Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1136 (2006) 10–18
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of intact human follicle-stimulating hormone preparations by reversed-phase high-performance liquid chromatography

Renan Fernandes Loureiro [a], João Ezequiel de Oliveira [a], Peter A. Torjesen [b], Paolo Bartolini [a], Maria Teresa C.P. Ribela [a,*]

[a] *Biotechnology Department, IPEN-CNEN, Av. Prof. Lineu Prestes 2242, Cidade Universitária, 05508-900 São Paulo, Brazil*
[b] *Hormone Laboratory, Aker University Hospital, 0514 Oslo, Norway*

Received 2 May 2006; received in revised form 6 September 2006; accepted 18 September 2006
Available online 17 October 2006

Abstract

A reversed-phase high-performance liquid chromatography (RP-HPLC) method for the qualitative and quantitative analysis of intact human follicle-stimulating hormone (hFSH) was established and validated for accuracy, precision and sensitivity. Human FSH is a dimeric glycoprotein hormone widely used as a diagnostic analyte and as a therapeutic product in reproductive medicine. The technique developed preserves the protein integrity, allowing the analysis of the intact heterodimeric form rather than just of its subunits, as is the case for the majority of the conditions currently employed. This methodology has also been employed for comparing the relative hydrophobicity of pituitary, urinary and two Chinese hamster ovary (CHO)-derived hFSH preparations, as well as of two other related glycoprotein hormones of the anterior pituitary: human thyroid-stimulating hormone (hTSH) and human luteinizing hormone (hLH). The least hydrophobic of the three glycohormones analyzed was hFSH, followed by hTSH and hLH. A significant difference ($p < 0.005$) was observed in t_R between the pituitary and recombinant hFSH preparations, reflecting structural differences in their carbohydrate moieties. Two main isoforms were detected in urinary hFSH, including a form which was significantly different ($p < 0.005$) from the pituitary and recombinant preparations. The linearity of the dose–response curve ($r = 0.9965$, $n = 15$) for this RP-HPLC methodology, as well as an inter-assay precision of less than 4% for the quantification of different hFSH preparations and a sensitivity of the order of 40 ng, were demonstrated. The chromatographic behaviour and relative hydrophobicity of the individual subunits of the pituitary and recombinant preparations were also analyzed. Furthermore, the molecular mass of individual hFSH subunits and of the heterodimer were simultaneously determined by matrix-assisted laser desorption ionization time-of-flight mass spectral analysis (MALDI-TOF-MS). The present methodology represents, in our opinion, an essential tool for the characterization and quality control of this hormone, that is not yet described in the main pharmacopoeias.

Keywords: Follicle-stimulating hormone; Reversed-phase HPLC; Glycohormone subunit; MALDI-TOF-MS

1. Introduction

Human follicle-stimulating hormone (hFSH), a glycoprotein hormone synthesized by the pituitary gland and formed by two non-covalently linked α and β subunits like the other related glycoproteins such as luteinizing hormone (hLH) and thyroid-stimulating hormone (hTSH), is critically involved in the maturation of ovarian follicles and in spermatogenesis. Considerable heterogeneity associated with different hFSH preparations has been reported, mainly related to the presence of different glycoforms [1–11]. Thus, the utilization of this hormone as a therapeutic agent in reproductive medicine requires a strict confirmation of the consistency of different batches of this product. Up to now, its quantitation has been mostly based on the classical Steelman & Pooley bioassay [12] modified by Van Hell et al. [13], or other in vitro bioassays [14–18] and on immunoassays [8,19–26], while its identification and analysis have been based mostly on sodium dodecyl sulfate poliacrylamide gel electrophoresis (SDS-PAGE) and Western blotting [3,7,27–30], whose limitations are well known [31–33]. More precise determinations have been obtained by isoelectric focusing [4,7,27–28,30,34–36] and capillary electrophoresis [36].

* Corresponding author. Fax: +55 11 38169231.
E-mail address: mtribela@ipen.br (M.T.C.P. Ribela).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.09.037

Studies of the effects of post-translational modifications, such as glycosylation, on the efficacy of recombinant FSH preparations were also reported [11,37–39]. The key role of these events in determining and modulating biological functions were also studied for the other three gonadotrophins, TSH [40], hLH and hCG [11].

Useful information with respect to purity, identity, potency and stability assessment of hFSH and pituitary hormones in general has been provided by high-performance liquid chromatography (HPLC) [41]. This methodology is the most frequently utilized in safety regulations and quality control strategies of biopharmaceuticals [42]. The assessment of hFSH vial content using size-exclusion HPLC (HPSEC) with a variability lower than 2% (filled-by-mass) is an alternative to the calibration of the final product based on in-vivo bioassays (filled-by-bioassay) that presents an intrinsic variability of up to 20% [43–46]. The filled-by-mass approach allows a more precise dosing of FSH content in different batches, being an important step towards the minimization of drug-related variability, which influences the response to hFSH treatment. The reversed-phase HPLC mode (RP-HPLC) is also very often utilized for the control of the manufacturing process, as shown in previous work on hTSH purification and characterization [47]. This methodology discriminates with basis on differences in hydrophobicity, being able to detect and quantify discrete modifications in the molecular structure of the analyzed product. RP-HPLC has been shown to be suitable for the identification of glycoprotein hormones such as pituitary-derived [48–52] or Chinese hamster ovary (CHO)-derived [33,53] human luteinizing hormone (hLH) and thyroid stimulating hormone (hTSH). While these two glycoprotein hormones were eluted as separate subunits (α and β) at low pH, they remained intact utilizing acetonitrile-based gradient elutions at neutral pH on silica-based C_4 columns [33,51,53]. However, the latter condition was shown to be inappropriate for hFSH, since a consistent partial dissociation of this hormone was observed at neutral pH. Most reports employing RP-HPLC for the analysis and characterization of either recombinant or pituitary hFSH identified only the dissociated subunits [7,34,50]. To circumvent this problem, Hiyama et al. [51] proposed the use of tandem RP-HPLC consisting of columns of different hydrophobicity strengths operating at neutral pH, allowing the separation of the three pituitary-derived glycoprotein hormones while maintaining their integrity. Under these conditions, however, the β subunit could not be resolved from the heterodimer.

Consistent with the high discriminating power, chemical specificity, sensitivity, robustness and simplicity of RP-HPLC, the present study describes, for the first time, a RP-HPLC method utilizing only one column (C_4) and a basic mobile phase that allows the analysis and characterization of the heterodimeric form of hFSH. Moreover, the method is capable of identifying the dissociated subunits that might be present in a pharmaceutical preparation as product-related impurities. These findings have been applied to the analysis and quality control of different preparations of hFSH, which has not yet been reported by the main pharmacopoeias.

2. Materials and methods

2.1. Chemicals and reagents

Water was obtained from a Millipore Milli-Q plus water purification system (Bedford, MA, USA).

Acetonitrile (HPLC-grade) Mallinckrodt Baker S.A. 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).

Recombinant hFSH (Gonal f 75 IU/vial) from Serono and recombinant hFSH (Puregon 100 IU/vial) from Organon were purchased from Expomed Comércio de Produtos Farmacêuticos (São Paulo, Brazil). Pituitary hFSH (NIDDK-hFSH-I-SIAFP-2), human alpha FSH (NIDDK-h(FSH-I-3), human beta FSH (NIDDK-h(FSH-I-SIAFP-2), pituitary hTSH (NIDDK-hTSH-SIAFP-B-2) and pituitary hLH (NIDDK-hLH-I-SIAFP-2) were obtained from the National Hormone and Pituitary Program (Torrance, CA, USA). A second preparation of pituitary hFSH, pit-hFSH NOR, was from Aker University Hospital (Oslo, Norway). Urinary hFSH (Metrodin HP 75 IU/vial) from Serono was kindly donated by Serono.

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

RP-HPLC was carried out with a Shimadzu Model SCL-10A HPLC apparatus with a SPD-10AV UV detector using a C_4-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 (Vydac 214 FSK 54) and with a silica precolumn (packed with LiChrosorb Si 60, 7.9–12.4 μm, Merck, Darmstadt, Germany) located between the pump and the injector. The latter, a silica saturator, was introduced by Riggin et al. [54] to protect the C_4-Vydac separation column from the dissolution of the silica by the mobile-phase. The column temperature was maintained at 25 °C. Detection was by UV absorbance at a wavelength of 220 nm and quantification was achieved by peak area determination against pituitary-hFSH (pit-hFSH NOR). Mobile phase A was 0.05 M ammonia phosphate buffer, pH 8.6, and mobile phase B was 50% acetonitrile plus 50% mobile phase A. A linear gradient of 30–100% B over 40 min was used at a flow-rate of 0.5 mL/min. Aliquots of 5–10 μL of pit-hFSH and of 150–250 μL of r-hFSH and u-hFSH were in general processed.

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

HPSEC was carried out with the same Shimadzu apparatus, processing 5–10 μL of pit-hFSH or 150–250 μL of r-hFSH and u-hFSH 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, 0.02 M sodium phosphate, pH 7.0, with a flow rate of 1.0 mL/min.

2.4. Protein determination

Total protein concentration was estimated utilizing Micro bicinchoninic acid (BCA) protein assay kit, according to the manufacturer's instruction (Micro BCA protein assay kit, Pierce, Rockford, IL, USA). As standard, solutions of pure bovine serum albumin (BSA), ranging from 0.5 μg/mL to 200 μg/mL, were used.

2.5. Mass spectrometry

The preparations of pit-hFSH NOR, u-hFSH Metrodin and r-hFSH Gonal f, containing their excipients, were embedded in a matrix consisting of a saturated solution of sinapinic acid in 50% acetonitrile/0.1% trifluoracetic acid. An aliquot of 10 μL (~20–30 pM of hormone) was air-dried and analyzed by MALDI-TOF using an Applied Biosystems Voyager-DE-STR, operated in linear positive-ion mode, with the following parameters: accelerating voltage, 20,000 V; grid voltage, 95.4%; guidewire voltage, 0.05% and high mass gate, 50,000. The laser power was altered from 1200 to 2000 and the laser beam was fired at multiple sites in a sample well by moving randomly the control joystick. Signals from 26 to 256 excitation pulses were accumulated and averaged to yield each recorded mass.

3. Results

In a previous study, a RP-HPLC methodology for the qualitative and quantitative analysis of human thyrotropin (hTSH) was set up in our laboratory [33]. Utilizing the same chromatographic conditions for pituitary-derived follicle stimulating hormone (hFSH), a partial dissociation (~70%) of the heterodimeric form into alpha and beta subunits was observed (Fig. 1A). The peaks with retention times (t_R) of 27.5 min and 35.7 min correspond to the pit-hFSH β and α subunits, respectively, as shown by comparing them with purified subunits of pit-hFSH NIDDK (Fig. 1B and C), illustrating that the β is less hydrophobic than the α subunit.

Under the same chromatographic conditions, the relative hydrophobicity of hFSH and of the other two heterodimeric glycohormones (hTSH and hLH) could be compared, illustrating their relative hydrophobicity in the following order: hLH > hTSH > hFSH (Fig. 2).

Optimized RP-HPLC elution conditions to maintain the integrity of the hFSH heterodimer were achieved by increasing the starting concentration of buffer B from 25 to 30% and the pH of buffer A from 7.0 to 8.6. In addition, the ionic composition of buffer A was altered by using ammonium instead of sodium phosphate. The latter modification was introduced to circumvent the interference of a "ghost" peak of the type of those described by Williams in his specific review [55]. Employing these conditions, dissociation of subunits did not occur and heterodimer recovery was close to 100%. The behavior of pituitary hFSH and of its subunits under the new elution conditions is depicted in Fig. 3. Utilizing these conditions, commercially available hFSH preparations of different origins, i.e.,urinary (u-hFSH) and CHO-derived (r-hFSH), could be compared with a highly purified pituitary preparation (pit-hFSH). In contrast to pit-hFSH, these preparations were found to be more heterogeneous. The elution profiles of the two recombinant preparations, Gonal F and Puregon (Fig. 4A and B) were nearly identical. The main isoforms showed identical retention times, which were significantly higher ($p < 0.005$) than that of the pituitary preparation (Table 1). On the other hand, urinary-derived hFSH presented

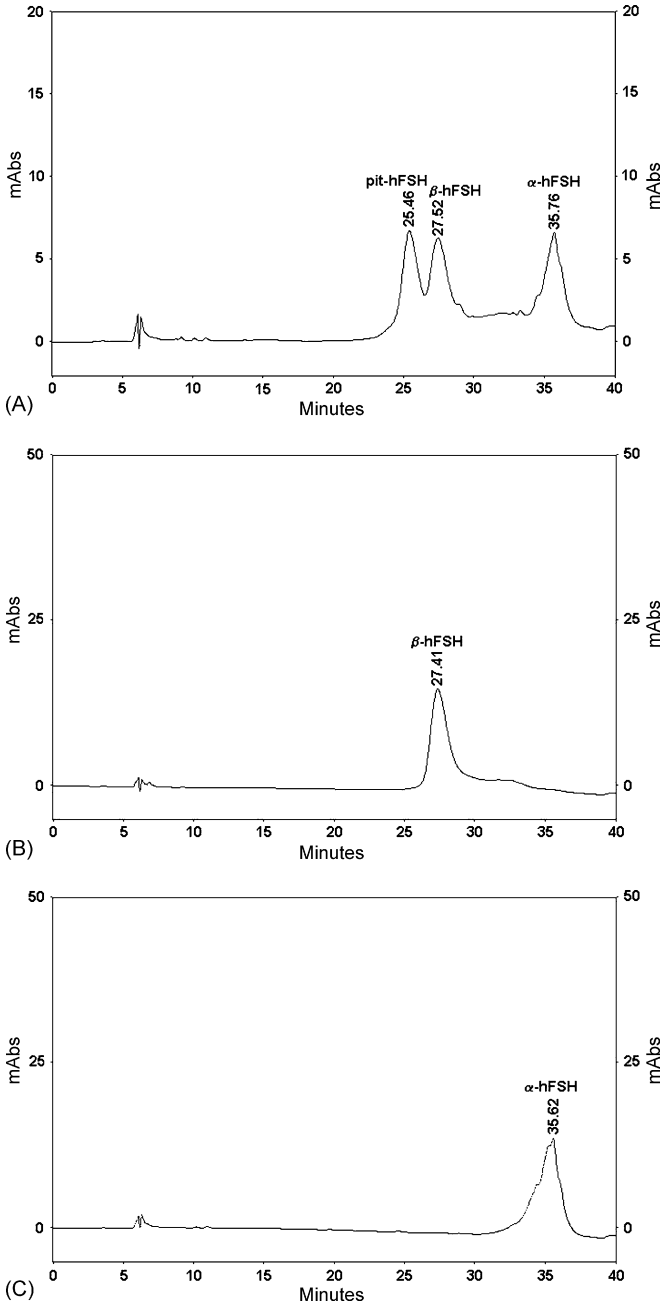

Fig. 1. RP-HPLC of pure pituitary hFSH (pit-hFSH NIDDK) and its subunits. Column: C4 Vydac, 214 TP54, 25 cm × 4.6 mm I.D., particle diameter 5 μm, elution at temperature 25 °C, flow rate 0.5 mL/min, λ = 220 nm. Eluent A: 0.05 M sodium phosphate buffer (pH 7.0); eluent B: 50% acetonitrile plus 50% eluent A. Elution over 40 min with a linear gradient of 25–100% B. (A) Pituitary hFSH, 5 μg; (B) pituitary β hFSH, 5 μg; (C) pituitary α hFSH, 5 μg. Observe that panel A has a different scale compared with panels B and C. In this and in the next experiments the amount indicated for each hormone is always based on the nominal content.

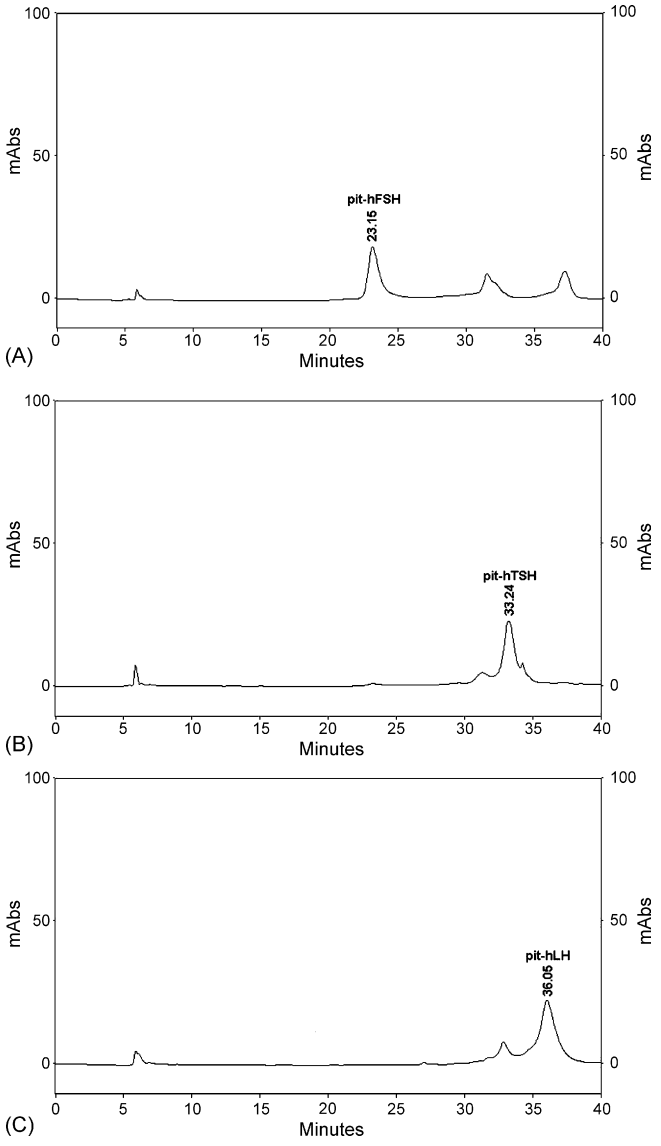

Fig. 2. RP-HPLC of pituitary glycohormones. The same chromatographic conditions as described in Fig. 1 were employed. (A) pit-hFSH NOR, 8.5 μg; (B) pit-hTSH NIDDK, 5 μg; (C) pit-hLH NIDDK, 5 μg.

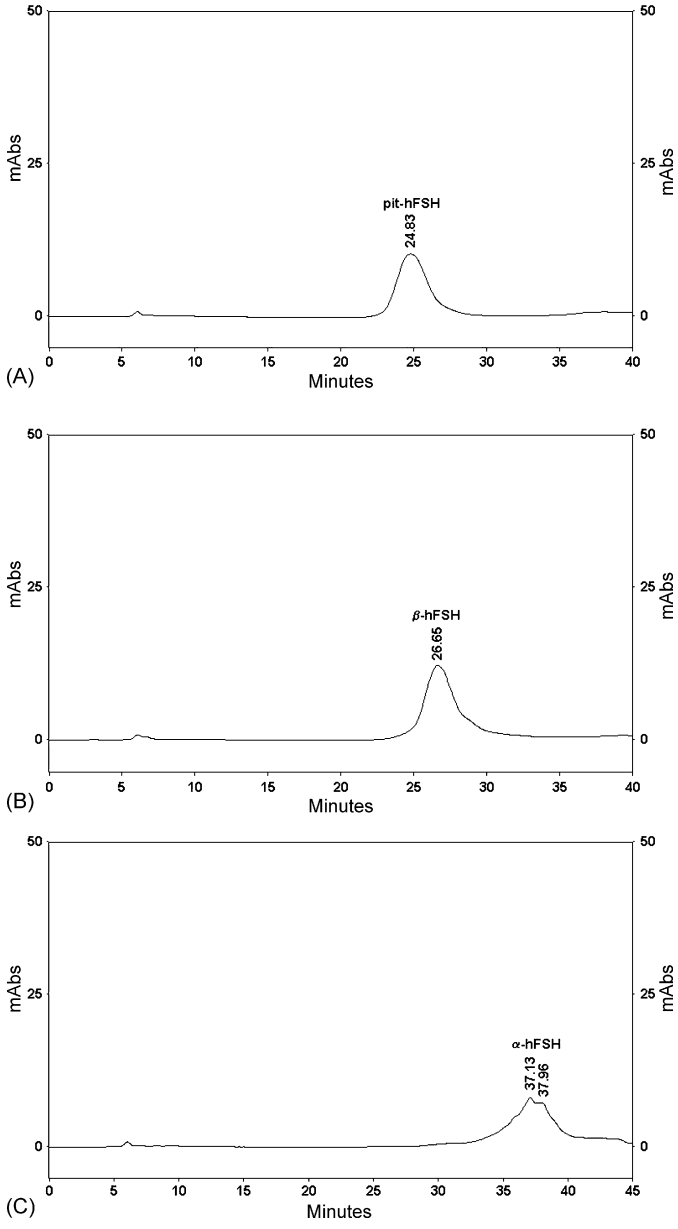

Fig. 3. New RP-HPLC elution conditions for pure pituitary hFSH (pit-hFSH NIDDK) and its subunits. Column: C4 Vydac, 214 TP54, 25 cm × 4.6 mm I.D., particle diameter 5 μm, elution at temperature 25 °C, flow rate 0.5 mL/min, λ = 220 nm. Eluent A: 0.05 M ammonia phosphate buffer (pH 8.6); eluent B: 50% acetonitrile plus 50% eluent A. Elution over 40 min with a linear gradient of 30–100% B. (A) 5 μg of pituitary hFSH; (B) 5 μg of pituitary β hFSH; (C) 5 μg of pituitary α hFSH.

two main isoforms (Fig. 4C), one of which (peak 1) had a lower and the other (peak 2) approximately the same retention time as the pituitary preparation. The lower retention time (t_R) of peak 1 was significantly ($p < 0.001$) different from the other preparations. The difference between urinary peak 1 and the main peak of recombinant or pituitary hFSH (∼6% and ∼3%), probably indicates important differences in their carbohydrate structures. Furthermore, Table 1 shows the excellent reproducibility of the inter-day t_R determinations, with a relative standard deviation of the order of 1% or less. It is important to point out that the peaks eluting at the beginning (t_R ∼ 5–10 min) of the chromatogram (Fig. 4) are due to different excipients that are present in these preparations.

When exposed to 3 M acetic acid overnight at 37 °C, these preparations dissociated completely into their subunits (∼98.2% recovery) as depicted for pit-hFSH in Fig. 5. The retention times of the heterodimers were significantly different ($p < 0.01$) from the retention times of the α and β subunits (Table 2). The good separation between the heterodimeric form and the corresponding subunits was accompanied by highly constant mean relative retention times ($t_{RR} = t_R$ subunit/t_R heterodimer): 1.100 ± 0.004 (RSD = 0.4%) and 1.517 ± 0.02 (RSD = 1.5%), respectively, for the β and α subunits of the pituitary and the two recombinant preparations (Table 2). The relative percentage yields of dissociated material in the peaks of the two subunits were 52.08% (β) and 47.92% (α) (Fig. 5), which is close to the theoretical yield based on mass determinations (54.8% for β and 45.2% for α) [34]. Unfortunately it was not possible to run the same

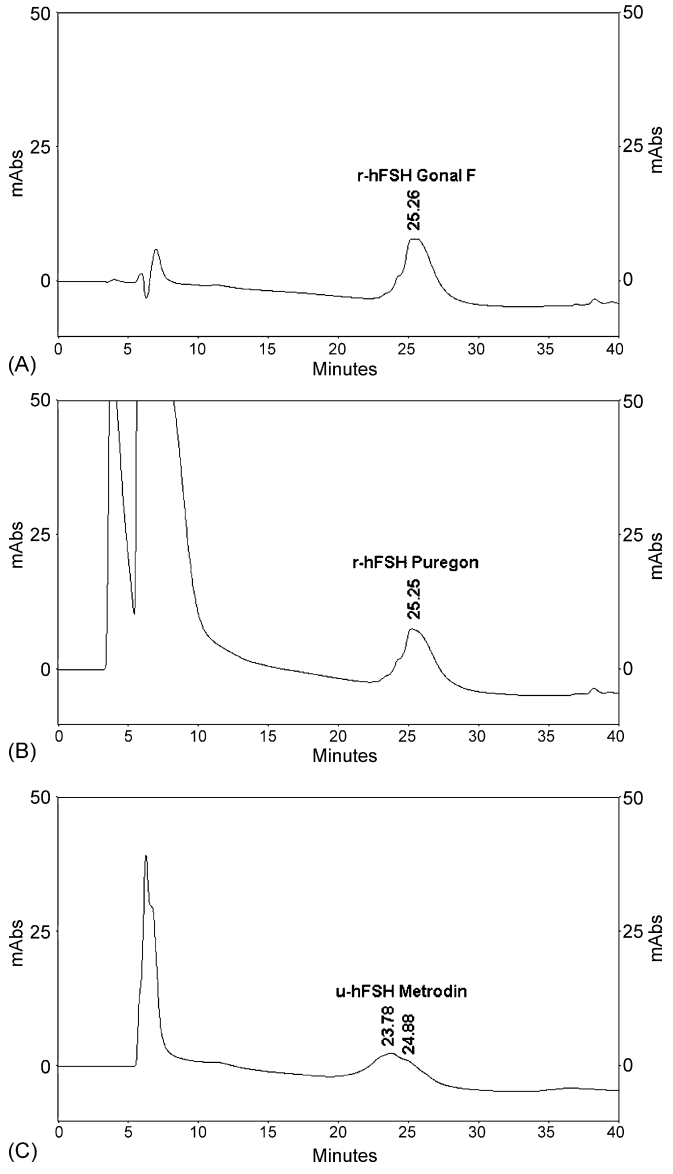

Fig. 4. RP-HPLC of different commercial hFSH preparations. The same chromatographic conditions as described in Fig. 3 were employed. (A) CHO-derived hFSH (Gonal F), 2.75 μg; (B) CHO-derived hFSH (Puregon), 3.4 μg; (C) urinary-derived hFSH (Metrodin), 2.75 μg.

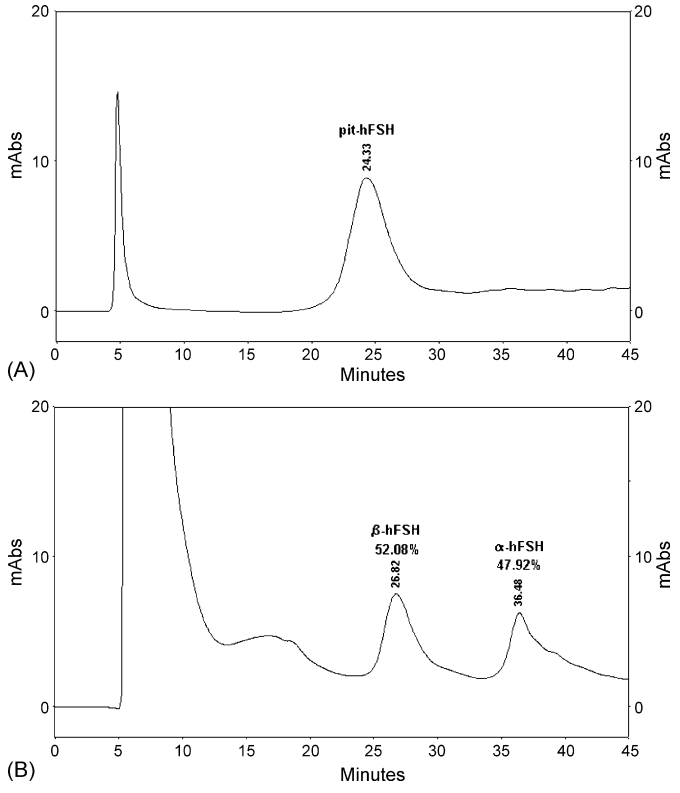

Fig. 5. RP-HPLC of pure pituitary hFSH NOR before(A) and after (B) overnight incubation with 3 M of acetic acid, at 37 °C. The same chromatographic conditions as described in Fig. 3 were employed.

experiment for u-hFSH due to the extremely limited amount of material available.

The same preparations were also analyzed by HPSEC (Fig. 6). The higher precision and reproducibility of the HPSEC methodology, as indicated by the lower RSD obtained, is shown in Table 3. A Student's *t*-test indicated a significant difference ($p < 0.001$) between the retention time of the urinary hFSH preparation and of the pituitary and recombinant FSH preparations. The urinary-derived preparation exhibited the shortest retention time, in accordance with its higher molecular mass found when the molecular masses of these preparations were determined by MALDI-TOF-MS. This method gave values of 32,527, 29,176 (average of two determinations) and 28,536, respectively, for the urinary, pituitary and recombinant hFSH (Fig. 7 and Table 4).

For quantitative RP-HPLC analysis, a pituitary preparation (pit-hFSH NOR) was utilized as an internal reference. The hFSH content of this preparation was confirmed by the BCA protein assay, which gave 574 ± 41 μg/vial ($n = 4$) against the declared content of 520 μg/vial based on specific absorbance determination. The chromatographic quality was also confirmed by

Table 1
Inter-day retention time statistics for pituitary, recombinant and urinary hFSH preparations analyzed by RP-HPLC

Sample	$t_R \pm SD$[a]	RSD[b] (%)	Difference from pituitary preparation (%)	No. of determinations
pit-hFSH NIDDK	24.62 ± 0.192	0.8	–	4
r-hFSH Gonal F	25.22 ± 0.062	0.3	+2.4	4
r-hFSH Puregon	25.33 ± 0.106	0.4	+2.9	4
u-hFSH peak 1	23.77 ± 0.226	1.0	−3.5	4
u-hFSH peak 2	25.02 ± 0.280	1.1	+1.6	4

[a] Median ± standard deviation.
[b] Relative standard deviation (RSD) expressed as percentage of the mean.

Table 2
Retention time of heterodimeric hFSH before dissociation, retention time of α-and β-subunits after dissociation and relative retention time (t_{RR}) of the α and β subunits with respect to heterodimeric hFSH, analyzed by RP-HPLC ($n = 2$)

SAMPLE	t_R Heterodimer	t_R β-Subunit	t_R α-Subunit	t_{RR}[a] β-Subunit	t_{RR}[a] α-Subunit
pit-hFSH NIDDK	24.43 ± 0.156	26.98 ± 0.160	36.63 ± 0.198	1.104	1.499
r-hFSH Gonal F	25.19 ± 0.129	27.62 ± 0.235	38.86 ± 0.214	1.096	1.543
r-hFSH Puregon	25.29 ± 0.070	27.85 ± 0.131	38.16 ± 0.127	1.101	1.509

[a] t_{RR}, relative retention time determined with respect to heterodimer t_R.

RP-HPLC, obtaining a single peak without the presence of contaminants (Fig. 8). A dose–response curve for hFSH determination via RP-HPLC, i.e., peak area versus known added amounts of hFSH in the range 0.5–11 μg, was also established, the equation of the calibration curve being: $Y_{a.u.} = 347.85X_{\mu g} + 59.53$, where "a.u." are arbitrary area units. The highly significant correlation coefficient ($r = 0.9965$; $p < 0.0001$ for $n = 15$) demonstrated the linearity of the response over the dose range analyzed. A mean value of 98.9 ± 2.93% with a relative standard deviation of 3% was observed in recovery determinations of hFSH-NIDDK, demonstrating the accuracy of this RP-HPLC methodology (Table 5).

Based on this calibration, a quantification of the four different preparations of hFSH was carried out in 3 independent assays (Table 6). An inter-day quantitative determination of pit-hFSH NIDDK, r-FSH Puregon, r-FSH Gonal F and u-hFSH Metrodin indicated that the precision of the assay system varied from 1.3% to 3.9%. A relatively good agreement of the RP-HPLC

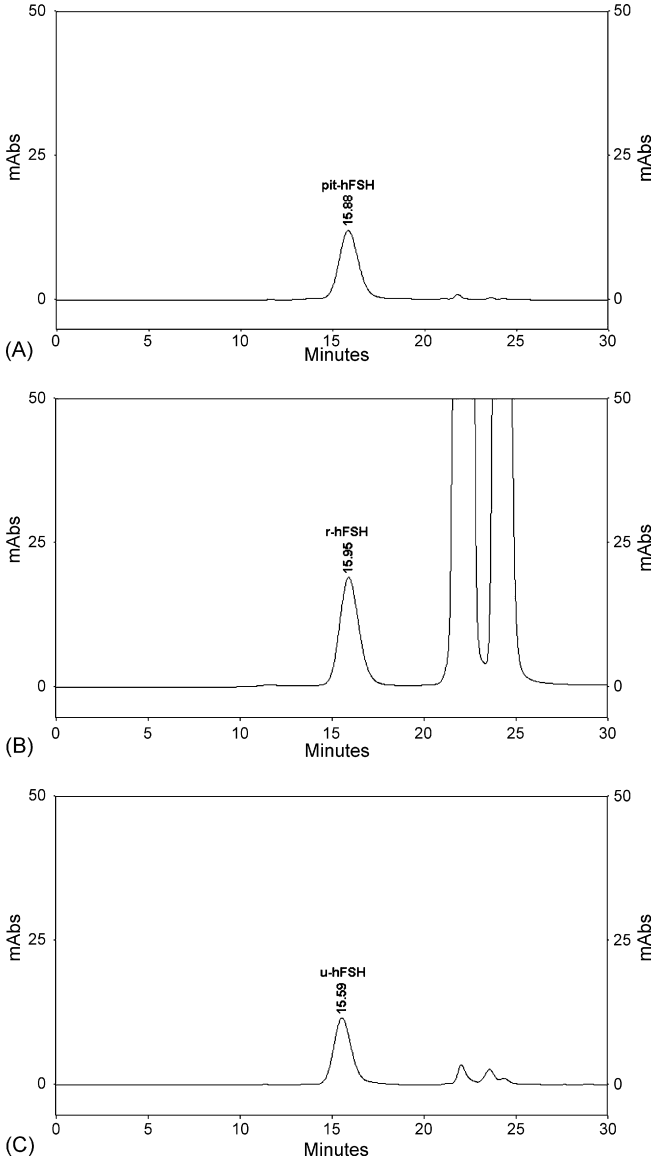

Fig. 6. HPSEC on a G2000 SW column of different hFSH preparations. (A) Pituitary hFSH (NIDDK); (B) recombinant hFSH (Puregon); (C) urinary hFSH (Metrodin).

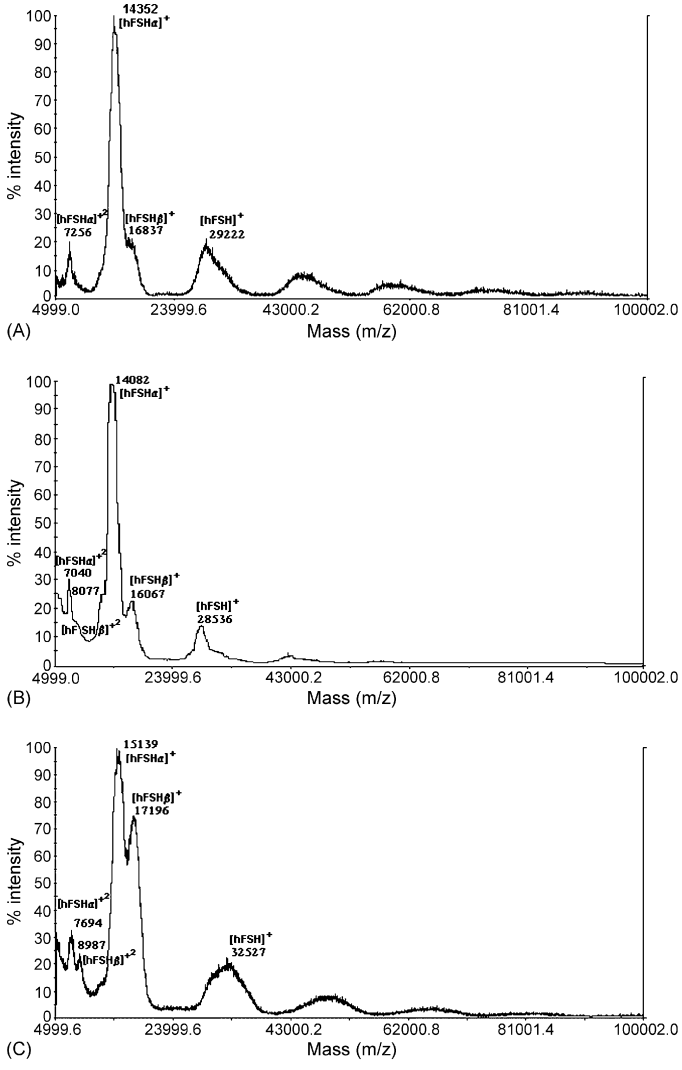

Fig. 7. MALDI-TOF-MS analyses of pituitary hFSH NOR (A), recombinant hFSH Gonal (B) and urinary hFSH (C).

Table 3
Inter-day retention time statistics for pituitary, recombinant and urinary hFSH preparations analyzed by HPSEC

Sample	$t_R \pm SD$[a]	RSD[b] (%)	Difference from pituitary preparation (%)	N° of determinations
pit-hFSH NIDDK	15.94 ± 0.050	0.3	–	4
r-hFSH Gonal F	16.00 ± 0.044	0.3	+0.4	4
r-hFSH Puregon	15.96 ± 0.097	0.6	+0.1	4
u-hFSH Metrodin	15.72 ± 0.012	0.1	−1.4	4

[a] Median ± standard deviation.
[b] RSD expressed as percentage of the mean.

Table 4
Relative molecular mass (Mr) of the heterodimer (α + β) and related subunits of different hFSH preparations, determined by Maldi-Tof mass spectrometry

Preparation	α-Subunit	β-Subunit	Heterodimer		Calc/Exp
			Experimental	Calculated α + β	
pit-hFSH NOR	14467[a]	16509[a]	29176[a]	30976[a]	1.06[a]
r-hFSH Gonal	14082	16067	28536	30149	1.06
u-hFSH Metrodin	15139	17196	32527	32335	0.99

[a] Average of two determinations.

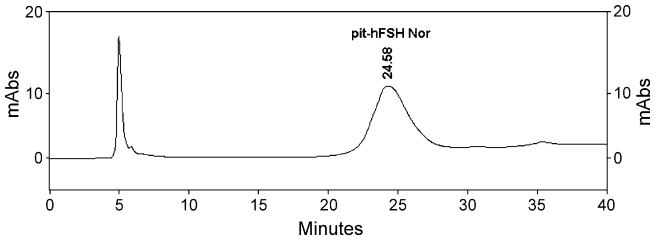

Fig. 8. RP-HPLC of 5 μg of pituitary hFSH NOR.

determinations with the declared content of the preparations was observed only for pit-hFSH NIDDK and u-hFSH Metrodin. For the two recombinant preparations, a high bias of 22% (Puregon) and 114% (Gonal F) relative to their nominal contents were observed. For Gonal F, a large difference from the nominal value (+75%), was also observed when the total protein content determination was carried out by the BCA protein assay, obtaining the value of 9.6 ± 1.43 μg/vial, $n = 4$. A better agreement (+6%) between BCA determination (10.6 ± 1.19 μg/vial, $n = 4$) and the declared content (10.0) was obtained for Puregon.

The RP-HPLC methodology developed here also proved to be quite sensitive, the minimal detectable dose calculated according to Rodbard's formulation [56] being 40 ng. The accuracy of this determination was also confirmed experimentally. In fact, when analyzed by RP-HPLC, a known amount of 40 ng of pit-hFSH (NIDDK) provided, in two determinations, an average value of 0.041 μg directly determined from the calibration curve.

Table 5
Recovery test of known amounts of pure pituitary hFSH quantified by RP-HPLC

Added hFSH (μg)	Peak area (a.u.)	Mean determined hFSH (μg)	Mean recovery (%)
0.55	239 ± 15.7	0.52	95
1.10	439 ± 4.0	1.09	100
2.21	816 ± 18.1	2.17	98
5.52	2028 ± 108.0	5.66	103
11.04	3879 ± 283.0	10.98	100

4. Discussion

An RP-HPLC methodology that preserves the heterodimer was established for the qualitative and quantitative analysis of hFSH preparations. For this purpose, the elution conditions previously described for hTSH [33] were modified and applied to the analysis of one pituitary, one urinary, and two commercial recombinant hFSH preparations. The new conditions were capable of detecting different degrees of heterogeneity in these

Table 6
Quantification of pure FSH preparations by RP-HPLC

Preparation	Nominal hFSH content[a] (μg/vial)	Determined hFSH content[b] (μg/vial)	RSD[c] (%)	Difference from nominal value (%)
pit-hFSH NIDDK	103.0	109 ± 1.7[d]	1.5	+6
r-hFSH Puregon	10.0	12.2 ± 0.2	1.3	+22
r-hFSH Gonal	5.5	11.8 ± 0.5	3.9	+114
u-hFSH Metrodin	5.5	5.4 ± 0.2	3.2	−2

[a] Based on declared vial content.
[b] Determined by RP-HPLC.
[c] RSD expressed as percentage of the mean.
[d] Standard deviation relative to $n = 3$ inter-day determinations.

preparations. The urinary preparation in particular exhibited a major peak whose t_R was found to be significantly different from that of the main form present in the other three preparations. This could be due to either a different glycoform or to an oxidized form of the hormone present in a significant amount. These findings are in agreement with those reported by Bergh et al. [57], who observed a percentage of oxidized forms four times higher in the same urinary preparation of hFSH than in r-hFSH Gonal F. The major RP-HPLC peak of the two recombinant preparations was also found to be significantly different from that of the pituitary hormone. Such heterogeneity is probably caused by the presence of different glycoforms, as described by several authors [4,8,36,58]. Although leading to partial hFSH dissociation, the conditions previously set up for hTSH were nonetheless useful for determining a hydrophobicity scale for pituitary glycoprotein hormones in the following order of decreasing strength: pit-hLH > pit-hTSH > pit-hFSH, confirming literature data [51]. As already reported for hTSH [47], subtle differences in hydrophobicity were also observed between pituitary, urinary and recombinant hFSH (Tables 1 and 2).

After several unsuccessful attempts, relatively mild dissociation conditions, based on overnight incubation at 37 °C in 3 M acetic acid, were also set up and provided a satisfactory dissociation yield of approximately 95%. This practical and flexible method may be adapted and applied to the dissociation of any recombinant or native heterodimeric glycoprotein hormone, allowing a direct characterization of each individual subunit. It is noteworthy that the t_R of heterodimeric hFSH has been found remarkably different from that of its related subunits, for either the pituitary or for the two recombinant preparations. This provides a direct identification of these hFSH-related impurities, whose absence must be demonstrated in a pharmaceutical product.

More accurate molecular mass determinations (Mr) were also carried out via MALDI-TOF mass spectral analysis for the pituitary, the urinary and one recombinant preparation of hFSH. As previously observed for hTSH [47], the simultaneous detection and analysis of heterodimer, α- and β-subunit is possible even for hFSH and the agreement between the experimental and calculated (by summation of the Mr of the two subunits) values was demonstrated. We would like to emphasize that the retention of a certain amount of heterodimer, obtained under the described MALDI-TOF-MS conditions, offers direct mass measurements without the need for chemical crosslinking. The higher Mr of urinary hFSH confirms less accurate molecular mass estimates obtained via HPSEC. The Mr of pit-hFSH (29176) and of rhFSH (28536) are relatively close to those previously reported by others using the same mass spectral technique, as reported in Table 1 of Mendonça et al. [47]. This indicates a relatively high inter-laboratory reproducibility for this methodology.

Finally, we would like to emphazise the good parameters of accuracy, precision and sensitivity shown in the validation of our method. For the quantification of the four hFSH preparations under analysis (Table 6), there is acceptable agreement with the declared content for pit-hFSH and u-hFSH, but a great bias for the case of the two recombinant preparations. Although the precise reason for this bias is not clear, there may be an interference from specific excipients present in the recombinant preparations.

In conclusion, the methodology reported herein may be extremely useful for the accurate analysis, identification and quality control of hFSH and its individual subunits.

Acknowledgements

This work was supported by FAPESP, São Paulo, Brazil and by the National Research Council (CNPq), Brasilia, Brazil (PCI 381970/01-9 and PQ 301520/91-7). We are grateful to Mr. Orlando V. Silva from Serono for the kind donation of Metrodin HP.

References

[1] E.D. Green, J.U. Baenziger, J. Biol. Chem. 263 (1988-A) 25.
[2] E.D. Green, J.U. Baenziger, J. Biol. Chem. 263 (1988-B) 36.
[3] P.G. Stanton, D.M. Robertson, P.G. Burgon, B. Schmauk-White, M.T.W. Hearn, Endocrinology 130 (1992) 2820.
[4] A. Cerpa-Poljak, L.A. Bishop, Y.J. Hort, C.K.H. Chin, R. DeKroon, S.M. Mahler, G.M. Smith, M.C. Stuart, P.R. Schofield, Endocrinology 132 (1993) 351.
[5] B. Rafferty, J.A. Mower, H.L. Ward, M. Rose, J. Endocrinol. 145 (1995) 527.
[6] A. Amoresano, R. Siciliano, S. Orru, R. Napoleoni, V. Altarocca, E. De Luca, A. Sirna, P. Pucci, J. Biochem. 242 (1996) 608.
[7] E. Loumaye, M. Dreano, A. Galazka, C. Howles, L. Ham, A. Munafo, A. Eshkol, E. Giudice, E. De Luca, A. Sirna, F. Antonetti, C.E. Giartosio, L. Scaglia, C. Kelton, R. Campbell, S. Chappel, B. Duthu, S. Cymbalista, P. Lepage, Hum. Reprod. Update 4 (1998) 862.
[8] S. Creus, Z. Chaia, E.H. Pellizzari, S.B. Cigorraga, A. Ulloa-Aguirre, S. Campo, Mol. Cell. Endocrinol. 174 (2001) 41.
[9] A. Ulloa-Aguirre, A. Maldonado, P. Damián-Matsumura, C. Timossi, Arch. Med. Res. 32 (2001) 520.
[10] J. Barrios-De-Tomasi, C. Timossi, H. Merchant, A. Quintanar, J.M. Avalos, C.Y. Andersen, A. Ulloa-Aguirre, Mol. Cell. Endocrinol. 186 (2002) 189.
[11] A. Gervais, Y.-A. Hammel, S. Pelloux, P. Lepage, G. Baer, N. Carte, O. Sorokine, J.-M. Strub, R. Koerner, E. Leize, A.V. Dorsselaer, Glycobiology 13 (2003) 179.
[12] S.L. Steelman, F.M. Pohley, Endocrinology 53 (1953) 604.
[13] H. Van Hell, R. Matthijsen, G.A. Overbeek, Acta Endocrinol. 47 (1964) 409.
[14] M.P. Van Damme, D.M. Robertson, R. Marana, E.M. Ritzen, Acta Endocrinol. 91 (1979) 224.
[15] X. Jia, A.J.W. Hsueh, Endocrinology 119 (1986) 1570.
[16] V. Padmanabhan, S.C. Chappel, I.Z. Beitins, Endocrinology 121 (1987) 1089.
[17] K. Dahl, X. Jia, A. Hsueh, Meth. Enzymol. 169 (1889) 414.
[18] A. Ulloa-Aguirre, A. Rees Midgley Jr., I.A. Beitins, V. Padmanabhan, Endocr. Rev. 16 (1995) 765.
[19] J. Seth, I. Hanning, R.R.A. Bacon, W.M. Hunter, Clin. Chim. Acta 186 (1989) 67.
[20] F. Jockenhovel, S.A. Khan, E. Nieschlag, J. Endocrinol. 127 (1990) 523.
[21] P.G. Burgon, D.M. Robertson, P.G. Stanton, M.T.W. Hearn, J. Endocrinol. 139 (1993) 511.
[22] A.E. Taylor, R.H. Khoury, W.R.J. Crowley, J. Clin. Endocrinol. Metab. 79 (1994) 240.
[23] M.P. Rose, R.E. Gaines-Das, J. Endocrinol. 158 (1998) 97.
[24] M.P. Rose, Clin. Chim. Acta 273 (1998) 103.
[25] R.L. Oliver, J.W. Kane, A. Waite, J.W.M. Mulders, W.R. Robertson, Clin. Endocrinol. 51 (1999) 681.
[26] G. Horsman, J.A. Talbot, J.D. McLoughlin, A. Lambert, W.R. Robertson, Hum. Reprod. 15 (2000) 1898.

[27] P. Van Wezenbeek, J. Draaijer, F. Van Meel, W. Olijve, in: D.J.A. Crommelin, H. Schellekens (Eds.), Clone to Clinic, Kluwer, Dordrecht, 1990, p. 245.
[28] W. De Boer, B. Mannaerts, in: D.J.A. Crommelin, H. Schellekens (Eds.), Clone to Clinic, Kluwer, Dordrecht, 1990, p. 253.
[29] K. Hakola, P. Van der Boogaart, J. Mulders, R. de Leeuw, W. Schoonen, J. Van Heyst, A. Swolfs, J. Van Casteren, I. Huhtaniemi, H. Kloosterboer, Mol. Endocrinol. 127 (1997) 59.
[30] N.A.K. Heum, S.C. Kim, K.S. Seo, S.H. Lee, W.B. Kim, K.C. Lee, J. Microbiol. Biotechnol. 15 (2005) 395.
[31] S.D. Rafferty, R.G. Das, Clin. Chem. 45 (1999) 2207.
[32] C.R.J. Soares, I.M.C. Camargo, L. Morganti, E. Gimbo, J.E. Oliveira, R. Legoux, P. Ferrara, P. Bartolini, J. Chromatogr. A 955 (2002) 229.
[33] J.E. Oliveira, F. Mendonça, C.N. Peroni, P. Bartolini, M.T.C.P. Ribela, J. Chromatogr. B 787 (2003) 345.
[34] C.M. Howles, Hum. Reprod. Update 2 (1996) 172.
[35] J.W.M. Mulders, M. Derksen, A. Swolfs, F. Maris, Biologicals 25 (1997) 269.
[36] P.L. Storring, R.E. Gaines Das, J.W.M. Mulders, M. Halder, Biologicals 30 (2002) 217.
[37] A. Ulloa-Aguirre, C. Timossi, J. Barrios-de-Tomasi, A. Maldonado, P. Nayudu, Biol. Reprod. 69 (2003) 379.
[38] C.Y. Andersen, L.G. Westergaard, M. van Wely, Reprod. Biomed. Online 9 (2004) 231.
[39] C. Wolfenson, J. Groisman, A.S. Couto, M. Hedenfalk, R.G. Cortvrindt, J.E.J. Smitz, S. Jespersen, Reprod. Biomed. Online 10 (2005) 442.
[40] R.A. Sendak, C. Ganesa, K.L. Lee, J.J. Harrahy, R. Théberge, C.J. Morgan, E.S. Cole, L.D. Kohn, R.J. Mattaliano, Thyroid 13 (2003) 1091.
[41] M.T.C.P. Ribela, P.W. Gout, J.E. Oliveira, P. Bartolini, Curr. Pharm. Anal. 2 (2006) 103.
[42] R.N. Rao, V. Nagaraju, J. Pharm. Biom. Anal. 33 (2003) 335.
[43] J.N. Hugues, I.C. Durnerin, Reprod. Biomed. Online 10 (Suppl. 3) (2005) 11.
[44] R. Bassett, Reprod. Biomed. Online 10 (2005) 169.
[45] C. Keck, R. Bassett, M. Ludwig, Reprod. Biomed. Online 11 (2005) 562.
[46] R. Driebergen, G. Baer, Curr. Med. Res. Opin. 19 (2003) 41.
[47] F. Mendonça, J.E. Oliveira, P. Bartolini, M.T.C.P. Ribela, J. Chromatogr. A 1062 (2005) 103.
[48] A.F. Bristow, C. Wilson, N. Sutcliffe, J. Chromatogr. 270 (1983) 285.
[49] B. Grego, G.S. Baldwin, J.A. Knessel, R.J. Simpson, F.J. Morgan, M.T.W. Hearn, J. Chromatogr. 297 (1984) 21.
[50] T.F. Parsons, T.W. Strickland, J.G. Pierce, Endocrinology 114 (1984) 2223.
[51] J. Hiyama, A.G.C. Renwick, J. Chromatogr. 529 (1990) 33.
[52] M.A. Chlenov, E.I. Kandyba, L.V. Nagornaya, I.L. Orlova, Y.V. Volgin, J. Chromatogr. 631 (1993) 261.
[53] E.S. Cole, K. Lee, K. Lauziere, C. Kelton, S. Chappel, B. Weintraub, D. Ferrara, P. Peterson, R. Bernasconi, T. Edmunds, S. Richards, L. Dickrell, J.M. Kleeman, J.M. McPherson, B.M. Pratt, BioTechnol 11 (1993) 1014.
[54] R.M. Riggin, G.K. Dorulla, D.J. Miner, Anal. Biochem. 167 (1987) 199.
[55] S. Williams, J. Chromatogr. A 1052 (2004) 1.
[56] D. Rodbard, Anal. Biochem. 90 (1978) 1.
[57] C. Bergh, C.M. Howles, K. Borg, L. Hamberger, B. Josefsson, L. Nilsson, M. Wikland, Hum. Reprod. 12 (1997) 2133.
[58] U.A. Vitt, H.J. Kloosterboer, U.M. Rose, J.W.M. Mulders, P.S. Kiesel, S. Bete, P.L. Nayudu, Biol. Reprod. 59 (1998) 854.