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Two-step chromatographic purification of recombinant human thyrotrophin and its immunological, biological, physico-chemical and mass spectral characterization

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Abstract

A purification strategy for rapidly obtaining recombinant human thyrotropin (rhTSH) was designed based on size exclusion and reversed-phase high-performance liquid chromatographic (HPLC) analysis, carried out on hTSH-secreting CHO cell conditioned medium. These analyses permitted the identification of the main contaminants to be eliminated. Considering that hTSH is highly hydrophobic and elutes only with the addition of organic solvents, hydrophobic interaction chromatography was adopted as the first purification step; this resulted in the elimination of, among others, the major contaminant. A second purification step, based on size exclusion chromatography, was then utilized, being effective in the elimination of other previously identified contaminating proteins. Useful purity, as high as 99% at the chemical reagent level, and recoveries (37%) were obtained by adopting this two step strategy, which also provided adequate material for physico-chemical, immunological and biological characterization. This included matrix-assisted laser desorption ionization time-of-flight mass spectral analysis (MALDI-TOF-MS), Western blotting analysis, in vivo biological assay, size-exclusion HPLC (HPSEC) and reversed-phase HPLC (RP-HPLC) analysis, which confirmed the integrity and bioactivity of our rhTSH in comparison with the only two reference preparations available at the milligram level of native (hTSH-NIDDK) and recombinant (Thyrogen) hTSH. Thyrogen and rhTSH-IPEN, when compared to pit-hTSH-NIDDK, presented more than twice as much biological activity and about 7% increased molecular mass by MALDI-TOF-MS analysis, an accurate heterodimer mass determination providing the M_r values of 29 611, 29 839 and 27 829, respectively. The increased molecular mass of the two recombinant preparations was also confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and HPSEC analysis. Comparing the two recombinant preparations, minor though interesting physico-chemical and biological differences were also observed.
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Keywords: Thyrotropin; Purification; HPLC; MALDI-TOF; Biological assay; SDS–PAGE

1. Introduction

Recombinant thyrotropin (rTSH) is a glycoprotein hormone used as a pharmaceutical with high clinical and aggregate value. Recent reports have demonstrated the successful use of this hormone in the follow-up and treatment of patients with thyroid cancer [1–6], as well as in several other medical applications [7,8]. Besides the clinical applications, important laboratorial use has also been described [9,10].

We have previously described the synthesis of rhTSH expressed in mammalian hosts (Chinese hamster ovary, CHO cells) after co-transfection with two dicistronic expression vectors [11]. Given the importance of this product, our goal in the present work was the purification and physico-chemical, immunological and biological characterization of this hormone in comparison with the only recombinant hTSH commercially available (Thyrogen) and with native pituitary human TSH (hTSH) from the National Hormone and Pituitary Program (USA).

Purification protocols described in the literature for rhTSH, and recently reviewed [12], generally consist of

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Table 1
Literature values of relative molecular mass (M_r) obtained via MALDI-TOF-MS analysis for human pituitary or placental glycoprotein hormones

Hormone	Reference	α-Subunit	β-Subunit	Heterodimer		
				Experimental	α + β	Calc/exp
plac-hCG	[25]	13 408	21 446	35 140	34 854	0.992
rhFSH	[26,27]	∼14 000	∼17 000		∼31 000	
pit-hFSH	[28]	14 377.1	13 450		27 827.1	

multi-step procedures involving conventional chromatographic methods such as affinity, ion-exchange (cationic or anionic) and gel filtration chromatography [13–15]. While there is a certain emphasis on dye affinity chromatography, none describe the utilization of hydrophobic interaction chromatography. In the present work, we combined the utilization of hydrophobic and gel filtration chromatography for the selective detection, identification and efficient purification of rhTSH expressed in CHO cells. This scheme was designed taking into account the physico-chemical properties of the proteins generally present in CHO culture supernatant, relying on literature information [16] and our analysis of the conditioned medium from hTSH-secreting CHO cells by size-exclusion HPLC (HPSEC) and by a RP-HPLC method developed in our laboratory [17]. Very few workers have utilized hydrophobic interaction chromatography in their protocols for hTSH purification. Torjesen [18] used Phenyl Sepharose after gel filtration and anion exchange chromatography for separating pituitary hTSH from hFSH, thus obtaining higher yields and purity when compared to previously described procedures [19]. Hiyama et al. [20] adopted Phenyl Sepharose chromatography to separate hTSH and hLH from hFSH still in pituitary extracts. Gadkari et al. [21] used hydrophobic interaction procedures for purifying recombinant glycoprotein hormones of the same family, hLH and hCG, obtained from Pichia pastoris. The elimination of an affinity chromatography step from the hTSH purification scheme is interesting for therapeutic applications because of the potential toxicity and safety problems related with the possible release of the immobilized ligand, that might contaminate the product [22].

Several physico-chemical, immunological and biological assays (in vitro or in vivo) have been described in the literature for recombinant hTSH characterization [7,14,23,24]. We used analogous physico-chemical and immunological methods, with the important addition of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), an extremely sensitive and accurate methodology whose application to molecular mass determination of pituitary and placental glycoprotein hormones has up to now been very limited. As indicated in Table 1 , as far as we know, only three other research groups have carried out a similar determination on these heterodimeric human glycoprotein hormones, namely pituitary and recombinant hFSH, placental chorionic gonadotropin and their α- and β-subunits. Mention should be made, however, of other extensive MALDI-TOF-MS analyses carried out by Bousfield et al. [29] for equine LH, FSH and CG α- and β-subunits structural characterization. For biological activity determination, rather than use one of the available in vitro bioassays, we chose instead to employ a modification [30] of the classical in vivo McKenzie method [31]. This assay has the advantage that it determines a potency parameter that takes into account all the integrated in vivo physiological responses, namely circulating half-life ($t_{1/2}$), receptor affinity and activation of intracellular signal transduction pathways, all of which are apparently influenced by glycosylation [32]. This is particularly important if we want to include effects due to the increased $t_{1/2}$ of highly sialylated CHO-derived hTSH molecules [15,33]. Besides the methodologies described, our work also emphasizes the important comparison between our product and two reference preparations of pituitary and recombinant hTSH available at the milligram level, as well as the frequently overlooked comparison between the native and the synthetic hormones.

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 Satelit (Araraquara, Brasil). All other chemicals were analytical reagent grade, purchased from Merck (São Paulo, Brazil) and Sigma (St. Louis, MO, USA). Antibody anti hTSH (MAb TC 14) was kindly provided by the National Institute for Biological Standards and Control (Hertforshire, UK). Chromatographic resins were purchased from Amersham Biosciences (São Paulo, Brazil). CHO cultivation medium (CHO-S-SFM II) was provided by Gibco-BRL (Gaithersburg, MD, USA). Recombinant hTSH (Thyrogen) from Genzyme (Framingham, MA, USA), lot no. 2750598, was purchased from Biobras (Montes Claros, Brasil). Pituitary hTSH (pit-hTSH, NIDDK-hTSH SIAFP-B-2) was kindly provided by Dr. A.F. Parlow of the National Hormone and Pituitary Program (Torrance, CA, USA). Solid-phase hTSH immunoradiometric assay (IRMA) was purchased from Skybio (Wyboston, Bedford, UK).

2.2. Cell cultivation

A clone, obtained in our laboratory (IPEN), derived from CHO DHFR$^-$ cells (mutant line DXB11) cotransfected with

the dicistronic vectors pEDdc-α and pEAdc-βTSH expressing human TSH [11], was cultured in 162 cm^2 flasks (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 10 mM methotrexate, which allows a selective pressure. Conditioned media (20 mL/flask) were harvested every day, a production lot (1–4 L) being obtained by pooling the harvested fluid from 10 to 20 days cultivation period.

2.3. Purification process

The purification process started with 1–4 L of hTSH-secreting CHO cell conditioned medium, which were concentrated 16× and diafiltered (against 20 mM phosphate buffer, pH 7.0) in a tangential flow filtration system (Millipore, Bedford, MA, USA) with a Pellicon membrane (M_r 10 000 cut-off). Following the addition of NaCl to this material to a final concentration of 1 M NaCl, the solution was loaded onto a 10 cm × 1.6 cm i.d. glass column packed with Phenyl-Sepharose CL 4B that had been previously equilibrated with 1 M NaCl, 20 mM phosphate buffer, pH 7.0. The bound proteins were eluted by washing with 5 column volumes of the following buffers: 1 and 0.6 M NaCl in 20 mM phosphate buffer, pH 7.0; 20 and 0.3 mM phosphate buffer, pH 7.0. The protein of interest was eluted with 40% ethanol in 0.3 mM phosphate buffer. This eluate was applied to a 90 cm × 2.6 cm i.d. Sephacryl S-100 column, equilibrated with 0.15 M NaCl in 20 mM sodium phosphate buffer. The hTSH peak resultant from this step was identified by IRMA, followed by addition of 1 mg/mL of lactose and lyophilization of the product for storage.

2.4. Protein determination

Total protein concentration was estimated by using bicinchoninic acid (BCA) and pure bovine serum albumin (BSA, Sigma, São Paulo, Brazil) as standard, according to the manufacturer's instruction (Micro BCA protein assay kit, Pierce, Rockford, IL, USA).

2.5. Immunoradiometric assay

The immunologic quantification of hTSH was carried out by an in-house, "sandwich" format, IRMA, utilizing a secondary hTSH standard calibrated against the International Standard of pituitary hTSH (WHO 80/558, 4.93 IU/mg) [9].

2.6. 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). A silica precolumn (packed with LiChrosorb Si 60, 7.9–12.4 μm, Merck, Darmstadt, Germany) was inserted between the pump and the injector. This silica precolumn (a silica saturator) was introduced by Riggin et al. [34] 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 analysis of peak area against pituitary-hTSH. Mobile phase A was 0.05 M sodium phosphate buffer (pH 7.0) and mobile phase B was 50% acetonitrile plus 50% mobile phase A. A linear gradient of 25–100% B over 40 min was used at a flow-rate of 0.5 mL/min.

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

HPSEC was carried out with the same Shimadzu apparatus, processing the samples 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. HPSEC was also used for the M_r determination of the three hTSH preparations, comparing their retention times with those of four standard proteins: bovine serum albumin (M_r 66 200), ovoalbumin (42 700), human growth hormone (22 400) and cytochrome *c* (12 500).

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Different hTSH preparations were analyzed by SDS–PAGE, carried out according to Laemmli's method [35] on a 15% polyacrylamide gel under non-reducing conditions. A low molecular mass electrophoresis calibration kit (Amersham Biosciences) was used for the molecular mass markers. Proteins were visualized by staining the gels with Coomassie Brilliant Blue G-250.

2.9. Western blotting

Following protein separation by SDS–PAGE, proteins were transferred onto a nitrocellulose membrane for immunoblot analysis, carried out according to Burnette [36]. Proteins were visualized using rabbit anti-human TSH, at a 1:100 dilution and ^{125}I-Protein A, prepared by the Chloramine T labelling technique [37].

2.10. "In vivo" bioassay

Relative biological activity of the purified hormone rhTSH-IPEN was determined by comparison with the International Reference Preparation of pituitary hTSH (pit-hTSH, NIDDK, SIAFP-B-2) and with rhTSH-Thyrogen, a commer-

cial preparation from Genzyme, employing an in vivo bioassay in which TSH-induced T_4 is measured after suppression of endogenous TSH by administration of T_3 [30]. Thirty-nine mice were utilized, three for each dose (1; 5; 10; 20 μg) of the three different TSH preparations, plus zero dose.

2.11. Mass spectrometry

MALDI-TOF-MS analyses of rhTSH-IPEN, rhTSH-Thyrogen and pituitary hTSH (NIDDK) were carried out by Commonwealth Biotechnologies, Richmond, VA, USA using sinapinic acid (SA) as the matrix and a Voyager-DE BioSpectrometry Workstation from Applied Biosystems (Foster City, CA, USA).

3. Results

The purification scheme developed in this work for obtaining pure, biologically active rhTSH (rhTSH-IPEN) was based on two chromatographic techniques (hydrophobic interaction chromatography and gel filtration) exploiting key differences in hydrophobicity and molecular mass between the contaminant proteins and hTSH, as observed in the crude conditioned medium. Mean rhTSH specific activity, determined in different production lots via RP-HPLC methodology (Table 2), was 3.2 ± 0.64 μg hTSH/μg protein × 100 (CV = 20%, n = 12). As shown by the HPSEC profile (Fig. 1A), hTSH, which was identified by IRMA in comparison with the retention time of the recombinant reference preparation, eluted after two major contaminants of higher molecular mass, with retention times (t_R) of 11.8 and 14.7 min, respectively. Some immunological activity was found to be associated with these peaks, suggesting that they might contain hTSH-related forms of higher molecular mass, such as aggregates or dimers. On the other hand, RP-HPLC analysis (Fig. 1B) showed that most of the contaminant proteins present in conditioned medium are less hydrophobic than hTSH, which eluted with a retention time of 32.8 min. A large peak with t_R = 41.3 min (out of the figure) is, in fact, due to gradient and culture medium components. In this case, the main protein contaminant corresponds to a peak with t_R = 30.4 min which, as will be shown below, appears to be the same material eluting with t_R = 14.7 min on HPSEC. Taking advantage of the differences in molecular size and hydrophobicity between TSH and the contaminants detected in the starting material, a two step (Phenyl Sepharose CL4B and Sephacryl S-100) purification process that led to >90% pure rhTSH was established.

Table 2
Specific activity of hTSH in different production lots carried out in 162 cm^2 flasks

Lot no.	hTSH[a] (μg/mL)	Protein[b] (μg/mL)	Specific activity (μg hTSH/μg protein) (%)
1	4.7	100.8	4.7
2	5.3	142.8	3.7
3	5.3	160.6	3.3
4	4.6	170.6	2.7
5	5.6	157.6	3.6
6	3.7	136.0	2.7
7	4.1	117.3	3.5
8	3.2	129.8	2.5
9	5.0	180.4	2.8
10	4.0	158.6	2.5
11	4.5	141.2	3.2
12	3.4	116.5	2.9

[a] Determined by RP-HPLC.
[b] Determined by BCA.

Fig. 2 presents the result of the first purification step, based on hydrophobic interaction chromatography on Phenyl Sepharose CL4B. Relatively high immunoactive protein peaks were obtained in three of the five elution steps (I, IV, V). Under the other two elution conditions (II, III) the protein peaks were small, but nonetheless immunologically active. The analysis of peak I by HPSEC and RP-HPLC (Fig. 3) showed that 1 M NaCl in 0.02 M sodium phosphate, pH 7.0, elutes most of the contaminant characterized by $t_R \sim 15$ min on HPSEC and 30 min on RP-HPLC. A small amount of intact hTSH was present in this step, in the region predicted by the reference preparation in both HPLC systems. The elution with 0.003 M sodium phosphate, pH 7.0 (peak IV of Fig. 2), eliminated a significant fraction of the contaminant that elutes with a t_R of ~11 min, as well as forms more hydrophobic than hTSH (Fig. 4). On the basis of the t_R of the

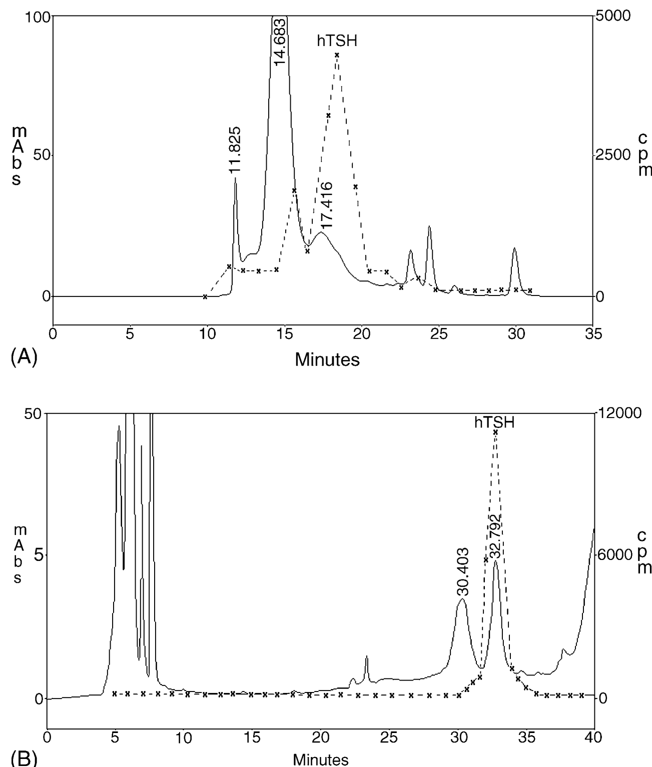


Fig. 1. HPLC profiles of a hTSH-secreting CHO cell conditioned medium: (A) HPSEC and (B) RP-HPLC. The retention times of the recombinant reference preparation in this experiment were 17.6 and 32.78 min, respectively, in the HPSEC and RP-HPLC analyses ((—) A_{220} and (- - -) hTSH IRMA).

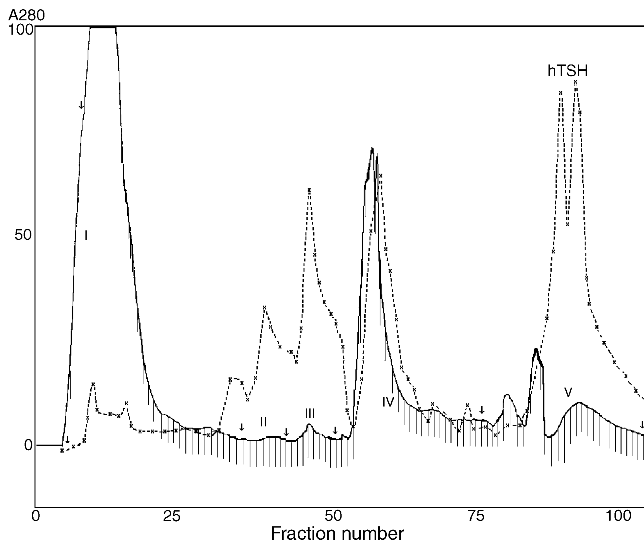


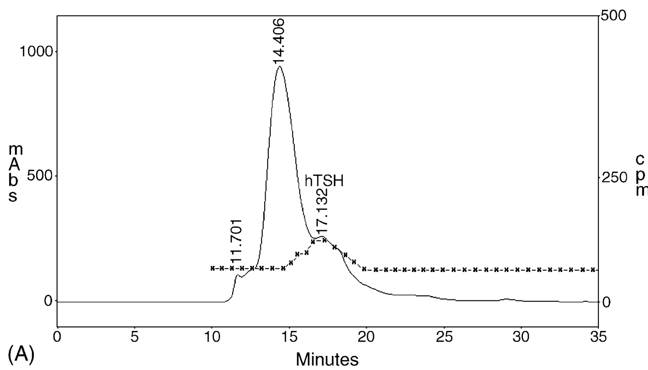
Fig. 2. Chromatogram resultant of the purification of hTSH-secreting CHO cell conditioned medium on Phenyl Sepharose CL 4B. Different elution steps, identified by arrows, using: (I) 0.02 M Na_2HPO_4, 1 M NaCl pH 7.0; (II) 0.02 M Na_2HPO_4 pH 7.0, 0.6 M NaCl; (III) 0.02 M Na_2HPO_4 pH 7.0; (IV) 0.3 mM Na_2HPO_4 pH 7.0; (V) 0.3 mM Na_2HPO_4 40% ethanol pH 7.0. ((—) A_{280} and (- - -) hTSH IRMA).

reference preparation, no intact hTSH appears to be present in this fraction. When analyzed on HPSEC and RP-HPLC, the hTSH that eluted with 40% ethanol (peak V), showed the profiles presented in Fig. 5. A good purification factor (25-fold) was achieved with hydrophobic interaction chromatography, although a considerable amount of high molecular mass material was still present in the sample. The recovery of hTSH in this step was estimated to be 62%.

The pool obtained from Phenyl Sepharose CL4B chromatography was further purified by gel filtration (Sephacryl S-100). Three peaks were obtained in this step, the main one presenting immunological activity. This peak was analysed by HPSEC and RP-HPLC (Fig. 6A and B). Considering the HPSEC profile, the main peak presented 94% purity. The same analysis, based on protein content determined by BCA, provided a 90% purity for this product. A more restrictive pool of this peak, eliminating the fraction on the left side where the interference of the contaminant with $t_R \sim 15$ min is higher, resulted in a higher purity (99%), when analyzed by HPSEC (Fig. 6C).

Characterization of the purified product was carried out by evaluating purity, identity, molecular mass, biological and immunological activity.

The electrophoretic mobility of the purified hTSH was compared with rhTSH-Thyrogen and pit-hTSH by SDS–PAGE under non-reducing conditions (Fig. 7A); one



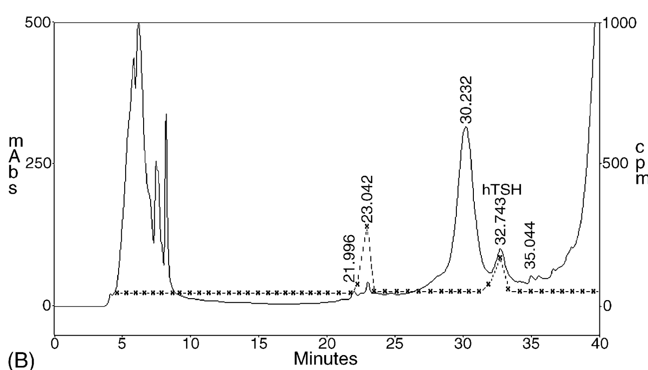
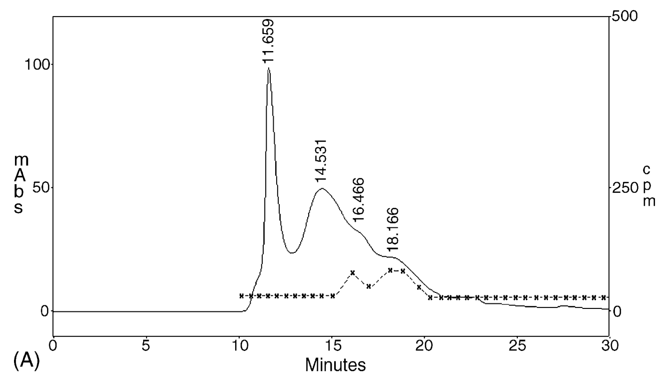


Fig. 3. HPSEC (A) and RP-HPLC (B) analyses of peak I eluted from Phenyl Sepharose CL 4B with 0.02 M Na_2HPO_4, 1 M NaCl, pH 7.0. The retention times of the recombinant reference preparation in this experiment were 17.15 and 32.81 min, respectively ((—) A_{220} and (- - -) hTSH IRMA).



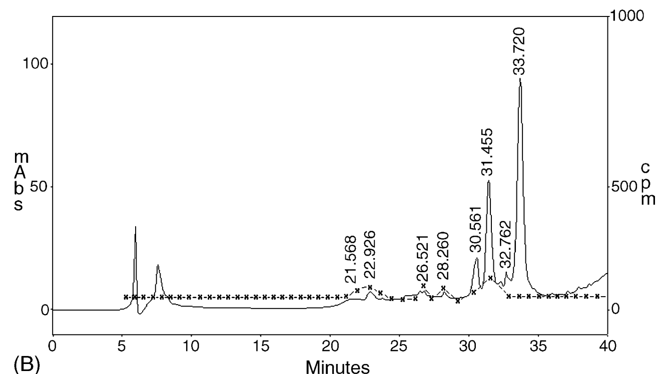


Fig. 4. HPSEC (A) and RP-HPLC (B) analyses of peak IV eluted from Phenyl Sepharose CL 4B with 0.3 mM Na_2HPO_4, pH 7.0. The retention times of the recombinant reference preparation in this experiment were 17.15 and 32.81 min, respectively ((—) A_{220} and (- - -) hTSH IRMA).

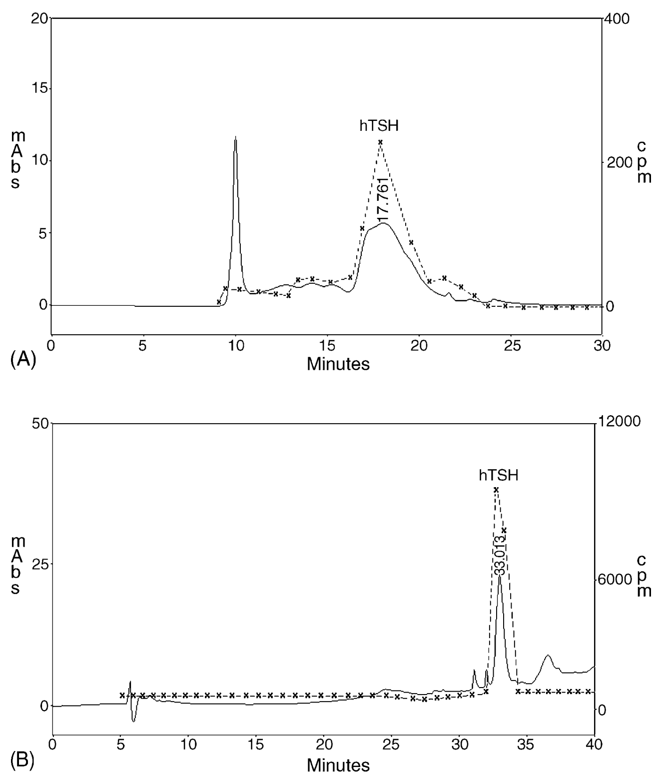


Fig. 5. Analysis of peak V eluted from Phenyl Sepharose CL4B with 40% ethanol in 0.3 mM phosphate buffer, pH 7.0: (A) HPSEC and (B) RP-HPLC. The retention times of the recombinant reference preparation in this experiment were 17.6 and 32.78 min, respectively ((—) A_{220} and (- - -) hTSH IRMA).

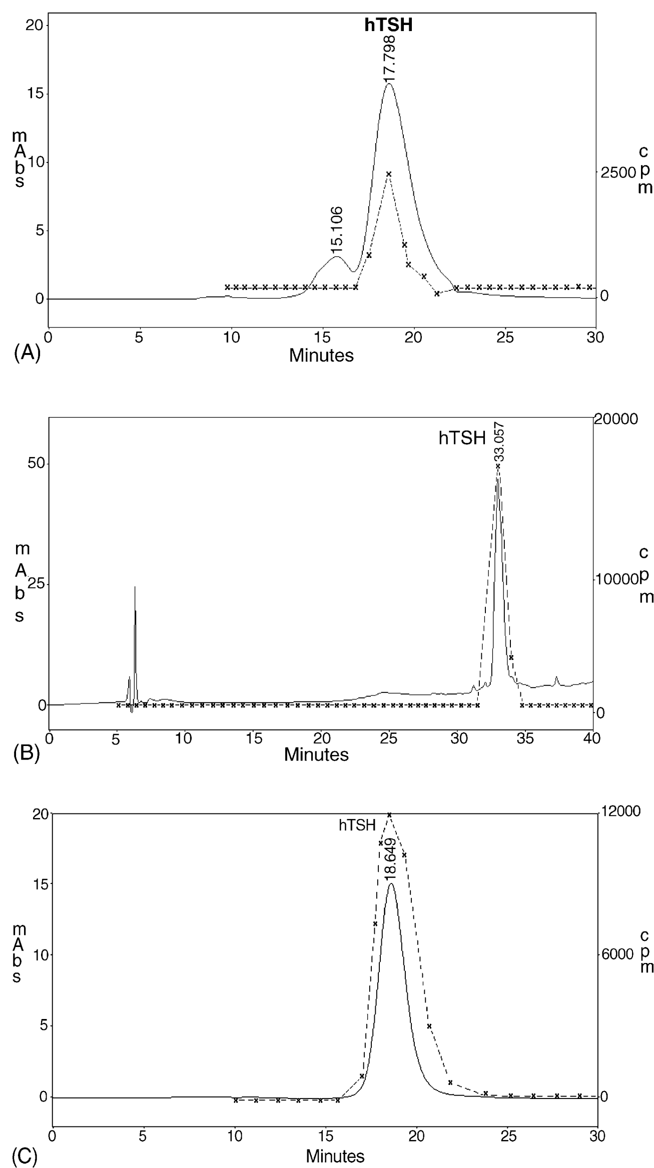


Fig. 6. Analysis of rhTSH eluted from Sephacryl S-100: (A) HPSEC and (B) RP-HPLC. The retention times of the recombinant reference preparation in this experiment were 17.6 and 32.78 min, respectively. (C) HPSEC of a more restrictive pool of the peak of rhTSH eluted from Sephacryl S-100. The retention time of the recombinant reference preparation in this experiment was 18.60 min ((—) A_{220} and (- - -) hTSH IRMA).

band, with an apparent M_r between 22 000 and 30 000, which is higher than pit-hTSH and lower than Thyrogen, was observed. Western blotting (Fig. 7B) confirmed the identity of the purified hTSH and the absence of significant amounts of altered forms.

The in vivo biological activity of rhTSH-IPEN was determined by a bioassay in mice based on the stimulation of thyroxin (T_4) by TSH. Typical dose-response curves for rhTSH-Thyrogen, pit-hTSH and rhTSH-IPEN are shown in Fig. 8. From four independent assays, using rhTSH-Thyrogen as reference with a potency of 4.0 IU/mg [38], the average bioactivity values for rhTSH-IPEN and pit-hTSH were 3.10 ± 0.68 IU/mg (R.S.D. = 21.9%) and 1.47 ± 0.36 IU/mg (R.S.D. = 24.5%), respectively. We could not use pit-hTSH as a standard for biological activity determination because this parameter is not declared for this preparation.

The IRMA dose-response curves of the three hTSH preparations, shown in Fig. 9, indicate a higher immunological activity for the two recombinant preparations, but with significant parallelism between the linear regions of the three analyzed curves.

A molecular mass (M_r) determination of the recombinant and pituitary hTSH preparations was carried out by HPSEC. The retention times (t_R) of four standard proteins were correlated with their molecular masses, providing the equation:

$$\log M_r = -0.1006 t_R + 3.2688, \qquad r = 0.9925 \quad (n = 4)$$

According to this equation M_r of 34 500, 32 600 and 31 700 respectively for hTSH-Thyrogen, hTSH-IPEN and hTSH-NIDDK are estimated.

MALDI-TOF-MS was also used to compare rhTSH-IPEN with Thyrogen and native pit-hTSH (Fig. 10). The average molecular masses for these three preparations and their subunits, determined in two independent assays, are shown in Table 3 . These data are in reasonable agreement with the results already obtained via SDS–PAGE, Western blotting and

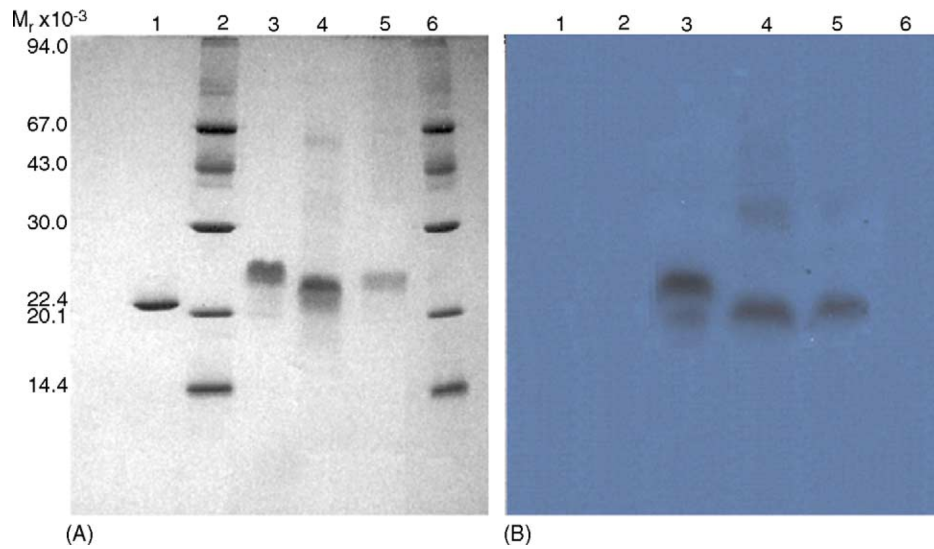


Fig. 7. (A) SDS–PAGE of rhTSH-Thyrogen (lane 3), pit-hTSH (lane 4) and rhTSH-IPEN (lane 5), loaded at 5 μg protein/lane and run on 15% polyacrylamide gels, stained with Comassie Brilliant Blue G. Molecular mass markers were loaded on lanes 1, 2 and 6. (B) Western-blotting was performed using anti-hTSH antiserum.

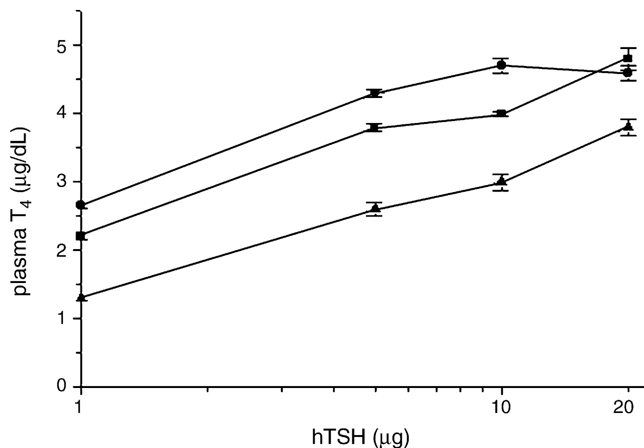


Fig. 8. Dose-dependent T_4 response curve obtained in TSH suppressed mice, 6 h after injection of rhTSH-Thyrogen (■), pit-hTSH (▲) and rhTSH-IPEN (●). All points are the mean of three animal determinations: the ranges for S.D. are indicated in the figure.

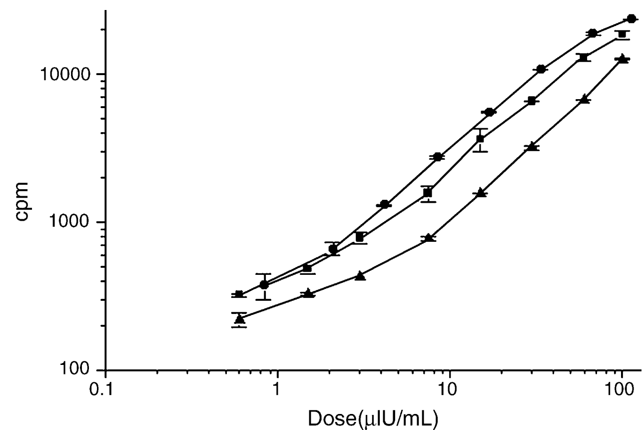


Fig. 9. Immunoradiometric dose–response curve obtained utilizing rhTSH-IPEN (●), rhTSH-Thyrogen (■) and pit-hTSH-NIDDK (▲) for the standard curve. All points are the mean of duplicate determinations: the ranges for S.D. are indicated in the figure.

HPSEC analysis, confirming a higher M_r for the recombinant preparations. The IPEN preparation presented a value of +7.2% molecular mass, relative to pit-hTSH-NIDDK, non-significantly different from the value of Thyrogen. It is also interesting to note the high interassay precision of the MALDI-TOF-MS determinations, the R.S.D. of which are, in general, below 1%, and the good agreement between the experimental values for the heterodimers and those calculated

Table 3
Average relative molecular mass (M_r) of the heterodimer and related subunits of different hTSH preparations, determined by MALDI-TOF mass spectrometry ($n = 2$ independent determinations), in comparison with the theoretical values provided by the literature

Preparation	α-Subunit (M_r ± S.D.)	β-Subunit (M_r ± S.D.)	Heterodimer (M_r ± S.D.)		
			Experimental	α + β	Calc./exp.
pit-hTSH (NIDDK)	13 018.5 ± 355.7	14 475.5 ± 21.8	27 829.0 ± 26.9	27 494.0	0.988
rhTSH (IPEN)	13 960.6 ± 55.9	15 359.2 ± 73.3	29 839.4 ± 208.7	29 319.8	0.983
rhTSH (Thyrogen)	13 859.5 ± 58.7	15 580.5 ± 0.71	29 611.5 ± 176.1	29 440.0	0.994
Theoretical values for Thyrogen[a]	13 820	15 840		29 660	

[a] Theoretical molecular masses are derived from the primary amino acid sequence with the contribution of carbohydrate structures added from the carbohydrate analysis [14].

by summation of the molecular masses of the two subunits. In addition, there is general agreement of the values obtained from MALDI-TOF-MS analysis for the heterodimers and the α- and β-subunits with those calculated by Cole et al. [14] for the Genzyme preparation. In this regard, it is obvious that the pituitary preparation presents the mass values that differ the most, due to the expected diversity in the carbohydrate structure synthesized by human thyrotropes instead of CHO cells. We should emphasize that all glycoproteins exhibit, in general, a great heterogeneity of carbohydrate chains, which is remarkably specific among glycoprotein hormones [7,25,39,40]. For a specific recombinant hormone, carbohydrate variability might arise either from the type of host cells used for the expression of the glycoprotein, from different cell cultivation and bioreactor conditions or from the presence of certain factors in the culture medium [12]. If we focalize CHO, the most widely used host cell for synthesis of therapeutic glycoproteins, glycosylation of rhTSH differs for example from that occurring in human thyrotropes, with respect to both the extent and type of glycosylation [14]. The overall level of glycosylation is higher for recombinant preparations and, since CHO cells lack *N*-acetylgalactosamine transferase and sulfo transferase, there is a consequent increase in the presence of sialylated galactose residues and no presence of *N*-acetylgalactosamine sulfate in the recombinant hormone. Another important difference between rhTSH and pit-hTSH is that the former seems to contain mainly biantennary and triantennary complex oligosaccharides, while the latter is reported to contain mainly biantennary complex structures [14,24,39,40].

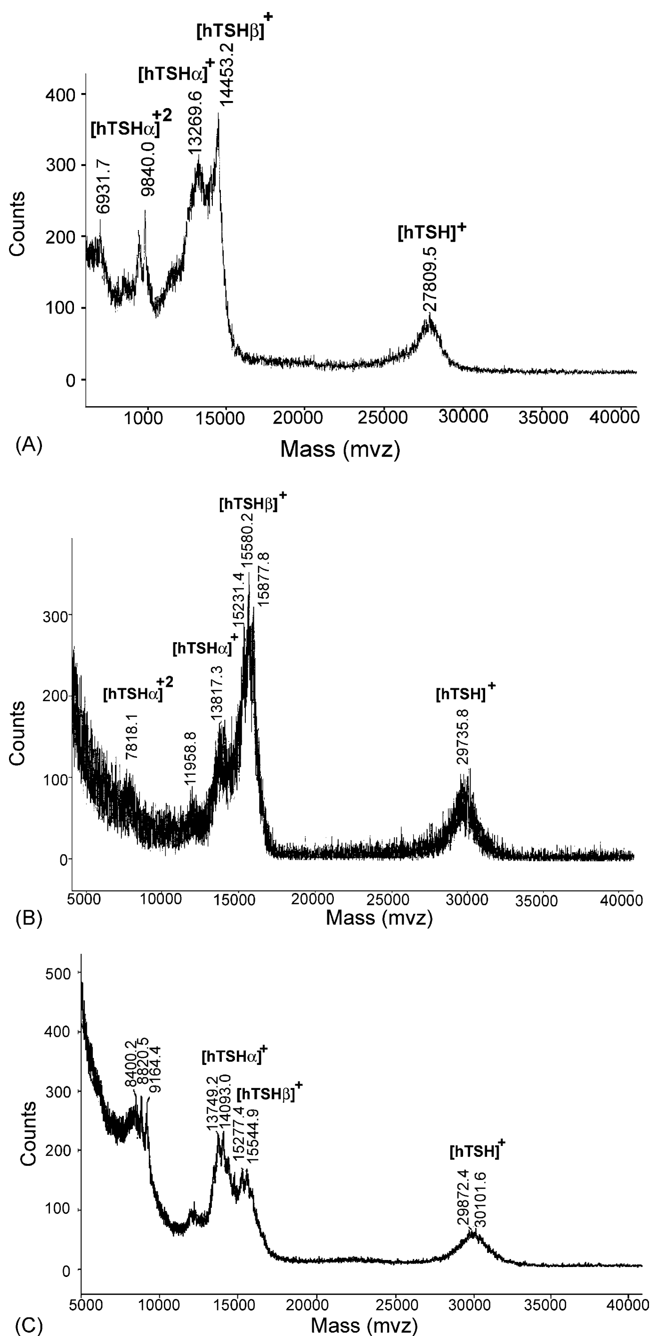


Fig. 10. MALDI-TOF-MS analyses of native hTSH (A), rhTSH-Thyrogen (B) and rhTSH-IPEN (C).

4. Discussion

Two well-standardized HPLC techniques, based on size exclusion and hydrophobic interactions, allowed good control of the production medium, as well as quite satisfactory lot reproducibility. The same analytical techniques were also used to detect the main contaminants and to choose and monitor the purification strategy. As a result, a rapid, two-step purification process, based on conventional and safe chromatographic matrices, was developed that provided rhTSH at the chemical reagent level, in 37% yield with a 90–99% purity range. In comparison with the only rhTSH purification process that has been described in detail [14], the present process started with material whose mass fraction was about 10-fold lower (0.03 versus 0.26 mg hTSH/mg total protein), obtaining, after two chromatographic steps, a purification factor of ∼28. Our strategy has thus been particularly successful when applied to quite poor crude material, in which the concentration of the protein of interest was determined by both quantitative and qualitative criteria, not normally considered in the more routinely used immunoassays [12].

For the first time, a newly synthesized rhTSH has been characterized by comparison with the highly purified reference preparation of pituitary hTSH from the National Hormone and Pituitary Program and with Thyrogen, the only recombinant preparation commercially available for clinical use. The IRMA dose–response curves of the two recombinant products exhibited a higher immunological activity, but still with significant parallelism in comparison with the pituitary reference preparations, confirming previous studies carried out on Thyrogen [9], which allows the use of rhTSH-IPEN for immunoassay reagent preparation. The in vivo bioactivity of the two recombinant hTSH preparations was also higher (about two-fold) than that of the pituitary preparation, rhTSH-

IPEN being about 20% less active than Thyrogen. This higher in vivo bioactivity of the recombinant products is probably due to the influence of the carbohydrate moiety, in particular sialic acid capping, on the metabolic clearance rate of the CHO-derived preparations, as emphasized by Szkudlinski et al. [7].

The HPSEC and SDS–PAGE characterization of the three preparations revealed differences in size in the following order: Thyrogen > rhTSH-IPEN > pit-hTSH. Confirming the tendency already reported in previous work [17], HPSEC indicated a mass increase of 8.8% and 2.8% for Thyrogen and rhTSH-IPEN, respectively, in comparison with pit-hTSH. The same mass increase could also be qualitatively observed by SDS–PAGE and Western blotting. Mass values of higher accuracy and precision were determined by MALDI-TOF-MS analysis, in which Thyrogen and rhTSH-IPEN presented mass increases of about 7%, in comparison with the pituitary preparation. The three experimental values obtained for Thyrogen (α-, β- subunits and heterodimer) presented differences of <1.6% relative to the theoretical values calculated by Cole et al. [14]. These same authors on the other hand obtained, by HPSEC, a M_r of 36 000, as compared to a value of 32 000 by low angle laser light scattering. Both values are in reasonable agreement with our M_r value of 34 500 determined for Thyrogen with a similar HPSEC methodology. All these estimates are, however, >7% above the values obtained by MALDI-TOF-MS analysis.

Starting with the pioneering work of Karas and Hillenkamp [41], this mass spectrometric technique has permitted, via the conversion of large macromolecules into intact gas-phase molecular ions, the analysis of proteins in general and of heterodimeric glycoproteins in particular. It has been reported that the quaternary structure of proteins can only be determined by MALDI-TOF-MS by matching particular matrix and solvent conditions [42,43]. We and others [25,44,45] have found that the presence of homo- and heterodimers is not only frequent, but sometimes difficult to avoid. The unexpected stability of the quaternary structure, at least in the case of hCG, has been attributed to the presence of large subunit surface areas (25–30% corresponding to approximately 4000 Å^2) which are buried by subunit binding and to the known "seat-belt" arrangement formed by the β-subunit C-terminal region wrapping around the α-subunit [46,47]. Thus, from an analytical point of view, we agree with Laidler et al. [25] that the possibility of simultaneous detection of heterodimer and α- and β-subunits increases enormously the power of MALDI-TOF-MS as an identification tool based on accurate molecular mass determination. Both our and literature data show that the peaks are wider and exhibit higher microheterogeneity in the case of these glycoprotein hormones [25,28,29,32,44,48] than for example those of highly purified human serum albumin preparations [45]. This may be due to variations in glycosylation and, in particular, in sialic acid content [25,44]. The data of Bousfield et al. [29], illustrating partial deglycosylation of equine LH α and FSH α resulting in slightly narrower peaks in MALDI-TOF-MS analysis, seem to confirm this. However, carbohydrate fragmentation may also occur as a consequence of post-ionization loss (post-source decay) of sialic acid, hexose and hexosamine residues due to a particular lability of these glycosidic bonds, which would introduce a negative bias into the mass determination [49,50]. The evident heterogeneity of our peaks cannot exclude this phenomenon. However, Laidler et al. [25] and our experimental values for the heterodimers agree within ∼1% with the values calculated by summing the α- and β-subunit mass values. Given also the agreement between the theoretical and experimental masses found for Thyrogen and its subunits, we think that an eventual bias is not significant enough to affect these determinations.

As recently emphasized by Flensburg and Belew [45] in the above mentioned work on MALDI-TOF-MS characterization of recombinant human serum albumin, this analytical tool is of increasing importance for characterizing gene products and for establishing the identity of, or key differences between, recombinant proteins and their natural counterparts, especially when these are intended for clinical use.

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References

[1] B.R. Haugen, E. Pacini, C. Reiners, M. Schlumberger, P.W. Ladenson, S.I. Sherman, D.S. Cooper, K.E. Graham, L.E. Braverman, M.C. Skarulis, T.F. Davies, L.J. DeGroot, E.L. Mazzaferri, G.H. Daniels, D.S. Ross, M. Luster, M.H. Samuels, D.V. Becker, H.R. Maxon, R.R. Cavalieri, C.A. Spencer, J. Clin. Endocrinol. Metab. 84 (1999) 3877.
[2] M. Luster, M. Lassmann, H. Haenscheid, J. Clin. Endocrinol. Metab. 85 (2000) 3640.
[3] M. Basaria, H. Graf, D.S. Cooper, Am. J. Med. 112 (2002) 721.
[4] R.J. Robbins, A.K. Robbins, J. Clin. Endocrinol. Metab. 88 (2003) 1933.
[5] E. Bombardieri, E. Seregni, C. Villano, G. Aliberti, F. Mattavelli, Tumori 89 (2003) 533.
[6] M. Torlontano, U. Crocetti, L. D'Aloiso, N. Bonfitto, A. Di Giorgio, S. Modoni, G. Valle, V. Frusciante, M. Bisceglia, S. Filetti, M. Schlumberger, V. Trischitta, Eur. J. Endocrinol. 148 (2003) 19.
[7] M.W. Szkudlinski, V. Fremont, C. Ronin, B.D. Weintraub, Physiol. Rev. 82 (2002) 473.
[8] C.H. Emerson, M.S.T. Torres, Biodrugs 17 (2003) 19.
[9] M.T.C.P. Ribela, A.C. Bianco, P. Bartolini, J. Clin. Endocrinol. Metab. 81 (1996) 249.
[10] B. Rafferty, R.G. Das, Clin. Chem. 45 (1999) 2207.
[11] C.N. Peroni, C.R.J. Soares, E. Gimbo, L. Morganti, M.T.C.P. Ribela, P. Bartolini, Biotechnol. Appl. Biochem. 35 (2002) 19.
[12] M.T.C.P. Ribela, P.W. Gout, P. Bartolini, J. Chromatogr. B 790 (2003) 285.

[13] N.R. Thotakura, R.K. Desai, L.G. Bates, E.S. Cole, B.M. Pratt, B.D. Weintraub, Endocrinology 128 (1991) 341.
[14] 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, Biotechnology 11 (1993) 1014.
[15] M.W. Szkudlinski, N.R. Thothakura, I. Bucci, L.R. Joshi, A. Tsai, J. East-Palmer, J. Shiloach, B.D. Weintraub, Endocrinology 133 (1993) 1490.
[16] A.T. Andrews, I. Noble, S. Keeratipibul, J.A. Asenjo, Biotechnol. Bioeng. 44 (1994) 29.
[17] J.E. Oliveira, F. Mendonça, C.N. Peroni, P. Bartolini, M.T.C.P. Ribela, J. Chromatogr. B 787 (2003) 345.
[18] P.A. Torjesen, Aker University Hospital, 0514 Oslo, Norway, Personal communication, 1989.
[19] P.A. Torjesen, T. Sand, N. Norman, O. Trygstad, I. Foss, Acta Endocrinol. 77 (1974) 485.
[20] J. Hiyama, A. Surus, A.G.C. Renwick, J. Endocrinol. 125 (1990) 493.
[21] R. Gadkari, R. Deshpande, R.R. Dighe, Protein Exp. Purif. 32 (2003) 175.
[22] N.E. Labrou, J. Chromatogr. B 790 (2003) 67.
[23] A. Hussain, C.A. Zimmerman, J.A. Boose, J. Froehlich, A. Richardson, R.S. Horowitz, M.T. Collins, R.W. Lash, J. Clin. Endocrinol. Metab. 81 (1996) 1184.
[24] M. Szkudlinski, M. Grossmann, H. Leitolf, B.D. Weintraub, Methods 21 (2000) 67.
[25] P. Laidler, D.A. Cowan, R.C. Hider, A. Keane, A.T. Kicman, Rapid Commun. Mass Spectrom. 9 (1995) 1021.
[26] C.M. Howles, Hum. Reprod. Update 2 (1996) 172.
[27] 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.
[28] W.J. Walton, V.T. Nguyen, V.Y. Butnev, V. Singh, W.T. Moore, G.R. Bousfield, J. Clin. Endocrinol. Metab. 86 (2001) 3675.
[29] G.R. Bousfield, V.L. Baker, R.R. Gotschall, V.Y. Butnev, V.Y. Butnev, Methods 21 (2000) 15.
[30] J. East-Palmer, M.W. Szkudlinski, J. Lee, N.R. Thotakura, B.D. Weintraub, Thyroid 5 (1995) 55.
[31] J.M. McKenzie, Endocrinology 63 (1958) 372.
[32] V.Y. Butnev, R.R. Gotschall, V.L. Baker, W.T. Moore, G.R. Bousfield, Endocrinology 137 (1996) 2530.
[33] M. Grossmann, R. Wong, N.G. Tech, J.E. Tropea, J. East-Palmer, B.D. Weintraub, M.W. Szkudlinski, Endocrinology 138 (1997) 92.
[34] R.M. Riggin, G.K. Dorulla, D.J. Miner, Anal. Biochem. 167 (1987) 199.
[35] U.K. Laemmli, Nature 227 (1970) 680.
[36] N.W. Burnette, Anal. Biochem. 112 (1981) 195.
[37] M.T.C.P. Ribela, Y. Murata, L. Morganti, D. Toniolo, P. Bartolini, J. Immunol. Methods 159 (1993) 269.
[38] B.R. Haughen, F. Pacini, C. Reiners, M. Schlumberger, P.W. Ladenson, S.I. Sherman, M.C. Skarulis, T.F. Davies, L.J. Degroot, E.L. Mazzaferri, G.H. Daniels, D.S. Ross, M. Luster, M.H. Samuels, D.V. Becker, H.R. Maxon, R.R. Cavalieri, C.A. Spencer, M.C. Ellin, B.D. Weintraub, E.C. Ridigway, J. Clin. Endocrinol. Metab. 84 (1999) 3877.
[39] E.D. Green, J.U. Baenziger, J. Biol. Chem. 263 (1988) 25.
[40] E.D. Green, J.U. Baenziger, J. Biol. Chem. 263 (1988) 36.
[41] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299.
[42] S.A. Carr, M.E. Hemling, M.F. Bean, G.D. Roberts, Anal. Chem. 63 (1991) 2802.
[43] Hillenkamp, M. Karas, R.C. Beavis, B.T. Chait, Anal. Chem. 63 (1991) 1193A.
[44] S.A. Butler, P. Laidler, J.R. Porter, A.T. Kicman, T. Chard, D.W. Cowan, R.K. Iles, J. Mol. Endocrinol. 22 (1999) 185.
[45] J. Flensburg, M. Belew, J. Chromatogr. A 1009 (2003) 111.
[46] A.J. Lapthorn, D.C. Harris, A. Littlejohn, J.W. Lustbader, R.E. Canfield, K.J. Machin, F.J. Morgan, N.W. Isaacs, Nature 369 (1994) 455.
[47] H. Wu, J.W. Lustbader, Y. Liu, R.E. Canfield, W.A. Hendrickson, Structure 2 (1994) 545.
[48] G.R. Bousfield, V.Y. Butnev, R.R. Gotschall, V.L. Baker, W.T. Moore, Mol. Cell. Endocrinol. 125 (1996) 3.
[49] R.J. Cotter, Anal. Chem. 64 (1992) 1027A.
[50] M. Karas, U. Bahr, K. Strupat, F. Hillenkamp, A. Tsarbopoulos, B.N. Pramanik, Anal. Chem. 67 (1995) 675.