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A pilot study on potency determination of human follicle-stimulating hormone: A comparison between reversed-phase high-performance liquid chromatography method and the *in vivo* bioassay

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) was compared with the classical Steelman–Pohley bioassay (BA), based on animal use, for the determination of human follicle-stimulating hormone (hFSH) biological activity. A linear relationship (BA_{IU} = 0.9925 RP-$HPLC_{IU}$ − 1.3165) with a highly significant correlation (r = 0.9371; p < 0.0001; n = 24) was found for these two methods for six hFSH preparations of different origins. The mean difference between the bioactivity predicted from RP-HPLC data via this equation and the mean of the bioactivities obtained with the two methods for six other hFSH preparations was −1.4%, with a 95% confidence interval of −9.3 to +6.6%. The precision of these parameters was 1.63% and 2.82%, respectively. These results demonstrate that RP-HPLC is a viable physical–chemical alternative to the use of an *in vivo* bioassay for hFSH potency determination, applicable also to hFSH Standards containing large amounts of human serum albumin.

1. Introduction

An important aspect in the quality control of therapeutic proteins is the assessment of their biological activity. For this purpose, animal models are most commonly used. Considering the ethical and political pressures on the use of animals and the nature of the assay, which can be imprecise and is in general costly, there is clearly a need for alternative assays that reduce the use of and/or replace such tests [1–4].

In this context, several physical–chemical methods have been reported in the literature as potential alternative assays for determining the biological activity of different proteins with greater precision and accuracy. As a case in point, size-exclusion high-performance liquid chromatography (HPSEC) was shown to be adequate for the determination of the potency of human growth hormone (hGH) [5,6] and this assay has been adopted by the principal pharmacopoeias as an alternative to the *in vivo* bioassay performed in hypophysectomized rats or dwarf "little" mice [7,8]. Similarly, a reversed-phase HPLC method (RP-HPLC) in combination with HPSEC was shown to correlate significantly with the *in vivo* bioassay for evaluating the potency of recombinant human granulocyte colony-stimulating factor (rhG-CSF) [9].

In the case of glycoproteins, pituitary hormones included, the replacement of tests based on animal use with physical–chemical methods is particularly challenging, especially considering the complexity of these molecules and the heterogeneity of their glycosylation. While some advocate the possibility of equivalency or at least complementarity between physical–chemical and *in vivo* methods [10], others believe that it is not possible to correlate a parameter derived from a global systemic measurement with a more targeted determination of an analyte [11]. Efforts have been made to develop tests for potency assessment that avoid the use of animals. There are already in the literature physical–chemical methods showing a good correlation with *in vivo* assays [12–17]. RP-HPLC was proposed as a possible alternative to the normocythaemic mice bioassay for the potency assessment of recombinant human erythropoietin [12]. The *in vivo* bioactivity of a therapeutic glycoprotein, human follicle-stimulating hormone (hFSH), used in the treatment of human infertility [18–20], could be predicted by using quantitative charge-based separation methods like isoelectric focusing and capillary zone electrophoresis. A highly significant correlation between the isoform distribution and the *in vivo* bioactivity was demonstrated by Mulders et al. [13,14] and by Storring et al. [15]. Still with regard to hFSH, HPSEC has been successfully employed instead of the bioassay in the production phase, for batch-to-batch consistency assessment and for precise drug delivery, an essential requirement for the achievement of the treatment objectives in clinical practice [16,17].

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Considering the advantages of speed, simplicity, and reliability of RP-HPLC, we chose to use a RP-HPLC method in this work for the quantification of different preparations of hFSH (CHO-, pituitary- and urinary-derived) in order to evaluate its applicability as a less expensive and more flexible alternative method to the classical Steelman and Pohley bioassay based on the rat ovarian weight gain method [21].

2. Materials and methods

2.1. Chemicals and reagents

Water was obtained from a Milli-Q Plus water-purification system (Millipore, Bedford, MA, USA). Acetonitrile (HPLC-grade, Mallinckrodt Baker) was purchased from Hexis (São Paulo, Brazil). All other chemicals were analytical reagent grade, purchased from Merck (São Paulo, Brazil) and Sigma (St. Louis, MO, USA).

2.2. Hormone preparations

Thirteen hFSH preparations were analyzed in this work: five recombinant preparations, CHO (Chinese hamster ovary)-derived (rhFSH-A, rhFSH-B, rhFSH-J, rhFSH-K, rhFSH-L), two pituitary (phFSH-C and phFSH-D), five urinary preparations (uhFSH-E, uhFSH-F, uhFSH-G, uhFSH-H, uhFSH-I) and the International Standard of rhFSH-WHO 92/642. For the biological assays, two standards were utilized: the International Standard of follicle-stimulating hormone (FSH) Recombinant, Human for Bioassay (WHO 92/642), and the International Standard of follicle-stimulating hormone (FSH) Pituitary, Human for Bioassay (WHO 83/575).

These preparations were obtained from: Aker University Hospital (Oslo, Norway), Ferring GmbH (Kiel, Germany), Institut Biochimique S.A. (IBSA) (Lugano, Switzerland), Laboratoires Serono S.A. (Aubonne, Switzerland), National Hormone and Pituitary Program (Torrance, CA, USA) and N.V. Organon (Oss, Netherlands). The WHO International Standards were from the National Institute for Biological Standards and Control (NIBSC, South Mimms, UK).

2.3. Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out with a Shimadzu Model SCL-10AHPLC apparatus with a SPD-10AV UV detector using a C4-Grace Vydac (Separations Group, Hesperia, CA, USA) 214 TP 54 column (25 cm × 4.6 mm I.D., pore diameter of 300 Å and particle diameter of 5 μm), coupled to a guard column (Grace Vydac 214 FSK 54), with a silica pre-column (packed with LiChrosorb Si 60, 7.9–12.4 μm, Merck, Darmstadt, Germany) located between the pump and the injector. The column temperature was maintained at 25 °C. Detection was by UV at 220 nm and quantification was achieved by peak area determination reported to the International Standard of rhFSH-WHO 92/642. Gradient solutions A and B were utilized, solution A being ammonium phosphate buffer (pH 8.6; 0.05 M) and solution B acetonitrile. The elution was performed with a linear gradient from A:B (85:15, v/v) to A:B (50:50, v/v) over 40 min, at a flow-rate of 0.5 ml/min. Aliquots of 5–10 μl of phFSH and 20–100 μl of rhFSH or uhFSH were, in general, processed.

2.4. High-performance size-exclusion chromatography (HPSEC)

HPSEC was carried out with the same Shimadzu apparatus, processing 20–50 μl of rhFSH on a Tosohaas (Montgomeryville, PA, USA) G2000 SW column (60 cm × 7.5 mm I.D. particle size of 10 μm and pore size of 125 Å) coupled to a 7.5 cm × 7.5 mm I.D. SW guard column. The mobile phase was 0.15 M NaCl, sodium phosphate buffer (pH 7.0; 0.02 M), with a flow rate of 1.0 ml/min.

Table 1
Inter-day hFSH determination of preparations of different origins, by RP-HPLC.

Preparation	FSH content[a] (μg/vial)	RSD[b] (%)
rhFSH-A	5.5 ± 0.32	5.8
rhFSH-B	9.8 ± 0.41	4.2
phFSH-C	4.8 ± 0.03	1.7
phFSH-D	6.7 ± 0.12	1.6
uhFSH-E	5.2 ± 0.30	5.8
uhFSH-F	10.3 ± 0.27	2.6

[a] Mean ± standard deviation (n = 4).
[b] Relative standard deviation.

2.5. Biological assays

hFSH activity was determined by the rat ovarian weight gain method of Steelman and Pohley [21] and the assay was conducted in accordance with the national protection laws on animal welfare. Briefly, 19–22 day old Sprague–Dawley immature female rats received once a day, subcutaneously, over three consecutive days, 0.5, 1 and 3 IU of hFSH. Autopsy was performed on the fourth day (72 h after the first injection). The ovaries were removed, dissected free of surrounding tissue, and weighed. The *in vivo* FSH bioactivity was calculated against the recombinant hFSH International Standard WHO 92/642 in the case of urinary- and CHO-derived hFSH or against the pituitary hFSH International Standard WHO 83/575, in the case of pituitary-derived samples.

Statistical analysis of the assay data was carried out according to Finney, by parallel line methods (3 × 3), using PLA 2.0 software (Stegmann System-beratung, Rodgau, Germany).

2.6. Statistical methods

The comparison between bioassay and RP-HPLC data was made by linear regression analysis and the agreement between the two methods was evaluated as described by Bland and Altman [22]. Differences between means were assessed by using Student's t-test (two-sided).

3. Results

The content of hFSH (μg per vial) in 6 preparations of different origins, CHO- (A and B), pituitary- (C and D) and urinary- (E and F) derived, was determined by a previously reported RP-HPLC technique (Table 1) [23]. An inter-day (n = 4) inter-ampoule variation < 6% was observed. Considering the lack of official standards suitable for physical–chemical testing, we reported our determinations relative to the hFSH international recombinant reference standard for biological assays from the National Institute for Biological Standards and Control (WHO 92/642), which presented the RP-HPLC chromatographic profile shown in Fig. 1A. It is noteworthy that the hFSH peak area shown in the figure, later proved to correspond to 2 μg of pure recombinant hFSH, as expected. This standard reference preparation could only be utilized for quantitative analysis carried out by RP-HPLC, considering the presence, in this preparation, of a high content (200:1) of human serum albumin (HSA), which completely obscures the peak of hFSH in HPSEC (Fig. 1B).

RP-HPLC profiles of the three groups of hFSH preparations analyzed in this study are shown in Fig. 2. Highly significant differences (p < 0.001) in FSH retention time (t_R) between recombinant (t_R = 26.42 ± 0.23, n = 8), pituitary (t_R = 25.19 ± 0.15, n = 8) and urinary (t_R = 24.46 ± 0.22, n = 8) preparations were found, reflecting differences in the carbohydrate moieties. The peak with t_R of

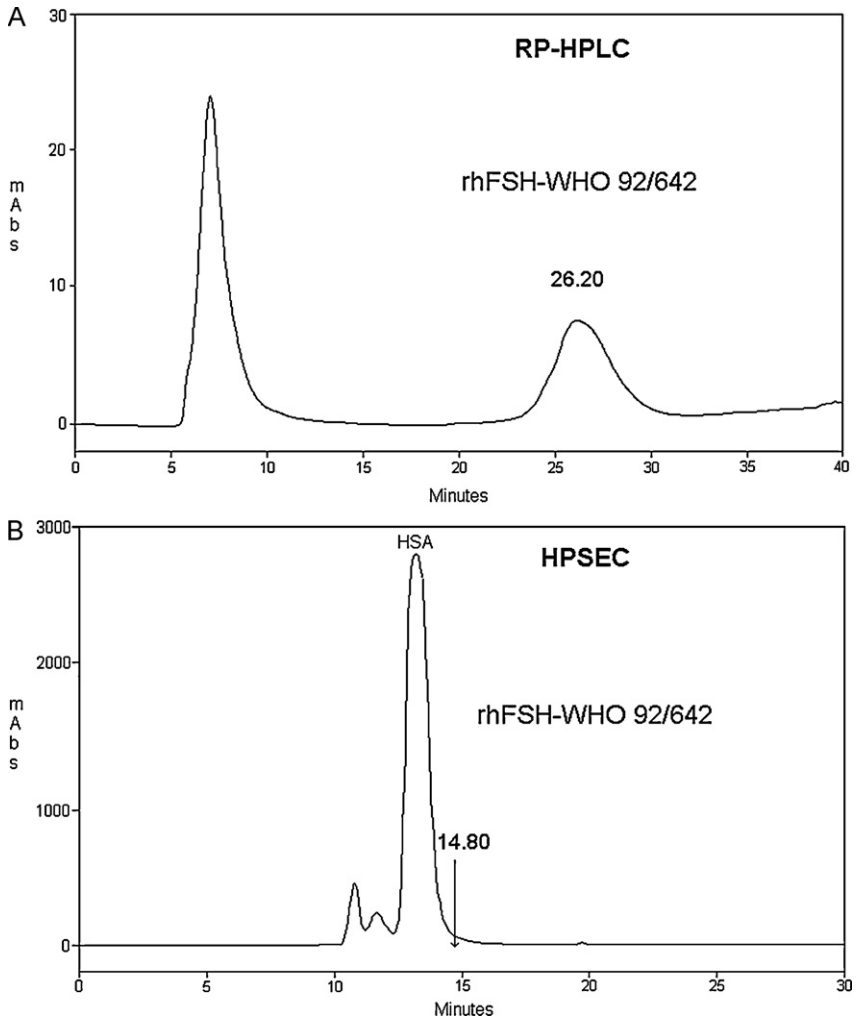

Fig. 1. HPLC profile of 2 μg of the recombinant hFSH reference standard for biological assays (WHO 92/642). (A) RP-HPLC. (B) HPSEC. The retention time of an rhFSH preparation that is free of HSA, run under identical conditions is indicated by the arrow.

~7 min corresponds to the excipient present in different amounts in the analyzed samples.

The potency estimates (International Units, IU per vial), by *in vivo* biological assay for recombinant and urinary preparations were determined against recombinant hFSH reference standard, WHO 92/642, while the pituitary preparations were determined against pituitary reference preparation WHO 83/575 (Table 2). For a better understanding and easier comparison, in the same Table the hFSH content that was determined by RP-HPLC in μg was expressed in IU because unitage system is still the one most commonly used in clinical practice. An average hFSH recovery of 108.3 ± 11.63% of the nominal content was estimated on the basis of the bioassay, as compared to an average recovery of 111.9 ± 20.09% by RP-HPLC (Table 2). When the potency determination by bioassay (BA_{IU}) was compared with hFSH quantification by RP-HPLC (RP-$HPLC_{IU}$), a linear relation was found ($BA_{IU} = 0.9925\ \text{RP-HPLC}_{IU} - 1.3165$), with a highly significant degree of correlation ($r = 0.9371$; $p < 0.0001$; $n = 24$).

To evaluate the potential of RP-HPLC for accurately predicting the *in vivo* bioactivity according to Bland–Altman analysis [22], a comparison between the *in vivo* bioactivity estimated by RP-HPLC from the above equation with that estimated by the Steelman and Pohley bioassay was carried out. Six samples that had not been used for the correlation analysis were employed (Table 3). This analysis revealed a mean percentage difference ($\bar{d}$) between the predicted and the mean of the bioactivities obtained by the two methods of −1.35%, well within the 95% confidence limits ($\bar{d} \pm 2\text{SD}$) of −9.3 to + 6.6% (Fig. 3). A Student's *t*-test performed on the average ± SD of the *in vivo* and predicted bioactivities indicated that the calculated *t*-value of 0.037 is much smaller than the theoretical *t*-value of 2.57, at the 0.05 significance limit for 5 degrees of freedom, indicating that the results of the two methodologies were not significantly different. To evaluate how precise the determinations were, the standard error of $\bar{d}$, defined as $\sqrt{\text{SD}^2/n}$ and the standard error of $\bar{d} \pm 2\text{SD}$, defined as $\sqrt{3\text{SD}^2/n}$, were determined as 1.63% and 2.82%, respectively. Then the 95% confidence interval for the bias $\left(\bar{d} \pm t\sqrt{\text{SD}^2/n}\right)$, for the lower limit of agreement (LL) and for the upper limit of agreement (UL) were calculated. All of these statistical parameters are shown in Table 4.

4. Discussion

Based on the findings of the present work, it can be concluded that physical–chemical analysis, via RP-HPLC, is indeed a viable alternative to *in vivo* bioassay for determining hFSH potency. This alternative can be useful, for example, in quality control of internet-obtained pharmaceuticals, being possibly able to detect counterfeited and/or substandard drugs [24]. Further work should be carried out in this direction and the current results should be complemented with samples of this type. Mass spectrometry (MS)

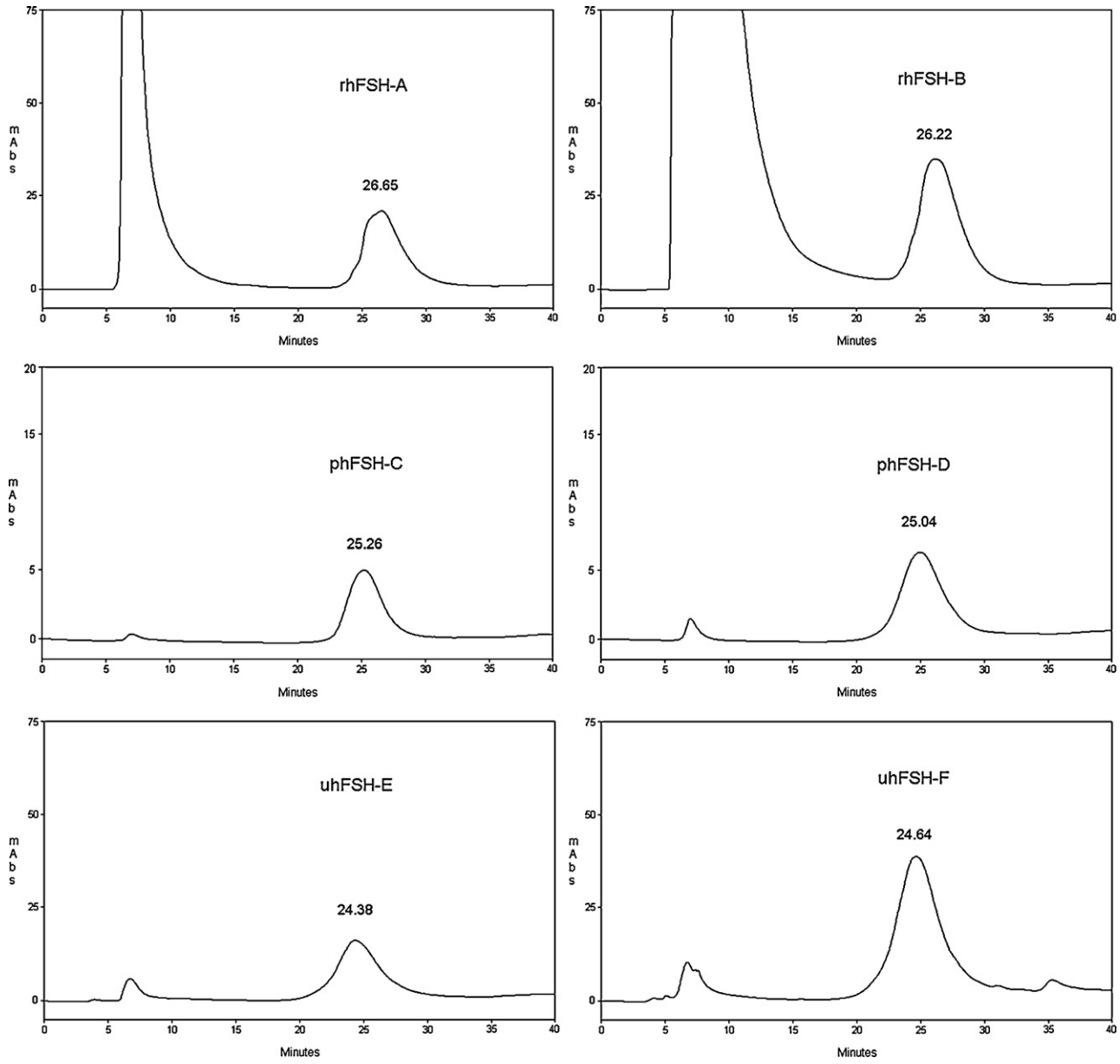

Fig. 2. RP-HPLC profile of different hFSH preparations: rhFSH-A (5.2 μg); rhFSH-B (10.3 μg); phFSH-C (1.2 μg); phFSH-D (1.7 μg); uhFSH-E (4.7 μg); uhFSH-F (11.1 μg).

evaluation would also be very interesting, especially for identifying glycosylation sites.

The RP-HPLC methodology employed, which was previously established in our laboratory and validated with respect to accuracy, precision, linearity and sensitivity, is, as far as we know, unique in the literature in its capacity to analyze intact hFSH, i.e., hFSH in its heterodimeric form [23]. Thanks to this methodology, we could also use an international standard of hFSH for biological assay (WHO 92/642) as the standard in our physical–chemical assay. Analysis of this standard via RP-HPLC demonstrated that the large amount of HSA present in the ampoule did not elute out of the column, under our running conditions, not interfering thus with hFSH determination, in a preparation in which it represents less than 1% of the total mass.

Table 2
Comparison between the quantification of hFSH preparations via *in vivo* bioassay and by RP-HPLC.

Preparation	hFSH nominal content[a] (IU/vial)	hFSH determined by *in vivo* bioassay (IU/vial)	hFSH determined by RP-HPLC[b] (IU/vial)
rhFSH-A	75	77.0	75.9
rhFSH-B	100	117.2	135.2
phFSH-C	85.6	80.7	92.1
phFSH-D	92.9	116.9	128.5
uhFSH-E	75	76.1	70.4
uhFSH-F	150	161.7	142.1

[a] Based on declared content.
[b] RP-HPLC determinations expressed in IU considering 13,800 IU/mg to be the potency of rhFSH-WHO 92/642 and 19184 IU/mg the potency of phFSH-WHO 83/575.

Table 3
Comparison between the biological activity determined by the *in vivo* bioassay and predicted by the equation via RP-HPLC analysis.

Preparation	Biological activity determined by *in vivo* bioassay (1) (IU/vial)	Biological activity predicted by RP-HPLC (2) (IU/vial)	Mean biological activity (1) and (2) (IU/vial)	Difference between predicted and mean biological activity *d* (IU)	Difference between predicted and mean biological activity *d* (%)
uhFSH-G	68.0	58.0	63.0	−5.0	−7.9
uhFSH-H	150.3	164.2	157.3	+6.9	4.4
uhFSH-I	74.0	70.3	72.2	−1.9	−2.6
rhFSH-J	119.0	116.4	117.7	−1.3	−1.1
rhFSH-K	116.6	114.2	115.4	−1.2	−1.0
rhFSH-L	115.8	116.1	116.0	+0.1	0.09
					$\bar{d} \pm SD = -1.35 \pm 3.99$

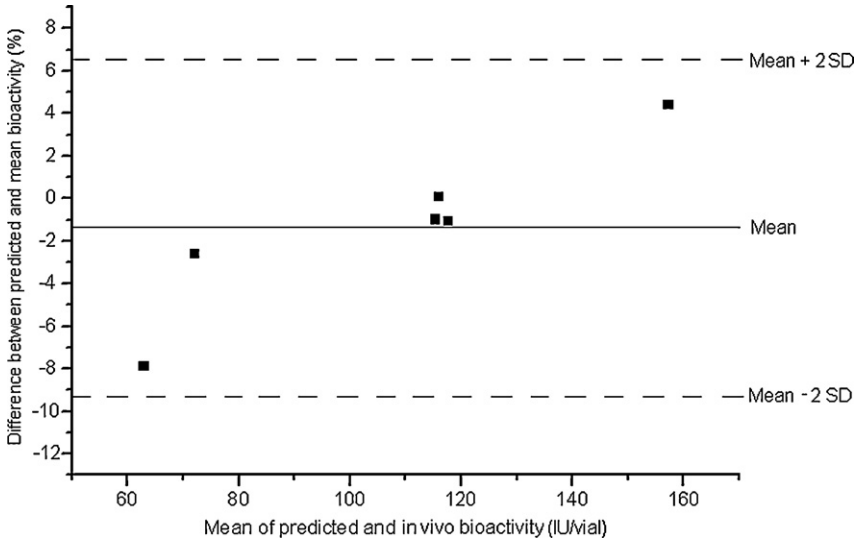

Fig. 3. Comparison between the RP-HPLC-predicted and *in vivo* bioactivity by plotting their percent difference against their mean.

Highly significant differences in FSH RP-HPLC retention time were found between recombinant, pituitary and urinary hFSH, probably reflecting the effect of structural differences in the carbohydrate moiety on the hydrophobicity of the glycoprotein molecule. Analogous observations were made for the other two pituitary glycoprotein hormones, TSH [25,26] and hLH [27]. The same RP-HPLC analytical approach was also shown to be useful for the detection of the presence of undesirable free subunits in preparations of the three glycoprotein hormones (hFSH, hTSH and hLH). This is an important pharmaceutical control, considering that the full hormonal activity is due only to the intact heterodimeric form [28].

A highly significant correlation ($p < 0.0001$) was found between the RP-HPLC results and the *in vivo* assay when analyzing recombinant, pituitary and urinary hFSH samples (see Table 2). This analysis, however, did not include partly-degraded samples which could present different degrees of *in vivo* activity. In this case, RP-HPLC should be able to detect the product alterations that cause loss of activity. For the validation of the regression model, Bland–Altman analysis [22] was carried out by utilizing six samples of hFSH preparations that had not been used to construct the correlation curve. For these six samples, the RP-HPLC-predicted and the *in vivo* bioassay data were not significantly different. An acceptable agreement (bias < 1.5%) between the two methodologies was found, the potency estimated by the former method being no less than 85% and no more than 109% of that estimated by the latter method. We can also observe that most of the calculated statistical parameters (see Table 4) were of the same order or even better than those found in the literature for analogous studies [13,14]. It is important to emphasize that this type of validation conforms to the guidelines of the "European Centre for Validation of Alternative Methods" (ECVAM) of the European Commission. It is recommended that this procedure for comparing data from different methods be used whenever possible [14,15,29].

Even though the RP-HPLC methodology is potentially capable of discriminating between different hFSH isoforms [30], we did not develop this possibility further, as has been done, for example, by Mulders et al. [13] and Storring et al. [15], applying either isoelectric focusing or capillary zone electrophoresis to evaluate the bioactivity of preparations consisting of different isoforms. The fact that, on a simple mass basis, our methodology was also able to predict biological activities with reasonable accuracy could be due to the fact that glycan or isoform distribution in general is not remarkably different between the various preparations tested or, alternatively, that the isoforms with the greatest bioactivity variation are present only in very small amounts. This might have also been the case in

Table 4
Statistical parameters, determined according to Bland and Altman [22], comparing the values of *in vivo* bioactivity with those predicted via RP-HPLC determinations.

Parameter	Determined value (%)
Mean difference ($\bar{d}$)	−1.35
Standard deviation (SD)	3.99
Range ($\bar{d} \pm 2SD$)	−9.3 to +6.6
$\sqrt{\frac{SD^2}{n}}$	1.63
$\sqrt{\frac{3SD^2}{n}}$	2.82
Range $\left(\bar{d} \pm t\sqrt{\frac{SD^2}{n}}\right)$	−5.5 to +2.8
Range $\left(LL \pm t\sqrt{\frac{SD^2}{n}}\right)$	−16.6 to −2.1
Range $\left(UL \pm t\sqrt{\frac{SD^2}{n}}\right)$	−0.65 to +13.9

other studies where bioactivity assessment for hFSH was also based on mass determination, such as those of Driebergen et al. [16] and Bassett [17].

A single type of physical–chemical analysis obviously cannot mirror all aspects of the *in vivo* biological activity of a hormone. In agreement with the "consistency approach" [29,31,32], which implies the use of specific tests to demonstrate equivalence of a product with another of proven efficacy, there is a tendency to accept alternative assays that could complement the classical *in vivo* bioassay. These can be used in routine determinations, after the initial validation of the process development in which a classical *in vivo* bioassay is recommended.

In conclusion, the employment of non-animal alternatives, such as RP-HPLC, can reduce the number of animals currently used to assure quality and efficacy of a pharmaceutical product. In this regard, the results of the present pilot study pave the way for possible replacement of *in vivo* bioassays in quality control protocols for the assessment of hFSH potency.

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