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Analysis of human luteinizing hormone and human chorionic gonadotropin preparations of different origins by reversed-phase high-performance liquid chromatography

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ABSTRACT

Specific reversed-phase high-performance liquid chromatography conditions are reported for the analysis of recombinant and native human luteinizing hormone (hLH) and human chorionic gonadotropin (hCG) preparations. Heterodimeric hLH, hCG and their α- and β-subunits migrated with significantly different retention times (t_R) in the following order of increasing hydrophobicity: α-hCG < α-hLH < hCG < hLH < β-hCG < β-hLH. Under these conditions, the main peak of three hCG preparations ran about 4% faster than the average t_R (38.35 ± 0.42 min; RSD = 1.1%) of four hLH preparations. Four heterogeneous urinary products were also analyzed, hLH, hFSH and hCG peaks being identified.

Quantitative analysis was validated for the homogeneous preparations and a highly linear dose–response curve ($r = 0.99998$; $p < 0.0001$; $n = 20$) used to assess the accuracy, precision and sensitivity of the analysis. Quantification of the different gonadotropins in the heterogeneous preparations was also carried out, but with limitations in accuracy.

1. Introduction

Human luteinizing hormone (hLH) is a heterodimeric glycoprotein hormone that is secreted by the gonadotrophs of the anterior pituitary gland in response to stimulation by luteinizing hormone-releasing hormone (LH-RH) from the hypothalamus. It is structurally and functionally related to human chorionic gonadotropin (hCG), which is secreted primarily by syncytiotrophoblasts in the human placenta. Both hormones bind to the same receptor, which is a transmembrane glycoprotein that belongs to the G-protein-coupled receptor superfamily and is present in the ovarian theca cells in females and in the testicular Leydig cells in males [1,2].

Despite the use of the same receptor, hLH and hCG have different functions. Multiple roles of luteinizing hormone are reported in the literature: LH participates in testicular and ovarian regulation, performs a critical role in follicular maturation, ovulation, corpus luteum development and maintenance and intervenes in the modification of the synthesis of steroid hormones, growth factors and cytokines [2,3]. CG, the hormone of pregnancy, maintains adequate levels of sex steroid synthesis by the corpus luteum until the placenta takes over this function. It also acts in trophoblast differentiation and in fetal nutrition through myometrial spiral artery angiogenesis [1,4]. Both hLH and hCG are glycoproteins, with molecular weights of 27.8 kDa [5] and 35.1 kDa [6], respectively, and have almost identical α-subunit and high cysteine content. The main structural difference between the two hormones is an additional 23 amino acid tail in the hCG β-subunit; this C-terminal peptide tail contains four additional O-linked carbohydrate side chains, each typically having two terminal sialic acid residues. Differences in their receptor affinities and in the clearance of these hormones were also found, the *in vivo* half-life of hCG being much longer (~3–10-fold higher) than that of hLH [7].

For many years, the available sources of exogenous LH activity for clinical use were either human pituitaries or human menopausal urine, which contains menopausal gonadotropin (hMG). Although the utilization of the former was suspended due to the inherent dangers associated with this type of biological material, the latter source is still being used. hMG preparations, containing follicle-stimulating hormone (FSH) and highly variable levels of LH, are often augmented with hCG, which mimics LH activity [8,9]. The long serum half-life of hCG, however, can result in accumulation of hCG bioactivity, with potentially detrimental effects on follicular development and oocyte quality [10,11]. These undesired effects of LH over-exposure associated with hCG can be prevented by the utilization of recombinant hLH (rhLH), which allows precise LH dosages for each different pathology [10,12].

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Various assay systems, based on biological and immunological methodologies, have been employed for the detection and determination of hLH and hCG [13]. Different physicochemical methods of analysis such as SDS-PAGE [5,9], isoelectric focusing [5,9] and capillary electrophoresis [14], have also been reported. Several HPLC modes have been applied in general to LH or to CG. Of these, reversed-phase high-performance liquid chromatography (RP-HPLC) on C_{18} or C_4 columns is perhaps the best method for isolation and analysis of LH or CG subunits [5,9,15–18], and for the determination of the heterodimeric forms of these hormones [19,20]. Hiyama and Renwick [20] used C_4 columns to separate intact hLH and hTSH and demonstrated the advantage of insertion of a small C_1 column for determination of hFSH heterodimer as well. Hoermann et al. [21] employed C_4 columns in a search for pituitary hCG β core fragment. Utilizing a C_{18} column, Birken et al. [22] isolated pituitary hCG for the first time and compared its subunits with urinary-derived hCG, and with pituitary-derived hLH subunits. The quality of recombinant products such as CHO-derived hLH [8] or *Pichia pastoris*-derived hCG [23] has also been evaluated by RP-HPLC on C_4 columns.

Although RP-HPLC has been widely used for the isolation and analysis of hLH and hCG, determination of their intact heterodimeric forms has only occasionally been reported, usually for pituitary-derived preparations. Apparently, an accurate quantitative RP-HPLC analysis has never been reported for these two hormones, despite the importance of hLH and hCG in clinical practice and the need for careful quality control of the products being administered. In the present work, we report specific RP-HPLC conditions for the identification and qualitative and quantitative analysis of these two hormones and of their subunits on a C_4 column. The method is used to analyze purified hormones and several heterogeneous hormone preparations of pituitary, urinary and recombinant origin.

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

Four hLH preparations were analyzed in this work, two pituitary (phLH-A and phLH-B) and two recombinant preparations: a commercial one (rhLH-C) and the International Standard of rhLH-WHO 96/602. Two commercial hCG preparations were also included: a urinary (uhCG-D) and a recombinant (rhCG-E) preparation, as well as the International Standard of uhCG-WHO 75/589. Four heterogeneous urinary preparations of hMG (hLH + hFSH): three commercial preparations (uhMG-F, uhMG-G and uhMG-H) and the International Standard of urinary hMG (uhMG-WHO 98/704), were then analyzed. Table 1 shows the specifications of these 11 samples.

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 Luteinizing Hormone (LH), Recombinant, Human for Bioassay (WHO 96/602).

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

Table 1
Specifications of the 11 gonadotropin samples analyzed.

Preparation	Origin	Product description
rhLH-WHO 96/602	CHO	Lutropin
phLH-A	Pituitary	Lutropin
phLH-B	Pituitary	Lutropin
rhLH-C	CHO	Lutropin
uhCG-WHO 75/589	Urine	Coriogonadotropin
uhCG-D	Urine	Coriogonadotropin
rhCG-E	CHO	Coriogonadotropin
uhMG-WHO 98/704	Urine	Menotropin
uhMG-F	Urine	Menotropin
uhMG-G	Urine	Menotropin
uhMG-H	Urine	Menotropin

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). A silica pre-column (packed with LiChrosorb Si 60, 7.9–12.4 μm, Merck, Darmstadt, Germany) was inserted between the pump and the injector. 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 referenced to the International Standard of rhLH-WHO 96/602.

For hLH and hCG, elution gradient of solutions A and B were utilized, solution A being sodium phosphate buffer (pH 7.0; 0.05 M) and solution B acetonitrile. The elution was performed with a linear gradient of A:B (87.5:12.5, v/v) to A:B (40:60, v/v) over 50 min, at a flow-rate of 0.5 ml/min. In general, aliquots of 5–10 μl of phLH, 150–250 μl of rhLH and 10–20 μl of rhCG or uhCG were processed. For hMG, solution A was ammonium phosphate buffer (pH 8.6; 0.05 M) and solution B acetonitrile. The elution was performed with a linear gradient of A:B (85:15, v/v) to A:B (40:60, v/v) over 40 min, then maintained at A:B (40:60, v/v) for an additional 10 min at a flow-rate of 0.5 ml/min. In general, aliquots of 50–200 μl of hMG and 5–10 μl of phLH, phFSH and uhCG were processed.

Peak tailing factors (T_f) were determined for the purified preparations of hLH, hCG and subunits, according to the definition: $T_f = A_{5\%h} + B_{5\%h}/2A_{5\%h}$.

2.4. Protein determination

Total protein concentration was estimated by utilizing bicinchoninic acid (Micro BCA protein assay kit, Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Solutions of pure bovine serum albumin (BSA), ranging from 0.5 to 200 μg/ml, were used as standard. All the samples analyzed, except samples A and B of phLH, were extensively dialyzed at 4 °C against 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M Na Cl.

2.5. Biological assays

For the identification of hFSH and hLH in hMG preparations, the eluted RP-HPLC peaks were tested for their hLH or hFSH *in vivo* bioactivities via two respective bioassays. The peaks correspond to the fractions eluted from HPLC in the range 21–27 min (hFSH) and in the range 32–39 min (hLH). hFSH activity was determined by the rat ovarian weight gain method. Briefly, 19–22-day

Table 2
Inter-day retention time (t_R) of different preparations of hLH, hCG and their α- and β-subunits, analyzed by RP-HPLC.

Sample	$t_R \pm SD$[a] (min)	RSD[b] (%)
rhLH-WHO 96/602	38.37 ± 0.177	0.5
phLH-A	38.87 ± 0.088	0.2
α-phLH-A	34.56 ± 0.080	0.2
β-phLH-A	45.34 ± 0.102	0.2
phLH-B	37.84 ± 0.226	0.6
rhLH-C	38.33 ± 0.199	0.5
uhCG-WHO 75/589	36.70 ± 0.146	0.4
uhCG-D	36.71 ± 0.121	0.3
rhCG-E	36.63 ± 0.131	0.4
α-rhCG-E	34.07 ± 0.180	0.5
β-rhCG-E	39.19 ± 0.038	0.1

[a] Mean ± standard deviation ($n = 4$ independent determinations).
[b] Relative standard deviation expressed as percentage of the mean.

old Sprague–Dawley female rats received 0.5, 1 and 3 IU/day of hFSH subcutaneously over 3 days. 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 relative to the recombinant hFSH International Standard WHO 92/642.

For hLH activity, the seminal vesicle weight gain assay was used. Briefly, 19–22-day-old Sprague–Dawley male rats received 1, 2 and 4 IU/day of hLH subcutaneously over 3 days. Autopsy was performed on the fourth day (72 h after the first injection). The seminal vesicles were removed, dissected free of surrounding tissue, and weighed. The *in vivo* LH bioactivity was calculated relative to the recombinant hLH International Standard WHO 96/602.

In both assays, 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. Subunit dissociation

hCG subunits were prepared by incubating the rhCG-E preparation (40 μg, dissolved in 100 μl phosphate buffered saline), overnight at 37 °C with 5.0 M acetic acid and applying the product of the dissociation reaction directly to a C_4 RP-HPLC column, as described [5]. Purified α- and β-subunits were analyzed by rechromatographing the corresponding eluted peaks on the same column. The α- and β-hLH subunits utilized in this work were from the National Hormone and Pituitary Program (Torrance, CA, USA).

3. Results

The RP-HPLC elution conditions were modified relative to those reported in previous work on hTSH and hFSH [5,24,25] in order to achieve a useful separation of hLH, hCG and their α- and β-subunits. Basically the modifications were concerning an increased final acetonitrile concentration (60%), prolonging the gradient time from 40 to 50 min. These modifications were tested on phLH-A and rhCG-E, the two preparations available in the largest amounts. The relative positions of each heterodimer and subunit in order of increasing hydrophobicity are: α-hCG < α-hLH < hCG < hLH < β-hCG < β-hLH. The statistics for the retention times of the six different molecular species, reported in Table 2, show that there is a highly significant difference between their t_R values ($p < 0.005$). This is particularly important because our primary objective in this work is the analysis and characterization of purified or semi-purified products. RP-HPLC peak tailing factors were determined for the purified preparations of hLH, hCG and their subunits and they ranged between 0.98 and 1.18.

Fig. 1 presents chromatograms for phLH-A, rhCG-E and their subunits. These show that α-hLH and β-hLH subunits are present as contaminants in the heterodimeric preparation and that a certain amount of heterodimer is present in β-hLH. Rechromatographing the purified heterodimers, under the same conditions, eliminated the possibility that these hormone-related impurities were artifacts due to RP-HPLC-induced dissociation. The fact that the β-subunit of rhCG presents two isoforms may be due to the absence of a N-linked glycan in one of them, as was reported based on SDS-PAGE analysis of the same hormone [9]. These data show that RP-HPLC can be an effective tool for detecting the presence of subunits as undesired hormone-related impurities in heterodimeric preparations.

The same RP-HPLC conditions were then used to analyze three other purified hLH preparations: a second native pituitary (phLH-B) and two recombinant preparations: the International Standard of rhLH-WHO 96/602, whose main function is to serve as standard for the bioassay of therapeutic rhLH products, and a commercial biopharmaceutical preparation (rhLH-C). Fig. 2 and Table 2 show that there is poor agreement between the t_R of the two pituitary preparations (1.03 min or ~3% difference). In contrast, the two recombinant preparations exhibit practically coincident t_R values, with a difference of only 0.04 min (0.1%). Analysis of human serum albumin (HSA) under the same conditions confirmed that the peak of rhLH-WHO 96/602 at $t_R = 44.8$ min (Fig. 2) is due to this protein, added to rhLH in large amounts (~200-fold) as a stabilizer. This means that, in addition to being available only in extremely small amounts (8.8 μg/ampoule), the primary standard for rhLH activity is also unsuitable as a standard for physicochemical characterization, in particular for quantitative analyses.

The International Standard of uhCG-WHO 75/589, a commercial urinary hCG (uhCG-D) and a recombinant preparation of the same hormone (rhCG-E) were then submitted to the same analysis (Figs. 1 and 2). It is noteworthy that rhCG-E is the purest preparation under analysis (also confirmed by high-performance size-exclusion chromatography, data not presented). The t_R values of the two hCG are also coincident (0.08 min or 0.2% difference). This hormone migrates approximately 4% faster (1.7 min) than the average of all homogeneous hLH preparations (38.35 ± 0.42 min, RSD = 1.1%, $n = 4$ preparations, Table 2). Moreover, like rhLH-WHO 96/602, uhCG-WHO 75/589 also contains large amounts of HSA, making protein quantification by BCA meaningless.

Four heterogeneous urinary preparations of human menopausal gonadotropin (hLH + hFSH) were also analyzed (Fig. 3). This required an additional modification of the RP-HPLC chromatographic conditions in order to avoid the hFSH dissociation that occurred under the previous conditions (see Section 2). Ammonium phosphate pH 8.6 was used instead of sodium phosphate buffer pH 7.0, while the initial concentration of acetonitrile went from 12.5 to 15%, reducing the gradient time of 10 min. An additional 10 min isocratic elution with 60% acetonitrile was carried out. The resultant chromatogram is illustrated in Fig. 3A for the International Standard of urinary hLH and urinary hFSH (uhMG-WHO 98/704). Three peaks were identified as hFSH, hCG and hLH on the basis of their t_R and of their *in vivo* bioactivity. The t_R values were comparable to those of the individual purified hormones chromatographed under identical conditions: 22.55 ± 0.18, 34.61 ± 0.22 and 38.04 ± 0.24 min, respectively, their difference being always significant ($p < 0.001$). Closer examination of this chromatogram suggests, however, that, in besides hFSH, there is a larger hCG and a smaller hLH fraction in the WHO 98/704 preparation (Table 3). This is consistent with the findings of van de Weijer et al. [9] in their analyses of one of these urinary preparations by a battery of physicochemical and immunological methods. Three commercial preparations of urinary hMG were also analyzed under these same chromatographic conditions and a presumptive peak of hLH could also be identified in uhMG-H (Fig. 3D, Table 3).

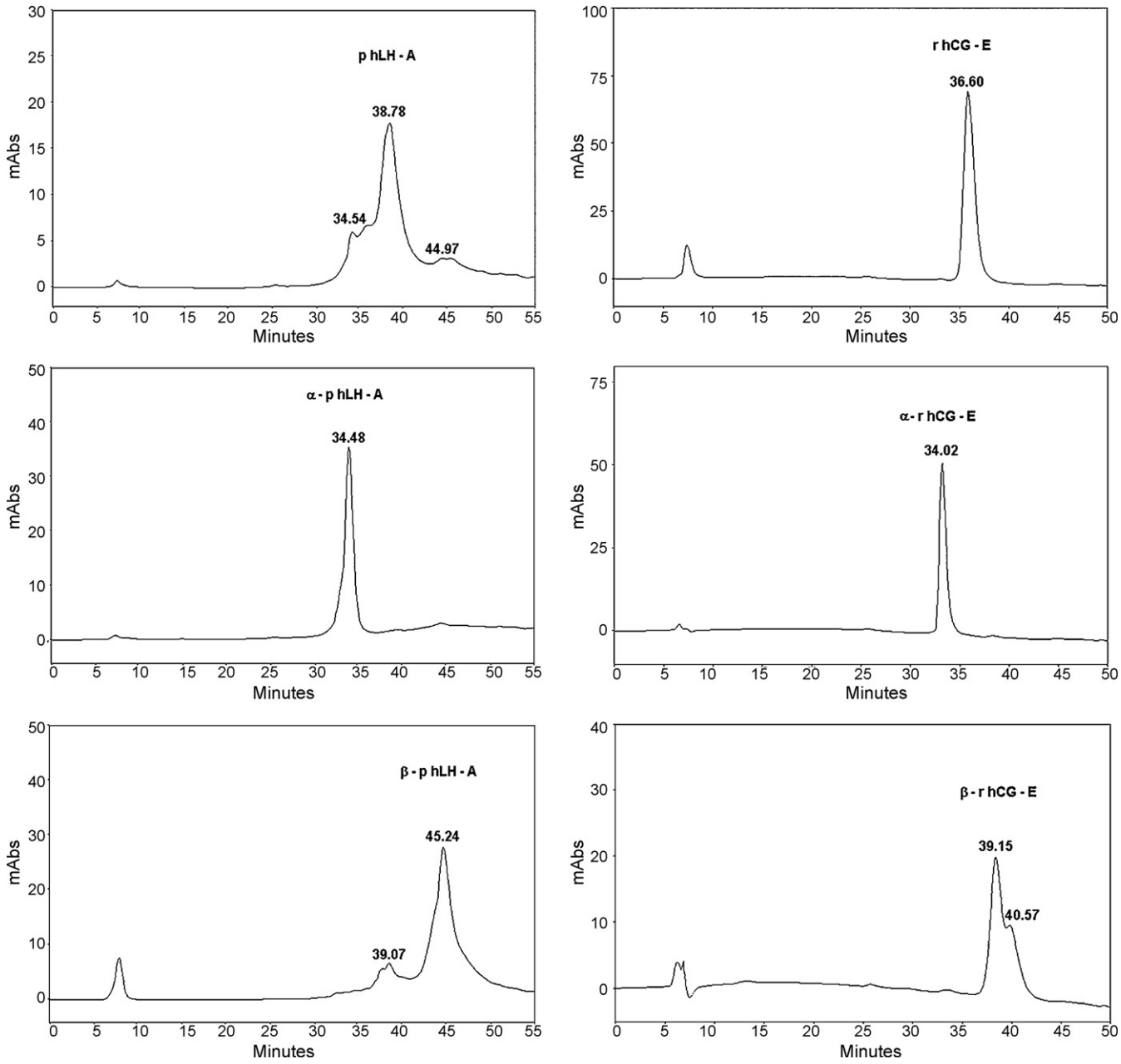

Fig. 1. RP-HPLC of phLH-A, rhCG-E and their subunits: phLH-A (2.5 μg); α-phLH-A (4 μg); β-phLH-A (7 μg); rhCG-E (5 μg); α-rhCG-E (2 μg); β-rhCG-E (2 μg).

Table 3
Inter-day retention time (t_R) of hFSH, hCG and hLH present in different hMG preparations, analyzed by RP-HPLC.

Sample	$t_R \pm SD$[a] (min)		
	hFSH	hCG	hLH
uhMG-WHO 98/704	24.29 ± 0.221	33.74 ± 0.166	37.38 ± 0.208
uhMG-F	22.70 ± 0.333	34.28 ± 0.294	
uhMG-G	23.08 ± 0.675	34.35 ± 0.221	
uhMG-H	24.21 ± 0.179	35.26 ± 0.239	37.36 ± 0.035
Inter-preparation average	23.57 ± 0.80	34.41 ± 0.63	37.37 ± 0.014
RSD[b]	3.4%	1.8%	0.04%

[a] Mean ± standard deviation ($n = 3$ independent determinations).
[b] Relative standard deviation.

The quantitative hLH analysis was validated only for homogeneous preparations. An unavoidable problem is that ca. 20% of the peak of the currently available International Standard (rhLH-WHO 96/602) overlaps, as shown in Fig. 2, with the peak of human serum albumin (HSA). In order to quantify the standard by RP-HPLC, we reported to a comparison of its peak with that of the pure commercial preparation rhLH-C. On the basis of $n = 5$ determinations, we calculated a correction factor for the systematic error that occurred in the determination of the peak area when the program integrated only the shaded area indicated in the figure. Using this correction factor (c.f. = 1.18 ± 0.061, RSD = 5.2%), together with the direct determinations, it was possible to define an internal response parameter in area units (au)/μg for all the seven homogeneous gonadotropin preparations, as reported in Table 4. For inter-comparison purposes, the total protein content of each preparation was determined with BCA. As expected, the higher response parameter was found for the three recombinant preparations (hLH

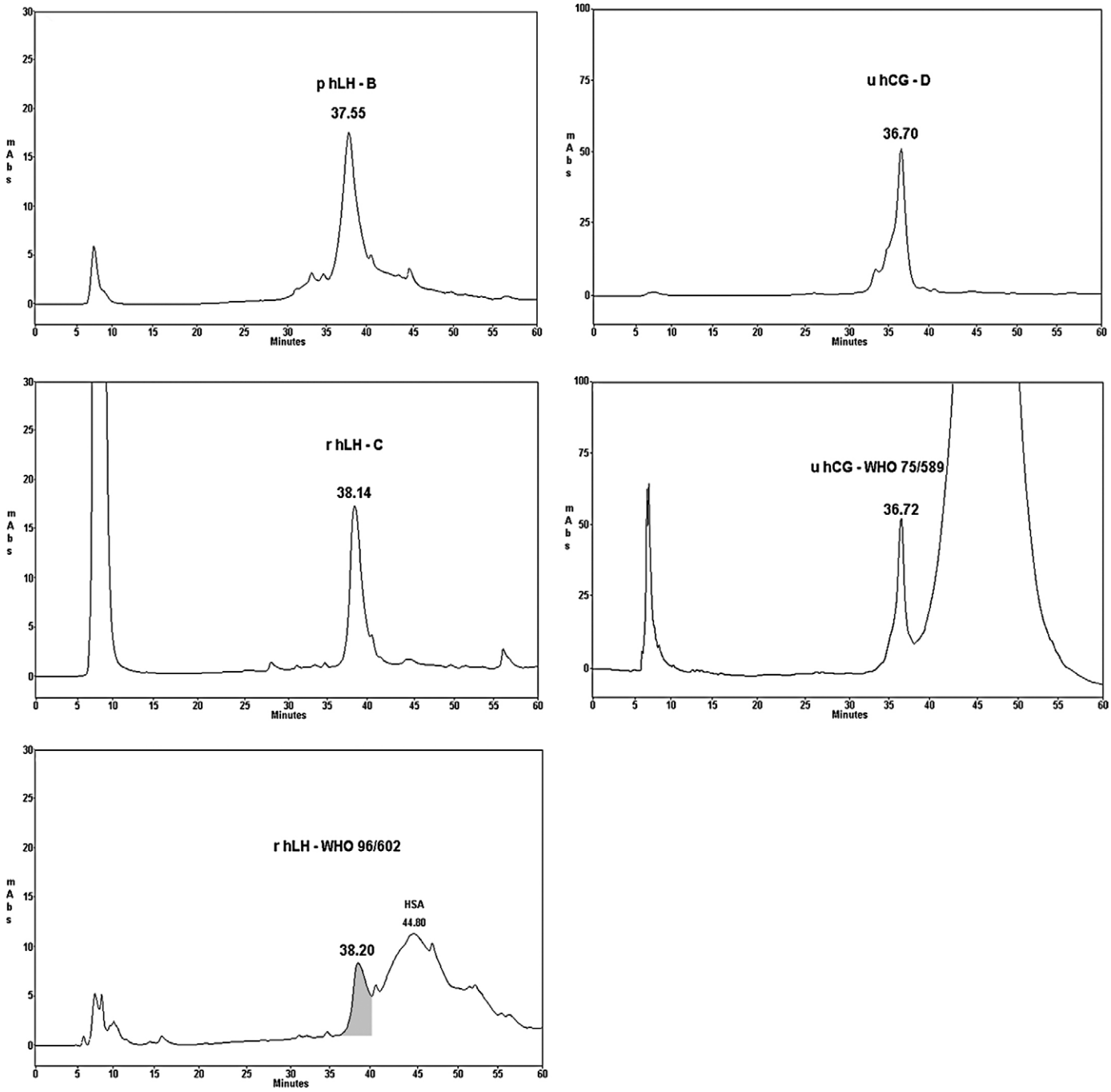

Fig. 2. RP-HPLC of different hLH and hCG preparations: phLH-B (4 μg); rhLH-C (2 μg); rhLH-WHO 96/602 (1.1 μg); uhCG-D (5 μg); uhCG-WHO 75/589 (5 μg).

and hCG), with an inter-preparation average of $\bar{X} = 931 \pm 83$ au/μg (RSD = 8.9%). This parameter was then used to normalize standard curves that were constructed with the hLH preparations available in larger amounts, such as phLH-A. For practical reasons, the hCG quantification is referenced to the same parameter established for hLH, assuming that both purified hormones have approximately the same specific absorbance at 220 nm, an assumption confirmed by the data in Table 4.

The dose–response curve for hLH was $Y_{au} = 959.4X_{\mu g} - 27.6$ ($r = 0.9999$; $p < 0.001$; $n = 20$) in the 0.14–8 μg range, with a calculated sensitivity of the order of 34 ng and a RSD for intra-day and inter-day precision that was always <1.55%. Accuracy, determined by a recovery test after addition of known amounts of hLH to the same buffer, was also of the order of 96–106%. The hLH or hCG contents of all preparations were determined by using this standard curve. The same was done via an analogous standard curve for the hFSH content, as previously described [25].

In Table 5, the quantitative analysis data for all the preparations studied in the present work are reported. A gonadotropin fraction of 100% was observed only for rhLH-C, a recombinant preparation from the same manufacturer that provided the purified material to NIBSC-WHO for preparing the International Standard (NIBSC Technical Sheet on rhLH-WHO 96/602, 18/01/2008). All other homogeneous preparations of either hLH or hCG provided mass fractions of the order of 65–87%. All the urinary hMG preparations, including the International Standard WHO 98/704, had similar gonadotropin fractions (34–43%), though with a great variety of protein content. It is noteworthy that they all had a declared hMG content of 75 IU of hFSH + 75 IU of hLH per vial, with a protein content varying from 30.4 to 909 μg/vial and, therefore, a potency

Table 4
Response parameters in area units (au)/μg of different preparations of hLH and hCG, analyzed by RP-HPLC.

Sample	Total protein (μg/ampoule)	Area (au/ampoule)	Response parameter (au/μg)
rhLH-WHO 96/602	8.80[a]	8511 ± 398	967 ± 45.3
phLH-A	3.18[b]	1924 ± 182	605 ± 57.4
phLH-B	5.48[b]	3820 ± 447	696 ± 81.5
rhLH-C	3.00[b]	2969 ± 212	990 ± 70.6
uhCG-WHO 75/589	70[a]	43,503 ± 2489	621 ± 35.6
uhCG-D	375[b]	266,033 ± 16,329	709 ± 44.0
rhCG-E	272[b]	227,553 ± 17,913	837 ± 66.4

[a] Declared content.
[b] Determined via BCA in the present work.

Table 5
Quantification of different hLH, hCG and hMG preparations by BCA and RP-HPLC, against the International Standard of rhLH-WHO 96/602 and rhFSH-WHO 92/642.

Preparation	Total protein (μg/ampoule)	hLH/hCG[a] (μg/ampoule)	hFSH[a] (μg/ampoule)	hLH/hCG + hFSH (μg/ampoule)	Gonadotropin fraction
rhLH-WHO 96/602	8.8[b]	–	–	–	1.00
phLH-A	3.18[c]	2.37	–	–	0.75
phLH-B	5.48[c]	4.17	–	–	0.76
rhLH-C	3.00[c]	3.02	–	–	1.01
uhCG-WHO 75/589	70[b]	45.7	–	–	0.65
uhCG-D	375[c]	277	–	–	0.74
rhCG-E	272[c]	237	–	–	0.87
uhMG-WHO 98/704	909[c]	146	216	362	0.40
uhMG-F	30.4[c]	8.2	4.9	13.1	0.43
uhMG-G	30.7[c]	4.7	7.1	11.8	0.38
uhMG-H	459[c]	47	111	158	0.34

[a] Determined by RP-HPLC.
[b] Declared content.
[c] Determined by BCA.

Table 6
hLH/hCG biopotency of all recombinant and urinary preparations.

Preparation	IU (ampoule)[a]	IU (mg)
rhLH-WHO 96/602	189	21,477[b]
rhLH-C	75	25,000[c]
uhCG-WHO 75/589	650	9,286[b]
uhCG-D	5000	13,333[c]
rhCG-E	6500	23,897[c]
uhMG-WHO 98/704	70	77[c]
uhMG-F	75	2,467[c]
uhMG-G	75	2,443[c]
uhMG-H	75	163[c]

[a] Declared by the manufacturer.
[b] Calculated based on the declared protein content.
[c] Calculated from BCA determinations (this work).

of 77–2500 IU/mg. This is quite far from the potencies of the three recombinant preparations (Table 6), whose values show excellent agreement (average bioactivity = 23,466 ± 1789 IU/mg; RSD = 7.6%). Moreover, the total protein content determined by us via the BCA method was always between −18% and +10% of the manufacturer's declared content, except for one preparation that showed 36% less protein content than declared. Three of the eleven preparations did not have declared total protein content, whereas large amounts of HSA had been added to two International Standards. Obviously, the quantitative analysis of the heterogeneous preparations cannot be considered to be accurate under the present conditions.

4. Discussion

Native pituitary, urinary and recombinant preparations of hLH and hCG and of their subunits have been compared for the first time by qualitative and quantitative RP-HPLC analysis. Specific chromatographic conditions provided a precise identification and, most of the time, even a good separation. Indeed, only the two α-subunits and hLH and β-hCG were not well resolved from each other. The significant difference in hydrophobicity between the two α-subunits of hLH and hCG confirms previous reports about their different glycosylation pattern. These α-subunits, even sharing the same aminoacid sequence, have different carbohydrate moieties determined by different steps of the glycosylation mechanism [5,17]. In the case of rhCG, the α- and β-subunits were efficiently prepared in our laboratory by applying a dissociation and characterization methodology already established in previous work [5].

Different RP-HPLC elution conditions for isolating and analyzing subunits and heterodimeric forms of TSH, FSH, LH and CG have been reported by various authors over the last 25 years [26]. The pioneering works were those of Bristow et al. [27], who detected the hTSH heterodimer for the first time by RP-HPLC, and of Parsons et al. [15], whose gradient setup was used for analyzing the dissociated subunits. Later modifications by several different authors permitted detection of the heterodimeric forms of these hormones as well [5,19,20,24,28–30]. As far as we know, only Loureiro et al. [25] succeeded in carrying out the separation, identification and analysis of hFSH heterodimer by simply adjusting RP-HPLC elution conditions.

In the case of hMG containing urinary hFSH and hLH, new RP-HPLC elution conditions had to be developed in order to avoid hFSH dissociation. However, in general, the hMG preparations were also found to contain hCG, added to compensate for a low hLH activity [9,31]. The newly adjusted conditions succeeded in identifying the three heterodimeric forms: hFSH, hCG and hLH in a complex mixture.

Seven homogeneous preparations of hLH and hCG were compared in the present study. As expected, the three that were obtained by DNA recombinant techniques were by far the purest. This allowed us to define an operational RP-HPLC internal response parameter, expressed in area units (au)/μg of the type already employed in previous work with other hormones [24,25]. This operational response parameter was quite similar for all three different recombinant preparations, which included hLH and hCG, that could be used for all quantifications and allowed an acceptable accuracy. The results of these quantitative analyses, showed

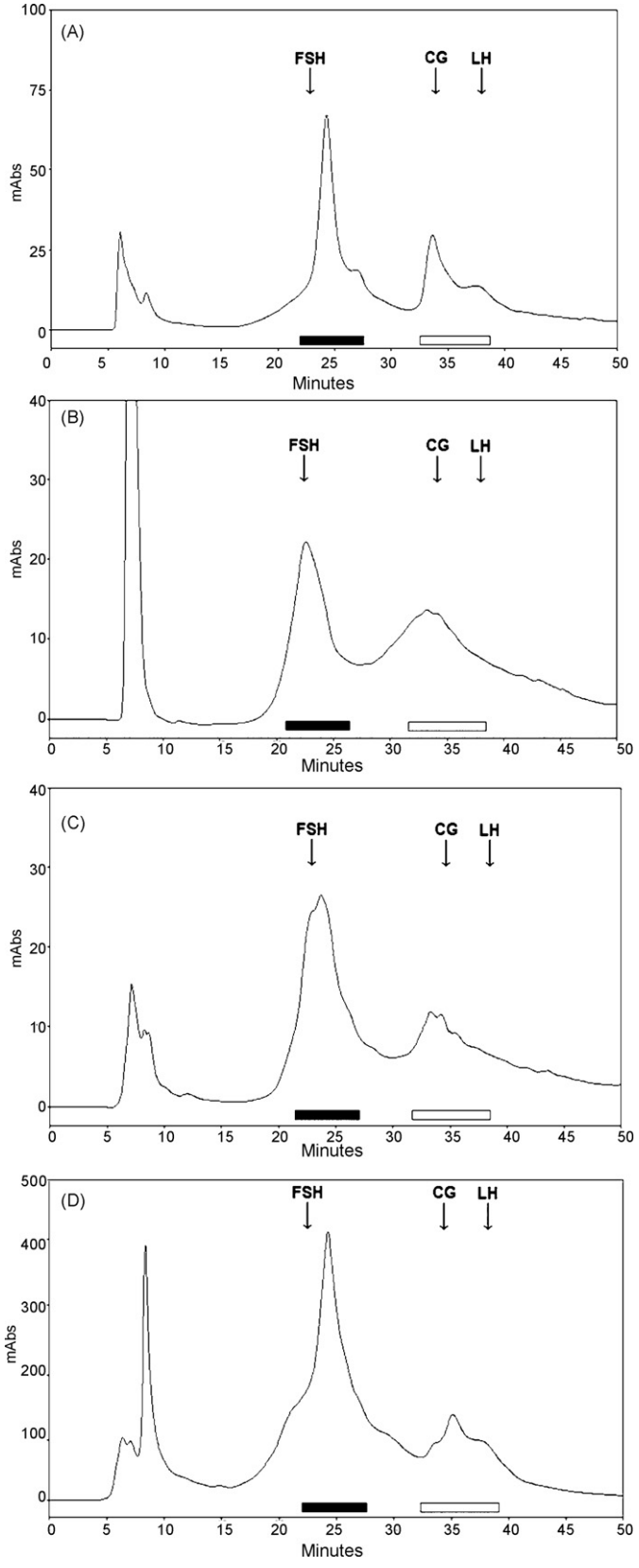

Fig. 3. RP-HPLC of heterogeneous urinary preparations of hMG: (A) uhMG-WHO 98/704; (B) uhMG-F; (C) uhMG-G; (D) uhMG-H. ■ , hFSH bioactivity; □ , hLH bioactivity. The retention times of pure phFSH, uhCG and phLH run under identical conditions are indicated by arrows.

that the recombinant preparations had a mass fraction of the order of 0.9–1.0, as opposed to mass fractions of the order of 0.75 for the urinary and the two pituitary preparations. The International Standard of uhCG-WHO 75/589 was apparently the least pure of all homogeneous preparations, probably because it consists of quite old and crude material, prepared exclusively for *in vivo* bioassays. This can also be inferred from the declared unitage and calculated biopotency (Table 6), which is substantially inferior to that of the three recombinant preparations. An unexpected difference (~1 min) was observed in the retention time of the main peaks of the two pituitary preparations of hLH, which suggests the presence of different purified isoforms, an hypothesis that could be verified by MS techniques.

Concerning the quantitative analysis of the heterogeneous preparations of hMG, it was difficult to carry out analogous assay validation tests. In this case, the importance of the RP-HPLC data is thus more qualitative. Reasonable agreement was found for the mass fraction (gonadotropins), of the order of 0.3–0.4, apparently unrelated to the declared hLH potency, which varied approximately 30-fold (see Table 6). Our data on a specific hMG preparation, in fact, confirm protein content, the addition of hCG, hLH non-detectability and the incomplete product purification, as reported by Giudice et al. [31] and by van de Weijer et al. [9].

On the basis of the data in Table 5, several inferences can be made concerning the products shown in Fig. 3. Endogenous hLH material appears to be present only in uhMG-WHO 98/704 and uhMG-H. If some endogenous hLH is present in uhMG-F and uhMG-G„ it is probably covered up by the bulk of proteins. In these two preparations, the amount of hCG determined by RP-HPLC (8.2 and 4.7 μg), together with the declared content of 75 IU of hLH/vial, corresponds to potencies of ~9100 and 16,000 IU/mg, respectively. This is a good indication of pure hCG addition. We have no ready explanation for the peaks that elute at the position of hCG in uhMG-WHO 98/704 and uhMG-H, i.e., in products whose potencies are ~15–50-fold lower than pure hCG. An hypothesis is that the added material was a crude extract containing impurities with approximately the same hydrophobicity as hCG.

In conclusion, we have developed two specific elution conditions that permit an accurate qualitative analysis by RP-HPLC. This permitted inter-comparison between different pharmaceutical and chemically purified preparations of hLH and hCG and of their subunits of pituitary, urinary and recombinant origin, as well as an accurate quantitative analysis of the seven preparations that were homogeneous. An attempt was also made to quantify the four heterogeneous preparations of urinary hMG whose purity was considerably lower and that contained hCG in addition to hLH and hFSH. Our task was further complicated by the fact that official standards suitable for physical–chemical testing are not available. Standard preparations of adequate purity, homogeneity and amount of material are urgently required for the development and applications of the physicochemical methods that are being progressively introduced by Regulatory Agencies and Pharmacopoeias to replace the expensive and imprecise bioassays based on animal use [32,33].

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