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Reversed-phase high performance liquid chromatography as an alternative to animal bioassay for human thyrotropin potency determination

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Reversed-phase high performance liquid chromatography (RP-HPLC) was compared to the *in vivo* bioassay (BA) based on TSH-induced T_4 determination in mice. A linear relationship ($BA_{\mu g} = 0.9790$ RP-$HPLC_{\mu g} - 0.052$) with a highly significant correlation ($r = 0.87$; $p < 0.001$; $n = 14$ preparations) was found between these two methods. The mean difference between the bioactivity predicted from RP-HPLC data *via* this equation and the mean of the bioactivities obtained with the two methods for 11 other hTSH preparations was −2.01%, with a 95% confidence interval of −13.13% to +9.11%. This analysis included 5 totally or partially-degraded samples, indicating a useful correlation between the two determinations and, as expected, a higher sensitivity of the physical–chemical method. Interestingly, a commercial hTSH lot did not show any alteration 2 years post expiration date. These results demonstrate that RP-HPLC is a novel and viable alternative to the use of *in vivo* bioassays for hTSH potency determination, thus avoiding or reducing animal use.

1. Introduction

In the production of recombinant proteins, in particular bioactive proteins, a constant concern is to monitor their potency throughout the production process. Animal bioassays are most commonly used for this purpose. However, when possible, the 3 Rs (refinement, reduction and replacement) philosophy should be respected, planning the optimization of the assay conditions and decreasing the number of animals needed for each potency test, as well as setting up non-animal methodologies that are able to predict the activity of the biopharmaceutical.

Such alternative strategies for potency assessment should provide biologically meaningful information and should be able to detect differences that may impact the *in vivo* bioactivity.[1] This approach is particularly challenging for glycoproteins, especially considering their structural complexity and the inherent heterogeneity due to their carbohydrate moiety. It has generally been agreed that no single method alone is able to include all possible effects involved in *in vivo* biological activity, a combination of different types of testing being the most feasible scenario to consider.[2] Alternative assays can partially replace animal testing, especially for monitoring the consistency of a recombinant protein production at all stages of the manufacturing process, ensuring a high quality of the final product.[3]

Several non-animal assays, including physical–chemical methods, have already been reported as alternative possibilities for biological activity evaluation of therapeutic glycoproteins.

Isoelectric focusing and capillary zone electrophoretic analysis were shown to predict the biological potency of human follitropin (hFSH) with precision and accuracy sufficient to discriminate between FSH preparations, which could meet the European Pharmacopoeia requirements for this hormone.[4–6] Size-exclusion liquid chromatography (HPSEC) was shown to be an appropriate alternative to bioassay for batch-to batch consistency assessment in the same hFSH production process and for its precise delivery.[7,8] Another physical–chemical testing strategy, reversed-phase high performance liquid chromatography (RP-HPLC), was shown by our research group to correlate well with *in vivo* bioassays for hFSH potency determination; the mean predicted potency estimated by RP-HPLC was no less than 85% and no more than 109% of that estimated by bioassay and the RP-HPLC method was proposed as a practical and useful assay for the quality control of internet-obtained pharmaceuticals and for the detection of counterfeit and/or substandard drugs.[9] RP-HPLC and HPSEC were also shown to be useful alternatives to the normocythaemic mice bioassay for

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erythropoietin potency assessment, the estimated content/potency determined by these methods being respectively 2% and 1% lower than those determined by *in vivo* bioassay, a difference that is statistically non-significant.[10]

Given the current interest in reducing and/or refining the currently required animal bioassays, the present work reports an RP-HPLC methodology that represents a potential alternative to the *in vivo* bioassay for testing the potency of human thyrotropin (hTSH). This hormone is a heterodimeric glycoprotein formed by two non-covalently linked subunits whose association is mandatory for expression of the biological activity. As a biopharmaceutical it is utilized especially for thyroid cancer management and for the evaluation of the thyroid reserve capacity.[11,12]

2. Materials and methods

2.1 Hormone preparations

Twenty-five different pituitary-derived (A and B) and recombinant (C–U) hTSH preparations were analyzed in this work. The pituitary preparations were obtained from the National Hormone and Pituitary Program (Torrance, CA, USA) and from the Oslo University Hospital (Oslo, Norway). Among the recombinant preparations, one is the commercial preparation, Thyrogen, from Genzyme Corporation (Framingham, MA, USA), while the others were from our laboratory at IPEN-CNEN/SP (São Paulo, Brasil), prepared as previously described.[13–16] The purity of all utilized preparations was >98%. All tested preparations were lyophilized and stored at 4 °C. The declared Thyrogen formulation included mannitol, sodium phosphate and sodium chloride. The pituitary preparations were pure. The IPEN preparations were lyophilized in 0.15 M NaCl, 0.02 M sodium phosphate buffer, pH 7.0. Five of the analyzed samples were potentially altered: one of them had a validity that had expired 2 years previously, another was subjected to five freezing and thawing cycles, while the other 3 samples were submitted to heating at different temperatures (100 °C, 65 °C, and 50 °C) for different times (5 min, 10 min, and 12 h respectively).

The recombinant International Standard of human Thyroid-Stimulating Hormone for bioassay, from the World Health Organization (WHO 03/192), was utilized in the assays.

2.2 *In vivo* bioassay (BA)

All assays were conducted in accordance with the national protection laws on animal welfare.

The biological activity of the different hTSH preparations was determined by an *in vivo* bioassay in BALB/c mice in which TSH-induced T_4 is measured after a 5 day suppression of endogenous TSH by T_3 administration.[17] A single dose (10 μg hTSH per mouse, injected intraperitoneally) and 10 mice were utilized for each hTSH preparation and for the standard.[14] Serum samples were obtained at 6 hours post injection. The serum T4 concentrations were determined using human T4 RIA kits (Siemens, Los Angeles, USA), according to the manufacturer's instructions.

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

As previously described,[18] RP-HPLC was carried out with a Vydac C4 column (300 Å pore size, 5 μm particle size, 25 cm × 4.6 mm I.D.) purchased from Grace-Vydac, connected to a Shimadzu HPLC apparatus. Chromatography was carried out at 25 °C with detection by UV absorbance at a wavelength of 220 nm. Solutions A and B were utilized in the mobile phase, solution A being sodium phosphate buffer (pH 7.0; 0.05 M) and solution B being 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 minutes was used.

2.4 High-performance size-exclusion liquid chromatography (HPSEC)

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 Tosohaas (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^{-1}, employing 0.15 M NaCl in sodium phosphate buffer (pH 7.0; 0.02 M) as the mobile phase.

2.5 Statistical analysis

The agreement between the hTSH potency determination by *in vivo* bioassay and the proposed alternative assay was evaluated following the Bland–Altman statistical methodology.[19,20] The difference between the values obtained by the two methods (d) and the corresponding mean and standard deviation (SD), defining the range between which 95% of the d values can be expected to lie ($\bar{d} \pm 2\mathrm{SD}$), were calculated. The standard error of the mean of $d(\sqrt{\mathrm{SD}^2/n})$ and the standard error of the 95% confidence limit $\sqrt{(3\mathrm{SD}^2/n)}$ were also calculated. The range that indicates the precision with which the mean of d was determined was calculated as $\bar{d} \pm t\sqrt{\mathrm{SD}^2/n}$. The precision of

Table 1 Comparison between the quantification of hTSH preparations *via in vivo* bioassay and by RP-HPLC

Preparation	hTSH determined by *in vivo* bioassay (μg per vial)	hTSH determined by RP-HPLC (μg per vial)
phTSH-A	5.2	3.7
rhTSH-C	11.3	10.0
rhTSH-D	11.1	10.7
rhTSH-E	9.0	9.3
rhTSH-F	7.6	9.7
rhTSH-G	4.4	5.8
rhTSH-H	4.4	4.0
rhTSH-I	8.8	8.6
rhTSH-J	8.2	9.9
rhTSH-K	7.9	8.8
rhTSH-L	10.0	8.0
rhTSH-M	3.6	5.4
rhTSH-N	9.5	9.0
rhTSH-O	4.0	5.1

$\mathrm{BA}_{\mu g} = 0.9790\ \mathrm{RP\text{-}HPLC}_{\mu g} - 0.052$, ($r = 0.8725$; $p < 0.001$; $n = 14$)

the lower confidence limit (LL) was calculated from the expression $LL \pm t\sqrt{3SD^2/n}$ and the precision for the upper confidence limit (UL) from $UL \pm t\sqrt{3SD^2/n}$. Student's t statistic with $n - 1$ degrees of freedom at the 0.05 significance limit was performed on the average ± SD of the *in vivo* and predicted bioactivities.

3. Results

The content of fourteen unaltered hTSH preparations was evaluated by RP-HPLC and by *in vivo* bioassay utilizing the WHO-03/192 International Standard for both systems (Table 1). This standard analyzed by RP-HPLC and by HPSEC presented a

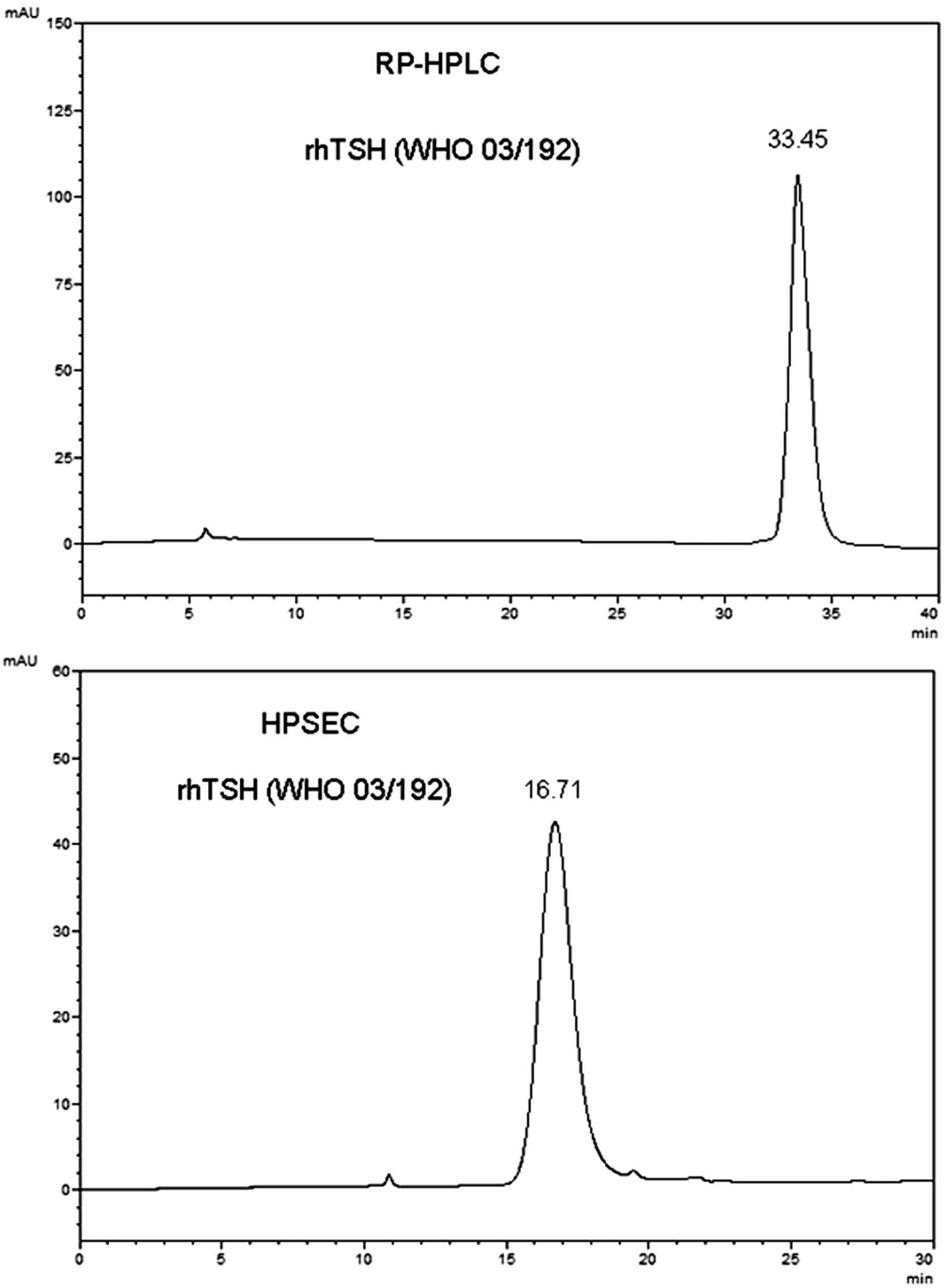

Fig. 1 HPLC profile of 5.0 μg of the International Standard of Recombinant Thyrotropin for biological assays (WHO-03/192): (A) RP-HPLC; (B) HPSEC.

unique peak, demonstrating its homogeneity and purity (Fig. 1). The content/potency of each ampoule was expressed in mass units since, for clinical applications, this biopharmaceutical is in general prescribed in mg. A linear relationship, with a highly significant correlation, was found between the RP-HPLC and the *in vivo* bioassay (BA) determinations: $BA_{\mu g} = 0.9790RP\text{-}HPLC_{\mu g} - 0.052$ ($r = 0.8725$; $p < 0.001$; $n = 14$).

The validity of this correlation was tested for 11 other different samples by comparison of the bioactivity predicted from RP-HPLC data *via* this equation with the mean of the bioactivities obtained by the two methods (Table 2). The difference (d) between these two parameters, for each sample, plotted against the mean of the bioactivities, led to the distribution shown in Fig. 2.

Bland and Altman statistics[19] were applied in the present study and the statistical parameters obtained, as defined in the Materials and methods section, are presented in Table 3.

The five potentially altered samples presented the RP-HPLC profiles shown in Fig. 3 and 4. In Fig. 3, the sample with expired validity (B) and the sample that was frozen and thawed 5 times (C) both showed the same profile as the control sample (A), with a recovery relative to the control of >94%. Likewise, the recovery derived from bioassay determinations was also complete (Table 4). The RP-HPLC profiles of samples heated at different temperatures showed different levels of degradation (Fig. 4). After heating the sample at 100 °C, a complete dissociation of the heterodimer into its α and β subunits occurred, without recovery of the heterodimeric form (Fig. 4B). This is in agreement with the data on total hTSH dissociation reported by Carvalho *et al.*:[21] 47.4% and 52.6% for α and β subunits, respectively; the alpha subunit is less hydrophobic than the beta subunit and its hydrophobicity is practically coincident with that of the heterodimer. As expected, no activity was detected by the *in vivo* bioassay for this sample as the hormone is only active when in its heterodimeric form. The heating of the same sample at lower temperatures (50 °C and 65 °C) provoked only partial dissociation (Fig. 4C and D). Although the α-subunit is not resolved from the heterodimer, its amount could be estimated stoichiometrically from the amount of β still present, the losses of the heterodimer being 12.4 and 21.6% at 50 °C and 65 °C, respectively (Table 4). A similar loss of activity (25.7%) was detected by bioassay for the sample heated at 65 °C. On the other hand, no detectable loss was observed in the bioassay of the sample heated at 50 °C, confirming the higher sensitivity of RP-HPLC for detecting relatively small alterations.

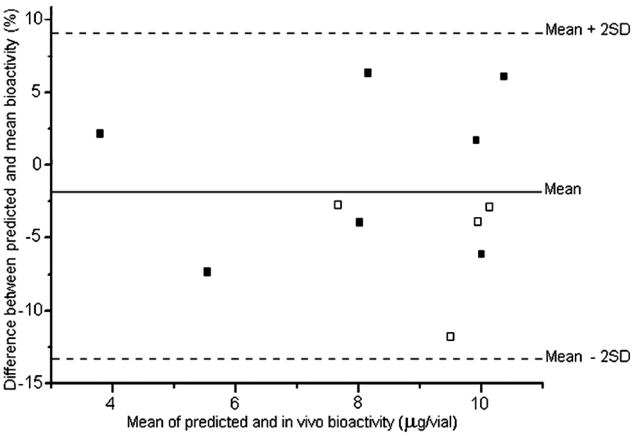

Fig. 2 Comparison between the activity predicted by RP-HPLC and the *in vivo* bioactivity, plotting as the percent difference *vs.* their mean. ■ different preparations or lots; □ potentially degraded samples.

4. Discussion

A suitable physical–chemical testing strategy (RP-HPLC) for hTSH potency assessment is proposed in this work as an alternative to *in vivo* bioassays. As far as we know, this is the first time that a chromatographic alternative was shown to efficiently substitute the *in vivo* bioassay for hTSH potency

Table 2 Comparison between the biological activity determined by the *in vivo* bioassay and predicted by the equation *via* RP-HPLC analysis

Preparation	Biological activity predicted by RP-HPLC (1) (μg per vial)	Biological activity determined by *in vivo* bioassay (2) (μg per vial)	Mean biological activity (1) and (2) (μg per vial)	Difference between predicted and mean biological activity d (μg)	Difference between predicted and mean biological activity d (%)
phTSH-B	5.1	5.8	5.5	−0.4	−7.3
rhTSH-P	11.0	9.7	10.4	0.6	5.8
rhTSH-Q	8.6	7.5	8.1	0.5	6.2
rhTSH-R	4.0	3.7	3.9	0.1	2.3
rhTSH-S	7.7	8.2	8.0	−0.3	−3.8
rhTSH-T	10.0	9.6	9.8	0.2	2.0
rhTSH-T (4 °C, 2 years post expiration date)	9.4	10.1	9.8	−0.4	−4.1
rhTSH-T (5 cycles of freezing and thawing)	9.9	10.4	10.2	−0.3	−2.9
rhTSH-U	9.4	10.5	10.0	−0.6	−6.0
rhTSH-U (50 °C, 12 h)	8.3	10.4	9.4	−1.1	−11.7
rhTSH-U (65 °C, 10 min)	7.4	7.8	7.6	−0.2	−2.6
					$\bar{d} \pm SD = 2.01 \pm 5.56$

Table 3 Statistical parameters, determined according to Bland and Altman, comparing the values of *in vivo* bioactivity with those predicted *via* RP-HPLC determinations

Parameter	Determined value (%)
Mean difference ($\bar{d}$)	−2.01
Standard deviation (SD)	5.56
Range ($\bar{d} \pm 2SD$)	−13.13 to +9.11
$\sqrt{SD^2/n}$	1.68
$\sqrt{3SD^2/n}$	2.90
Range $\left(\bar{d} \pm t\sqrt{SD^2/n}\right)$	−5.76 to +1.4
Range $\left(LL \pm t\sqrt{3SD^2/n}\right)$	−19.60 to −6.65
Range $\left(UL \pm t\sqrt{3SD^2/n}\right)$	+2.64 to +15.58

determination, thus reducing the dependence on animal use, which is a strong worldwide tendency. In this respect, the results are in the direction of combining or eventually replacing *in vivo* bioassays in recombinant hTSH quality control protocols. This is in line with the recommendations of various regulatory agencies that *in vivo* assays be employed only in cases where insufficient structural information is available and the desired analysis cannot be performed by other techniques that do not involve the use of animals.[22]

The biological approach is intrinsically the most appropriate method of determining the potency of a given hormone.[23] Nonetheless, even though physical–chemical analytical methods may not completely reflect the *in vivo* action of the hormone, they can still complement the classical *in vivo* bioassay and represent an extremely useful and important alternative to animal bioassay.

The comparison between the results obtained by *in vivo* bioassays and those predicted by RP-HPLC analysis for 11 hTSH samples (intact and totally- or partially-degraded) that were not used to construct the correlation curve showed an acceptable agreement (bias ~ 2%); the potency estimated by RP-HPLC was no less than 80% and no more than 115% of that estimated by the bioassay method. The Bland–Altman statistics for hTSH were similar to those obtained in our previous work on hFSH in which slightly better parameters were obtained: bias of −1.35%, with a potency no less than 85% and no more than 109% of that estimated by *in vivo* bioassay.[9]

The agreement obtained for hTSH samples subjected to thermal treatment demonstrated the capacity of the alternative assay to detect product alterations that cause a loss of activity. Loss of activity was found for the samples heated at 100 °C and 65 °C employing both assay systems. In these samples, RP-HPLC was able to identify the total or partial dissociation of the heterodimer into subunits, which by themselves have no biological activity. In samples heated at 50 °C, a slight dissociation was also detected by RP-HPLC, but not in the *in vivo* bioassay, showing the higher sensitivity of RP-HPLC for the detection of structural alterations of the protein. We emphasize here the importance of a previous study carried out in our laboratory by Carvalho *et al.*[21] on the identification and characterization of

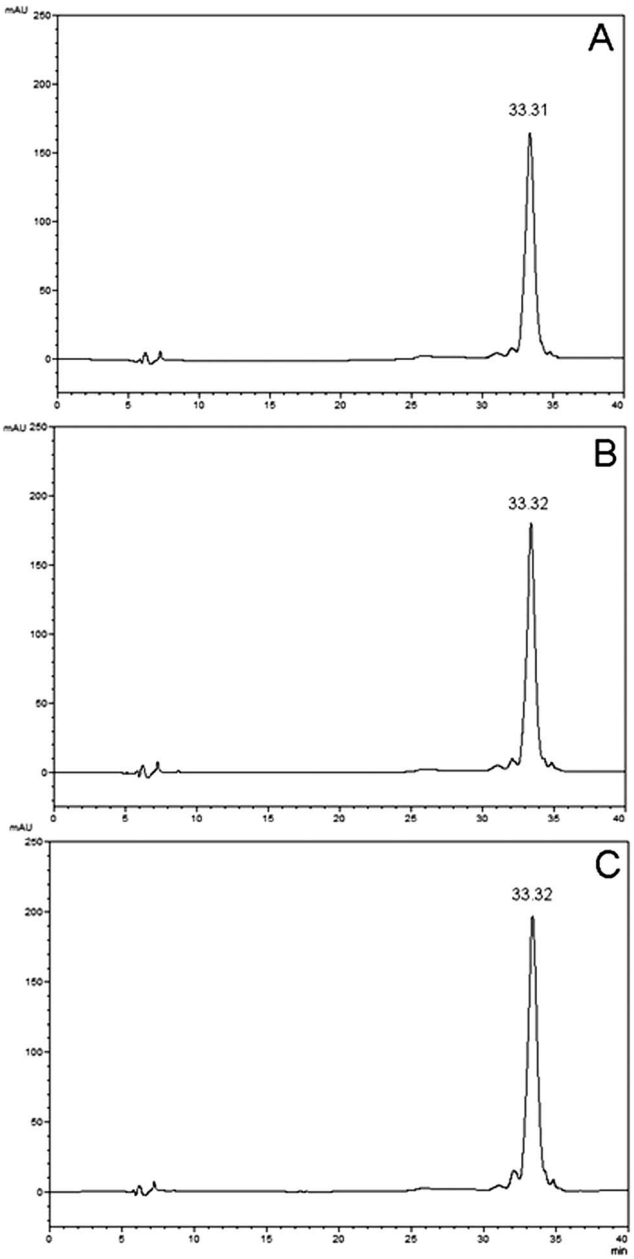

Fig. 3 RP-HPLC profile of the rhTSH-T preparation under different conditions: (A) control; (B) 2 years post expiration date, always kept at 4 °C; (C) submitted to 5 cycles of freezing and thawing.

the α and β subunits of pituitary hormones, which allowed the quantification of the remaining heterodimeric portion of the heated samples.

Also noteworthy is the high stability found for the commercial sample of hTSH, which was stored at 4 °C and analyzed two years after its expiration date; no detectable degradation was found by either the *in vivo* bioassay or by the proposed RP-HPLC method. The sample subjected to five cycles of freezing and thawing also remained unaltered, as confirmed by the two methodologies. Other authors also reported the high biological stability of this commercial recombinant hTSH preparation, under different conditions, when tested in a cell culture

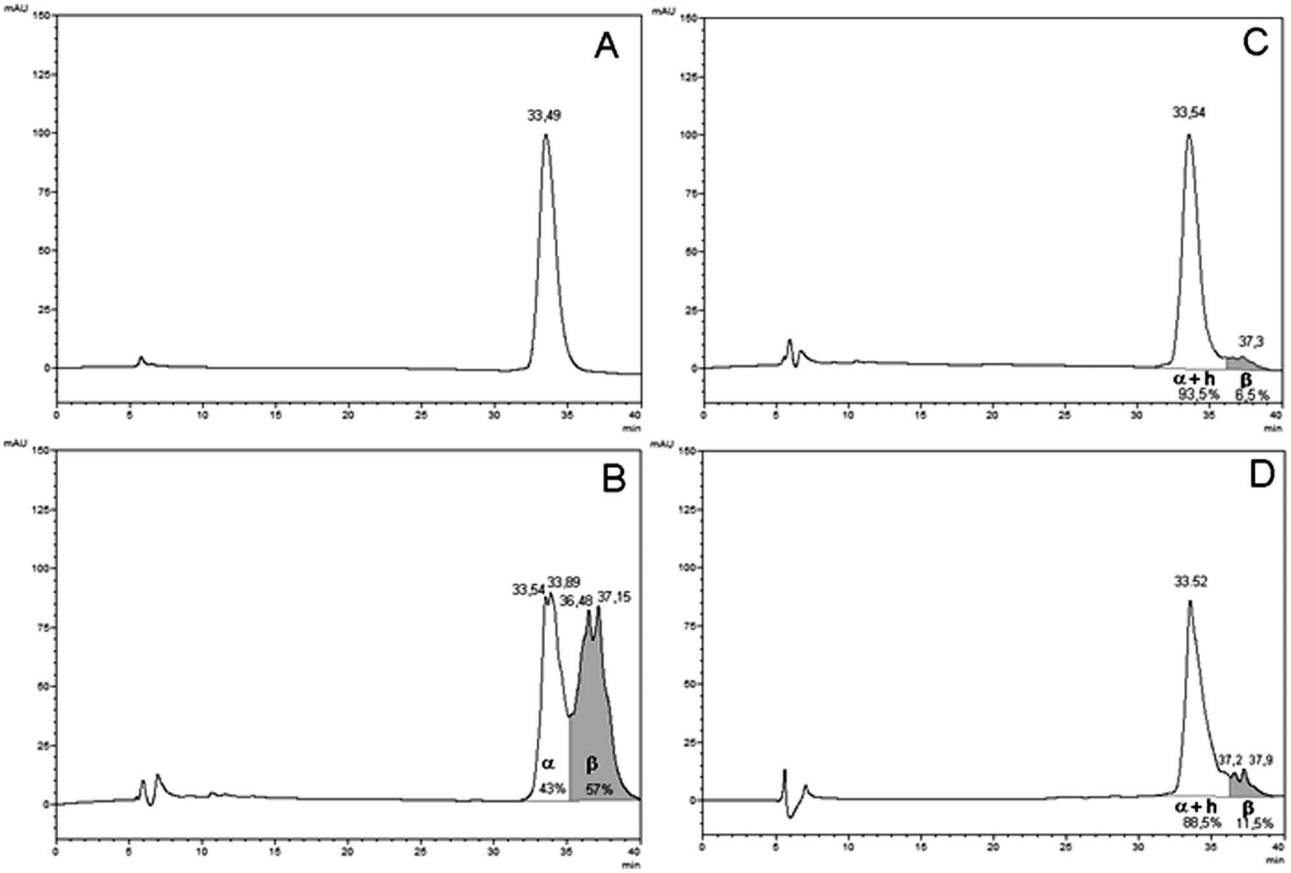

Fig. 4 RP-HPLC profile of the rhTSH-U preparation treated as follows: (A) control; (B) heated at 100 °C for 5 minutes; (C) heated at 50 °C for 12 hours; (D) heated at 65 °C for 10 minutes.

Table 4 Comparison between the quantification of potentially degraded hTSH preparations *via in vivo* bioassay and by RP-HPLC[a]

	hTSH determined by RP-HPLC		hTSH determined by *in vivo* bioassay	
Preparation	(μg per vial)	(% recovery)	(μg per vial)	(% recovery)
rhTSH-T (control)	10.3	___	9.6	___
rhTSH-T (4 °C, 2 years post expiration date)	9.7	94.2	10.1	105.2
rhTSH-T (5 cycles of freezing and thawing)	10.2	99.0	10.4	108.3
rhTSH-U (control)	9.7	___	10.5	___
rhTSH-U (50 °C, 12 h)	8.5	87.6	10.4	99.0
rhTSH-U (65 °C, 10 min)	7.6	78.4	7.8	74.3
rhTSH-U (100 °C, 5 min)	N.D.	0	N.D.	0

[a] N.D. = not detectable.

bioassay based on FRTL-5 cells.[24] In our opinion, the optimized formulation and lyophilization conditions of this preparation allow its long-term stability, although some effects of increasing oxidation should be noticed after several years of storage, as reported by Sendak *et al.*[25] We further emphasize that the RP-HPLC assay proposed here was validated following the ECVAM (the European Centre for Validation of Alternative Methods) guidelines and recommendations.[26,27] This methodology in combination with others, such as isoelectric focusing[28] or capillary zone electrophoresis,[29] can be a powerful tool for batch-to-batch variability evaluation and for recombinant glycoprotein quality assessment along the production process.

The potency determination of products intended for pharmaceutical use is very important, especially considering the lack of precision and accuracy in biological measurements, which can compromise the effectiveness of a treatment with unpredictable consequences for human health. Physical–chemical assays provide more accurate and precise determinations and can thus be used in the production process after an initial validation against a recommended *in vivo* bioassay. The present

work thus contributes to a more strict control of the potency of pituitary hormones with the perspective of reducing both the assay time and eliminating the necessity of employing animal-based *in vivo* assays.

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References

1 A. R. Mire-Sluis, *Pharm. Res.*, 2001, **18**, 1239–1246.
2 M. J. Schiffelers, B. J. Blaauboer, C. F. Hendriksen and W. E. Bakker, *ATLEX*, 2012, **29**, 287–300.
3 B. Metz, F. Brunel, C. Chamberlin, J. van der Gun, M. Halder, W. Jiskoot, G. Kersten, O. van Opstal, J. W. Petersen, S. D. Ravetkar, K. Redhead, M. Schwanig, E. S. Wilhelmsen, W. F. Vann and C. Hendriksen, *ATLA, Altern. Lab. Anim.*, 2007, **35**, 323–331.
4 J. W. Mulders, M. Derksen, A. Swolfs and F. Maris, *Biologicals*, 1997, **25**, 269–281.
5 J. W. Mulders, H. Wijn, F. Theunissen, P. Machielsen and P. Janssen, *Pharm. Pharmacol. Commun.*, 1999, **5**, 51–55.
6 P. L. Storring, R. E. Gaines Das, J. W. Mulders and M. Halder, *Biologicals*, 2002, **30**, 217–234.
7 R. Driebergen and G. Baer, *Curr. Med. Res. Opin.*, 2003, **19**, 41–46.
8 R. Bassett, *Reprod. BioMed. Online*, 2005, **10**, 169–177.
9 B. E. Almeida, J. E. Oliveira, R. Damiani, S. L. Dalmora, P. Bartolini and M. T. C. P. Ribela, *J. Pharm. Biomed. Anal.*, 2011, **54**, 681–686.
10 R. Schutkoski, R. B. Souto, G. Z. Calegari, L. I. Elsenbach, L. P. Porto, V. Schramm, F. P. Stamm and S. L. Dalmora, *Anal. Methods*, 2013, **5**, 4238–4243.
11 M. K. Chen, I. Doddamane and D. W. Cheng, *Curr. Opin. Oncol.*, 2010, **22**, 6–10.
12 S. J. Bonnema and L. Hegedus, *Endocr. Rev.*, 2012, **33**, 920–980.
13 C. N. Peroni, C. R. J. Soares, E. Gimbo, L. Morganti, M. T. C. P. Ribela and P. Bartolini, *Biotechnol. Appl. Biochem.*, 2002, **35**, 19–26.
14 J. E. Oliveira, R. Damiani, P. Bartolini and M. T. C. P. Ribela, *J. Chromatogr. A*, 2007, **1164**, 206–211.
15 R. Damiani, J. E. Oliveira, K. Vorauer-Uhl, C. N. Peroni, E. G. Vianna, P. Bartolini and M. T. C. P. Ribela, *Protein Expression Purif.*, 2009, **67**, 7–14.
16 R. Damiani, B. E. Almeida, J. E. Oliveira, P. Bartolini and M. T. C. P. Ribela, *Appl. Biochem. Biotechnol.*, 2013, **171**, 1658–1672.
17 J. East-Palmer, M. W. Szkudlinski, J. Lee, N. R. Thotakura and B. D. Weintraub, *Thyroid*, 1995, **5**, 55–59.
18 J. E. Oliveira, F. Mendonça, C. N. Peroni, P. Bartolini and M. T. C. P. Ribela, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2003, **787**, 345–355.
19 J. M. Bland and D. G. Altman, *Lancet*, 1986, **1**, 307–310.
20 B. Garthoff, C. F. M. Hendriksen, A. Bayol, D. Goncalves, A. Grauer, R. Deleeuw, J. Vannoordwijk, M. Pares, R. Pirovano, M. Rieth, H. Ronneberger, J. M. Spieser, P. Storring, K. Vosbeck and H. Weichert, *ATLA, Altern. Lab. Anim.*, 1995, **23**, 699–712.
21 C. M. Carvalho, J. E. Oliveira, B. E. Almeida, E. K. M. Ueda, P. A. Torjesen, P. Bartolini and M. T. C. P. Ribela, *J. Chromatogr. A*, 2009, **1216**, 1431–1438.
22 J. C. Madden, M. Hewitt, K. Przybylak, R. J. Vandebriel, A. H. Piersma and M. T. Cronin, *Regul. Toxicol. Pharmacol.*, 2012, **63**, 140–154.
23 A. F. Bristow, *Philos. Trans. R. Soc., A*, 2011, **369**, 4004–4013.
24 R. Lin, V. Hogen, S. Cannon, K. M. Marion, M. S. Fenton and J. M. Hershman, *Thyroid*, 2010, **20**, 1139–1143.
25 R. A. Sendak, C. Ganesa, K. L. Lee, J. J. Harrahy, R. Théberge, C. J. Morgan, E. S. Cole, L. D. Kohn and R. J. Mattaliano, *Thyroid*, 2003, **13**, 1091–1101.
26 A. P. Worth and M. Balls, *ATLA, Altern. Lab. Anim.*, 2004, **32**, 623–629.
27 M. Leist, N. Hasiwa, M. Daneshian and T. Hartung, *Toxicol. Res.*, 2012, **1**, 8–22.
28 Q. Zhou, S. Park, S. Boucher, E. Higgins, K. Lee and T. Edmunds, *Anal. Biochem.*, 2004, **335**, 10–16.
29 Y. Alahamad, N. T. Tran, J. Duboeuf, A. Gregoire, I. Rance and M. Taverna, *Electrophoresis*, 2009, **30**, 2347–2354.

