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Journal of Chromatography B, 787 (2003) 345–355
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of Chinese hamster ovary cell-derived recombinant thyrotropin by reversed-phase liquid chromatography

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Received 7 May 2002; received in revised form 23 September 2002; accepted 26 November 2002

Abstract

A reversed-phase high-performance liquid chromatography (RP-HPLC) methodology for the qualitative and quantitative analysis of human thyrotropin (hTSH) in CHO cell conditioned medium and in purified preparations has been set up and validated for accuracy, precision and sensitivity. A recovery test indicated a bias of less than 2% and intra-day and inter-day quantitative determinations presented relative standard deviations (RSD) always <7%, while sensitivity was 0.2 μg (RSD=5.6%). The novel methodology was applied to the study of the best cultivation conditions and was able to detect a significant difference in retention time (t_R) between pituitary and recombinant hTSH, probably reflecting the influence of the heterogeneity of the carbohydrate moiety on the hydrophobic properties of the molecule.

Keywords: Chinese hamster ovary cells; Thyrotropin

1. Introduction

The advent of recombinant DNA technology in the last two decades has made available practically unlimited amounts of biosynthetic proteins for clinical use. Among these is recombinant human thyrotropin (rec-hTSH), a heterodimeric glycoprotein hormone consisting of non-covalently linked alpha and beta subunits, whose synthesis in CHO cells has been described by different authors [1–3]. The great clinical potential of rec-hTSH for diagnostic and therapeutic purposes is especially related to thyroid carcinoma, a malignancy whose successful follow-up and treatment is essentially based on hTSH-stimulated ^{131}I uptake and organification by thyroid epithelial cells [4–12].

Thus, a variety of analytical techniques, mostly based on its physico-chemical properties, have been recommended for hTSH identity, purity and potency determinations. These complement the classical biological and immunological assays, whose accuracy limitations are well known [13,14], particularly since the glycosylation pattern may affect the antigenic structure of the molecule [15,16]. Among these techniques, reversed-phase high-performance liquid chromatography (RP-HPLC), which exploits the hydrophobic properties of molecules in the separation process and offers a high level of accuracy and sensitivity [17–19], has been widely used for the characterization and quality control of recombinant proteins in general [20] and hormones in particular

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1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00965-0

[1,21–24]. Besides being applied to the analysis of numerous heterologous proteins in their purified form, this technique has also been used for the qualitative and quantitative determination of recombinant products directly in fermentation broth matrices [14,25,26] and for monitoring the purification and folding process [23,27].

The utilization of RP-HPLC for the purification, characterization and analysis of pituitary-derived hTSH has been reported by several authors. Using mostly C_{18} silica packing, Bristow et al. [28] and Grego and Hearn [29] set up preparative and semi-preparative methodologies with the purpose of optimizing resolution and recoveries of the heterodimeric form of the hormone. With the same type of stationary phase, Parsons et al. [30] carried out the separation and detection of the different subunits of bovine and human glycoprotein hormones, hTSH included, at the 10 μg level; they also described the large scale isolation of as much as 100 mg of dissociated hormone. Using Vydac C_4 columns, Hiyama et al. [31,32] and Chlenov et al. [33] reported analytical and preparative procedures, with either partial or total separation of hTSH subunits from the undissociated form at the 10–20 μg level. Up to now, however, no specific RP-HPLC procedure has been described and validated for the qualitative and quantitative analysis of hTSH in general and of recombinant hTSH in particular, that is also applicable in CHO cell conditioned medium. The major challenge of this approach is certainly the co-elution of host cells and medium contaminants with the product of interest. This is in general circumvented in an RP-HPLC design, as pointed out by others [26,34,35], by exploiting the relative hydrophobicity of the components, by sample preparation prior to analysis and by column selection or optimization of mobile phase composition and elution conditions. The heterologous protein can thus be followed during cultivation, allowing better control of parameters that can influence product quality and permitting optimization of the production process, modifying or even avoiding an unnecessary time-consuming and expensive purification.

In the present work, therefore, we start from chromatographic conditions described by Chlenov et al. [33], for the isolation and characterization of partially purified mixtures of glycoprotein hormones, to develop and validate a practical and rapid RP-HPLC methodology for the qualitative and quantitative analysis of hTSH directly in CHO cell conditioned medium. This method can also be applied to the purification process and to the quality assessment of the purified form of the hormone. Together with high-performance size-exclusion chromatography (HPSEC), this technique will also be particularly useful for determination of the identity, purity and potency of this hormone, whose importance in clinical diagnosis and therapy is rapidly increasing [36,37].

2. Material 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) was purchased from Satelit (Araraquara, SP, Brazil). All other chemicals were analytical reagent-grade, purchased from Merck (São Paulo, Brazil) and Sigma (St Louis, MO, USA). CHO expression media were provided by Gibco–BRL (Gaithersburg, MD, USA). Recombinant hTSH (rec-hTSH IPEN) was prepared in our laboratory [3] and recombinant hTSH from Genzyme (Thyrogen) was purchased from Biobras (Montes Claros, MG, Brazil). Pituitary hTSH (pit-hTSH, NIDDK-hTSH SIAFP-B-2) was kindly provided by Dr. A.F. Parlow from the National Hormone and Pituitary Program (Torrance, CA, USA).

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

RP-HPLC was carried out with a Shimadzu Model SCL-10A HPLC apparatus coupled to 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. The column temperature was maintained at 25 °C. Detection was by UV absorbance at a wave-

length of 280 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 to 100% B over 40 min was used at a flow-rate of 0.5 ml/min. Aliquots of 200 μl of conditioned CHO medium and of 5–10 μl of pure hTSH were in general processed.

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

HPSEC was carried out with the same Shimadzu apparatus, processing 50 μl of rec-hTSH IPEN and 5- to 10-μl aliquots of Thyrogen or pit-hTSH 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.

2.4. Cell cultivation

The most productive rec-hTSH clone derived from CHO DHFR$^-$ cells (mutant line DXB11) cotransfected with the dicistronic vectors pEDdc-α and pEAdc-βTSH [3] was cultured in 162 cm^2 flasks (Corning Costar Corporation, Cambridge, MA, USA) at 37 °C in a humidified 5% CO_2 incubator. Transfected cells were grown in different media optimized for the production of recombinant proteins expressed in CHO cells, such as CHO-S-SFM II with and without nucleosides and a synthetic (CD CHO) medium. These media are low-protein and serum-free, while the absence of nucleosides (hypoxanthine and thymidine) or the addition of 0.1 μ*M* methotrexate allows a selective pressure. Conditioned media (20 ml/flask) were harvested every day during 10–20 days. Employing this same cell culture process, a blank crude cell extract was prepared with non-transfected CHO cells (DXB11).

2.5. Protein determination

Total protein concentration was estimated using the Micro BCA protein assay (Pierce Chemical Co), using a standard curve ranging from 0.5 to 200 μg/ml, constructed with a solution of pure BSA (Sigma).

2.6. hTSH immunological determination

The immunological quantification of hTSH was carried out by an in-house ''sandwich'' format IRMA, using a secondary hTSH standard calibrated against the International Standard of pituitary hTSH (WHO 80/558, 4.93 IU/mg) [38].

2.7. Sensitivity calculation

The detection limit (sensitivity) of the RP-HPLC methodology was calculated on the basis of the standard deviation ($n=3$ replicate determinations) of the lowest dose used (0.625 μg) and of the zero dose (only blank addition) using a *t*-test (one-sided, $P=0.05$) according to Rodbard's formulation [39]:

$$Y_{\min} = Y_0 + ts_p\sqrt{\frac{1}{n_0}+\frac{1}{n_1}}$$

where

$$s_p = \sqrt{\frac{s_0^2(n_0-1)+s_1^2(n_1-1)}{n_0+n_1-2}}$$

and where $Y_{\min}$ = minimal detectable response (peak area); Y_0 = response of the zero dose; n_0 = number of replicates of the zero dose; s_0 = standard deviation of the zero dose; n_1 = number of replicates of the lowest dose used; s_1 = standard deviation of the lowest dose used; t = Student's *t*-test value.

The minimal detectable dose ($X_{\min}$) was then calculated via the calibration curve.

3. Results

An adequate resolution between the original medium or host (CHO) constituents and hTSH was achieved using an RP-HPLC C_4 column at neutral pH with a gradient of acetonitrile, as shown in Fig. 1. This figure presents chromatograms of a non-transfected CHO cell conditioned medium (blank), pure recombinant TSH (Thyrogen) added to the blank and hTSH-secreting CHO cell conditioned

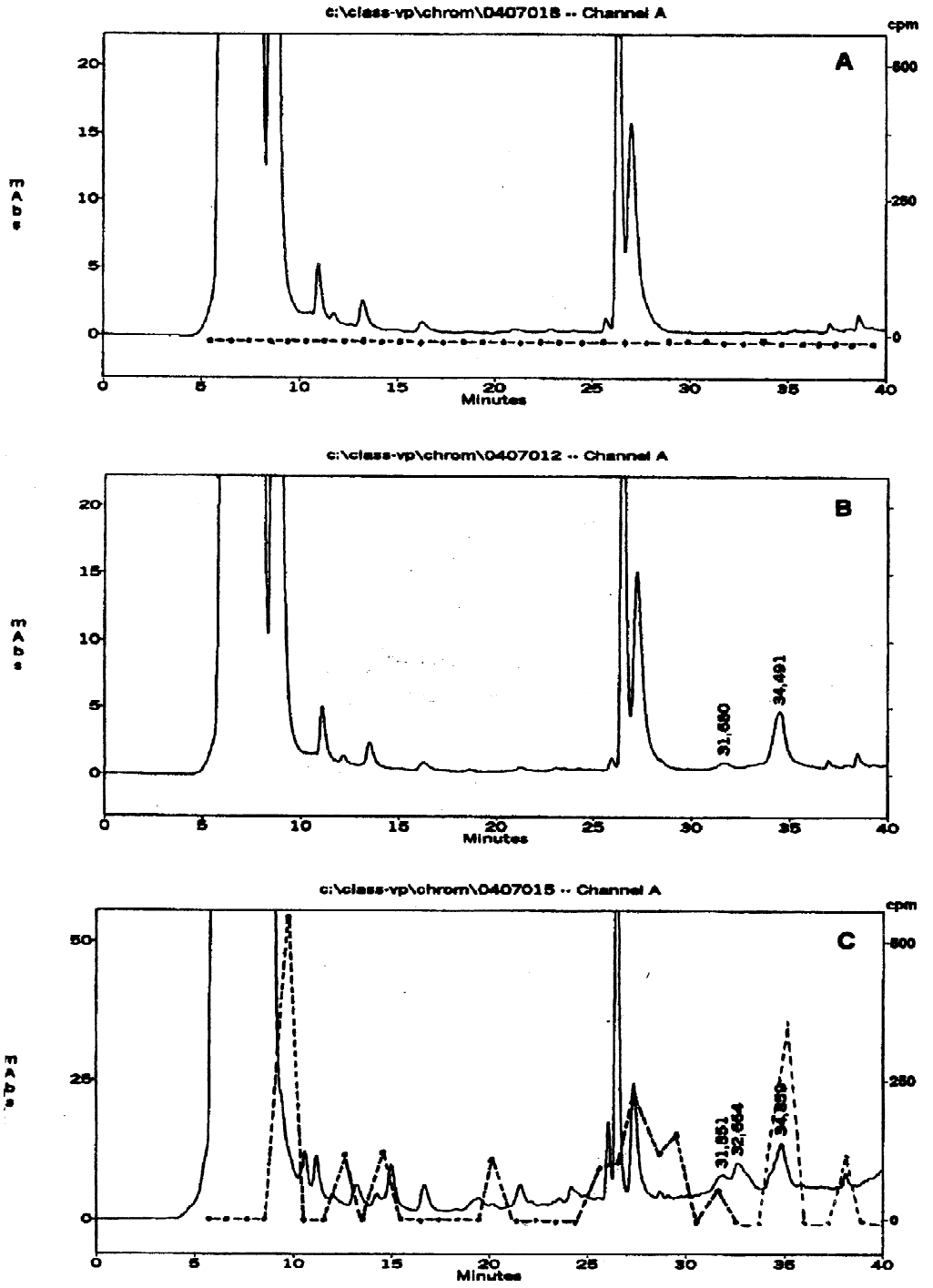

Fig. 1. RP-HPLC on a C_4 Vydac column of CHO conditioned media. (A) Medium from non-transfected cells (blank). (B) Same blank medium with the addition of 10 μg of hTSH-NIDDK. (C) Medium from hTSH-secreting cells (production medium). A_{280nm} (solid line); hTSH activity (solid circle ornament on line) determined by IRMA.

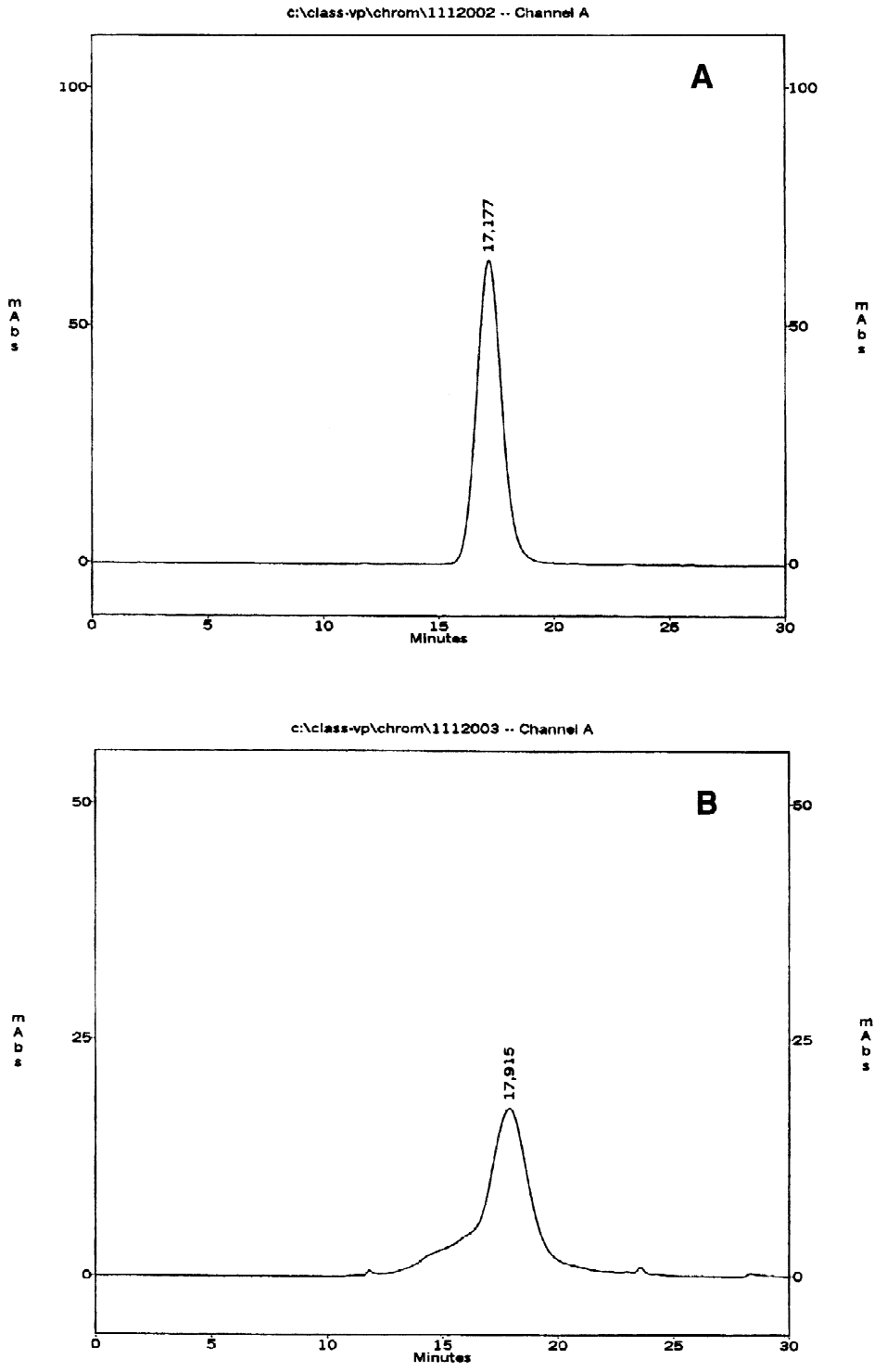

Fig. 2. HPSEC on a G2000 SW column of pure hTSH preparations. (A) Recombinant hTSH (Thyrogen), 10 μg. (B) Pituitary hTSH (NIDDK), 10 μg.

medium (production medium). The complete peak of unmodified hTSH is eluted at a t_R of 33–36 min, with altered forms starting to appear at a t_R of ~31 min. TSH is thus eluted in a practically clean area of the chromatogram, even though several extra peaks can be observed in the production medium. Some of these latter peaks have immunological activity and deserve further study. The purity and potency of Thyrogen, the only commercially available preparation of rec-hTSH, are compared with a pituitary reference preparation (pit-hTSH from NIDDK) in Fig. 2. In this case, the analysis of an equal nominal amount of the two preparations was based on high-performance size-exclusion chromatography (HPSEC), a classical physico-chemical approach for qualitative and quantitative assessment of purified proteins. As we can observe, the pituitary hTSH preparation presents a slightly higher t_R (~4%) and, apparently, two unresolved higher M_r forms that are not present in the recombinant preparation.

The present RP-HPLC methodology for the quantification of hTSH in conditioned medium was validated by analyzing the following parameters: accuracy, linearity of the calibration curve, precision and sensitivity. A recovery test was carried out by adding known amounts of pure recombinant hTSH (Thyrogen) to a conditioned medium and quantifying it against the reference preparation (pit-hTSH from NIDDK). The mean recovery of hTSH was 97.4±1.83%, with a relative standard deviation (RSD) of 1.9% ($n=12$, Table 1).

A good accuracy for hTSH determination was thus demonstrated, the correlation between added and recovered hTSH being highly significant ($r=0.9999$; $P<0.001$, for $n=6$) with a bias inferior to 2%: $Y_{recov}=0.9838X_{added}-0.0279$.

Table 1
Recovery test of known amounts of pure recombinant hTSH added to a CHO cell conditioned medium

Added hTSH (μg)	Peak area (a.u.)	Determined hTSH (μg)	Recovery (%)
20	879.0	19.60	98.0
10	448.7	9.96	99.6
5	215.9	4.81	96.2
2.5	105.9	2.36	94.4
1.25	55.1	1.23	98.4
0.625	27.3	0.61	97.6

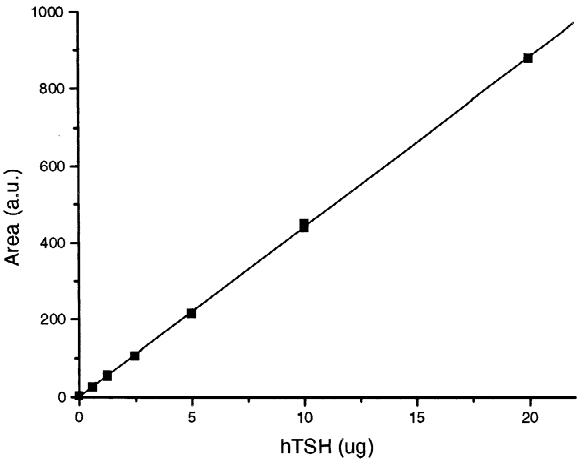

Fig. 3. Calibration curve for hTSH determination by RP-HPLC, relating peak area with known added amounts of the pure pituitary hormone.

The calibration curve (added hTSH vs. peak area), in the range from 0.625 to 20 μg, is shown in Fig. 3. The equation of the calibration curve is: $Y_{au}=44.006X_{\mu g}+1.1248$, where ''au'' are arbitrary area units. The highly significant correlation coefficient ($r=0.9999$; $P<0.0001$ for $n=15$) confirmed the linearity of the response obtained in this range.

The intra- and inter-day precision of the RP-HPLC system was evaluated in triplicate, on a single day and on three different days with three hTSH samples under the same conditions. These were prepared by adding pure hTSH to a crude cell extract with concentrations ranging from 3 to 50 μg/ml (A, B, C). The data in Table 2 show intra-day and inter-day relative standard deviations of less than 2.5 and 7.2%, respectively. Determinations with lower, but still useful levels of precision were also obtained

Table 2
Intra- and inter-day precision determination of different levels of hTSH by RP-HPLC

Sample	Intra-day[a] (μg/ml)	Inter-day[a]	
		Internal standard (μg/ml)	Dose–response curve (μg/ml)
A	3.04±2.52[b]	3.17±4.19[b]	3.17±5.52[b]
B	6.15±2.15	6.25±7.17	5.35±12.55
C	50.0±1.06	49.8±0.53	49.0±3.42

[a] All data refer to three determinations.
[b] RSD, expressed as a percentage of the mean, $n=3$.

when the same samples were analyzed inter-day using the calibration curve shown in Fig. 3, without the need of running a reference preparation each time.

The sensitivity of the method obtained according to Rodbard's formulation was 0.205 μg (or 1.02 μg/ml), with an intra-assay relative standard deviation of 5.6%. The accuracy and precision of this theoretically calculated lowest detectable amount of hTSH was confirmed experimentally. A known amount of 0.200 μg was analyzed under the same conditions, providing a value of 0.196±0.006 μg (RSD=3.1%) determined on the calibration curve.

In Table 3, an example is given of how the RP-HPLC methodology could be successfully applied to the choice of the best medium for cultivation and to monitor the secretion conditions of hTSH in the production medium. Under our conditions, SFM with nucleosides+MTX seems to be the best medium for CHO culture, with no great difference in hTSH expression over the course of the useful cultivation period of 17 days in the example shown (Fig. 4).

An intra-day qualitative analysis based on RP-HPLC retention times of different hTSH preparations was carried out on pituitary hTSH(NIDDK) and on the two recombinant products available to us, rec-hTSH-Genzyme and rec hTSH-IPEN (Table 4). The intra-day strategy was adopted as a function of the intrinsic inter-day variation in the retention time of RP-HPLC, which is extremely sensitive to small differences in the composition of the mobile phase. A Student's *t*-test indicated a highly significant difference ($P<0.001$) between the retention time of pituitary and both recombinant hTSH preparations. Thus, rec-hTSH-Genzyme has a retention time that is 1.22% lower than that of the pituitary preparation,

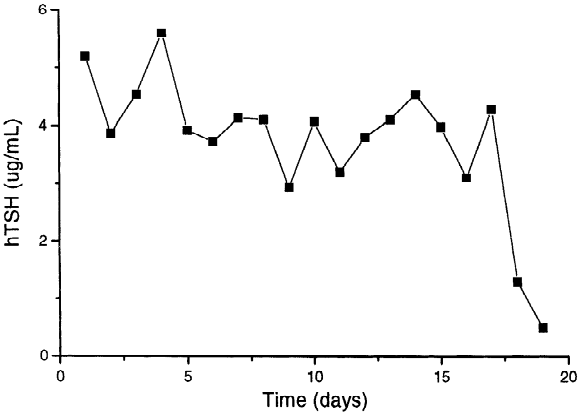

Fig. 4. hTSH level in CHO production media (SFM with nucleosides+10 μ*M* MTX) collected on different days along the cultivation period, carried out in T-flasks (162 cm^2).

while in the case of rec-hTSH-IPEN this difference is 0.91%. The two recombinant products therefore appear to be more similar to each other, even though their retention difference (0.31%) is also highly significant ($P<0.001$). Analogous statistically significant differences were found when the same three preparations were analyzed by HPSEC, probably reflecting a significant difference in molecular size (Table 5). In the case of HPSEC, an inter-day strategy was adopted as a function of the high precision obtained even with this design.

In Fig. 5, an example of the application of the present RP-HPLC methodology to the analytical comparison of the two highly purified reference preparations of hTSH (pituitary and recombinant) is reported. The recombinant preparation is apparently more homogeneous, presenting less isoforms (<5%) than the pituitary preparation, while retention times confirmed the same difference (1.2%) already found (Table 4).

Table 3
hTSH and protein content of different types of media (Gibco) used in recombinant protein production from CHO cells

Medium	hTSH[a] (μg/ml)	Total protein[b] (μg/ml)	Specific activity % (μg hTSH/μg protein)
CD without nucleosides	0.70	62.5	1.12
SFM without nucleosides	2.04	123.8	1.65
SFM with nucleosides+MTX	2.74	121.2	2.26
SFM with nucleosides	2.40	115.8	2.07

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

Table 4
Intra-day retention time statistics for three hTSH preparations analyzed on RP-HPLC

Sample	$t_R \pm SD$[a] (min)	RSD[b] (%)	Difference from the native preparation (%)	No. of determinations
Pituitary hTSH (NIDDK)	32.88±0.036	0.11	–	6
Recombinant hTSH (Thyrogen)	32.48±0.028	0.09	−1.22	7
Recombinant hTSH (IPEN)	32.58±0.060	0.06	−0.91	10

[a] Median±standard deviation.
[b] RSD expressed as percentage of the mean.

4. Discussion

A RP-HPLC methodology for the direct qualitative and quantitative analysis of rec-hTSH in CHO cell conditioned medium has been set up. Our approach was based on the unpublished observation that, by classical hydrophobic interaction chromatography (i.e. Phenyl Sepharose) the hTSH molecule was found to be more hydrophobic than most of the proteins present in the chosen CHO culture media. This behaviour was reproduced in practice in the RP-HPLC methodology. Under these conditions, we also tried to introduce the minimum number of manipulations in the sample that could qualitatively or quantitatively influence our protein of interest. We found it to be essential, however, in order to reach a higher sensitivity and resolution compared to Chlenov's conditions, to introduce a fourfold decrease in the flow-rate (0.5 ml/min instead of 2.0 ml/min). This confirmed the experimental observation of Riggin et al. [21] who found increased column efficiency and resolution with decreasing flow-rate. Better resolution was also obtained via a twofold decrease in the ionic strength of the mobile phase, using 0.05 *M* instead of 0.1 *M* phosphate buffer, with the pH slightly increased from 6.8 to 7.0. We also observed that spectrophotometric detection at 280 nm instead of 220 nm significantly decreased the interference of the components of the medium. The technique obviously can also be applied to the characterization of the final, purified product. Together with HPSEC, presented here for comparison, it represents one of the very few physico-chemical methods that can be used for an accurate analysis of this hormone. Each of these two techniques can, of course, detect the presence of different types of isoforms [21,40]. The applicability of this RP-HPLC technique to rec-hTSH determination in crude media or in partially purified products makes it ideal for planning and following each step of the purification process.

The novel methodology has been validated with respect to accuracy, precision and sensitivity. This last parameter is of particular interest, since it indicates a detection limit not obtained previously for hTSH by physico-chemical techniques. This limit was confirmed by both a theoretical approach and a practical recovery test. The quantification method has proved to be so robust that a predetermined standard curve with a high linearity range provided useful determinations with an acceptable precision without running a new standard each time. This is

Table 5
Inter-day retention time statistics for three hTSH preparations analyzed on HPSEC

Sample	$t_R \pm SD$[a] (min)	RSD[b] (%)	Difference from the native preparation (%)	No. of determinations
Pituitary hTSH (NIDDK)	17.61±0.070	0.40	–	5
Recombinant hTSH (Thyrogen)	17.16±0.034	0.20	−2.56	5
Recombinant hTSH (IPEN)	17.39±0.065	0.37	−1.25	5

[a] Median±standard deviation.
[b] RSD expressed as percentage of the mean.

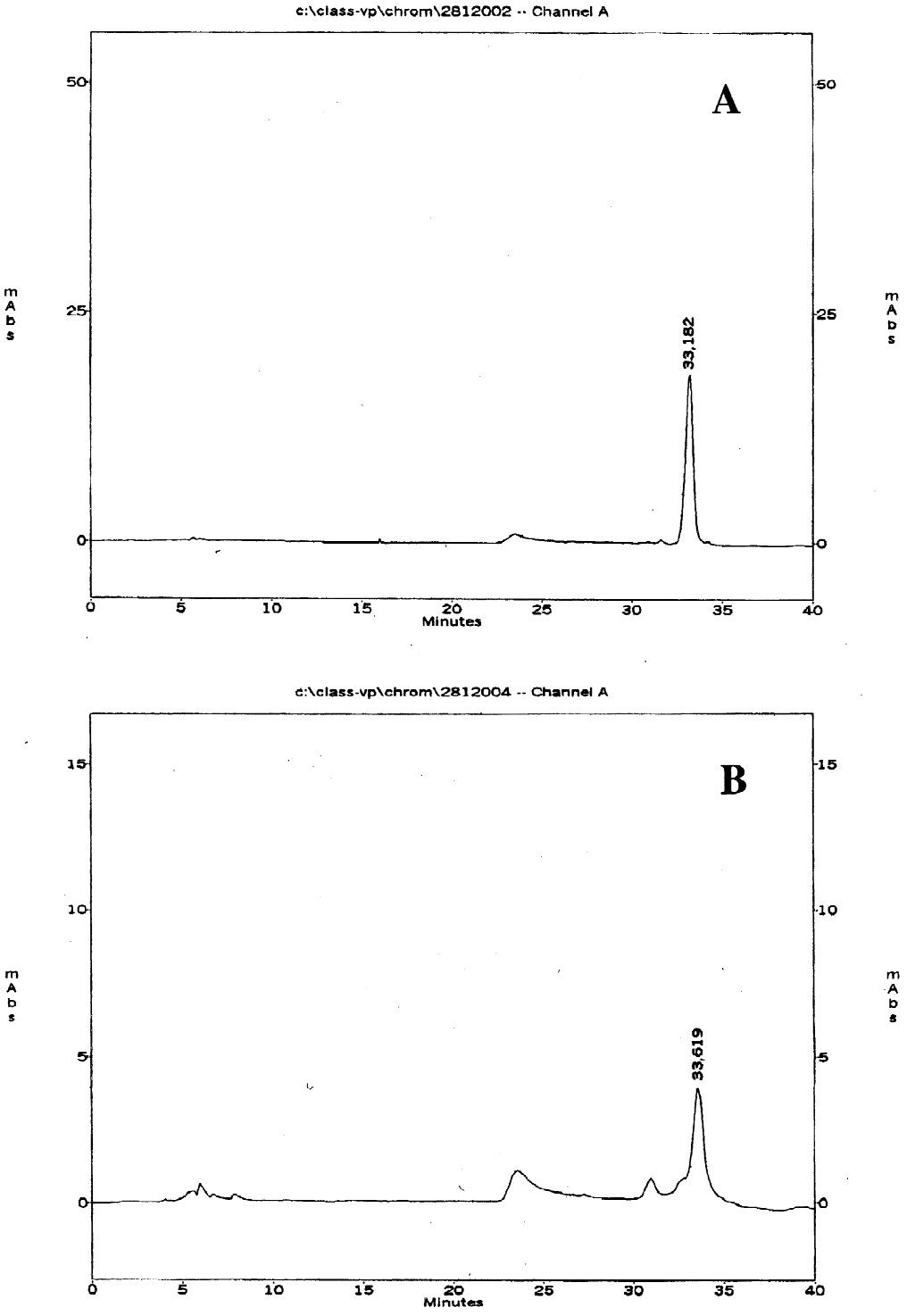

Fig. 5. RP-HPLC on a C_4 Vydac column of pure hTSH preparations. (A) Recombinant hTSH (Thyrogen), 10 μg. (B) Pituitary hTSH (NIDDK), 10 μg.

particularly important in view of the high cost and limited availability of purified hTSH. It might also be useful to consider the utilization of micro-HPLC, a miniaturized separation system which offers advantages over classical HPLC such as the use of very small samples and a higher mass sensitivity [41]. Standard or reference preparations of recombinant or pituitary-derived hTSH are very rare and precious and almost never exceed the hundreds of micrograms scale. For this reason, we present a chromatographic comparison by classical HPSEC and the present RP-HPLC technique of the only pituitary reference preparation of hTSH now available on this scale and of the only recombinant hTSH now commercially available. As far as we know, a similar comparison has never been reported before.

From a qualitative point of view, the method proved to be extremely sensitive at detecting very fine structural differences, probably related to the carbohydrate moiety. As pointed out by Rassi [42], there is evidence that carbohydrate microheterogeneity contributes to some extent to molecular retention in RP-HPLC. Carbohydrate variability can arise both from the host cell type being used for the heterologous glycoprotein expression [15,38,43,44] and from different cultivation and bioreactor conditions or the presence of specific factors in the culture medium [45–48]. RP-HPLC retention time, a type of hydrophobicity index, could be used as a rapid, highly sensitive identity and quality test for such variability [14,34,42]. Other authors have reported that differences in the oligosaccharide structures of glycoprotein hormones do not affect their RP-HPLC elution properties [30,31]. Our data, showing a significant difference in t_R between a well-known reference preparation of pituitary hTSH (NIDDK) and a completely characterized [1] preparation of recombinant hTSH (Thyrogen), whose structural differences are apparently only related to their carbohydrate moiety [15], tend to modify this concept.

Other applications of the technique, such as the choice of the best culture medium, qualitative and quantitative follow-up of rec-hTSH secretion during cultivation and monitoring purification steps, have demonstrated their effectiveness. The use of RP-HPLC is thus likely to improve greatly the quality, reproducibility and efficiency of the costly and delicate production process of rec-hTSH.

Acknowledgements

This work was supported by FAPESP, São Paulo, Brazil (projects 99/01628-4 and 00/09008-4), by National Research Council (CNPq), Brasilia, Brazil (projects RHAE 381970/01-9 and PQ 301520/91-7). We are grateful to José Maria de Souza for valuable and skilled technical assistance.

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