Journal of Pharmaceutical and Biomedical Analysis 63 (2012) 160–164

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Qualitative and quantitative reversed-phase high performance liquid chromatographic analysis of glycoprotein hormones in the presence of a large excess of human serum albumin

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ARTICLE INFO

Article history:
Received 30 November 2011
Received in revised form 27 January 2012
Accepted 29 January 2012
Available online 6 February 2012

Keywords:
RP-HPLC
HSA
Glycoprotein hormones
Excipient
WHO standards

ABSTRACT

The present work describes reversed-phase high performance liquid chromatographic methodologies (RP-HPLC) for the qualitative and quantitative analysis of the human glycoprotein hormones thyrotropin (hTSH), follitropin (hFSH), choriogonadotropin (hCG) and lutropin (hLH) in the presence of a large excess (up to 250:1) of human serum albumin (HSA). Chromatographic profiles with a good separation between the hormone and HSA were obtained by using a C4 column and specific gradient elution conditions for each hormone. Parameters such as resolution factor, tailing factor and relative retention time, were determined, and are useful for the evaluation of the quality of the separation obtained between the active pharmaceutical ingredient and the excipient present in the final formulation. The potential of each method for quantification of both HSA and the hormone was also demonstrated. Besides furnishing chromatographic quantifications that can substitute for in vivo bioassays and animal use, the chromatograms also provide a direct panorama of the quality and heterogeneity of the protein of interest.

1. Introduction

Long-term stability is a quality requirement for both pharmaceutical proteins and reference standards, and addition of stabilizers is crucial for attaining this goal [1]. In the final preparations, the active pharmaceutical substance is, in general, present in extremely limited amounts as compared to the large amounts of excipients. These are added to prevent adsorption of proteins to the vial, increase their stability, and protect them during storage. Common excipients include sugars (e.g., saccharose, lactose, trehalose), amino acids (e.g., methionine, arginine, glycine), polyols (e.g., mannitol), polymers (e.g., poloxamer P188), detergents (e.g., polysorbate 20), salts (e.g., sodium chloride, sodium phosphate) and proteins [2].

Human serum albumin (HSA), in particular, has been quite successfully employed as a protein excipient. The stabilizing, antioxidant and cryoprotective properties of HSA prevent losses due to adsorption and degradation, which correlate with reduced bioactivity and enhanced immunogenic reactions [3]. A major drawback of the presence of this excipient is its interference with many analytical techniques. Suitable analytical methods that permit an adequate discrimination between excipients and protein thus need to be developed. For this reason, several chromatographic and electrophoretic methods that attempt to circumvent this limitation have been reported in the literature. Bietlot and Girard [4], utilizing high-performance capillary electrophoresis (HPCE), analyzed recombinant human erythropoietin (r-hEPO) in the presence of HSA by adding 1 mM nickel chloride to the electrophoretic buffer, obtaining a complete separation of the two proteins. In this case, the added metal ions interacted selectively with HSA, decreasing its electrophoretic mobility. Wilczynska et al. [5] described a reversed-phase high performance liquid chromatographic (RP-HPLC) procedure for the quantitative estimation of r-hEPO in biopharmaceutical products formulated with HSA. A suitable resolution between albumin, r-hEPO and related proteins was established utilizing a C18 column and a gradient profile developed specifically for this purpose. Qian et al. [6] developed a high performance size exclusion chromatographic (HPSEC) method to evaluate HSA stability over the three year shelf-life of a pharmaceutical preparation of interferon alfa-2b (IFN). For this purpose, several key factors that affect HPSEC selectivity and chromatographic performance were optimized, such as column type, mobile phase, sample preparation (concentration, diluents, method of mixing), injection volume and flow rate. Eertmans et al. [7] developed and validated a RP-HPLC method that allows a quick and reliable determination of the HSA content in assisted reproductive techniques-related media, without the interference of other matrix ingredients. They utilized a C4 column at 40 °C and gradient elution over 20 min, with HSA

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doi:10.1016/j.jpba.2012.01.039

eluting within the first 10 min. Another approach for eliminating the interference of excipients in biopharmaceuticals analysis is to remove them from the pharmaceutical formulations or to extract the active protein. Lara-Quintanar et al. [8] proposed the removal of HSA from r-hEPO formulations by immunochromatography in their capillary electrophoretic method. Liu et al. [9] isolated recombinant follitropin (r-hFSH) with high purity and yield from a pharmaceutical preparation formulated with multiple excipients. The authors adopted a purification strategy involving anion exchange chromatography followed by desalting, utilizing a centrifuge device with a 10 kDa cut off ultrafiltration membrane.

Besides pharmaceutical preparations, International Standards and Reference Reagents, including those prepared by the National Institute for Biological Standards and Control (NIBSC) on behalf of World Health Organization (WHO), are also frequently lyophilized in the presence of a large excess of a protecting/stabilizing protein such as HSA [10–12]. This is mostly due to the fact that these precious purified, controlled and calibrated reagents are used to prepare thousands of ampoules in which they are present in very small amounts. These preparations are intended for use as standards for in vivo and in vitro bioassays in which the extraneous protein is practically inert. Nonetheless, this hampers the utilization of such standards for chromatographic testing, a type of assay that is progressively substituting bioassays because of higher precision and lower cost. In addition, physical chemical tests provide an alternative to the use of animals, avoiding the associated ethical considerations [13]. In this context, the use of the same reference standard for monitoring the content/potency of therapeutic products in both types of assays is extremely important.

The focus of this work was, therefore, the development of high resolution RP-HPLC techniques for the analysis of reference preparations of human glycoprotein hormones, specifically hFSH, hLH, hTSH and hCG, in the presence of up to 250-fold larger amounts of human serum albumin. For this purpose, three new chromatographic conditions were developed by introducing modifications to the five conditions already set up by our research group for glycoprotein hormone analysis by RP-HPLC [12,14–16]. Besides allowing the quantitative chromatographic determination of these specific hormones, these methodologies also provide a real-time panorama of the quality and heterogeneity of the protein of interest.

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

The WHO International Standards for glycoprotein hormones utilized in this work were from the National Institute for Biological Standards and Control (NIBSC, South Mimms, UK): International Reference Preparation of Thyroid Stimulating Hormone (TSH) Pituitary, Human, for Immunoassay (WHO 80/558); International Standard of Follicle-Stimulating Hormone (FSH) Recombinant, Human, for Bioassay (WHO 92/642); International Standard of Luteinizing hormone (LH) Recombinant, Human, for Bioassay (WHO 96/602); International Standard of human Chorionic Gonadotropin (CG) Urinary (WHO 07/364). Commercial recombinant preparations from Genzyme Corporation (Framingham, MA, USA) and from Laboratoires Serono S.A. (Aubonne, Switzerland) were also utilized: Thyrogen (r-hTSH), Gonal-f (r-hFSH), Luveris (r-hLH) and Ovidrel (r-hCG). In Table 1, the declared units and/or mass contents are reported, together with the calculated specific bioactivities.

Table 1
Bioactivities of all recombinant and native preparations.

Preparation	Hormone mass (μg/ampoule)	Unitage (IU/ampoule)	Specific activity (IU/mg)
p-hTSH (WHO 80/558)	7.5[a]	0.037[a]	4.9
r-hFSH (WHO 92//642)	10[a]	138[a]	13,800
u-hCG (WHO 07/364)	13.7[b]	162[a]	11,825
r-hLH (WHO 96/602)	8.8[a]	189[a]	21,477
r-hTSH (Thyrogen)	1100[a]	–	4–12[a]
r-hFSH (Gonal-f)	5.5[a]	75[a]	13,636
r-hCG (Ovidrel)	250[a]	6500[a]	26,000
r-hLH (Luveris)	3.0[c]	75[a]	25,000

[a] Declared by the manufacturer.
[b] Calculated with basis on a MW of 35.1 kDa [17] from the declared content of 0.39 nmol.
[c] Calculated from BCA determinations [12].

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

RP-HPLC analyses of the WHO glycoprotein hormones (hFSH, hLH, hTSH and hCG), formulated with HSA were carried out with a Shimadzu Model SCL-10A HPLC 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 (1 cm × 4.6 mm I.D.). 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 and detection was by UV absorbance at 220 nm. Gradient solutions A and B were utilized, solution A being sodium phosphate buffer (pH 7.0; 0.05 M) and solution B acetonitrile. For hFSH and hTSH elution, linear gradients from A:B (87.5:12.5, v/v) to A:B (50:50, v/v) over 40 min and from A:B (50:50, v/v) to A:B (35:65, v/v) over 20 min were utilized. For hLH elution, linear gradients from A:B (73:27, v/v) to A:B (58:42, v/v) over 60 min and from A:B (58:42, v/v) to A:B (35:65, v/v) over 20 min were utilized. For hCG, linear gradients from A:B (75:25, v/v) to A:B (50:50,v/v) over 50 min and from A:B (50:50, v/v) to A:B (35:65, v/v) over 20 min were used. Aliquots of 20–125 μl of these preparations were processed, at a flow-rate of 0.5 ml/min. To evaluate the quality of the separation between HSA and the hormones, the resolution factor (Rf) was determined, according to definition that considers the peak asymmetry [18]. In general, Rf > 1.5 is indicative of complete resolution, allowing an accurate integration of the individual peaks and their quantification.

$$\mathrm{Rf} = \frac{2(t_2 + w_2/2(1 - 1/\mathrm{Tf}_2) - t_1 - w_1/2(1 - 1/\mathrm{Tf}_1))}{w_1 + w_2}$$

where t_2 and t_1 are the retention times of HSA and the hormone, respectively, w_2 and w_1 are the peak width at baseline of the eluted HSA and hormone peaks, respectively, and Tf_2 and Tf_1 are the tailing factors of HSA and hormone, respectively.

The degree of peak asymmetry was estimated via the tailing factor, determined according to the definition:

$$\mathrm{Tf} = \frac{a + b}{2a}$$

where a and b are the peak widths of the left and right sides, respectively, measured at the baseline, with $w = a + b$. The peak is symmetrical when Tf is 1.00.

Table 2
System suitability parameters determined for RP-HPLC analysis of WHO glycoprotein hormones: retention time (t_R), peak width (w), tailing factor (Tf), relative retention time (T_{RR}) and resolution factor between each hormone and HSA (Rf).

Preparation	Hormone			HSA			T_{RR}	Rf
	t_{R1}[a] (min)	w_1 (min)	Tf_1	t_{R2}[a] (min)	w_2 (min)	Tf_2		
p-hTSH (WHO 80/558)	33.61 ± 0.12	1.98	1.0	46.42 ± 0.05	3.24	1.2	0.72	5.02
r-hFSH (WHO 92//642) α	32.32 ± 0.29	2.72	1.2	46.62 ± 0.35	4.71	1.3	0.69	3.95
r-hFSH (WHO 92//642) β	23.29 ± 0.19	2.43	1.3				0.50	6.01
u-hCG (WHO 07/364)	24.87 ± 0.11	4.10	1.1	51.37 ± 0.25	8.29	1.2	0.48	4.36
r-hLH (WHO 96/602)	30.00 ± 0.16	11.40	1.6	72.12 ± 0.12	18.28	1.1	0.42	2.72

[a] Mean ± standard deviation (n = 3).

An index of relative retention of the hormones (T_{RR}) was determined according to the definition:

$$T_{RR} = \frac{t_R \text{ hormone}}{t_R \text{ HSA}}$$

For the determination of the HSA content, a calibration curve was constructed by plotting the HSA peak area (in arbitrary units) as a function of the amount of added HSA (μg), over the range of 5–200 μg. For hormone quantification, highly purified commercial recombinant preparations, already characterized in previous work in our laboratory [12,14,15], were utilized as internal reference preparations, since the WHO standards containing hormone + HSA were considered to be the unknown preparations in this study. In the case of r-hFSH quantitation, the determination was done by considering peak areas of the two subunits. The results were then compared with those obtained via a previously set up RP-HPLC condition that is able to determine the undissociated heterodimeric peak [13,15]. Both HSA and the hormones were determined in three independent assays.

3. Results

Specific RP-HPLC conditions were set up that provided useful separations between the huge peak of HSA, present in the WHO standard preparations, and the peaks of hTSH, hFSH, hLH or hCG (see Section 2). Under optimal chromatographic conditions for each hormone, the heterogeneous formulations, analyzed in three RP-HPLC assays, showed good separation between the two peaks (Fig. 1). All peaks presented retention times with a relative standard deviation (RSD) < 1%, tailing factors (Tf) 1 < Tf < 1.6 and resolution factor (Rf) > 2 (Table 2). These system suitability parameters are in agreement with the Food and Drug Administration (FDA) validation requirements [19]. The lowest resolution of the hormone relative to HSA was found for hLH, probably because it presents the highest degree of asymmetry (Tf = 1.6). Conversely, the peak corresponding to hTSH was symmetrical, with Tf = 1, while the resolution relative to HSA was the second highest (Rf = 5.02), even though the two peaks eluted quite close to each other (T_{RR} = 0.72).

The HSA present in the WHO ampoules of p-hTSH, r-hFSH, u-hCG and r-hLH (Table 3) was determined by utilizing the constructed calibration curve:

$$Y_{u.a.} = 223.34X_{\mu g} - 124.69, \quad r = 0.9994\,(n = 6)$$

The hormone contents in the WHO ampoules, also shown in Table 3, were determined relative to the previously characterized reference preparations, which served as secondary standards. The inter-day quantitative determinations indicated a relative standard deviation (RSD) of 0.95–4.5%. The minimal detectable amount, determined according to the Rodbard definition of sensitivity [20], was in the order of 6–20 ng for the four hormones. The mean percent recoveries of HSA were 97.6 ± 9.1 relative to the stated nominal HSA contents. Concerning the hormone contents in the ampoules, the mean percent recovery was 103.4 ± 5.5 for recombinant preparations of hFSH and hLH; however, a significant discrepancy was found for p-hTSH and u-hCG, a recovery of only 56% and 53% being observed, respectively.

4. Discussion

An important issue in biopharmaceutical drug quality control is the availability of chromatographic tools that permit the accurate analysis of the active principle without interference from other components of the final drug formulation with similar hydrophobic, ionic and/or spectrophotometric characteristics. In this context, the three RP-HPLC methodologies developed here for the qualitative and quantitative analysis of hTSH, hFSH, hCG and hLH in the presence of substantial excesses of HSA successfully permit the quantitative and qualitative analysis of all of the proteins present in each formulation. For the first time, these hormones could be analyzed merely by altering and optimizing the chromatographic conditions, without the need for salt addition, extraction or protein removal typical of other approaches in the literature [4,8,9], thus providing a powerful instrument for identification and quality control of these precious preparations. Albumin is not used as an excipient in the recombinant human glycoprotein hormones preparations currently on the pharmaceutical market, but we believe that improved separations between hormone and albumin are useful as a model for qualitative and quantitative analysis of glycoprotein hormone in the presence of excipients. They can also provide an initial characterization of recombinant hormone before final separation of a major albumin-like contaminant, which is very common in the CHO cell serum-free media.

Reproducible chromatographic profiles, with a good separation between the hormone and HSA (Rf > 2), were achieved using a C4 column and gradient elution conditions optimized for each

Table 3
Quantitative analysis of glycoprotein hormones and HSA in WHO ampoules.

Preparation	Hormone			HSA		
	Nominal amount (μg)	Found amount[a] (μg)	RSD (%)	Nominal amount (mg)	Found amount[a] (mg)	RSD (%)
p-hTSH (WHO 80/558)	7.5	4.22 ± 0.04	0.95	1.0	1.06 ± 0.02	1.89
r-hFSH (WHO 92//642)	10.0	10.72 ± 0.21	1.96	2.0	2.10 ± 0.06	2.86
u-hCG (WHO 07/364)	13.7	7.20 ± 0.25	3.47	2.0	1.79 ± 0.08	4.47
r-hLH (WHO 96/602)	8.8	8.76 ± 0.23	2.63	2.0	1.80 ± 0.05	2.78

[a] Mean ± standard deviation (n = 3).

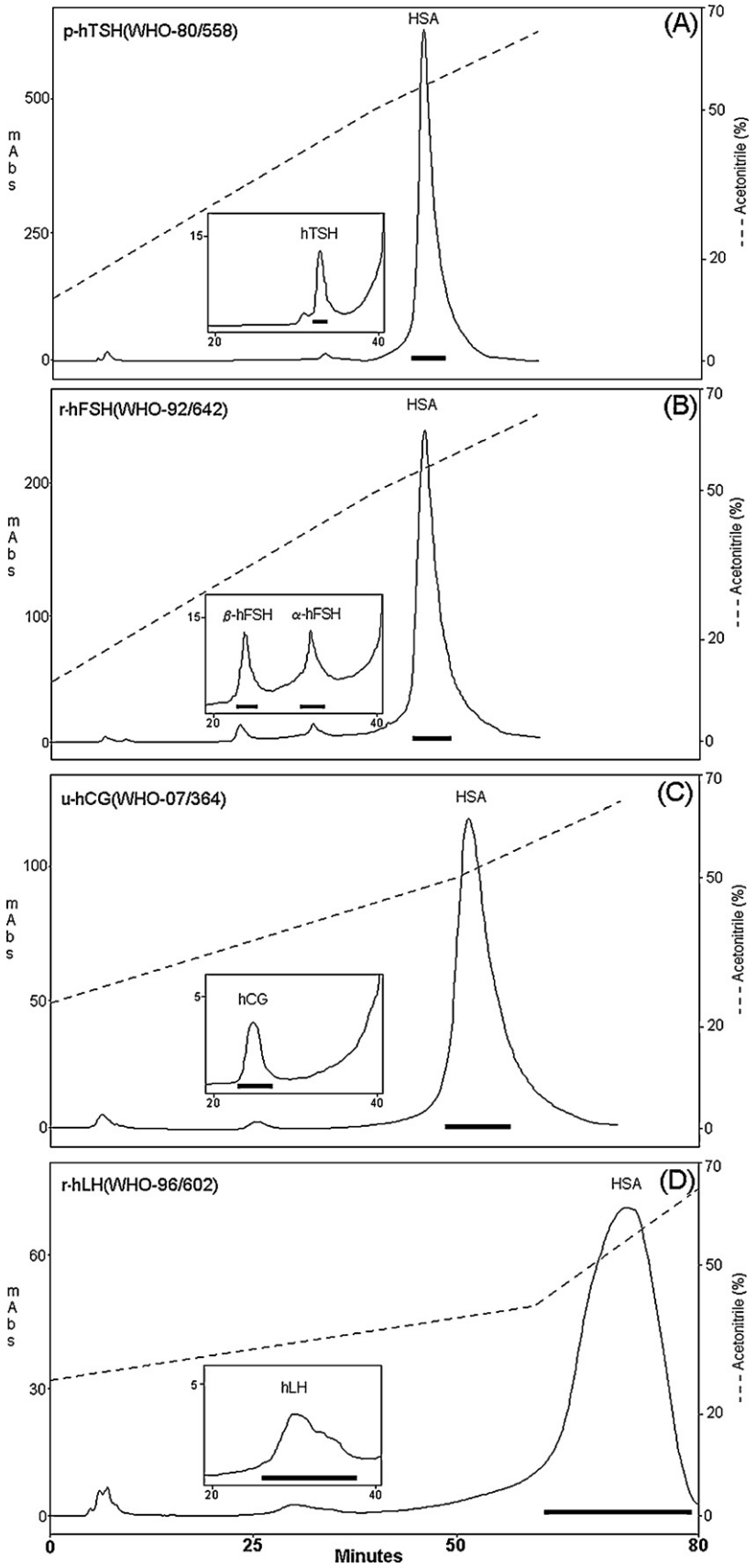

Fig. 1. RP-HPLC profile of different WHO International Standard preparations with HSA/hormone ratio of 200–250: (A) p-hTSH (WHO-80/558); (B) r-hFSH (WHO-92/642); (C) u-hCG (WHO-07/364); and (D) r-hLH (WHO-96/602).

hormone. In the methods previously developed for hLH and hCG in our laboratory [12], lower T_{RR} and poorer resolutions between the hormone and HSA had been obtained. An approximately 2-fold improvement in both of these parameters was achieved thanks to the current methodologies. Parameters such as the resolution factor, tailing factor and relative retention time were found to be suitable in general for evaluating the chromatographic resolution and selectivity between the active protein and the excipient. The sensitivities, determined for the methodologies described, were excellent for all four hormone preparations.

Quantitative estimation of the hormones in the International Standard formulations showed very good agreement with the declared content for the recombinant hormones (r-hFSH and r-hLH). However, for urinary-hCG and pituitary-hTSH, a significant bias (∼50%) was found, probably due to the much lower specific activity of these native pituitary and urinary preparations compared to the recombinant ones. We emphasize, for example, that a recent recombinant hTSH Standard (WHO 03/192) has a bioactivity of 10.9 IU/mg against the 4.9 IU/mg of p-hTSH (WHO 80/558). The less pure native preparations were quantified against highly purified recombinant preparations and therefore a correspondent lower mass was found, per unit. Thus, the RP-HPLC strategy described herein is able to reveal the correct relationship between biological activity and real amount of hormone present. It is worth mentioning that hFSH quantitation was possible considering both subunits area combined, since well-defined peaks were obtained for each subunit. However, when these areas are not as easy to define, as for example for pituitary hFSH subunits, the hormone quantitation could utilize the RP-HPLC condition previously developed in our laboratory for the determination of intact heterodimeric hFSH [15]. Our RP-HPLC strategy was also successfully employed for HSA quantification and allowed accurate determination of the amount of HSA present in the drug formulations. In addition, the complete chromatographic separation of the hormone from the protein excipient also provided a qualitative estimate of the hormone under analysis and of its possible heterogeneity.

In conclusion, this work demonstrates the enormous potential of RP-HPLC for the separation and the quantitative and qualitative evaluation of protein excipients and hormones, underlining its importance as a chromatographic method for the rapid analysis of standards, reference preparations and biopharmaceutical in general.

Acknowledgements

This work was supported by FAPESP, São Paulo, Brazil (11/07289-0) and by the Brazilian National Research Council (CNPq), Brasilia, Brazil (PQ 303839/2008-2 and PQ 300473/2009-5).

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