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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