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Practical reversed-phase high-performance liquid chromatography method for laboratory-scale purification of recombinant human thyrotropin

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Abstract

A small, semi-preparative C_4 RP-HPLC column was used to set up the conclusive laboratory-scale purification of Chinese hamster ovary-derived human thyrotropin (hTSH), after a preliminary concentration-purification of an extremely dilute and poorly (∼0.6 μg hTSH/mL; mass fraction = 0.35%) conditioned medium on a cation exchanger. Several fractions of this eluate were repeatedly injected on the semi-preparative column, obtaining, in a single run (<1 h chromatographic time), a concentrated pool (∼1.2 mg/mL) of highly purified hTSH that could be further concentrated to >3 mg/mL and then efficiently lyophilized. The overall recovery in the rapid RP-HPLC purification step, including concentration and lyophilization, was of the order of 80%. The final product, when tested via a precise, single-dose *in vivo* bioassay, confirmed that it did not suffer any loss of bioactivity. This same methodology can be easily adapted to the small-scale purification of other recombinant products, even when obtained from genetically modified organisms at extremely low concentrations and mass fractions.

Keywords: Reversed-phase HPLC; Thyrotropin; Protein purification; DNA recombinant; *In vivo* bioassay

1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely used for the analytical characterization and quality control of recombinant proteins and biopharmaceuticals in general and of human pituitary hormones in particular [1]. Considering that physical-chemical testing is particularly recommended by the major pharmacopoeias, our laboratory has developed RP-HPLC-based analytical methods for human growth hormone (hGH) [2], human prolactin (hPRL) [3], human thyrotropin (hTSH) [4] and human follitropin (hFSH) [5] using this HPLC mode together with high-performance size-exclusion chromatography (HPSEC), which was also used to follow all hGH and hTSH purification steps [6,7].

Although accuracy, sensitivity, speed, resolving power and versatility are well known RP-HPLC characteristics, its use and importance for purification purposes has, up to now, been quite underestimated [8]. Recombinant interleukin and colony-stimulating factor (CSF) [9], hirudin [10], insulin [11], β-interferon [12], and insulin-like growth factor [13,14] are all biopharmaceuticals that have been purified by using RP-HPLC. More recently, milligram-level loadings of microcystins were successfully purified via C_{18} RP-HPLC [15], while a methodology based on a C_4 preparative column was applied to interferon α purification from *Escherichia coli* (*E.coli*) inclusion bodies [16]. Hodges and coworkers [17–20] in particular have made extensive use of preparative RP-HPLC, employing C_8 columns with internal diameters of up to 50 mm and loadings of up to 5.7 g of different synthetic peptides and native or recombinant proteins. In the field of recombinant pituitary hormone downstream processing, in addition to a patent for hGH purification [21], the only other example, which we would like to emphasize, is the utilization of a strategic C_{18} RP-HPLC separation as one of the final polishing steps for eliminating some specific contaminants in hFSH purification [22]. In this case, the crude hFSH material had a mass fraction of ∼2% and an overall purification factor of 41 had already been reached after the gross purification phase involving three conventional columns.

With regards to hTSH purification, reported strategies involve conventional chromatographic methods such as hydrophobic, dye affinity, ion-exchange and gel filtration chromatography [7,23–26]. As far as we know, an approach utilizing RP-HPLC

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as an effective purification step on a preparative scale was never described for this hormone. We decribe here the utilization of a small (25 cm × 10 mm I.D.) semi-preparative C_4 RP-HPLC column for the conclusive laboratory-scale purification of Chinese hamster ovary (CHO)-derived hTSH, starting from an extremely dilute and impure (i.e., with a very low mass fraction) crude material obtained in T-flasks. Particular care was taken to check the biological activity of the final product, especially considering the presence of acetonitrile in the mobile phase, a solvent that is considered to be potentially threatening to the stability of complex proteins.

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 of analytical reagent grade purchased from Merck (São Paulo, Brazil) and Sigma (St. Louis, MO, USA).

Chromatographic resins were purchased from GE Healthcare Life Sciences (São Paulo, Brazil). CHO cells cultivation medium (CHO-S-SFM) was provided by Gibco-BRL (Gaithersburg, MD, USA). Recombinant hTSH (Thyrogen), produced by Genzyme (Framingham, MA, USA) was purchased from Collect (São Paulo, Brasil). Solid-phase hTSH immunoradiometric assay (IRMA) and monoclonal anti-hTSH antibody were purchased from Netria (London, UK), while the Reference Preparation of pituitary hTSH (pit-hTSH, NIDDK, SIAFP-B2) was obtained from Dr. A.F. Parlow of the National Hormone and Pituitary Program (Torrance, CA, USA).

2.2. Cell cultivation

A clone, obtained in our laboratory, derived from CHO DHFR-cells (mutant line DXB11), cotransfected with the dicistronic vectors pEDdc-α and pEAdc-βTSH and expressing human TSH [27], was cultured in T-flasks (225 cm^2, from Corning Costar, Cambridge, MA, USA) at 37 °C in a humidified 5% CO_2 incubator. Transfected cells were grown in CHO-S-SFM II medium with nucleosides (hypoxanthine and thymidine) and with the addition of 0.1 μM methotrexate, which allows a selective pressure. Conditioned media (40 mL/flask) were harvested every day over the 30-day cultivation period.

2.3. Purification process

A typical purification process begins with several 1 L fractions of CHO cell conditioned medium, each fraction coming from 3 days harvesting. This material, after pH adjustment to 5.0, was loaded onto a 10 cm × 2.6 cm I.D. glass column packed with SP-Sepharose fast flow (FF) that had been previously equilibrated with 50 mM NaCl, 20 mM sodium acetate buffer, pH 5.0. After washing with 5 column volumes of this buffer, the protein of interest was eluted with a linear gradient (from 50 mM to 250 mM NaCl), at a flow-rate of 200 mL/h. The resultant pool (∼150 mL) was stored at −20 °C. Thus, for example, a total of 10 L conditioned medium from ten separate chromatographic steps will provide ∼1.5 L of collected pools that can be dialyzed and re-chromatographed on the same SP-Sepharose column, obtaining a selected pool of ∼80 mL. This was further concentrated to 10 mL via an Amicon Ultra-15 centrifugal filter device (Millipore). Multiple injections of this pool (5 × 2 mL), through a 2.5 mL injection loop, were carried out on a semi-preparative Vydac C4, 214 TP510 column (300 Å pore size, 5 μm particle size, 25 cm × 10 mm I.D.) purchased from Grace-Vydac (Hesperia, CA, USA), connected to a Shimadzu Model SCL-10A HPLC apparatus. Eluent A was 0.05 M sodium phosphate buffer, pH 7.0, and eluent B was made with 50% acetonitrile plus 50% eluent A. The elution was carried out with a linear gradient of 25–100% B, over 40 min, at 25 °C. A flow-rate of 2.5 mL/min and detection by UV absorbance at a wavelength of 220 nm were used. The final pool was eventually concentrated, dialyzed against 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 5 mg/mL glycine and lyophilized.

2.4. Analytical HPLC

The resultant pools of each purification step, containing the product of interest, were analyzed by RP-HPLC and HPSEC, utilizing the same Shimadzu HPLC apparatus employed for the semi-preparative run. In both analyses, Thyrogen was utilized as the reference preparation for the quantification of the unknown samples.

For RP-HPLC, a C4-Vydac 214TP54 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) was utilized. The running conditions were the same as described for the semi-preparative column, except for the flow-rate (0.5 mL/min).

For HPSEC, 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 was utilized. The mobile phase utilized was 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.0, at a flow-rate of 1.0 mL/min.

2.5. Protein determination

Total protein content was determined by the classical method of Lowry et al. [28] or by BCA protein assay, using bicinchoninic acid (Micro BCA protein assay kit, Pierce, Rockford, IL, USA). Pure bovine serum albumin (BSA, Sigma, São Paulo, Brazil) was used as standard in both methods.

2.6. Immunoradiometric assay

An immunoradiometric assay was utilized for the identification of hTSH after each purification step. IRMA was carried out by an in-house, "sandwich" format, utilizing a secondary hTSH standard calibrated against the Interna-

tional Standard of pituitary hTSH (WHO 80/558, 4.93 IU/mg) [29].

2.7. "In vivo" bioassay

The biological activity of the recombinant purified hormone (r-hTSH-IPEN) was evaluated by an *in vivo* bioassay in which TSH-induced T_4 is measured after suppression of endogenous TSH by administration of T_3 in Balb C mice. We employed a modification of the *in vivo* East-Palmer method [30], utilizing a single dose (10 μg/mouse) and 6 mice for each preparation. The choice of this unique dose was based on the results obtained in previous works in which 10 μg of hTSH always provided a reproducible response in the linear part of the dose-response curve [7,27]. The biological activity was determined by comparison with the Reference Preparation of pituitary hTSH (NIDDK) and with Thyrogen, a putative recombinant reference preparation, whose potency was 4 IU/mg [7].

3. Results

CHO conditioned medium containing approximately 0.6 μg hTSH/mL, determined by IRMA, with a mass fraction of the order of 0.35% was applied to a SP-Sepharose FF chromatographic column obtaining (with ~84% recovery) a greatly enriched pool, with a hTSH mass fraction of 12.7% and, consequently, a purification factor of 36-fold (Fig. 1A and Table 1). As described in Materials and Methods, starting from approximately 10 L of CHO conditioned medium, one ends up with ~80 mL (125× concentration) SP-Sepharose pool, that could be further concentrated to 10 mL (1000× overall concentration) via filter device centrifugation and then applied to preparative RP-HPLC. Obviously, the whole purification process can be carried out with only 1 L of medium (~0.6 mg hTSH), but with much lower yields: ~24% (data not shown) instead of the 70% overall recovery obtained in the present work.

Before RP-HPLC purification, the selected pool was analyzed by HPSEC and RP-HPLC. The presence of contaminants with higher molecular mass (t_R = 12.4 min and 15.7 min) and with a lower hydrophobicity (t_R < 10 min) is illustrated in Figs. 2A and 3A. The preparative RP-HPLC purification step is shown in Fig. 1B, where it is possible to observe, in the first part of the chromatogram, 5 "waves" reflecting the five successive 2 mL sample applications. These are probably due to low hydrophobicity material. Even under these conditions, the narrow peak of hTSH showed practically the same t_R as the reference preparation: 2 μg dissolved in 2 μL. It is of interest that this entire purification step lasted for less than 1 h. HPSEC and RP-HPLC quality control of the pool obtained by preparative RP-HPLC is presented in Figs. 2B and 3B, showing purities of >99% in both analytical systems. HPSEC

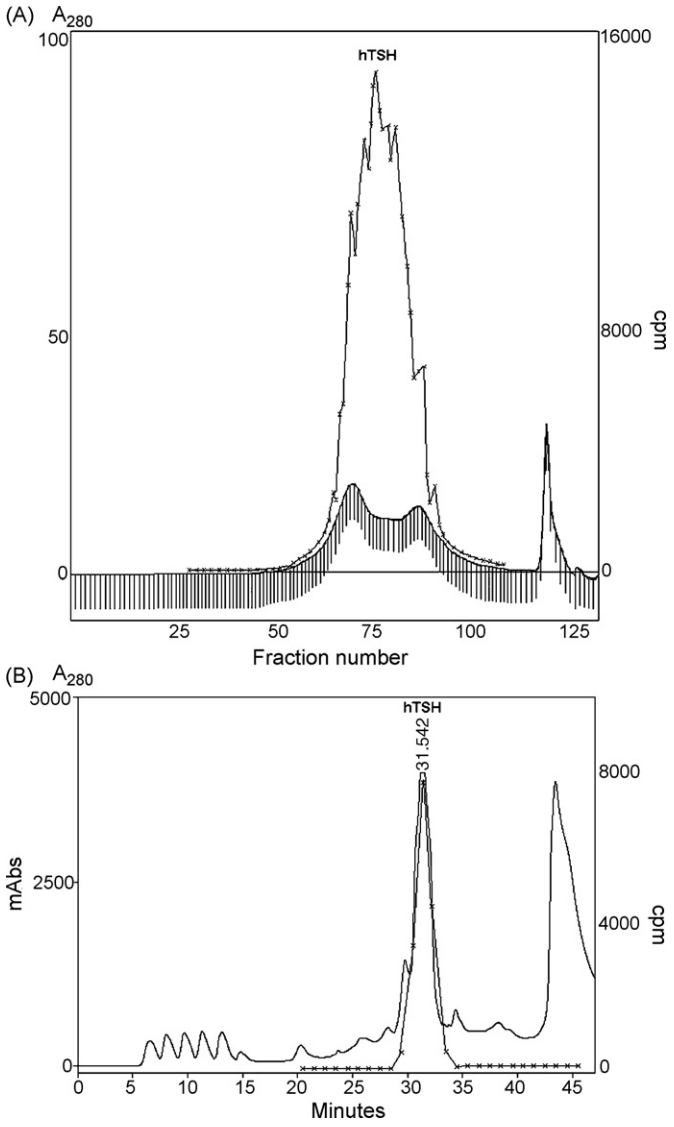

Fig. 1. Purification strategy for the laboratory production of CHO-derived hTSH on: (A) SP Sepharose FF (10 cm × 2.6 cm I.D.). (B) Semi-preparative RP-HPLC (25 cm × 10 mm I.D.): 5 × 2 mL sample application in a single run. (—) A_{220}; (×–×–×) immunoactivity determined via hTSH IRMA. In a parallel run, the hTSH reference preparation (2 μg/2 μL) presented a t_R = 31.3 min.

Table 1
Recombinant hTSH purification table, starting from approximately 10 L of CHO-conditioned medium

Purification step	Total protein[a] (mg)	hTSH (mg)	Step yield (%)	Mass fraction (%)
Conditioned medium	1604	5.67[b]	—	0.35
Cationic exchanger eluate	37.4	4.76[c]	84	12.7
RP-HPLC eluate	2.84	2.95[c]	83	104

[a] Estimated by BCA assay or Lowry method.
[b] Estimated by RP-HPLC.
[c] Estimated by HPSEC.

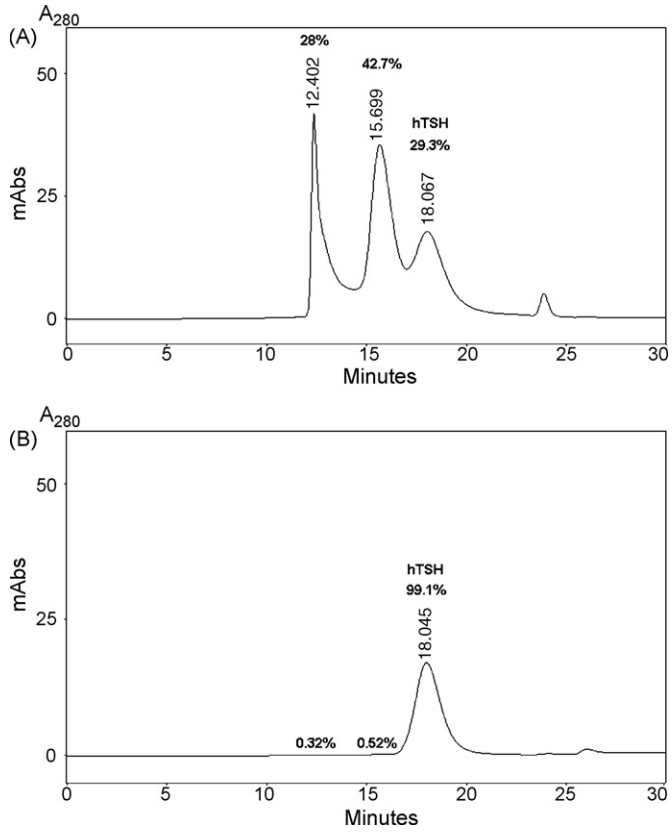

Fig. 2. HPSEC profiles of the pools coming from the two purification steps: (A) cation-exchange chromatography on SP-Sepharose FF. (B) Semi-preparative RP-HPLC. The retention time of the recombinant reference preparation of hTSH in this experiment was 18.06 min.

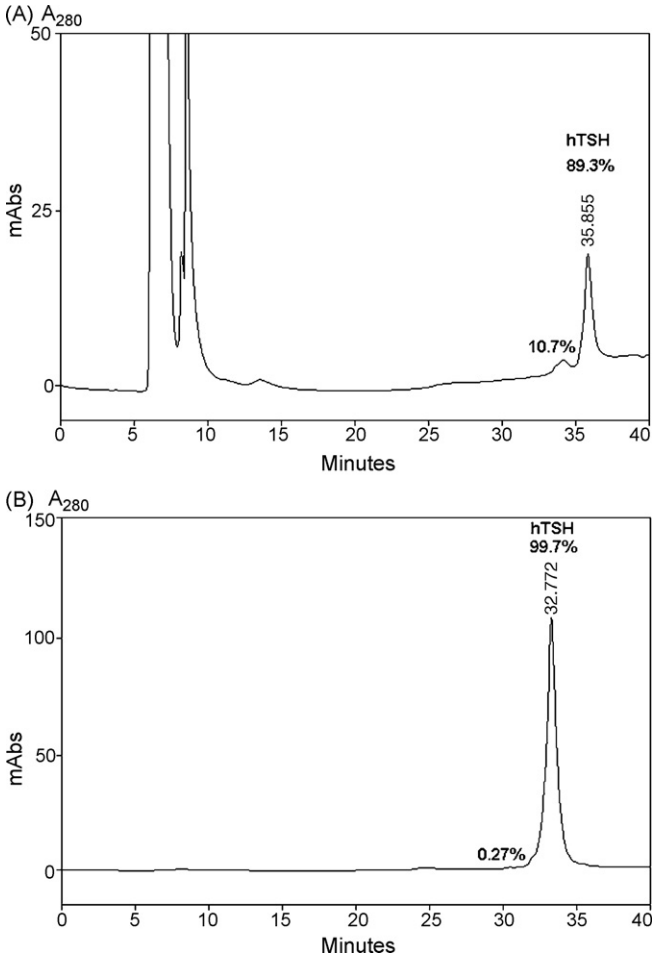

Fig. 3. RP-HPLC profiles of the pools coming from the two purification steps: (A) cation-exchange chromatography on SP-Sepharose FF. (B) Semi-preparative RP-HPLC; the retention time of the recombinant reference preparation of hTSH in this last experiment was 32.83 min.

was also used for the final hTSH quantification before and after lyophilization of the product, providing, for example, in the experiment reported in Table 1, a concentration of 1.2 mg hTSH/mL. When necessary, the product can be further concentrated to >3 mg/mL by filter device centrifugation. After dialysis against a buffer containing glycine, the product was successfully lyophilized with practically no loss and very little increase in the product-related impurities (<1.5% for HPSEC and <3.5% for RP-HPLC).

From Table 1, based on mass fractions, it is possible to calculate the purification factors for each chromatographic step: 36× for cation-exchange chromatography and 8× for RP-HPLC. This can be somehow misleading, apparently indicating a higher purification power for the first column, a conventional cation exchanger already utilized as the first column by different authors for CHO-derived TSH or LH purification [24,25,31]. As a matter of fact, the first step of a process is normally able to provide a higher purification factor, especially when starting from crude material with a quite low mass fraction of the protein of interest. We proved this experimentally by just repeating an analogous purification scheme in which the semi-preparative RP-HPLC was used as a first chromatographic step (data not shown). Under these conditions, semi-preparative RP-HPLC presented in fact a purification factor of 228×, confirming its great resolving power. On such a small semi-preparative column, however, it is only possible to apply relative small sample volumes and amounts of protein, factors that argue against its use in the initial step.

The single-dose *in vivo* bioassay, based on thyroxine stimulation in mice [32], showed that, compared to Thyrogen, our purified hTSH presented a relative potency of 1.13 (Table 2), i.e., an absolute bioactivity of 4.5 ± 0.98 IU/mg. This value, however, was not found to be significantly different from that of 4.0 IU/mg declared for Thyrogen [7,33].

Table 2
In vivo bioassay: levels of T_4 determined by RIA after hTSH administration to T_3-treated mice

Preparation	Mice number	$T_4 \pm SD$ (μg/dL)[a]	RSD[c] (%)
Saline	6	n.d.[b]	–
hTSH-NIDDK	6	2.0 ± 0.31	15.5
Thyrogen	6	3.2 ± 0.40	12.5
hTSH-IPEN	6	3.6 ± 0.78	21.7

[a] Each value is the average of 6 mouse sera determinations, each serum being analyzed in duplicate.
[b] Non detectable.
[c] Relative standard deviation.

4. Discussion

A practical, cheap and rapid semi-preparative RP-HPLC purification step, included after a conventional cation exchange-chromatography, has provided practically pure hTSH (>99%), whose biological potency was indistinguishable from that of the purest recombinant preparation. Starting from 1.6 g of extremely impure and dilute crude material in 10 L of medium (hTSH mass fraction = 0.35%), after the first concentration-purification step, ∼37 mg of protein (hTSH mass fraction = 12.7%) could be applied to RP-HPLC, thus providing ∼3 mg of pure hTSH (mass fraction = 100%). It is noteworthy that the protein load, dissolved in 10 mL and repeatedly injected onto the column in a single run, did not affect at all the retention time of the peak of hTSH, where practically all of the immunoactivity was localized. The great purification power of this small, semi-preparative column was also demonstrated by a simple experiment in which the same crude material was shown to be enriched 228-fold when purified directly through this first column. Also of interest is the enormous volume concentration (4000×) that was obtained by these two chromatographic steps, without considerable hTSH aggregation or activity losses.

Comparing our results with the only other hTSH purification process that is reported in details [24], where the crude material contained 25 μg hTSH/mL at a mass fraction of 26%, we note that, even starting with 42-fold less concentrated and 74-fold less pure material, it was possible to obtain a product that was at least as pure as that obtained by Cole and coworkers after the same number of purification steps. These authors employed two more columns for the final polishing required for attaining the biopharmaceutical "injection grade".

The starting material for this purification process was also ∼8-fold less concentrated and ∼8-fold less pure than that we used in previous work according to a different purification procedure [7]. In the present work, we obtained practically the same purity in less than one half the processing time and with much higher yields: 70% versus 37%. It is also important to emphasise the good bioactivity of the final product, which was not significantly different from the recombinant reference preparation of hTSH, even after a particularly critical RP-HPLC purification in the presence of up to 25% acetonitrile in the mobile phase. The new bioassay scheme, based on a relatively high number of replicates of a unique dose, provided a precision that is quite good for an *in vivo* response, with coefficients of variation of the order of 15%, in general.

In laboratory work, when processes are not yet well controlled and when different hosts, methods, reagents and conditions are tested, it is very common to obtain low secretion yields and mass fractions. We believe that this type of process can be easily adapted to different proteins of pharmaceutical or biotechnological interest, being very useful for obtaining, in a short time and at low cost, enough pure material that it can be fully characterized and used for further studies and preliminary applications. This will pave the way for the consequent utilization of preparative RP-HPLC in downstream processing of these same products, keeping in mind that, already in 1999, approximately one-third of the worldwide market for US Food and Drug Administration-approved recombinant biotherapeutics was also based on this technique for their purification [8].

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