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Journal of Chromatography A, 955 (2002) 229–236

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Reversed-phase high-performance liquid chromatography method for the determination of prolactin in bacterial extracts and in its purified form

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Received 18 September 2001; received in revised form 1 March 2002; accepted 1 March 2002

Abstract

Reversed-phase high-performance liquid chromatography methodology for the determination of human prolactin (hPRL) in bacterial periplasmic space or in purified preparations has been developed. The technique, based on the high hydrophobicity of the hPRL molecule, allows its separation from the bulk of bacterial proteins. The precision for periplasmic shock fluid analysis was characterized by relative standard variations of 3–7% for intra-day and of 3–25% for inter-day determinations. Accuracy, evaluated by recovery tests, was of the order of 90%, a calibration curve being constructed with the use of a lyophilized osmotic shock fluid extract, which provided a stable, readily prepared internal reference. Sensitivity was of the order of 0.5 μg of hPRL. The methodology developed also provided a tool for comparing the hydrophobicity of glycosylated and non-glycosylated prolactin molecules obtained from several different species and of different preparations of native or biosynthetic human prolactin.

Keywords: Prolactin; Hormones; Peptides; Proteins

1. Introduction

Prolactin (PRL) is a polypeptide hormone of molecular mass 23 000 with a single chain of 199 residues and three disulfide-bonded loops. It is one of the most versatile hormones in terms of biological actions, more than 100 different effects having being documented [1]. PRL is, however, best known for its stimulation of lactation and its regulatory roles in the growth and differentiation of the mammary gland and in reproduction [2]. The human hormone is produced mainly by the lactotrope cells of the anterior pituitary in the form of a precursor with a sequence of 227 amino acids; an enzymatic cleavage occurs at position 28, giving the native form of human (h) PRL. Post-translational modified variants, such as cleaved, phosphorylated and glycosylated PRL are also present in the hypophysis [1,3]. hPRL is present in the pituitary gland only in very limited amounts (0.1–0.2 mg) [4,5]. Its purification process has always been quite laborious, providing in general only low yields of a quite labile protein [4,6–8]. Only recently has the production of this hormone in larger scale become possible with the use of DNA

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00229-7

recombinant technology, which provides both greater amounts and a more homogeneous product [9–18]. Following extraction and purification of hPRL from either human pituitaries or genetically engineered host cells, the hormone is usually quantified by immunoassay or sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with or without immunoblotting, relying on confirmation of its mitogenic activity in the Nb2 lymphoma cell proliferation assay [19]. The accurate immunoassay quantification of a mixture of isoforms, however, has always been a very critical issue, especially with regard to the choice, availability and comparability of suitable standards, antibodies and assay methods in general [20,21]. Regarding, for example, glycosylated (G-hPRL) and non-glycosylated (NG-hPRL) prolactin, a recent WHO International Collaborative Study involving 15 expert laboratories, including ours, showed that NG-hPRL exhibited one to three times the immunoactivity of G-hPRL, depending upon the assay system used [22]. SDS–PAGE, immunoblotting and the Nb2 bioassay, though extremely useful, have also well known limitations concerning accuracy and precision. As far as we know, no accurate physico–chemical method for hPRL determination has been described previously. The experimental evidence that hPRL and hGH have similar hydrophobic properties [8] and that hGH is more hydrophobic than the majority of *E. coli* proteins, from which it can be easily resolved by isocratic RP-HPLC [20,23,24], suggested to us that this methodology could be useful for quantitating hPRL in bacterial extracts and in its purified form, and should provide a rapid means of identification and qualitative analysis. The purpose of the present work is to describe our implementation of such a methodology, that can be utilized to follow the hPRL production and purification process, as well as in all studies and applications related to this hormone, whose importance in research, diagnosis and therapy is rapidly increasing [16,25–28].

2. Materials and methods

2.1. Chemicals and reagents

Water was obtained from a Millipore ‘‘Milli-Q plus’’ water purification system (Bedford, MA, USA). Acetonitrile and *n*-propanol (HPLC grade) were from Mallinckrodt (Paris, KY, USA). All other chemicals were analytical reagent grade. Lyophilized recombinant authentic human prolactin derived from *Escherichia coli* periplasmic extracts, as well as recombinant glycosylated (G-hPRL) and non-glycosylated (NG-hPRL) prolactin produced by genetically modified CHO cells, were produced in this laboratory. The vector used for hPRL expression in CHO cells, (pEDdc) was kindly donated by the Genetics Institute (Cambridge, MA, USA), while the construction of hPRL bacterial expression vector and the selection of hPRL-secreting CHO cells lines was carried out in collaboration with Sanofi Recherche (Toulouse, France) [16]. A highly purified pituitary hPRL preparation (hPRL-RUS), non-glycosylated porcine PRL (NG-pPRL) and glycosylated porcine PRL (G-pPRL) were kindly provided by Dr. A. Bulatov (National Research Center for Endocrinology, Moscow, Russia). A second preparation of highly purified pituitary hPRL (hPRL-NOR) was kindly donated by Dr. P. Torjesen (Aker University Hospital, Oslo, Norway). Human pituitary prolactin preparations from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK-hPRL-RP-2, NIDDK-hPRL-I-8, and NIDDK-hPRL-SIAFP-B-3), rabbit anti-hPRL antiserum (NIDDK-anti-hPRL-3) and glycosylated human and ovine prolactin (G-oPRL) were kindly donated by Dr. A.F. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA. Ovine PRL (oPRL, 100 IU/vial) was purchased from Sigma (St. Louis, MO, USA). The international standard for growth hormone, human, for bioassay, coded 80/505, the First International Standard for Somatropin (recombinant DNA-derived human growth hormone) coded 88/624 and the chemical reference standard (CRS) of recombinant hPRL-CRS, were kindly provided by the National Institute for Biological Standards and Control (South Mimms, UK).

2.2. Osmotic shock

Periplasmic-osmotic shock fluid was obtained by the method of Koshland and Botstein [29]. Briefly, a volume of fermentation broth medium with 40.0 A_{600} units was harvested by centrifugation at 3000 *g*

for 5 min. All subsequent steps were carried out at 4 °C in an ice bath. Pellets were resuspended in 0.4 ml of ice-cold 10 m*M* Tris–HCl, pH 7.5, containing 20% (w/v) sucrose. Then 13 μl of 0.5 *M* EDTA, pH 8.0, were added and incubation on ice was continued for 10 min. The cells were then centrifuged and the pellet rapidly resuspended by vigorous agitation in 0.4 ml of cold 1 m*M* Tris–HCl, pH 7.5, solution. The mixture was incubated for 10 min on ice and then centrifuged again for 5 min. The supernatant was removed and saved as the periplasmic fraction.

2.3. RP-HPLC

A Shimadzu Model SCL-10A HPLC apparatus coupled to a SPD-10AV UV detector (Shimadzu, MD, USA) was used, employing the Class VP software, also from Shimadzu. The column was a C_4 Vydac 214TP54 (25 cm×4.6 mm I.D., pore diameter of 300 Å and particle diameter of 5 μm) with a guard column (Vydac 214FSK54) between the sample injector and the main column and a silica precolumn packed with LiChrosorb Si-60, 7.9–12.4 μm (Merck, Darmstadt, Germany) located between the pump and the injector. All Vydac columns were purchased from The Separation Group (Hesperia, CA, USA). The mobile phase consisted of 71% Tris–HCl buffer (50 m*M*, pH 7.5) and 29% *n*-propanol, as described by Dalmora et al. [24], with a flow-rate of 0.5 ml/min, detector wavelength at 220 nm, column temperature maintained at 45 °C and a sample volume of 25–200 μl. Intra-day RSD for the retention time (t_R) determination of a given prolactin isoform was 0.3–0.8%.

Since inter-day variation increased significantly (RSD up to 4.6%) due to the difficulty of precisely reproducing the same mobile phase [30], we used a relative retention time (t_{RR}), for inter-day comparisons, calculated on the basis of the hGH t_R of each day, where $t_{RR\text{-}x} = t_{R\text{-}x}/t_{R\text{-}hGH}$ for a given form x.

2.4. Precision determination

The precision of the quantitative determination was calculated using different periplasmic shock fluids on an intra-day and inter-day basis. The intra-day RSD was the result of a triplicate determination on the same sample run with the same mobile phase, prepared daily. The inter-day precision was obtained using the mean intra-day values determined over a period never exceeding 1 month. The values, in micrograms, were obtained against a previously calibrated lyophilized internal reference preparation (irp), run on the same day, and whose long-term stability was confirmed by assaying singly against frozen aliquots of the NIDDK-hPRL preparation.

2.5. Gel electrophoresis and Western-blot analysis

Discontinuous SDS–PAGE using 12% polyacrylamide gels was performed under non-reducing conditions as described [16]. Gelcode ® Blue Stain Reagent (Pierce, Rockford, IL, USA) was used for staining and the molecular mass markers were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Western-blot analysis was performed using ^{125}I-labelled Protein A [16].

2.6. Amino acid analysis

Protein quantitation was achieved by amino acid analysis. Aliquots (100–500 μg) of growth hormone, prolactin and bovine serum albumin (BSA) (Sigma), used as a reference preparation, were hydrolyzed in gas phase 6 *M* HCl at 110 °C for 20 h and the amino acids were analysed with an Amino Quant system (Agilent, Palo Alto, CA), which is based on pre-column derivatization with *ortho*-phthalaldehyde or 9-fluorenylmethylchloroformate. A certified preparation of the amino acids routinely found in proteins (250 pmol) was run as a standard. The determination was carried out on three samples of each protein. BSA vials provided a protein content of 95% (±7.3% RSD, $n=3$), compared to the nominal value.

2.7. Radioimmunoassay

Human PRL immunoactivity determination was carried out as previously described [14] using NIDDK reagents and hPRL-RUS for radioiodination. Human blood-based Immunoassay Quality Controls (Dade® Tri-level, Baxter Diagnostics, Deerfield, IL, USA) were used for each standard curve.

2.8. UV absorbance measurements

The absorbance coefficient ($A^{0.1\%}$) was determined at different wavelengths (λ) using a nominal concentration of 10 μg/ml ($\lambda = 220$ nm) or of 100 μg/ml ($\lambda = 276$, 279 and 280 nm) in an Ultrospec III (Pharmacia-LKB, Uppsala, Sweden) spectrophotometer. Before each measurement, the samples were centrifuged at 16 000 *g* for 5 min. Readings at 220 nm were carried out against aqueous buffer or 29% *n*-propanol buffer (RP-HPLC mobile phase) at room temperature.

3. Results

Examples of RP-HPLC chromatograms of osmotic shock fluids from an *E. coli* strain, transformed or not with a hPRL expression vector, are presented in Fig. 1. Together with the protein ($A_{220\ nm}$), the hPRL immunoactivity profile is also shown. It is evident that the non-transformed strain fails to present any interfering immunoactivity and that the hPRL peak is well-resolved from the bulk of bacterial proteins. The purity of the hPRL peak, after RP-HPLC elution of an osmotic shock fluid, can be appreciated in Fig. 2.

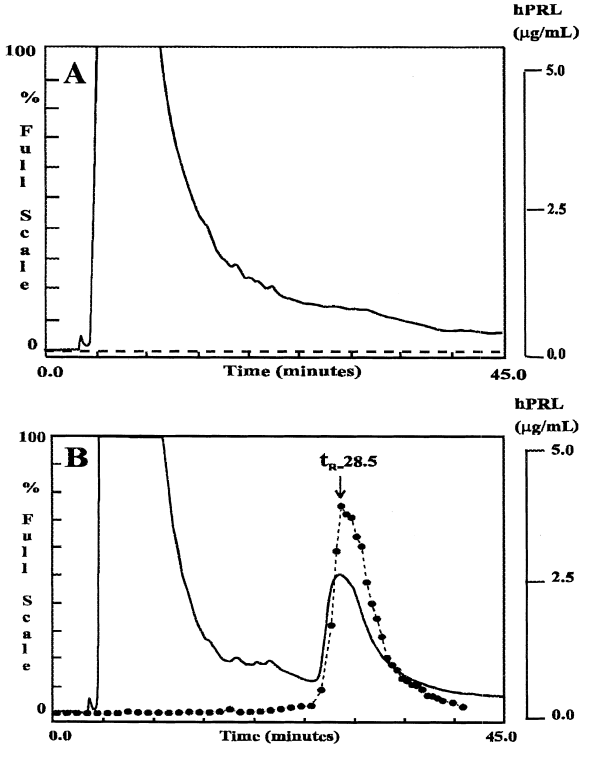

Fig. 1. Isocratic RP-HPLC on a C4 Vydac 214 TP 54 column of different osmotic fluids obtained from: (A) *E. coli*, non-transformed strain (RB791); (B) Same strain, transformed with hPRL expression vector. Sample volume: 200 μl. —— A_{220}, - - - hPRL activity determined by RIA against NIDDK-HPRL-RP1.

In Table 1 the retention times (t_R) of different prolactin preparations (including native and biosynthetic, glycosylated and non-glycosylated) derived from various species are reported. The values reported in the table, directly related to the hydrophobicity of the molecule, are an example of intra-day determination carried out in comparison with the well-known International Standards of pituitary and recombinant human growth hormone (rhGH). As specified in Materials and methods, inter-day comparisons were performed on the basis of the calculated t_{RR} values, for which the inter-day RSD never exceeded 1.5% ($n = 3$) for any given specific isoform. In Fig. 3, typical chromatograms for rhGH, pit-hPRL, rhPRL and oPRL are also shown. Except for rhPRL from lyophilized periplasmic extract, the presence of earlier eluting minor peaks can be observed.

Lyophilized periplasmic extracts obtained from an *E. coli* strain that had been transformed with an hPRL expression vector were used as irp after calibration against the highly purified preparation hPRL-NIDDK. The latter provided the dose-response curve:

$Y_A = 672X_W - 73$ ($n = 5$; $r = 0.9987$; $P < 0.001$),

where A is the peak area and W represents the dose (μg) of hPRL-NIDDK, while, for example, one of the lyophilized preparations provided the curve:

$Y_A = 51.4X_V - 8.3$ ($n = 6$; $r = 0.9995$; $P < 0.001$),

where V represents the volume (μl) withdrawn from a 100 μl solution of the whole ampoule content. Comparing the two curves provided, for this particular irp, an ampoule content of 7.74 μg of hPRL. Three different lyophilized ''irp'' have been prepared up to now in our laboratory, always obtaining comparable precision and stability. To confirm the accuracy of the method, a recovery test was carried out by adding known amounts of a purified recombinant preparation (hPRL-CRS) to a bacterial shock

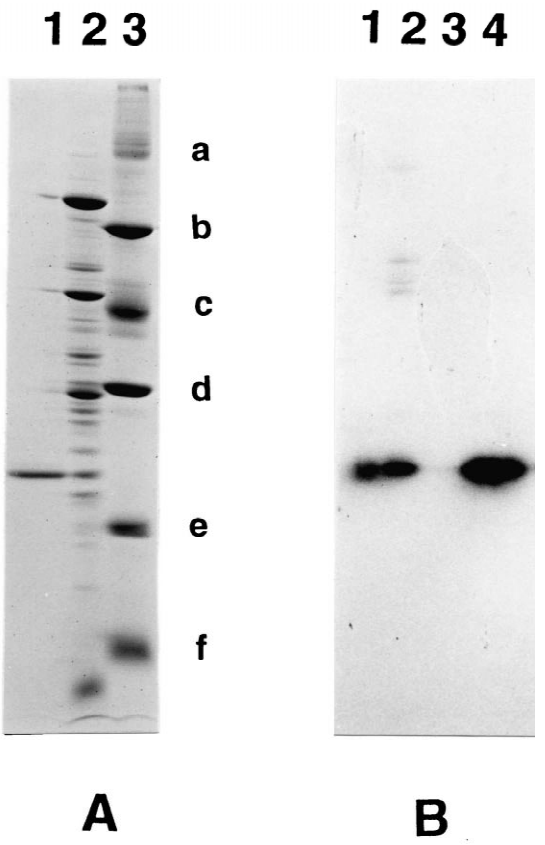

Fig. 2. SDS–PAGE (A) and Western-blot (B) analysis, under non-reducing conditions, of rhPRL extracted from periplasmic-osmotic shock fluid and purified by RP-HPLC. (A) Lanes: 1=rhPRL eluted from RP-HPLC (t_R=28.5 min), 1 μg; 2=osmotic shock fluid, 10 μl; 3=molecular mass marker: a, phosphorylase *b* (M_r 97 000); b, BSA (66 000); c, ovoalbumin (45 000); d, carbonic anhydrase (30 000); e, trypsin inhibitor (20 100); f, α-lactalbumin (14 400). (B) Lanes: 1=hPRL-NIDDK-SIAFP-B3, 500 ng; 2=osmotic shock fluid, 5 μl; 3=1st peak eluted from RP-HPLC (t_R~10 min, see Fig. 1), 25 μl; 4=rhPRL eluted from RP-HPLC (t_R=28.5 min), 2 μg.

fluid obtained with the non-transformed *E. coli* strain. Recoveries averaged 94% and correlation between added and recovered hPRL was highly significant, as indicated by the linear regression equation:

$$Y_{recov} = 0.931X_{added} + 0.046 \quad (r = 0.9995; \quad P < 0.001 \text{ for } n = 11).$$

An analogous dose-response curve was also determined for hGH by using the International Standard WHO-88/624 and a comparable slope was found (690 area units/μg). The spectrophotometric absorbance of the two proteins was also compared at several different wavelengths: 220, 276, 279 and 280 nm (Table 2). Absorbance at 220 nm is utilized by our detector; the declared absorbance coefficient at 276 and 279 nm of hGH [31] and for hPRL [32] are $A^{1\%}_{276\ nm}$=8.18 and $A^{1\%}_{279\ nm}$=8.39, respectively; absorbance at 280 nm is quite commonly used for protein determination.

Table 1
Retention times (t_R) of different prolactins on isocratic RP-HPLC and relative retention times (t_{RR}) determined against the International Standard of rhGH

Sample	Source	t_R (min)	t_{RR}
rhGH	(WHO 88/624)	32.2	–
pit-hGH	(WHO 80/505)	32.3	1.00
pit-hPRL	(NIDDK)	27.6	0.86
pit-G-hPRL	(NIDDK)	23.1	0.72
pit-hPRL	(RUS)	30.2	0.94
pit-hPRL	(NOR)	30.3	0.94
rhPRL	(*E. coli*)	30.7	0.95
rhPRL	(CHO)	30.0	0.93
rG-hPRL	(CHO)	19.9	0.62
oPRL	(Sigma)	24.0	0.74
G-oPRL	(NIDDK)	19.5	0.60
pPRL	(RUS)	28.1	0.87
G-pPRL	(RUS)	18.3	0.57

The amino acid composition analysis provided amounts of 89% (±6.5% RSD) for the hGH and 58.4% (±6.1% RSD) for the hPRL preparation, relative to the nominal values. The absorbance coefficients for the two hormones were thus calculated and found to be comparable, even though the values obtained at 276 and 279 nm are higher than literature values (Table 2).

Finally, the precision of the method was determined for periplasmic extracts, covering the range 0.5–3 μg of hPRL, for both the intra-day and the inter-day (up to a 3-month time period) assays (Table 3). The value of 0.5 μg hPRL, obtained with an inter-day RSD=25%, was considered to be the ''working sensitivity'' of the method. It must be emphasized that most of these data were obtained employing a lyophilized periplasmic extract as reference preparation, the long-term stability of which could be checked for about 6 months before running out of samples. This provided a reproducible inter-day response, with no decreasing tendency, of

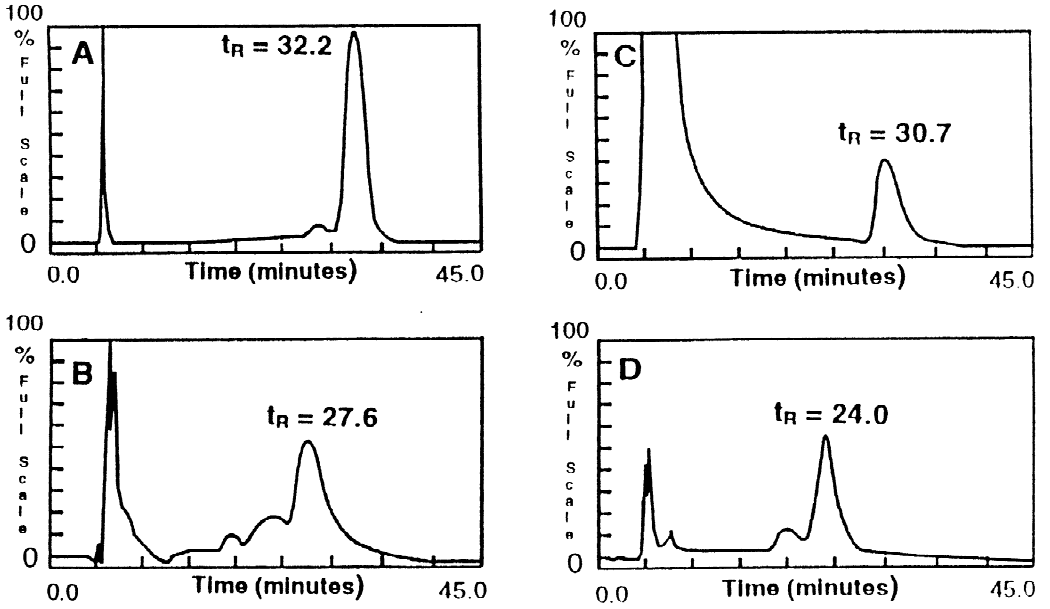

Fig. 3. Typical chromatograms (RP-HPLC) of some of the products whose t_R are presented in Table 1. The amount of applied protein is based on the nominal vial content. (A) rhGH (WHO 88/624), 10 μg; (B) pit-hPRL (NIDDK, SIAFP-B-3), 20 μg; (C) lyophilized periplasmic extract containing bacterial hPRL (irp), 6 μg; (D) ovine PRL (Sigma), 10 μg.

8.15±1.17 μg (RSD=14.3%, $n=22$) per ampoule. Batch-to-batch variation, determined via the intra-day application of 5 μg hPRL samples, was defined by a RSD of 2.9% ($n=4$).

Table 2
Absorbance coefficients ($A^{0.1\%}$) determination at several different wavelengths (λ) for the purified preparation of hPRL (NIDDK-SIAFP-B-3), compared with the International Standard of rhGH (WHO 88/624)

λ (nm)	hPRL-NIDDK[a] ($A^{0.1\%}$)	rhGH-WHO[a] ($A^{0.1\%}$)
280	0.89	0.90
279	0.89	0.92
276	0.88	0.92
220 (aqueous buffer)[b]	12.92	13.61
220 (29% *n*-propanol)	13.74	13.72

[a] Sample concentration determined by amino acid composition analysis.

[b] hPRL–NIDDK was dissolved in 0.01 *M* sodium hydrogen carbonate; rhGH-WHO in water, taking into account that the ampoule contents included, in addition to 2 mg of the hormone, 2 mg of glycine, 2.5 mg sodium hydrogen carbonate, 2 mg lactose and 2 mg mannitol.

4. Discussion

An isocratic RP-HPLC method has been developed for the determination of prolactin in purified preparations and directly in osmotic shock fluids. This appears to be the first report describing a qualitative and quantitative physico–chemical analysis of this hormone under non-denaturing conditions. Following the pioneering work on RP-HPLC by

Table 3
Intra- and inter-day determination of hPRL by RP-HPLC in different periplasmic shock fluids

Shock No.	Single day[a] (μg)	Inter-day[a] (μg)
1	2.94±2.9[b]	3.04±4.4[b]
2	2.89±2.4	2.81±3.4
3	1.89±1.7	1.91±15.1
4	0.72±6.3	0.66±8.6
5	0.48±5.2	0.51±25.1

[a] All data refer to a 3-month time period, and were obtained against the ''irp'' of hPRL.

[b] RSD, expressed as percentage of the mean, $n=3$.

Karger et al. [33], Hancock and co-workers [34,35] and Horváth and Melander [36], this methodology has been widely used for hGH analysis and characterization [20,24,30,37–43]. Setting up such RP-HPLC methodology, however, has always been quite difficult for prolactin, primarily due to its limited availability. Pituitary extracted hPRL is extremely precious and labile [15,32,44], while the authentic recombinant form of this hormone, secreted in the *E. coli* periplasmic space, has been expressed only at very low levels up to now [14,15]. The present study was made possible thanks to the utilization of a hPRL-secreting *E. coli* strain that provides much higher expression levels (data not shown) and whose periplasmic extracts contain relatively high concentrations of the hormone. In fact, even with the availability of much greater amounts of purified hPRL in our laboratory, these lyophilized crude extracts provide an inexpensive, readily prepared, stable and reliable internal reference preparation, which has the additional advantage of not having been submitted to long and potentially strenuous purification procedures. The latter characteristic is quite important for accurately identifying the fundamental molecular form of hPRL. It would have been more appropriate to compare our preparations to the recently established First WHO Reference Reagent for PRL, human, recombinant, 97/714 [22]. Our laboratory took part in this study but we agreed not to publish data on this Reference Reagent until the results of the study are published.

RP-HPLC is well known for having a great efficiency for resolution of chemically modified forms of hGH [30,37–39]. It is therefore an extremely useful tool for detecting and possibly identifying the isomers of prolactin, as can be observed, for example, in the analysis of hPRL-NIDDK or ovine PRL-Sigma (Fig. 3B). As already noted, in the case of the International Standard of rhGH [24] earlier eluting minor peaks could be attributed to sulfoxide and desamido derivatives. At the same time, the retention times of the main peak of 11 prolactin preparations, all of different origin or composition (Table 1), constitute a hydrophobicity index that can be quite useful for their identification, characterization and purification [23]. Under our conditions, all forms of prolactin are, in general, less hydrophobic than hGH. The glycosylated forms are 1.2–1.5 times less hydrophobic than the non-glycosylated forms and there is very good agreement between the t_R of hPRL (DNA recombinant- or pituitary-derived) from different origins, with the exception of the NIDDK preparation. As shown in Table 1, the same agreement also occurs for the two hGH preparations. This hydrophobic character can thus be used not only for more accurate determination of the proportion of glycosylated prolactin present in heterogeneous preparations, but also for the study of differences in isoform distribution between pituitary and recombinant prolactins of different origins [13,16]. These potential applications of the present physical chemical methodology can, with the necessary modifications, be extended to other proteins and glycoproteins, e.g. recombinant human thyroid-stimulating hormone (rhTSH), whose characterization is still mostly based on immunological and biological techniques [45].

The performance of the method was positively characterized with regard to accuracy, precision and sensitivity. The slope of the dose-response curve relating the amount of pituitary prolactin (NIDDK) to arbitrary area units was found to be comparable to that obtained with the International Standard of rhGH. The same agreement was found via spectrophotometric determination of the UV absorbance coefficients of these two proteins at 220 nm ($A^{0.1\%}_{220\ nm}$), the wavelength routinely utilized, as recommended by the European Pharmacopoeia [46], for the pharmaceutical control of somatropin for injection.

Acknowledgements

This work was supported by FAPESP, São Paulo, Brazil (project 95/9719-8, 99/08942-6 and 01/01769-9), CNPq, Brasília, Brazil (project 301520/91-7 and 260025/96.8) and the International Atomic Energy Agency (IAEA) (Technical Cooperation Project BRA 2/012), Vienna, Austria. We are grateful to Mr. Eric Ueda and Mr. José Maria de Sousa for valuable and skilled assistance.

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