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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Human thyroid-stimulating hormone synthesis in human embryonic kidney cells and related N-glycoprofiling analysis for carbohydrate composition determination

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Abstract
A strain of embryonic human kidney cells (HEK293) was transiently co-transfected with the expression vectors coding for the α- and β-subunits of human thyroid-stimulating hormone (hTSH), and, for the first time, a human cell-derived recombinant hTSH was synthesized and extensively characterized. The purification strategy involving two steps provided an overall yield of 55% and a purity level > 90%. The purified material (hTSH-HEK) was analyzed and compared to a CHO-derived recombinant preparation (hTSH-CHO) and to a pituitary-derived (hTSH-Pit) preparation. The three preparations showed an equivalent purity (> 95%) with a hTSH-HEK molecular mass 2.1% lower than that of hTSH-CHO and 2.7% higher than that of hTSH-Pit. Remarkable differences were found in the carbohydrate moiety, the lowest sialic acid content and highest fucose content being observed in hTSH-HEK. In vivo biological activity was confirmed for the three preparations, the hTSH-HEK bioactivity being 39 and 16% lower than those of hTSH-CHO and hTSH-Pit, respectively. The hTSH-HEK circulatory half-life ($t_{1/2}$) was also shorter than those of hTSH-CHO (1.5-fold) and hTSH-Pit (1.2-fold). According to these findings, HEK-293-derived hTSH can be considered to be useful for clinical applications, in view as well of its human origin and particular carbohydrate composition.

Keywords Thyroid-stimulating hormone · HEK293 cells · N-glycan composition · In vivo bioactivity · Circulatory half-life · MALDI-TOF-MS

Introduction

A major concern in the synthesis of pharmaceutical recombinant proteins is to obtain proteins that are more and more similar to the natural ones. This similarity is particularly critical in the case of glycoproteins for therapeutic use, since differences in glycosylation can affect the structure, biological activity, and pharmacological properties; an inappropriate glycosylation can result in a nonfunctional product with unwanted clearance and immunogenicity (Brooks 2006; Dumont et al. 2016). Therefore, appropriate human cell lines, which are expected to have post-translational modifications more similar to their natural counterparts and less immunogenicity, should be the best hosts of choice for the production of high-quality, human-like recombinant proteins (Durocher and Butler 2009; Fliedl et al. 2015). Among these, human embryonic kidney cells (HEK293) are often used to express recombinant proteins obtained by either transient or stable transfection (Picanço-Castro et al. 2013). However, the suitability of the use of these cells should be based on a case-by-case basis, taking into account the protein of interest, the cell line, and vector properties (Swiech et al. 2012; Böhn et al. 2015). It has been reported that the glycosylation induced by HEK-293 cells presents differences in relation to other mammalian cells currently used for human therapeutic protein production, such as Chinese hamster ovary cells (CHO) (Picanço-Castro et al. 2013; Butler and Spearman 2014). These differences are primarily due to species- and organ-specific glycosylation machinery and their impact on the effectiveness of the therapeutic product must be considered. Very recent reports have in fact shown the highly efficient, rapid, and consistent production of antibody (Ding et al. 2017) and of hyperglycosylated IFN-α_2 with an improved therapeutic efficiency (Gugliotta et al. 2017) via transient gene expression in HEK293 cells. Sialic acid and

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fucose linkages and the presence of bisecting GlcNAc residues differ between CHO- and HEK293-derived glycoproteins due to the lack, in CHO cells, of some sugar-transferring enzymes, such as α2,6 sialyltransferase, α1,3/4 fucosyltransferase, and bisecting N-acetylglucosamine transferase (Durocher and Butler 2009). Another difference between these cells is the absence in human cells of the residue N-glycolylneuraminic acid (Neu5Gc) that occurs due to the inactivity, caused by a mutation, of the enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), responsible for converting CMP-Neu5Ac into CMP-Neu5Gc (Picanço-Castro et al. 2013). On the other hand, CHO cells synthesize this oligosaccharide. Hence, proteins derived from CHO cells potentially contain non-human glycan structures and this may induce undesired immunogenicity in humans. Immunogenic reactions can reduce the efficiency of a biopharmaceutical because of its rapid clearance by the immune system or the impossibility of repeating the administration of the drug since it can potentially cause immune response.

In the present study, the suitability of the use of a human host cell (HEK293 cells) to produce recombinant human thyroid-stimulating hormone (hTSH) was evaluated. Human thyroid-stimulating hormone is a glycoprotein hormone composed of two non-covalently attached subunits (α and β) with 92 amino acids and 5 disulfide bridges in the α-subunit and 118 amino acids and 6 disulfide bridges in the β-subunit, the two subunits only having biological activity when assembled (Ribela et al. 2006; Carvalho et al. 2009). This hormone is produced by the thyrotropes, in the anterior pituitary gland, and stimulates the thyroid gland to secrete the thyroid hormones thyroxine (T4) and triiodothyronine (T3), which are essential for regulating metabolism. The main recombinant product available on the market is derived from CHO cells (Thyrogen®). Recombinant hTSH has several applications in the diagnostic and therapeutic field and has been mainly used to evaluate thyroid function, in the treatment of multinodular goiter and in the detection and treatment of thyroid cancer (Szkudlinski et al. 2002; Damiani et al. 2013; da Silva et al. 2016). The hTSH α-subunit has two N-glycosylation sites, on asparagine A_{sn}^{52} and A_{sn}^{78}, and the β-subunit has one glycosylation site on A_{sn}^{23}. One of the most common terminations of human glycoproteins of pituitary origin, sulfate, is not present in CHO cell-derived products since sulfotransferase, the enzyme responsible for sulfating N-acetylgalactosamine, is also absent (Ribela et al. 2017).

Practically, all commercially available recombinant hTSH is expressed in CHO cells. The native hTSH form (pituitary-derived) and recombinant hTSH derived from a murine host cell (CHO cells) were therefore compared in this work with the HEK293-derived preparation, with regard to their identity, purity, glycidic portion, and biological behavior.

Material and methods

Construction of expression vectors

The cDNAs of hTSH α- and β-subunits, previously utilized in our laboratory for the synthesis of CHO cell-derived hTSH (Peroni et al. 2002; Damiani et al. 2009), were amplified by the polymerase chain reaction (PCR) utilizing the specific sense and antisense primers of each subunit at the concentration of 10 mM, and 1 U of Taq DNA Polymerase High Fidelity (Life Technology, Carlsbald, CA, USA), and purified using the PCR Clean-UP System from Promega (Madison, WI, USA) and the Exo-SAP-IT enzyme from AFFY Metrix (Santa Clara, CA, USA). The α (621 bp) and β (460 bp) subunit cDNAs were inserted into the commercial expression vector pcDNA™ 3.4-TOPO (6011 bp), purchased from Life Technology (Carlsbald, CA, USA). The expression vectors were then amplified and purified using the Nucleobond® Xtra-midi kit from Macherey-Nagel (Duren, Germany).

Cell line, culture conditions, and transfection

HEK-293 T cells (ATCC®CRL-11268™), that constitutively express the large T antigen of the simian virus 40 (SV40), were grown in static culture, being adherently cultured in 10 mL of Dulbecco's modified Eagle's medium (DMEM) (Life Technology, Carlsbald, CA, USA), supplemented with 10% fetal bovine serum, in a 75-cm^2 T-flask, at a seeding density of ~ 3×10^6 cells/flask, in a 5% CO_2 humidified incubator, at 37 °C. When cells reached a confluence level of 80%, they were transiently transfected, using the cationic lipid Lipofectamine (Life Technology, Carlsbald, CA, USA). Transfections were performed using 16 μg DNA, in the same proportion (1:5) of α and β as utilized by Peroni et al. (2002), and 16 μL of the transfection reagent, for 30 min, at 25 °C. Transfected cells were maintained in culture during 5 days, this period being determined with basis on volumetric hTSH productivity. The conditioned culture medium was harvested and replaced daily, with fresh serum-free medium.

Purification process

The purification process used in this study followed a protocol previously set up in our laboratory (Oliveira et al. 2007). The process consists of two chromatographic steps, the first being a classical ion exchange chromatography of the cationic type, followed by a high-efficiency liquid chromatography on reversed phase. The first step was performed on an AKTA Purifier system (GE Healthcare, Uppsala, Sweden) and the second step on a HPLC apparatus (Shimadzu SCL-10A, MD, USA). Before the first step, a concentration of the conditioned medium was carried out on a tangential flow filtration

system (Millipore, Bedford, MA, USA) utilizing a Pellicon XL membrane of 5 kDa cutoff.

In a typical purification, the concentrated conditioned medium, after its pH was adjusted to 5.0, was loaded at a flow rate of 38 cm/h on a SP-Sepharose Fast Flow (SPFF) column (10 cm × 2.6 cm I.D.), previously equilibrated with 0.02 M sodium acetate, pH 5.0, 0.05 M NaCl. After several washes (5 column volumes) with the equilibration buffer, hTSH was eluted with a linear NaCl gradient. The NaCl concentration in the mobile phase was increased from 0.05 to 0.25 M. The fractions containing hTSH, as determined by IRMA (5 mL/fraction), were pooled.

The pool of fractions containing hTSH was concentrated to 10 mL in an Amicon Ultra-15 centrifuge filter device (Millipore, Bedford, MA, USA) and loaded on a semi-preparative reversed-phase 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). The column, connected to a HPLC system, was maintained at 25 °C. Two buffers, A and B, were used in the mobile phase (buffer A: 0.05 M sodium phosphate, pH 7.0, and buffer B: 50% A + 50% acetonitrile). Proteins were eluted with a linear gradient of 25 to 100% buffer B, over 40 min. A flow rate of 191 cm/h and detection by UV at a wavelength of 280 nm were used. An additional dialysis step in 0.02 M sodium phosphate, pH 7.0, 0.15 M NaCl was carried out on the resultant hTSH pool.

Hormone preparations

Three hTSH preparations of different origins were compared: the recombinant preparation derived from human embryonic kidney cells (HEK293), synthesized, purified, and characterized in the present work (hTSH-HEK), a recombinant CHO-derived (hTSH-CHO), and a native pituitary-derived (hTSH-Pit) preparation, both previously analyzed in our laboratory (Ribela et al. 2017).

Immunoradiometric assay

Human TSH immunoradiometric assay (IRMA) was carried out following a protocol previously set up in our laboratory utilizing a reference preparation calibrated against the International Standard of pituitary hTSH (WHO 80/558, 4.93 IU/mg) (Ribela et al. 1996). Briefly, ^{125}I-monoclonal antibody (60,000 cpm), reference (0.15–100 μUI/mL), or unknown preparation and cellulose-coupled polyclonal antibody (1.25 mg) were incubated on a rotary mixer, at room temperature. After 16 h, a centrifugation at 5000*g* for 30 min was carried out for the separation of the ^{125}I-antibody-bound hormone, which was detected in an automatic well-type NaI (Tl) gamma counter (2470 Wizard2™ from PerkinElmer, Waltham, MA, USA).

Total protein determination

Total protein concentration was estimated utilizing a Micro BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Solutions of pure bovine serum albumin (0.5–200 μg/mL) were used as standard. All the samples analyzed were dialyzed at 4 °C against 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl.

High-performance size-exclusion liquid chromatography

High-performance size-exclusion liquid chromatography (HPSEC) was carried out on a G2000 SW column (60 cm × 7.5 mm I.D., particle size of 10 μm, and pore size of 125 Å), purchased from Tosoh Bioscience (Montgomeryville, PA, USA), connected to a Shimadzu Model SCL-10A HPLC apparatus. Detection was by UV absorbance at 220 nm with a flow rate of 1.0 mL/min, employing 0.15 M NaCl in 0.02 M sodium phosphate buffer, pH 7.0, as the mobile phase.

Analytical reversed-phase high-performance liquid chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out on a Vydac C4 214 TP54 column (25 cm × 4.6 mm I.D., 5 μm particle size, and 300 Å pore size) purchased from Grace-Vydac (Separation Group, Hesperia, CA, USA), connected to a Shimadzu HPLC apparatus. Chromatography was carried out at 25 °C with detection by UV absorbance at a wavelength of 220 nm. Gradient solutions A and B were utilized, solution A being 0.05 M sodium phosphate buffer, pH 7.0, and solution B acetonitrile. For hTSH elution, a linear gradient from A/B (87.5:12.5, *v*/*v*) to A/B (50:50, *v*/*v*) over 40 min was used, as described (Oliveira et al. 2003).

In vivo bioassay

The biological activity of the three hTSH preparations of different origins was evaluated by an in vivo bioassay in BALB/c male mice, weighing between 22 and 30 g, in which TSH-induced T4 is measured after a 5-day suppression of endogenous TSH by T3 administration. Saline solution (200 μL saline solution per mouse) or a single dose of each hTSH preparation (10 μg hTSH in 200 μL saline per mouse) was i.p. injected to groups of 10 animals. After 6 h post injection, serum samples were obtained via retro orbital plexus and serum T4 concentrations (μg/dL) were determined using a human T4 ELISA kit (Foresight, San Diego, CA, USA), according to the manufacturer's instructions.

Pharmacokinetic studies

Single doses of hTSH-HEK (2 μg in 200 μL saline) were administrated to two groups of BALB/c mice (five males per group) via i.p. injection. Blood samples were obtained at 30, 60, and 90 min post injection from one group and at 120, 180, 240, and 300 min post injection from the other group. Serum hTSH concentrations were determined by IRMA, in duplicate per animal and expressed as the percentage of the maximum hTSH concentration. The study was repeated twice.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

The molecular mass of hTSH-HEK preparation (approximately 100 μg of sample) was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a Voyager DE BioSpectrometry Workstation system (Applied Biosystems Sciex, Framingham, MA, USA), operated in the linear positive ion mode, using a saturated solution of sinapinic acid (SA) in 50% acetonitrile and 0.1% trifluoroacetic acid as the matrix. The spectrum was obtained by the accumulation of 100 shots and was calibrated with an external standard (ProMix 3; LaserBiolabs). This analysis was performed in the laboratory Proteodynamics SARL (Riom, France).

N-Glycan structure analysis

N-glycoprofiling was performed after glycosidase digestion and permethylation of N-glycans. Samples were denatured in 0.5% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol (90 °C, 10 min) and deglycosylated with 20 U of peptide-N-glycosidase F (PNGase F, Promega, Madison, WI, USA) (37 °C, 15 h) in 50 mM phosphate buffer, pH 7.5, after having added 22 μL of NP 40 (Sigma-Aldrich Chimie S.a.r.I., Lyon, France) to avoid PNGase denaturation. N-glycans were purified on an Ultra Clean SPE Carbograph column (Alltech, Deerfield, IL, USA) and, after elution with 25% acetonitrile containing 0.1% TFA, they were lyophilized before permethylation. To control whether the hTSH-HEK carries sulfated N-glycans, a specifically adapted permethylation protocol based on Yu et al. (2009), using sodium hydroxide, dimethyl sulfate (DMSO), ICH_3 procedure was used to allow separation of sulfated and non-sulfated or phosphorylated N-glycans eventually present. After derivatization, the reaction products were purified on C18 Sep Pak Plus (Waters, Milford, MA, USA) by step-elution with 25 and 75% acetonitrile. The 25% acetonitrile fraction should contain sulfated glycans, while non-sulfated and phosphorylated glycans are found in the 75% acetonitrile fraction. Eluted N-glycans were lyophilized. The purified permethylated glycans of both acetonitrile elutions were solubilized with 50:50 water:methanol mixed with 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg/mL) (LaserBiolabs, Sophia-Antipolis, France) and spotted on a MALDI-plate. Positive ion reflectron MALDI mass spectra were acquired on a VOYAGER DE PRO mass spectrometer (AB Sciex, Framingham, MA, USA). The spectra were obtained by accumulation of 500 shots and were calibrated with an external standard (PepMix 4; LaserBiolabs). This analysis was performed in the laboratory Proteodynamics SARL (Riom, France). The glycoprofiling and the relative percent intensity of each determined glycan were used to calculate the average N-glycan mass that is present in the hTSH molecule. Through this stoichiometric approach, the contribution of each monosaccharide to each glycan can also be calculated, as detailed in a previous work (Capone et al. 2015). Having determined the average glycan mass (AGM) on the basis of glycoprofiling and the total molecular mass of the glycoprotein by MALDI-TOF-MS, the site occupancy can be calculated according to the relation:

$$\mathrm{AGM} \times \textit{Number of Occupied sites} = \textit{Mass of Carbohydrate Moiety},$$

where the mass of the carbohydrate moiety in the hTSH molecule can be obtained from the relation:

$$\textit{Mass of Carbohydrate Moiety} = \textit{Molecular mass} - \textit{hTSH protein backbone}$$

The hTSH protein backbone is 24,330 Da, as determined by Cole et al. (1993) from the analysis of the amino acid sequence of recombinant hTSH (Thyrogen®) and of its individual subunits. Knowing the occupancy, it is then possible to determine each monosaccharide (or sulfate)/hTSH molar ratio.

Results

The average hTSH-HEK concentration (mean ± SD), obtained over 5 days and resulting from four independent transfections, is shown in Fig. 1a. The highest hTSH-HEK concentration (1.01 ± 0.45 μg/mL) was attained at 72 h. After 5 days, the hTSH-HEK level decreased approximately 40% (0.61 ± 0.14 μg/mL). During this period, transfected HEK293T cells were growing at a rate of 1.2×10^6 cells/day and % viability varied from 99.7 to 95.5% (Fig. 1b). Based on these parameters, the specific productivity was calculated (Fig. 1c).

The material harvested from 48 to 120 h was pooled (3209 mL), concentrated ~12-fold by tangential filtration (271 mL), and purified by two chromatographic steps. In ion-exchange chromatography, the range of eluted fractions

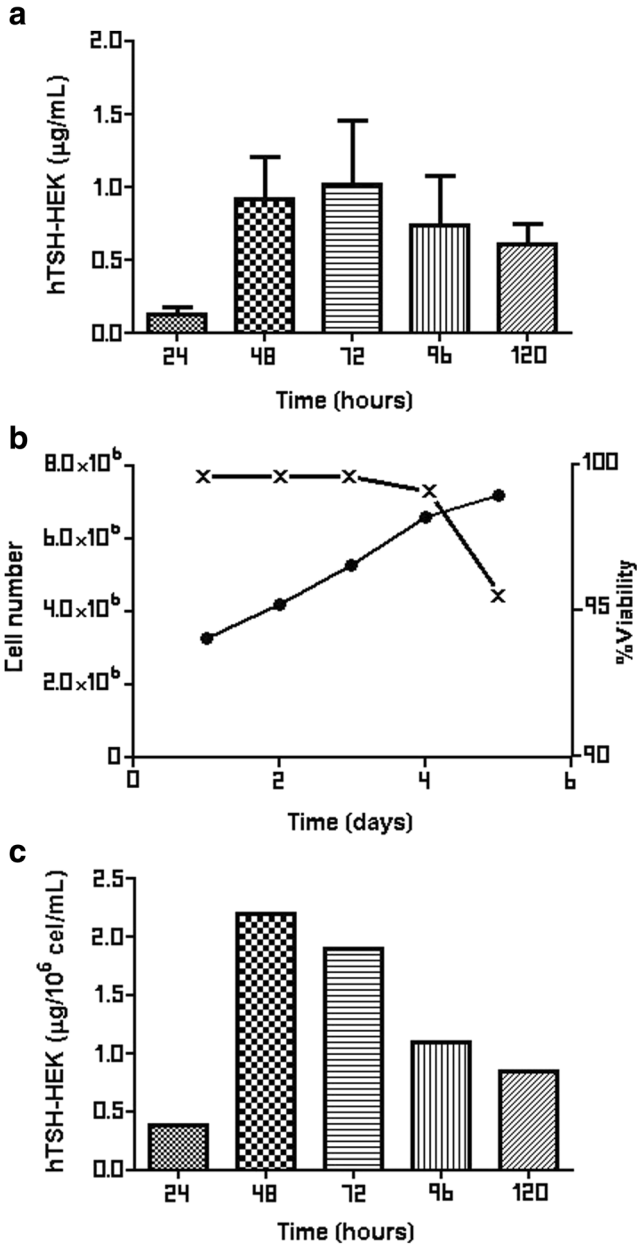

Fig. 1 **a** Yields of hTSH-HEK (mean ± SD, µg/mL) during 5 days of culture. **b** Viable cell number (black circle) and % of viability ("x" symbol) during the same 5 days. **c** Specific productivity (µg/10^6 cells/day) during the same 5 days

pooled for the next purification step was selected on the basis of hTSH immunological activity. The peak eluted from semi-preparative reversed-phase chromatography was dialyzed and analyzed by size-exclusion HPLC (Fig. 2d) and reversed-phase HPLC (Fig. 2h), showing 97% purity.

A complete view of the purification process is shown in Table 1. The overall recovery yield of the process was 55.2%. The highest enrichment of hTSH (71-fold) was attained by ion-exchange chromatography. In this step, the highest loss (~ 37%) also occurred. A low mass fraction (< 1%) was found in the conditioned medium. In fact, the size-exclusion HPLC (Fig. 2a) and reversed-phase HPLC (Fig. 2e) profiles of conditioned medium show a large amount of impurities together with the protein of interest, whose presence could only be identified by immunoradiometric detection. The progressive elimination of the contaminants in the different purification steps could be followed by these HPLC techniques (Fig. 2). The purified product (hTSH-HEK) was characterized with regard to purity, molecular mass, glycidic portion, biological activity, and pharmacokinetics and compared to a recombinant commercial preparation obtained from CHO cells (hTSH-CHO) and to a native pituitary-derived preparation (hTSH-Pit).

Concerning the relative molecular mass (Mr), MALDI-TOF mass spectrometry allowed the simultaneous detection of the monomers (α- and β-subunit) and of the heterodimer of hTSH-HEK (Fig. 3). These forms, in three independent assays, presented an average molecular mass (mean ± SD) of 13,833 ± 8.17 Da (CV = 0.06%) for the α-subunit, of 15,469.1 ± 4.14 Da (CV = 0.03%) for the β-subunit, and of 29,531.8 ± 121.5 Da (CV = 0.41%) for the heterodimer. The sum of the two subunits (29,302.1 Da) agrees with the experimental value found for the heterodimer ($\alpha + \beta$/het = 0.992), with a difference of < 1%. Figure 3 also shows the formation of α-subunit homodimers (α-α = 27,480.2 ± 339.6 Da) and the presence of charged 2^+ ions of the α-subunit (α/2 = 6992.7 ± 198.2 Da). In these cases, the difference between the calculated and experimentally determined values was still < 1%. Comparing the α + β hTSH-HEK mass (29,302) with the previously determined masses (Ribela et al. 2017) of hTSH-CHO (29,921) and of hTSH-Pit (28,524), it was found that the hTSH-HEK molecular mass was 2.1% lower than the hTSH CHO mass and 2.7% higher than the hTSH-Pit mass. In this comparison, we utilized the values of α + β mass addition, instead of the heterodimer mass values, due to the higher resolution and narrower peaks of the subunits, and considering that this type of calculation has always shown, in our hands, a higher precision and accuracy. Considering that the mass of the protein backbone of hTSH is 24,330 Da (Cole et al. 1993), the glycidic portion in the hTSH-HEK molecule corresponds to 17%, thus presenting a 2.3% higher carbohydrate mass than in hTSH-Pit and a 1.7% lower carbohydrate mass than in hTSH-CHO.

When hTSH of different origins was analyzed by HPSEC and RP-HPLC, hTSH-HEK always presented a retention time (t_R) higher than hTSH-CHO and than hTSH-Pit in the following order: t_R CHO < t_R Pit < t_R HEK (Table 2). In HPSEC, the differences between the t_R of the two preparations of human origin were not significant. However, the difference between the t_R of hTSH-HEK and hTSH CHO was significant ($p < 0.05$). Considering RP-HPLC, there was a difference of ~ 1 min in the t_R of hTSH-HEK and that of the other two preparations, but this difference was not significant. The HPSEC profiles of these preparations showed the same degree of purity for the three preparations (Fig. 4).

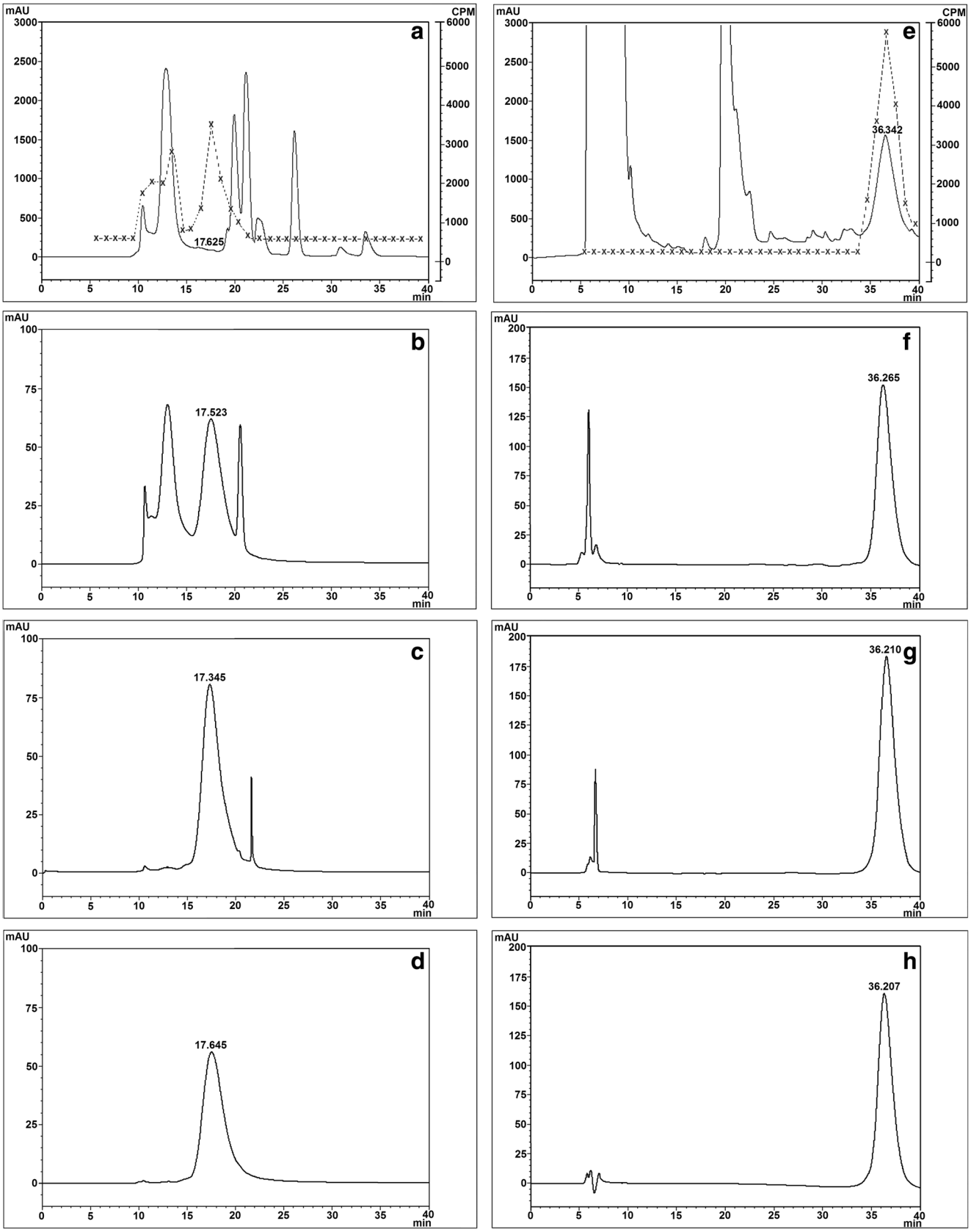

Fig. 2 HPSEC (**a**–**d**) and RP-HPLC (**e**–**h**) profiles of the different purification steps. **a**, **e** Conditioned medium. **b**, **f** Cationic exchange chromatography eluate. **c**, **g** Reversed-phase chromatography eluate. **d**, **h** Dialyzed product

Table 1 Purification process of hTSH-HEK

Step	Volume (mL)	hTSH (μg/mL)	Total protein (μg/mL)	Step recovery (%)	Mass fraction (%)
Concentrated conditioned medium	271	11.3[a]	1616	–	0.70
Ion-exchange chromatography	10	194.3[a]	391	63.4	49.7
Reversed-phase chromatography	13	135.2[b]	145	90.6	93.2
Dialysis	14.1	119.9[b]	132.5	96.2	90.5
				Overall recovery 55.2%	

[a] Quantification by RP-HPLC

[b] Quantification by HPSEC

A total of 24 different structures were identified in HEK-derived hTSH, all of them of the complex type (Table 3). Only two of these structures had a relative intensity superior to 7.4%. These two most relevant structures were tri-antennary with one galactose terminal residue, with or without fucose. Only three of the hTSH-HEK structures were common to hTSH-CHO and six of them were common to hTSH-Pit. The number of glycans identified in hTSH-HEK was lower than that observed in hTSH-Pit (38 different glycan structures) and higher than those observed in hTSH-CHO (15 different glycan structures). In the last two preparations, complex glycans were predominant and small amounts of hybrid structures were also found, 3.2 and 1.2% in the pituitary- and CHO-derived preparations, respectively. The proportion of the two most abundant structures varied in the hTSH preparations of different origins. They correspond to 45.3% of the total structures in hTSH-HEK, 28.1% in hTSH-Pit, and 68% in hTSH-CHO. Considering that the identification of the N-glycan structures was done based on MALDI-TOF glycan mass determination, six isomers were observed in HEK- and pituitary-derived material, which contained different combinations of GlcNAc and GalNAc that could not be distinguished under the adopted conditions. We observed, however, that hTSH-HEK, even being of human origin, does not contain either GalNAc or sulfate. Consequently, the structures 22, 31, 33, 39, 43, and 47 are undoubtedly those referred in Table 3. However, some inaccuracy could exist in the interpretation of the glycan structures 23, 32, 34, 40, 44, and 48 of hTSH-Pit as reported in Ribela et al. (2017), due to the approximation and rejection criteria adopted in that paper to choose the proper N-glycan structure. Considering that three out of these six glycans present in hTSH-Pit had intensities < 1%, while the other three had intensities varying between 1.3 and 4.2%, we believe that the possible misinterpretation would not greatly affect the general conclusions.

All structures of hTSH-CHO present galactose (Gal), with a large predominance (82.8%) of structures with 2 Gal residues. A lower number of glycans with Gal residue (1–3) were found in the two preparations of human origin, with predominance of structures with one galactose (Table 4(A)). In hTSH-

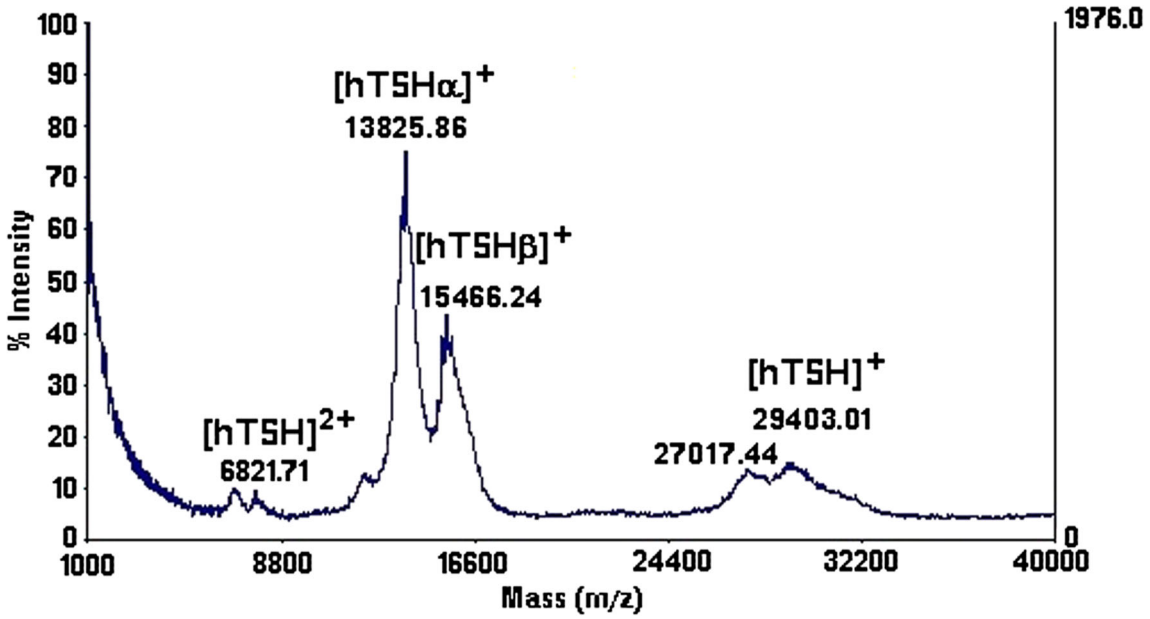

Fig. 3 MALDI-TOF mass spectrum of hTSH-HEK

Table 2 HPSEC and RP-HPLC retention times (t_R, mean ± SD) of hTSH preparations of different origins

Preparation	t_R in HPSEC (min)	t_R in RP-HPLC (min)	HPSEC relative difference hTSH-HEK (%)	RP-HPLC relative difference hTSH-HEK (%)
hTSH-HEK	17.50 ± 0.21	35.74 ± 0.67	–	–
hTSH-Pit	17.08 ± 0.08	34.82 ± 0.62	− 2.4	− 2.6
hTSH-CHO	16.31 ± 0.03	34.47 ± 0.66	− 6.8	− 3.6

HEK, structures with fucose were very frequent (63%), while in hTSH-Pit and hTSH-CHO, they appeared at a much lower incidence, 35.2 and 11.9%, respectively (Table 4(A)). A remarkable difference between the preparations of different origins concerned the frequency of structures with sialic acid. Very few structures present sialic acid in hTSH-HEK, with hTSH-CHO being the preparation with the highest presence of sialic acid (Table 4(A)). Bi-, tri-, and tetra-antennary structures were found in hTSH-HEK, with predominance of the tri-antennary structures (Table 4(B)). On the other hand, in hTSH-CHO and hTSH-Pit, the bi-antennary structures were predominant and tetra-antennary structures were absent only in hTSH-CHO (Table 4(B)). The mole numbers of monosaccharides (Fuc, GalNAc, GlcNAc, Gal, Man, SA) were calculated considering their mass contribution to the different structures of the hTSH preparations (Table 5). hTSH-HEK presents the higher amount of fucose, ~7-fold higher than hTSH-CHO and ~3-fold higher than hTSH-Pit. Concerning GalNAc and sulfate, only hTSH-Pit presented these species. The mole number of Gal in hTSH-HEK was 62.1% higher than in hTSH-Pit and 42.9% lower than in hTSH-CHO. Regarding Man, hTSH-HEK and hTSH-CHO present about the same mole number while hTSH-Pit presents 21.6% less than hTSH-HEK. Sialic acid was 11.8-fold and 31.3-fold lower in hTSH-HEK than in hTSH-Pit and hTSH-CHO, respectively.

Maximum mice plasmatic concentration after intraperitoneal administration of hTSH-HEK was attained at 60 min. After 5 h, practically 86% of the administered hTSH was already cleared. Analyzing the kinetic behavior of the three preparations of different origins, it was observed that the hTSH-HEK circulatory half-life ($t_{1/2}$) was 1.5- and 1.2-fold shorter than for hTSH-CHO and hTSH-Pit, respectively (Table 6). While the maximum hTSH-CHO concentration occurred at 90 min, for both preparations of human origin, the maximum hTSH concentration occurred at 60 min, generating a lower exposure of the organism to the hormone.

Biological activity, evaluated by an in vivo bioassay based on T4 stimulation by hTSH administration, is shown in Table 6. Biological activity was confirmed for the three preparations of different origins, the hTSH-HEK bioactivity being 39 and 16% lower than that of hTSH-CHO and hTSH-Pit, respectively. The native preparation (hTSH-Pit) was also less active than hTSH-CHO, by ~ 27%.

Discussion

In the current study, a biologically active human thyroid-stimulating hormone with proper quality attributes was expressed in embryonic human kidney cells HEK293T (hTSH-HEK). Up to now, most recombinant hTSH preparations reported in the literature have been derived from Chinese hamster ovary cells (hTSH-CHO), in particular in investigations from the National Institute of Health (NIH, Bethesda, MD, USA), from Genzyme Corporation (Framingham, MA, USA), and from our research group (Szkudlinski et al. 1993; Cole et al. 1993; Peroni et al. 2002). Thyrogen® from Genzyme is in fact the main hTSH biopharmaceutical now available on the market. Although the synthesis of hTSH in HEK293 was reported in 1988, paving the way for pioneering studies on glycohormone carbohydrate patterns (Wondisford et al. 1988; Grossmann et al. 1995), this is the first time that hTSH expressed in these cells has been extensively purified and characterized, with comparison to the native pituitary and to the most widely used biopharmaceutical preparation.

An efficient transient expression system, utilizing the commercial vector TOPO pc DNA 3.4 into which we introduced the genes of α- and β-subunits of hTSH, in the same proportion (1:5) as described by Peroni et al. (2002), was utilized for hTSH-HEK expression.

Under the transfection conditions utilized in this work, a concentration of hTSH-HEK in conditioned medium of 0.95 μg/mL and a specific productivity of 2.1 $\mu g/10^6$ cell/day were obtained. The latter value is comparable to that reported by Damiani et al. (2009) for human-like sialylated hTSH obtained from a CHO strain in which the sialic acid binding could be in $\alpha_{2,3}$ and $\alpha_{2,6}$ configuration. This secretion level, however, was lower than that reported by Cole et al. (1993) (4.5 $\mu g/10^6$ cells/day) or by Peroni et al. (2002) (7.2 $\mu g/10^6$ cells/day) for hTSH derived from regularly transfected CHO cells, but was nonetheless sufficient for a proper purification and characterization.

The purification process started from 438 mg of crude material dissolved in 3.2 L of culture medium containing 3.06 mg of hTSH (mass fraction = 0.7%; hTSH concentration = 0.95 μg/mL), then concentrated to 271 mL. After two chromatography steps, approximately 1.7 mg of hTSH with the purity of a chemical reagent (> 90%) was obtained. In the

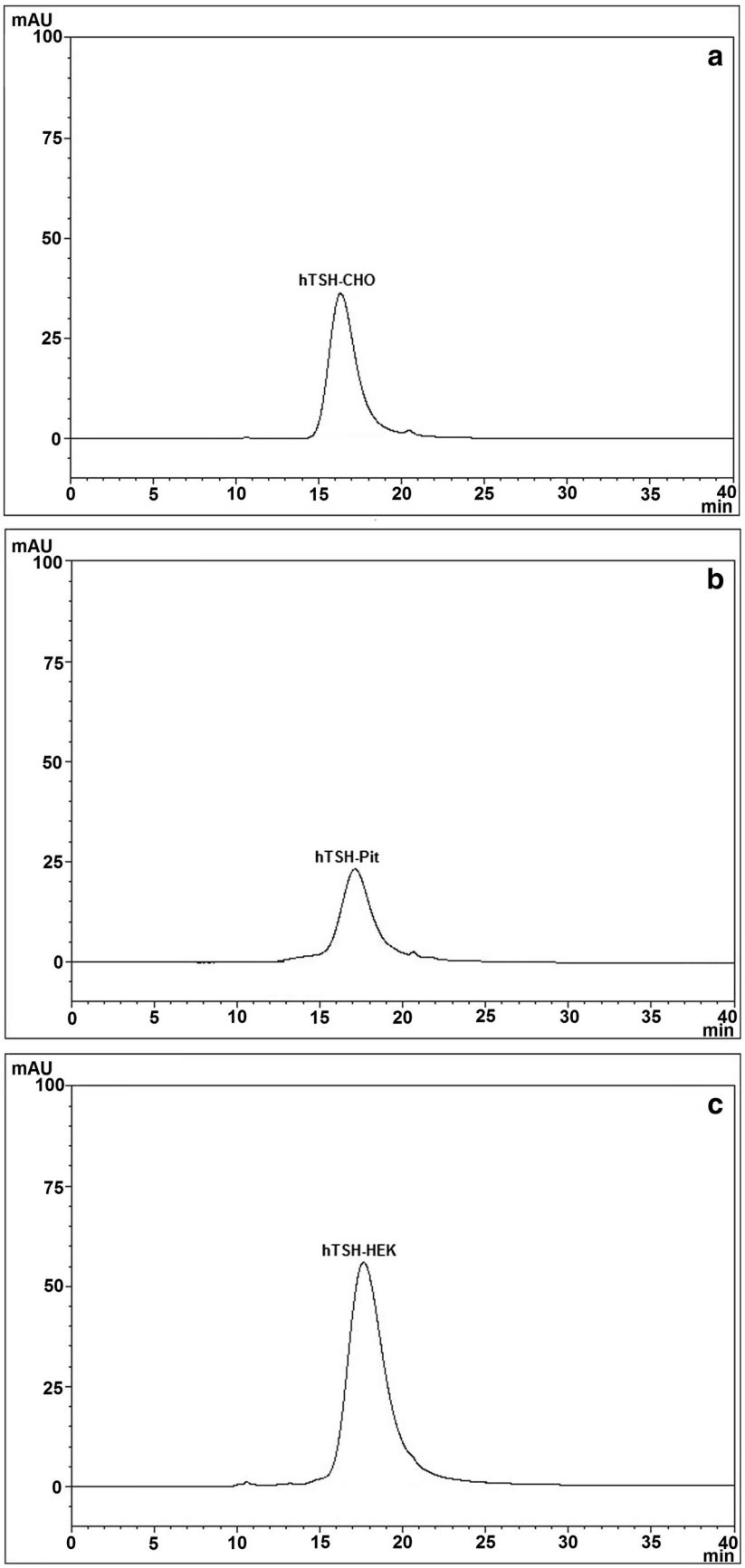

Fig. 4 HPSEC profiles of purified hTSH preparations. **a** CHO-derived hTSH. **b** Pituitary-derived hTSH. **c** HEK-derived hTSH

present work, the same purification process described by Oliveira et al. (2007) for the purification of CHO-derived hTSH was utilized. In both cases, a product with adequate purity was obtained, even considering that, in the present work, the initial material was 1.6-fold more concentrated (0.95 versus 0.60 μg/mL) and 2-fold more pure (mass fraction 0.70 versus 0.35%) than that reported by Oliveira et al. (2007). The overall recovery in the present work was lower (55 versus 70%), although still useful for our purposes.

MALDI-TOF mass spectrometry has been shown to be a very important tool for evaluating the identity and exact molecular mass of a product, being very useful for identifying key differences existing between preparations derived from different hosts (Sandra et al. 2014). The hTSH-HEK mass (29,302 Da), determined by MALDI-TOF MS, was lower than the hTSH-CHO mass. This fact was also evident in the analysis of these two preparations by size exclusion HPLC, where a difference of retention time of ~ 7% was observed, with hTSH-CHO eluting early, reflecting a higher mass. In relation to the hTSH-Pit molecular mass (28,524 Da), determined by MALDI-TOF-MS, the hTSH-HEK mass was 2.7% higher. Comparing these two preparations by HPSEC, hTSH-Pit eluted first, but the difference between their retention times (2.4%) was not significant.

N-glycan profiling showed 24 different structures for hTSH-HEK, most of which (80%) were not present in the other two hTSH preparations analyzed. Very few of them (6.6%) were common to hTSH-CHO and 16.8% to hTSH-Pit. The two most relevant structures of hTSH-HEK were not detected in the other two preparations. Only 3.4% of the structures were in common between the three preparations. With regard to the two predominant N-glycans present in the three preparations, we can only say that the most abundant glycan structure of hTSH-Pit corresponds to the second more abundant glycan of hTSH-CHO. While 95.3% of the glycans in hTSH-HEK were neutral, in hTSH-Pit and hTSH-CHO, only 49.2 and 5.8% of them were uncharged. Gugliotta et al. (2017) also reported a higher (8-fold) proportion of neutral glycans in a hyperglycosylated interferon-α2b mutein (IFN4N) produced in HEK293 compared to the CHO-derived product. A very low sialylation level was found for hTSH-HEK in comparison with the native and CHO-derived preparations. It has previously been reported in the literature that sialylation is highly cell specific (Zhang et al. 2010). Böhn et al. (2015) detected a 5.5-fold lower sialic acid content in the human coagulation factor VII derived from HEK293 cells (0.57 SA/mol) compared to the CHO-derived product (3.14 SA/mol). Gugliotta et al. (2017) reported a 2.3-fold greater sialic acid content in IFN4N produced in CHO cells (10.7 SA/mol) than in that produced in HEK293 cells (4.7 SA/mol). Zhang et al. (2010) showed, by isoelectric focusing (IEF), differences in the sialylation between CHO- and HEK293-derived erythropoietin, the last presenting a lower sialylation degree. Croset et al. (2012) analyzed 12 proteins of molecular mass varying from 10,000 to 53,000 Da expressed in CHO and HEK293 cells by IEF and detected that CHO-derived proteins had more acidic isoforms than HEK293-derived proteins, indicating a lower amount of sialic acid in HEK-derived preparations. In previous work with glycosylated human prolactin (G-hPRL) expression in CHO cells, we indeed found 1.34 mol of SA/mol of G-hPRL and only 0.020 mol/mol of native pituitary G-hPRL (Capone et al. 2015). The amount of sialic acid of a glycoprotein is determined by two opposing processes: intracellular addition of sialic acid by sialyltransferases and extracellular removal of sialic acid by enzymatic cleavage via sialidase. Intracellular addition of sialic acid involves the enzymatic transfer of sialic acid from the precursor nucleotide (CMP-sialic acid) to the available galactose on the structure of the emerging glycan that is linked to the newly synthesized protein. Factors that may influence the degree of sialylation include the availability of CMP-sialic acid, the activity of sialyltransferase and galactosyltransferase, and the galactose availability on the structure of the emerging glycan (Jing et al. 2010). The decreased amount of sialic acid in hTSH-Pit may be associated with the decreased availability of galactose. In fact, it was observed that the molar ratios SA/Gal of this preparation (0.71) and of hTSH-CHO (0.67) are of the same order, as was previously reported (Ribela et al. 2017). This, however, does not occur in hTSH-HEK, where this ratio is ~20-fold lower (0.037), suggesting that the decrease of sialic acid may also have occurred due to the action of sialidases.

Another very significant difference observed in the preparations analyzed was the largest amount of fucose in hTSH-HEK. This was also observed by Böhn et al. (2015), who reported 5-fold more fucose in a preparation of coagulation factor VII derived from HEK cells than in that derived from CHO cells.

Concerning the antennarity of the glycan structures of the three analyzed hTSH preparations, while predominantly bi-antennary structures occur in hTSH-CHO and in hTSH-Pit, in hTSH-HEK, the prevalence was of tri-antennary structures, a difference of 55 and 64.8% with hTSH-CHO and hTSH-Pit, respectively. This is, in our opinion, the only remarkable difference observed between the glycan structures found in hTSH-HEK and those reported by Gugliotta et al. (2017) for HEK293-derived IFN-α_2.

Structures containing N-acetylgalactosamine (GalNAc) and sulfate, which occur in the native preparation, were not found in hTSH-HEK, even considering that HEK293 is a cell of human origin.

As far as we know, very few considerations in the literature point to a specific influence of a certain type of glycan or even a certain monosaccharide on biological activity or physiological function. However, the role of terminal modifications such as sialic acid and sulfate has been well discussed.

Table 3 N-Glycan structures of hTSH preparations of different origins

	Glycan mass—H_2O	N-Glycan structure	Relative intensity (%)		
			hTSH-HEK	hTSH-Pit[a]	hTSH-CHO[a]
1	1298.96	$(GlcNAc)_2 + (Man)_3\ (GlcNAc)_2$	0.3	0.0	0.0
2	1378.43	$(GalNAc)_1(GlcNAc)_1(Sulf)_1 + (Man)_3\ (GlcNAc)_2$	0.0	5.1	0.0
3	1444.53	$(GlcNAc)_2\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.6	0.3	0.0
4	1460.53	$(Gal)_1(GlcNAc)_2 + (Man)_3\ (GlcNAc)_2$	2.5	0.4	0.4
5	1501.56	$(GlcNAc)_3 + (Man)_3\ (GlcNAc)_2$	3.5	0.0	0.0
6	1524.49	$(GalNAc)_1\ (GlcNAc)_1\ (Sulf)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.5	0.0
7	1540.49	$(Gal)_1\ (GalNAc)_1\ (GlcNAc)_1\ (Sulf)_1 + (Man)_3\ (GlcNAc)_2$	0.0	5.2	0.0
8	1548.54	$(NeuAc)_1\ (Gal)_1(GlcNAc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.8	0.0
9	1581.51	$(GalNAc)_1\ (GlcNAc)_2\ (Sulf)_1 + (Man)_3\ (GlcNAc)_2$	0.0	6.7	0.0
10	1606.59	$(Gal)_1\ (GlcNAc)_2\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	7.4	1.0	0.0
11	1622.58	$(Gal)_2\ (GlcNAc)_2 + (Man)_3\ (GlcNAc)_2$	3.2	0.0	5.0
12	1647.61	$(GlcNAc)_3\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	1.9	1.8	0.0
13	1663.61	$(Gal)_1\ (GlcNAc)_3 + (Man)_3\ (GlcNAc)_2$	15.4	0.0	0.0
14	1686.54	$(GalNAc)_1\ (GlcNAc)_1\ (Sulf)_1\ (Fuc)_1 + (Man)_4\ (GlcNAc)_2$	0.0	0.7	0.0
15	1694.60	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.1	0.0
16	1702.54	$(GalNAc)_1\ (GlcNAc)_1\ (Sulf)_1 + (Man)_5\ (GlcNAc)_2$	0.0	2.5	0.0
17	1710.60	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_1 + (Man)_4\ (GlcNAc)_2$	0.0	0.0	1.2
18	1727.62	$(GalNAc)_1\ (GlcNAc)_2\ (Sulf)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.4	0.0
19	1743.56	$(Gal)_1\ (GalNAc)_1(GlcNAc)_2\ (Sulf)_1 + (Man)_3\ (GlcNAc)_2$	0.0	3.6	0.0
20	1751.62	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_2 + (Man)_3\ (GlcNAc)_2$	0.0	1.3	0.5
21	1768.64	$(Gal)_2\ (GlcNAc)_2\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	3.5	0.6	0.0
22	1809.67	$(Gal)_1\ (GlcNAc)_3\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	29.9	0.0	0.0
23	1809.67	$(Gal)_1\ (GlcNAc)_2(GalNAc)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	0.8	0.0
24	1825.66	$(Gal)_2\ (GlcNAc)_3 + (Man)_3\ (GlcNAc)_2$	6.7	0.0	0.0
25	1850.69	$(GlcNAc)_4\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	3.0	0.0	0.0
26	1864.55	$(GalNAc)_2\ (GlcNAc)_2\ (Sulf)_2 + (Man)_3\ (GlcNAc)_2$	0.0	3.4	0.0
27	1889.62	$(Gal)_1\ (GalNAc)_1\ (GlcNAc)_2\ (Sulf)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	4.6	0.0
28	1897.68	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_2\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.0	0.0
29	1913.68	$(NeuAc)_1\ (Gal)_2(GlcNAc)_2 + (Man)_3\ (GlcNAc)_2$	0.9	0.9	36.1
30	1930.65	$(GalNAc)_2\ (GlcNAc)_2\ (Sulf)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.6	0.0
31	1954.70	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_3 + (Man)_3\ (GlcNAc)_2$	1.9	0.0	0.0
32	1954.70	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_2(GalNAc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	0.7	0.0
33	1955.72	$(Gal)_1\ (GlcNAc)_3\ (Fuc)_2 + (Man)_3\ (GlcNAc)_2$	6.2	0.0	0.0
34	1955.72	$(Gal)_1\ (GlcNAc)_2(GalNAc)_1\ (Fuc)_2 + (Man)_3\ (GlcNAc)_2$	0.0	0.8	0.0
35	1971.72	$(Gal)_2\ (GlcNAc)_3\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	3.9	0.0	0.0
36	1996.75	$(GlcNAc)_4\ (Fuc)_2 + (Man)_3\ (GlcNAc)_2$	2.5	0.0	0.0
37	2010.60	$(GalNAc)_2\ (GlcNAc)_2\ (Sulf)_2\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	4.4	0.0
38	2012.75	$(Gal)_1\ (GlcNAc)_4\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.6	0.0	0.0
39	2028.74	$(Gal)_2\ (GlcNAc)_4 + (Man)_3\ (GlcNAc)_2$	1.0	0.0	0.0
40	2028.74	$(Gal)_2\ (GlcNAc)_3(GalNAc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	1.3	0.0
41	2034.66	$(NeuAc)_1\ (Gal)_1\ (GalNAc)_1\ (GlcNAc)_2\ (Sulf)_1 + (Man)_3\ (GlcNAc)_2$	0.0	14.0	0.0
42	2059.73	$(NeuAc)_1\ (Gal)_2\ (GlcNAc)_2\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	0.0	3.4
43	2100.76	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_3\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	1.2	0.0	0.0
44	2100.76	$(NeuAc)_1\ (Gal)_1\ (GlcNAc)_2(GalNAc)_1\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	0.0	4.2	0.0
45	2116.76	$(NeuAc)_1\ (Gal)_2\ (GlcNAc)_3 + (Man)_3\ (GlcNAc)_2$	0.7	0.0	0.0
46	2117.78	$(Gal)_2\ (GlcNAc)_3\ (Fuc)_2 + (Man)_3\ (GlcNAc)_2$	0.0	0.0	0.5
47	2174.80	$(Gal)_2\ (GlcNAc)_4\ (Fuc)_1 + (Man)_3\ (GlcNAc)_2$	1.7	0.0	0.0

Table 3 (continued)

	Glycan mass—H_2O	N-Glycan structure	Relative intensity (%)		
			hTSH-HEK	hTSH-Pit[a]	hTSH-CHO[a]
48	2174.80	$(Gal)_2$ $(GlcNAc)_3(GalNAc)_1$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	1.4	0.0
49	2180.72	$(NeuAc)_1$ $(Gal)_1$ $(GalNAc)_1$ $(GlcNAc)_2$ $(Sulf)_1$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	3.5	0.0
50	2190.79	$(Gal)_3$ $(GlcNAc)_4$ + $(Man)_3$ $(GlcNAc)_2$	0.9	0.0	0.0
51	2204.77	$(NeuAc)_2$ $(Gal)_2$ $(GlcNAc)_2$ + $(Man)_3$ $(GlcNAc)_2$	0.0	14.1	31.9
52	2245.80	$(NeuAc)_2$ $(Gal)_1$ $(GalNAc)_1$ $(GlcNAc)_2$ + $(Man)_3$ $(GlcNAc)_2$	0.0	2.3	0.0
53	2278.81	$(NeuAc)_1$ $(Gal)_3$ $(GlcNAc)_3$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.0	2.7
54	2350.83	$(NeuAc)_2$ $(Gal)_2$ $(GlcNAc)_2$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	2.1	5.3
55	2424.87	$(NeuAc)_1$ $(Gal)_3(GlcNAc)_3$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.0	0.5
56	2553.91	$(NeuAc)_2$ $(Gal)_2$ $(GlcNAc)_3$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.5	0.0
57	2569.90	$(NeuAc)_2$ $(Gal)_3$ $(GlcNAc)_3$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.0	6.9
58	2684.97	$(NeuAc)_1$ $(Gal)_3$ $(GalNAc)_1$ $(GlcNAc)_4$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.8	0.0
59	2715.96	$(NeuAc)_2$ $(Gal)_3$ $(GlcNAc)_3$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.0	1.5
60	2743.01	$(Gal)_3$ $(GlcNAc)_4$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.6	0.0	0.0
61	2831.03	$(NeuAc)_1$ $(Gal)_3(GalNAc)_1$ $(GlcNAc)_4$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	1.0	0.0
62	2861.00	$(NeuAc)_3$ $(Gal)_3$ $(GlcNAc)_3$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.6	3.5
63	3007.06	$(NeuAc)_3$ $(Gal)_3$ $(GlcNAc)_3$ $(Fuc)_1$ + $(Man)_3$ $(GlcNAc)_2$	0.0	0.9	0.7
Average mass (Da)			1860.8	1931.3	2128.2

[a] Ribela et al. 2017

These residues greatly influence the in vivo half-life of glycohormones; sialylation protects them against clearance, while sulfation leads to a more rapid clearance rate upon binding to a receptor on the surface of liver endothelial cells (Boime and Ben-Menahem 1999; Szkudlinski et al. 2002; Ribela et al. 2017). In fact, regarding the circulatory half-life, it was observed that the $t_{1/2}$ of hTSH-HEK was the lowest of the three preparations analyzed, but, however, the closest to the $t_{1/2}$ of the native human hormone. This is possibly related to the sialylation level of these preparations, because sialic acid bound to the protein prevents its recognition by the liver asialoglycoprotein receptors that are directly responsible for the metabolic clearance of a protein, the clearance rate being inversely proportional to the degree of sialylation (Szkudlinski et al. 1993). Thus, hTSH-HEK, which has the smallest amount of sialic acid, was the preparation that cleared more rapidly from the circulation. These data, in fact, showed a highly significative correlation between $t_{1/2}$ and the amount of sialic acid (mol/mol): $r = 0.9996$, $p < 0.001$. Our findings concerning the shorter time required to reach the maximum hTSH-HEK concentration (60 min) in comparison with hTSH-CHO (90 min) agree with recently reported literature data. In fact, Gugliotta et al. (2017) observed that CHO-derived IFN4N took three times longer to reach the maximum

Table 4 Relative intensity of different types of glycans (A) and antennarity (B) in hTSH of different origins

(A)			
Glycans	hTSH-HEK (%)	hTSH-Pit[a] (%)	hTSH-CHO[a] (%)
Galactosylated	88.2	70.5	100
Fucosylated	63	35.2	11.9
Sialylated	4.7	50.8	94.2
(B)			
Antennarity	hTSH-HEK (%)	hTSH-Pit[a] (%)	hTSH-CHO[a] (%)
Mono-antennary	–	17.9	1.2
Bi-antennary	18.4	73.7	82.6
Tri-antennary	71.3	6.5	16.5
Tetra-antennary	10.3	1.8	–

[a] Ribela et al. 2017

Table 5 Molar ratio of monosaccharides or sulfate per hTSH molecule (mol/mol), determined on the basis of N-glycoprofiling analysis

Preparation	Monosaccharide (mol/mol hTSH)						
	Fuc	GalNAc	GlcNAc	Gal	Man	SA	Sulfate
hTSH-HEK	2.20	0	14.22	3.21	8.72	0.12	0
hTSH-Pit[a]	0.77	1.84	8.59	1.98	6.84	1.41	1.54
hTSH-CHO[a]	0.30	0	11.04	5.63	8.08	3.76	0

[a] Ribela et al. 2017

Table 6 Pharmacokinetic parameters and biological activity (mean T_4 level ± SD) of hTSH preparations of different origins

Preparation	T_{max} (min)	$t_{1/2}$ (min)	AUC (μg min/mL)	T_4 (μg/dL)
hTSH-HEK	60	74.3 ± 18.03	12,194 ± 2033.2	2.98 ± 0.33
hTSH-Pit	60	89.7 ± 0.42	14,270 ± 2147.1	3.55 ± 0.52
hTSH-CHO	90	115.1 ± 3.12	16,155 ± 1398.1	4.86 ± 0.77

plasma concentration than the same HEK-derived product (7.2 and 2.7 h, respectively).

In the present study, biological activity was also impacted by sialylation levels. The activity levels of hTSH-HEK were lower than those of hTSH-CHO and hTSH-Pit, but still the closest to that of the human hormone. This result is consistent with that obtained from the pharmacokinetic study, in which hTSH-HEK resulted in the lowest exposure of the organism (AUC = 12,194 μg min/mL). Also, hTSH-Pit was less active than hTSH-CHO, which is in agreement with literature reports (Thotakura et al. 1991; Szkudlinski et al. 1993; Mendonça et al. 2005; Oliveira et al. 2007; Damiani et al. 2013). The present data showed a high and significant correlation between the biological activity and the circulatory half-life (r = 0.9965, $p < 0.001$) and with the amount of sialic acid (r = 0.9983, $p < 0.001$). Preparations with low sialic acid content remain less time in traffic compared to those with higher sialic acid content and therefore their in vivo biological activity is lower. Nevertheless, hTSH-CHO (the most widely used therapeutic preparation), which according to our data is 1.8-fold more sialylated than hTSH-Pit, is 1.4-fold more bioactive but, on the other hand, hTSH-HEK, which is ~11-fold less sialylated, is only 1.2-fold less bioactive than the natural hormone. We believe that other N-glycan structures, not well identified yet, are in charge of preserving this important bioactivity. It is worth noting the accuracy of the biological assay performed in this study, with a coefficient of variation always < 16%, a variability considered small for in vivo bioassays.

In conclusion, the results obtained in the present study show that the use of HEK293T cells as a host is a viable alternative for recombinant human thyroid-stimulating hormone expression. The product synthesized meets the criteria of identity, purity, and potency required to support its use as a chemical reagent, or for in vitro use (as an immunoassay reagent), as well as in bioassays and biodistribution or structure-function studies. After the development of proper safety studies and preclinical assays, it can represent a valid alternative for clinical applications. Being of human origin, we expected greater similarities between hTSH-HEK and the human native hormone (hTSH-Pit). Some similarities were, however, found in the pharmacokinetic parameters (T_{max} and $t_{1/2}$), in the in vivo bioactivity, and, up to a point, in the fucosylation and sialylation of the N-glycan structures. The practicality in quickly obtaining the product via transient transfection is also of great interest for the development of studies on this and other human hormones, used as biopharmaceuticals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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