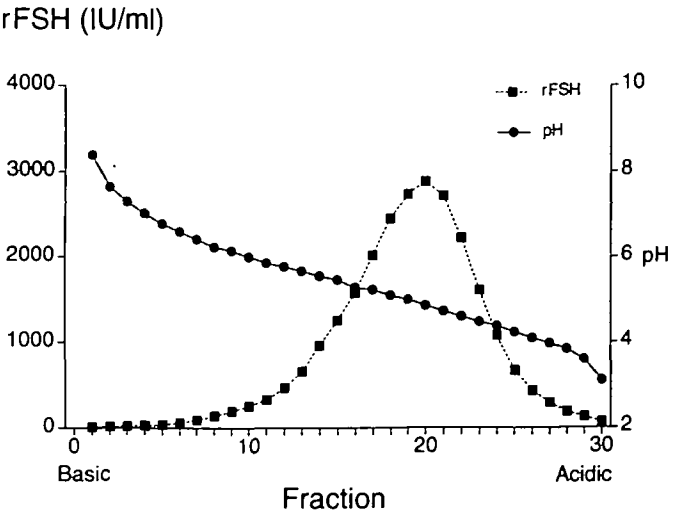

Figure 1. Isohormone profile of immunoreactive follicle stimulating hormone (FSH) in recombinant FSH (batch 517) after preparative free flow focusing. Fractions were collected and FSH immunoreactivity was determined by FSH-enzyme immunoassay.

Separation of rFSH isohormones

The different isohormones of rFSH were separated based on their isoelectric point (pI) using recycling free flow focusing (RF3; Rainin Instrument Co. Inc., Emeryville, CA, USA). After focusing 30 fractions of 3 ml each were collected and analysed for immunoreactive FSH, using a two-site FSH-enzyme immunoassay (EIA) (Mannaerts *et al.*, 1991). In this FSH immunoassay the different rFSH isohormone fractions displayed comparable specific immunoactivities (results not shown) which indicate that all isohormones of rFSH are recognized equally well. This also implies that the biological comparison of the rFSH isohormones based on FSH-EIA activity (see below) is not affected by differences in immunoreactivity.

As shown in Figure 1, a typical batch of rFSH displayed a distribution of FSH immunoreactivity from pI 6 to 4. Alternating fractions or pools of fractions were used for either the characterization of the carbohydrate chains or for the biological characterization.

Characterization of the carbohydrate chains of rFSH isohormones

Monosaccharide analysis (Kamerling and Vliegenthart, 1989) of the isohormone fractions revealed significant differences in the carbohydrate moieties of the isohormones. As expected an increasing relative amount of NeuAc (sialic acid) was correlated with a decreasing pI. The results also indicated that with decreasing pI the absolute carbohydrate content increases (Table I).

To further investigate carbohydrate features, the isohormones were dissociated into the α- and β-subunits by reverse phase–high performance liquid chromatography (RP-HPLC). Sub-

Table I. The molecular carbohydrate composition, given relative to Man (3.0), of the recombinant follicle stimulating hormone (rFSH) isohormone fractions. The carbohydrate content is expressed as a percentage (w/w); rFSH starting material has been included as reference

Fraction	pI	Monosaccharide					Carbohydrate content (%)
		Man	Fuc	Gal	GlcNAc	NeuAc	
16	5.30	3	0.5	2.2	3.8	1.1	33.8
18	5.10	3	0.5	2.2	4.1	1.5	35.5
20	4.87	3	0.4	2.3	4.2	1.8	34.3
22	4.66	3	0.4	2.4	4.7	2.3	34.6
24–26	4.27	3	0.5	2.5	4.4	2.2	45.6
Starting material		3	0.4	2.6	4.8	2.0	36.9

pI = isoelectric point.

Table II. Relative amount (%) of neutral, mono-, di-, tri- and tetrasialo carbohydrate chains of the recombinant follicle stimulating hormone (rFSH) isohormone fractions; rFSH starting material has been included as reference

Subunit	Fraction	pI	Neutral	Monosialo	Disialo	Trisialo	Tetrasialo
α	16–17	5.25	14	42	36	6	2
	20–21	4.77	13	33	45	8	1
	24–25	4.34	4	17	51	26	2
β	16–17	5.25	15	33	39	10	3
	20–21	4.77	4	25	47	21	3
	24–25	4.34	1	7	36	40	16
	26–28	4.05	2	3	26	42	27
Starting material			12.6	43.3	33.4	8.6	2.0

pI = isoelectric point.

sequently the *N*-linked carbohydrate chains were released from the protein moiety (Damm *et al.*, 1989), fractionated (Damm *et al.*, 1987) and a profile analysis was performed (Baenziger and Green, 1988). This study revealed that all isohormone fractions contain neutral, monosialo, disialo, trisialo and tetrasialo oligosaccharides; however, isohormone fractions with a low pI value have a relatively high content of tri- and tetrasialo oligosaccharides and a relatively low content of neutral and monosialo oligosaccharides. For isohormone fractions with a high pI value the situation is reversed (Table II).

The profiles revealed that the β-linked oligosaccharides are more heavily sialylated and branched than the α-linked oligosaccharides. Thus, rFSH isohormones with a low pI value have a high sialic acid/galactose ratio and are rich in tri- and tetra-antennary *N*-linked carbohydrate chains as compared to the high pI isohormones.

In-vitro biological properties of rFSH isohormones

The receptor binding activity of the rFSH isohormone fractions was established by receptor displacement studies. In these studies calf testis membranes were used as FSH receptor source and ^{125}I-labelled human pituitary FSH as radiolabelled ligand (Mannaerts *et al.*, 1991). In addition, the in-vitro bioactivity was determined using a Sertoli cell bioassay based on the induction of aromatase activity in immature rat Sertoli cells by FSH (Mannaerts *et al.*, 1987). In both in-vitro assays the isohormone fractions were compared in terms of immunoreactivity measured by FSH-EIA.

When expressed in terms of receptor binding affinity/immunoreactivity (RBA/I) ratio and in-vitro bioactivity/immunoreactivity in-vitro (B/I) ratio the isohormones displayed an increasing activity with increasing pI (Table III). When the pI increased from 4.27 to 5.49, the RBA/I ratio increased from 1.1 to 1.9, whereas the in-vitro B/I ratio increased from 0.9 to 2.9. In comparison, the RBA/I ratio and in-vitro B/I ratio of the corresponding starting material were 1.3 and 1.4 respectively.

Table III. Receptor binding activity (RBA), in-vitro bioactivity and in-vivo bioactivity, expressed in terms of RBA/I, in-vitro bioactivity/immunoreactivity (B/I) and in-vivo B/I ratio respectively, of recombinant follicle stimulating hormone (FSH) isohormone fractions and corresponding starting material

Fraction	pI	RBA/I ratio	In-vitro B/I ratio	In-vivo B/I ratio
14–15	5.49	1.9	2.9	0.08
17	5.22	1.7	1.8	0.13
19	4.99	1.4	2.0	0.34
21	4.75	1.3	1.2	0.82
23	4.51	1.1	1.3	1.41
24–26	4.27	1.1	0.9	1.81
Starting material	–	1.3	1.4	0.77

pI = isoelectric point.

These results indicate that the relative basic isohormones exhibit the highest receptor binding activity and intrinsic bioactivity, the difference between the most basic and most acidic isohormone fractions being 2–3-fold. These results are in agreement with earlier observations on rat FSH isohormones (Ulloa-Aguirre *et al.*, 1988).

In-vivo bioactivity of rFSH isohormones

The in-vivo bioactivity of the rFSH isohormone fractions was established in the rat Steelman–Pohley assay (Steelman and

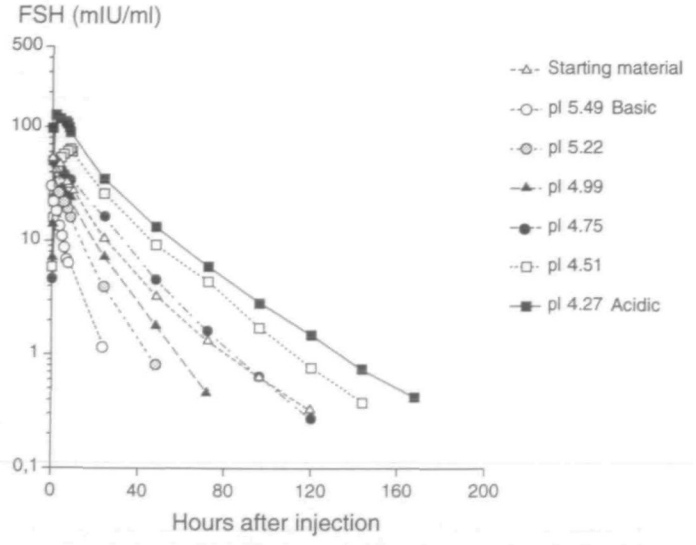

Figure 2. Plasma concentrations of immunoreactive follicle stimulating hormone (FSH; IU/l) in Beagle dogs (n = 2) after single i.m. injection of rFSH isohormone fractions with pI's ranging from 4.27–5.49 and starting material at a dose level of 20 IU/kg in terms of FSH immunoreactivity determined by FSH-enzyme immunoassay.

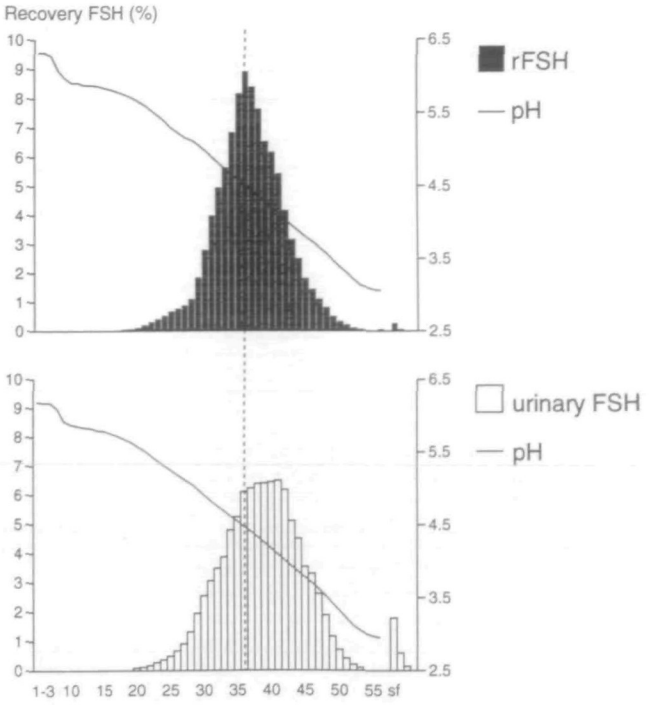

Figure 3. Representative chromatofocusing profiles of immunoreactive follicle stimulating hormone (FSH) in recombinant FSH and urinary FSH. Fractions were collected and FSH immunoreactivity was determined by FSH-enzyme immunoassay. sf = salt fraction.

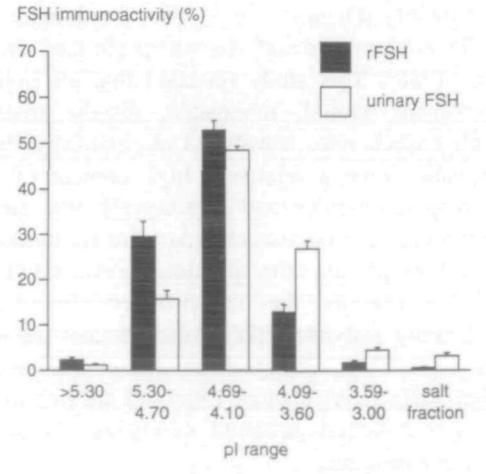

Figure 4. Mean follicle stimulating hormone (FSH) immunoreactivity per pI range of 13 batches rFSH and 10 batches urinary FSH. Values represent mean ± SD.

Pohley, 1953) after dosing in terms of immunoreactivity measured by FSH-EIA.

When expressed in terms of in-vivo B/I ratio, the in-vivo bioactivity displayed a remarkable increase with increasing acidity (Table III). With decreasing pI from 5.49–4.27 the in-vivo B/I ratio increased from 0.08–1.81, which represents a more than 20-fold increase. The in-vivo B/I ratio of the starting material was 0.77, which is in between the in-vivo B/I ratio of fractions 19 and 21 (intermediate acidity), corresponding with the peak of the isohormone profile (Figure 1).

Pharmacokinetic behaviour of rFSH isohormones

The pharmacokinetic behaviour of the rFSH isohormone fractions was compared in female Beagle dogs after a single i.m. administration of 20 IU/kg in terms of FSH immunoreactivity measured by FSH-EIA. The rFSH starting material was included as reference. Plasma FSH concentrations were determined using a two-site FSH time-resolved fluoroimmunoassay (Delfia®; Pharmacia, Roosendad, The Netherlands), which does not recognize endogenous dog FSH. After administering the single i.m. dose, the rFSH isohormones showed remarkable differences in clearance (Figure 2).

With increasing acidity from pI 5.49–4.27, the area under plasma-concentration curve (AUC(0–∞); extent of absorption) increased from 186 to 2841 IU/h/l and the clearance rate decreased from 82 to 5.9 ml/h/kg (Table IV). For both parameters a clear correlation was found with the pI of the isohormone fraction.

The maximum plasma concentrations of immunoreactive FSH (C_{max} = rate of absorption) did not differ for the isohormone fractions with pIs ranging from 5.49 to 4.75. Only the two most acidic fractions displayed a higher C_{max}. For the time at which the immunoreactive FSH concentrations peaked (T_{max}) there was no clear difference among the different isohormone fraction. The terminal elimination half life ($T_{\frac{1}{2}}$-elim) could not be estimated for the most basic fraction (pI 5.49) due to the very rapid clearance of this fraction. However, from pI 5.22–pI 4.27, the $T_{\frac{1}{2}}$-elim increased from 11.8 to 24.1 h, suggesting a more than 2-fold difference in T $_{\frac{1}{2}}$-elim between the most basic and acid fractions.

For the AUC (0–∞) and clearance rate the values of the rFSH starting material were in between those of isohormone fraction 19 and 21 (intermediate acidity), corresponding with with the peak of the isohormone profile (Figure 1).

In contrast, the $T_{\frac{1}{2}}$-elim of the starting material corresponds

Table IV. Pharmacokinetic parameters of immunoreactive follicle stimulating hormone (FSH) after single dose i.m. administration of recombinant FSH isohormones and starting material to female Beagle dogs (n = 2) at a dose level of 20 IU/kg in terms of immunoreactive FSH

Fraction	pI	C_{max} (IU/l)	T_{max} (h)	AUC(0–∞) (IU/h/l)	Clearance rate (ml/h/kg)	$T_{\frac{1}{2}}$-elim (h)
15	5.49	36.7	0.8	186*	82.0	ne
17	5.22	44.9	1.7	500	34.5	11.8
19	4.99	29.0	5.0	558	26.1	12.3
21	4.75	39.6	4.1	1026	14.5	17.8
23	4.51	65.5	7.0	1814	8.8	20.5
24–26	4.27	130.0	2.5	2841	5.9	24.1
Starting material		56.8	2.0	915	16.3	21.4

* = AUC (0–24)
ne = could not be estimated.
pI = isoelectric point; C_{max} = rate of absorption; T_{max} = time at which the immunoreactive FSH concentrations peaked; AUC(0–∞) = area-under-plasma-concentration curve; $T_{\frac{1}{2}}$-elim = terminal elimination half life.

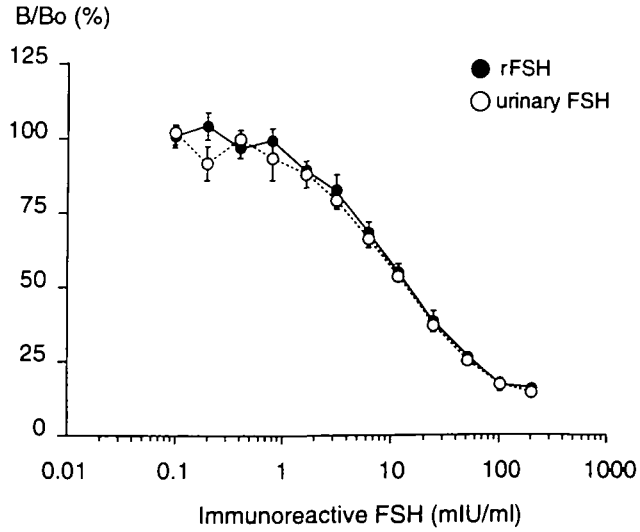

Figure 5. Displacement of [^{125}I]-human pituitary follicle stimulating hormone (FSH) binding to calf testis FSH receptors by increasing concentrations of recombinant FSH and urinary FSH. Values represent the mean of triplicates ± SD.

with the $T_{\frac{1}{2}}$-elim of the two most acidic fractions, suggesting that such acidic fractions have the greatest contribution to the elimination half-life of an intact FSH preparation.

Thus, for the rFSH isohormone fractions a clear correlation was found between pI and pharmacokinetic behaviour. With increasing acidity the extent of absorption and elimination half-life increases and the clearance rate decreases.

These results are in agreement with those obtained for rat FSH isohormones (Blum and Gupta, 1985), showing that acidic isohormones display a long plasma residence time whereas a short plasma residence time was found for the basic isohormones.

Conclusion

After preparative isoelectrofocusing rFSH exhibited an isohormone profile ranging from pI 4.0–6.0. Carbohydrate analysis revealed that isohormones with a low pI contain more heavily sialylated and more highly branched carbohydrate chains than the high pI isohormones. The relatively basic isohormones displayed a higher receptor binding activity and intrinsic bioactivity than the relatively acidic isohormones, the difference being 2–3-fold. In contrast, the in-vivo bioactivity of the acidic isohormones was more than 20-fold higher than that of the basic isohormones. This higher in-vivo bioactivity of the acidic isohormones is in agreement with their higher extent of absorption, lower clearance rate and longer elimination half-life in comparison with the basic isohormones.

Comparison of rFSH and urinary FSH

Isohormone comparison

Since the isohormone composition of FSH is important for its biological properties, in particular kinetic behaviour and consequently in-vivo bioactivity (see above), the isohormone profile of rFSH was compared with that of urinary human FSH (Metrodin; Serono) by using chromatofocusing (Matikainen *et al.*, 1994).

rFSH exhibited a distribution of FSH immunoreactivity between pI 5.69–3.21 with a peak at pI 4.55 ± 0.12 (mean ± SD, 13 batches) and >50% recovery between pI 4.1–4.7 (total recovery 95%). This broad focusing range is similar to that of natural FSH (Zaidi *et al.*, 1981; Ulloa-Aguirre *et al.*, 1988). It is also similar to that of another recombinant, CHO cell-derived human FSH preparation (Cerpa-Poljak *et al.*, 1993).

When comparing rFSH (13 batches) with urinary FSH, the latter focusing between pI 5.58–3.8 with a peak at pI 4.29 ± 0.13 (mean of 10 batches), it was found that rFSH contains an ~2-fold lower percentage of relative acidic isoforms (pI <4.1; 14.7 compared with 31.4%) and an ~2-fold higher percentage of relatively basic isoforms (pI ≥4.7; 32.0 compared with 17.0%) (Figures 3 and 4).

The biological consequence of these differences in isohormone composition will be further discussed by comparing several in-vitro and in-vivo biological properties of rFSH (Batch IP 190/0824; 166 IU/ampoule in-vivo bioactivity in terms of IS 70/45) and urinary FSH (Metrodin; Batch 91B21; 75 IU/ampoule in-vivo bioactivity in terms of IS 70/45).

In-vitro biological properties of rFSH and urinary FSH

The receptor binding activity of rFSH was examined in receptor displacement studies using calf testis membranes and ^{125}I-labelled human pituitary FSH (Mannaerts *et al.*, 1991). rFSH inhibited the receptor binding of iodinated FSH in a dose-dependent manner (Figure 5).

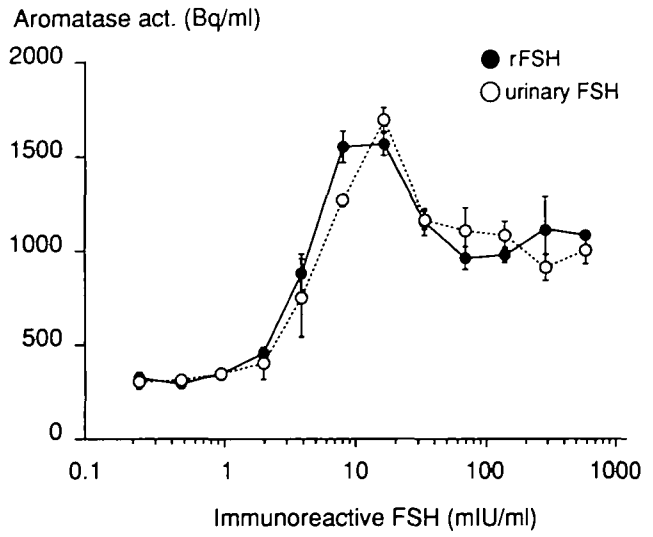

Figure 6. Dose-dependent stimulation of aromatase activity in immature rat granulosa cells by recombinant follicle stimulating hormone (FSH) and urinary FSH. Values represent the mean of triplicates ± SD.

When equal amounts of immunoreactive FSH were added, rFSH and urinary FSH displayed similar dose-inhibition curves. Hence, the receptor binding affinity/immunoreactivity ratio of both preparations is similar. Likewise, rFSH exhibited dose-inhibition curves similar to those of a pituitary FSH reference preparation (IS 83/575, Mannaerts *et al.*, 1991).

The induction of aromatase activity in granulosa cells (Overes *et al.*, 1992) obtained from immature rats by rFSH and urinary FSH was investigated to compare their intrinsic activities. In this assay recombinant as well as urinary FSH increased aromatase activity in an identical dose-dependent manner when equal amounts of immunoreactive FSH were tested (Figure 6).

Both preparations also induced a similar maximal response, which was followed by a decline. This decline in aromatase activity at high concentration is likely due to very high levels of cAMP (Overes *et al.*, 1992). The identical dose-response curves in the granulosa cell bioassay are in agreement with previous results showing that rFSH and urinary FSH produce similar dose-response curves in a Sertoli cell bioassay based on the induction of aromatase activity in immature rat Sertoli cells (Mannaerts *et al.*, 1991).

The results of the in-vitro studies show that the receptor binding affinity and intrinsic bioactivity of rFSH and urinary FSH are similar. Hence, the relatively small difference in isohormone profile does not affect these biological properties.

In-vivo biological properties of rFSH and urinary FSH

In-vivo bioactivity in intact rats

In the standard Steelman–Pohley assay, rFSH induced a linear log-dose response parallel to that of a calibrated urinary in-house reference. The specific bioactivity of rFSH (relative to IS 70/45) is ~10 000 IU/mg protein, the mass of protein being measured by amino acid analysis (Moore and Stein, 1951).

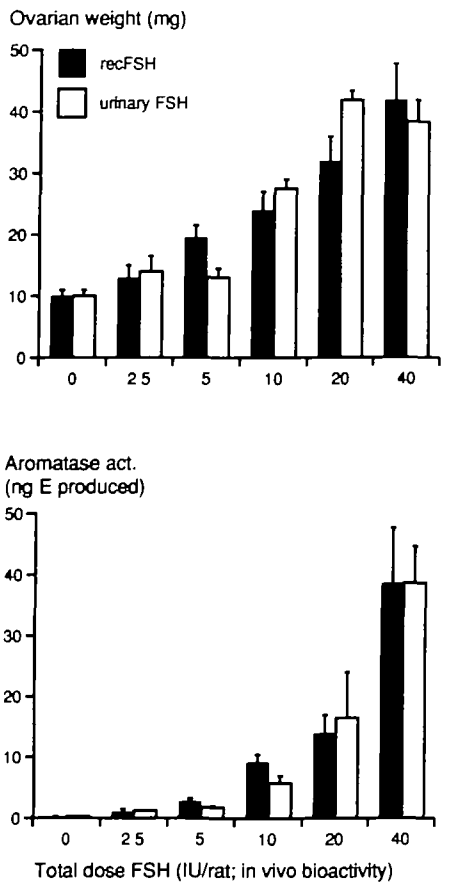

Figure 7. Effect of increasing doses of recombinant follicle stimulating hormone (FSH) and urinary FSH on ovarian weight and aromatase activity in immature hypophysectomized female rats (n = 5–6). Doses refer to in-vivo bioactivity. Values represent mean ± SD.

In-vivo bioactivity in immature hypophysectomized rats

The in-vivo efficacy of rFSH and urinary FSH was investigated in immature, hypophysectomized female rats (Mannaerts *et al.*, 1991; Overes *et al.*, 1992). This animal model can be considered analogous to infertile human subjects with low endogenous FSH and LH levels.

When given as a cumulative dose of FSH between 2.5–40 IU (in-vivo bioactivity assessed in the Steelman–Pohley assay), rFSH and urinary FSH increased ovarian weight and ovarian aromatase activity in a similar dose-dependent manner (Figure 7).

In comparison with vehicle-treated animals maximum stimulation represented a 4- and 100-fold increase in ovarian weight and ovarian aromatase activity respectively, for both preparations. Neither rFSH nor urinary FSH affected the plasma oestradiol concentrations. This is likely due to the absence of LH activity since in the same model rFSH supplemented with increasing doses of HCG resulted in an HCG dose-dependent increase in plasma oestradiol (Mannaerts *et al.*, 1991).

The efficacy of rFSH was also assessed by gross histological examination of the ovaries. rFSH dose-dependently increased the number of large follicles, and a gradual shift of small antral to large preovulatory follicles, were observed. The latter

Table V. Pharmacokinetics of immunoreactive follicle stimulating hormone (FSH) after single-dose i.v. and i.m. administration of recombinant (rFSH) and urinary FSH (50 IU/kg in terms of in-vivo bioactivity) in rats[a]. Data represent mean ± SD

Preparation	Dose and route (IU/kg)	C_{max} (IU/L)	T_{max} (h)	AUC(0-∞) (IU/h/l)	nAUC(0-∞)* (IU/h/l)	$T_{\frac{1}{2}}$-elim (h)	F** (%)
rFSH	50 i.v.	–	–	1066 ± 298	126 ± 41	5.7 ± 0.9	–
urinary FSH	50 i.v.	–	–	917 ± 238	150 ± 45	6.2 ± 1.0	–
rFSH	50 i.m.	23.4 ± 10.4	7.5 ± 2.6	466 ± 223	54 ± 26	11.4 ± 3.9	42
urinary FSH	50 i.m.	28.2 ± 8.7	6.3 ± 2.0	498 ± 142	73 ± 22	10.4 ± 3.8	49

[a]Data for rats represent the averaged data of male and female animals.
*nAUC(0-∞) represent immunoreactive dose-normalized values.
**absolute bioavailability calculated from individual real dose AUC values in reference to i.v. administration.
– = values not calculated.
For other abbreviations, see Table IV.

ovulated after a bolus injection of 10 IU HCG. rFSH also diminished the incidence of atresia, in particular in the smallest size class follicles. Supplementation with HCG caused a considerable shift in the size of the follicles and reduced the incidence of atresia (Mannaerts *et al.*, 1994).

From these in-vivo studies it can be concluded that, when dosed in terms of in-vivo bioactivity assessed in the Steelman–Pohley assay, the efficacy of rFSH and urinary FSH in immature hypophysectomized rats is similar.

Pharmacokinetic behaviour in the rat

The pharmacokinetic behaviour of rFSH and urinary FSH was compared in male and female Wistar rats after single i.v. and i.m. administration at a dose level of 50 IU/kg in terms of in-vivo bioactivity. Immunoreactive FSH plasma concentrations were measured with a two-site FSH immunoradiometric assay (IRMA; Medgenix, Amersfordt, The Netherlands).

Single dose i.v. administration revealed no differences in terms of $T_{\frac{1}{2}}$-elim (~6 h) and AUC(0-∞) (~1000 IU/h/l) between rFSH and urinary FSH (Table V).

After single dose i.m. administration both rFSH and urinary FSH displayed a slow absorption into the circulation. For rFSH as well as urinary FSH plasma concentrations of immunoreactive FSH peaked at 6–8 h after injection (T_{max}; 7.5 compared with 6.3 h). Likewise, the rate of absorption (C_{max}: 23.4 versus 28.2 IU/l) and extent of absorption (AUC(0-∞); 466 versus 498 IU/h/l) were similar. The absolute bioavailability after single-dose i.m. administration was also similar (42 compared with 49%).

Comparison of a single-dose i.m. and i.v. administration revealed that elimination half-lives differed ~2-fold (11 versus 6 h respectively), for both FSH preparations (Table V). It is assumed that the terminal declining phase in the plasma-levels-versus-time curve after i.m. dosing does not reflect true elimination but rather relatively slow absorption of FSH from the i.m. injection site (flip-flop effect).

Based on their pharmacokinetic behaviour rFSH and urinary FSH can be considered to be bioequivalent in the rat. This is not surprising since both preparations were administered in terms of in-vivo bioactivity calibrated in the rat Steelman–Pohley assay.

However, when normalizing the AUC(0-∞) values for the immunoreactive dose, significantly lower values for rFSH emerged (Table V). This is probably due to the fact that rFSH contains less acidic isoforms than urinary FSH, hence higher quantities of immunoreactive hormone are needed to achieve the same in-vivo bioactivity. Likewise, when comparing another recombinant human FSH preparation (Gonal-F; Serono) with urinary FSH (Metrodin) a similar significant difference in AUC was found in *Cynomolgus* monkey (Porchet *et al.*, 1993).

Table VI. Pharmacokinetics of immunoreactive follicle stimulating hormone (FSH) after single dose i.v. administration of recombinant FSH (rFSH) and urinary FSH (25 IU/kg in terms of in-vivo bioactivity) in female Beagle dogs. Data represent mean ± SD

Preparation	C_{max} (IU/l)	AUC(0-∞) (IU/h/l)	nAUC(0-∞)* (IU/h/l)	$T_{\frac{1}{2}}$-elim.
rFSH	283 ± 43	148 ± 340	7.2 ± 0.4	27.9 ± 3.7
urinary FSH	302 ± 60	2550 ± 500	11.7 ± 1.0	30.4 ± 0.9

*nAUC(0-∞) represent immunoreactive dose-normalized values.
For other abbreviations, see Table IV.

Pharmacokinetic behaviour in dog

The pharmacokinetic behaviour of rFSH and urinary FSH was also compared in female Beagle dogs. For this purpose Beagle dogs were injected i.v. at a dose level of 25 IU/kg in terms of in-vivo bioactivity and plasma concentrations of immunoreactive FSH were measured by FSH-Delfia.

Single dose i.v. administration revealed no differences between rFSH and urinary FSH with respect to C_{max} (283 compared with 302 IU/l) or $T_{\frac{1}{2}}$-elim (27.9 compared with 30.4 h). In contrast, both the AUC(0-∞) and immunoreactive dose-normalized AUC(0-∞) of rFSH were significantly lower than those of urinary FSH (Table VI).

These results indicate that with respect to the extent of absorption, rFSH and urinary FSH are not bioequivalent in Beagle dogs. These results are in agreement with those obtained in humans (Mannaerts *et al.*, 1996), showing that Puregon and Metrodin are comparable with respect to elimination half-life (30–40 h) but are not bioequivalent. As in dogs, the bioavailability of rFSH after single dose administration was lower than that of urinary FSH. This difference in bioavailability is probably due to the fact that the relatively basic isohormones, which are more prevalent in rFSH, are cleared more rapidly than the relatively acidic isohormones.

In contrast to dogs and humans, rFSH and urinary FSH were bioequivalent in rats. This can be explained by the fact

that both preparations were dosed in terms of in-vivo bioactivity calibrated in the rat Steelman–Pohley assay. Thus, when calibrated in the rat, it is not surprising that rFSH and urinary FSH display a similar pharmacokinetic behaviour and consequently a comparable in-vivo efficacy in immature hypophysectomized rats (see above).

However, in species other than rat, such as dog and human, rFSH and urinary FSH are not bioequivalent. In addition, the elimination half-life of FSH is much longer in dogs and humans compared with rats. This might indicate that the rat Steelman–Pohley assay is not a valid model to predict the FSH bioactivity in species other than rat.

This is strongly supported by the fact that although the bioavailability of Puregon in humans is lower than that of Metrodin, a large prospective randomized IVF trial demonstrated a significantly higher number of oocytes, embryos and on-going pregnancies using fewer ampoules for a shorter treatment period for Puregon in comparison with Metrodin (Out *et al.*, 1995, 1996).

Summary

By using preparative isoelectrofocusing the isohormones of a CHO-derived recombinant FSH were separated and characterized with respect to structural and functional features. Carbohydrate analysis revealed that FSH isohormones with a low pI contain more heavily sialylated and more branched oligosaccharides than isohormones with a high pI. The most basic isohormones exhibited a 2–3 fold higher receptor binding activity and intrinsic bioactivity than the most acidic isohormones. However, the acidic isohormones displayed a more than 20-fold higher in-vivo bioactivity in rats due to a much lower clearance rate.

A comparison of the isohormone profile of rFSH and urinary FSH (Metrodin) revealed that rFSH contains about 2-fold more relatively basic isohormones with pI $\geq$4.7 and 2-fold less relatively acidic isohormones with pI $<$4.1.

Results of in-vitro studies show that the receptor binding affinity and intrinsic bioactivity of both preparations are similar, which indicates that the difference in isohormone composition does not affect these biological characteristics. This is consistent with the fact that the difference in receptor binding affinity and intrinsic bioactivity of the most basic and most acidic isohormones of rFSH is only 2–3-fold. Therefore, it cannot be expected that a relatively small difference in isohormone profile will have a serious impact on these in-vitro biological properties.

Also the in-vivo efficacy and the pharmacokinetic behaviour of rFSH and urinary FSH in the rat were similar. This again is not surprising since both preparations were compared in terms of in-vivo bioactivity calibrated in the rat Steelman–Pohley assay. This implies that for rFSH, containing more rapidly cleared basic isohormones than urinary FSH, higher immunoreactive quantities will be needed to achieve the same in-vivo biological activity. This was confirmed by the fact that immunoreactive dose-normalization of the AUC(0-∞) resulted in significantly lower values for rFSH when compared with urinary FSH.

In dogs, the bioavailability of rFSH was lower than that of urinary FSH. This is in agreement with the higher percentage of relatively basic isohormones in rFSH. This suggests that the pharmacokinetic behaviour of FSH in rats and dogs is different, which is supported by the much longer elimination half-life in dogs.

From these results it can be concluded that although rFSH contains more relatively basic isohormones than urinary FSH (Metrodin), both FSH preparations displayed comparable in-vitro and in-vivo biological properties in the rat. The observed differences in pharmacokinetic behaviour in dogs and rats indicate that the rat Steelman–Pohley assay might not be a valid model for the prediction of the FSH bioactivity in species other than rat, which is supported by clinical studies showing that Puregon is more efficacious than Metrodin.

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