A REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) METHOD FOR THE DETERMINATION OF HUMAN PROLACTIN (hPRL) IN OSMOTIC SHOCK FLUIDS AND IN PURIFIED PREPARATIONS

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Abstract

A reversed-phase high-performance liquid chromatography (RP-HPLC) methodology for the determination of human prolactin (hPRL) secreted in bacterial periplasmic space or in purified preparations has been set up. The technique is based on the high hydrophobicity of the hPRL molecule that allows its separation from the bulk of bacterial proteins. The precision obtained for periplasmic shock fluid analysis was defined by coefficients of variation of 3-5% for intra-day and of 3-24% 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 and easy to prepare internal reference preparation. Sensitivity was of the order of 0.8 µg of hPRL/200 µL (CV = 24%). The set up methodology also provided a tool for comparing the hydrophobicity of several prolactin molecules, either glycosylated or non-glycosylated, obtained from different species as well as of different preparations of native or biosynthetic human prolactin.

1. RP-HPLC of osmotic shock fluid from an E. coli strain, transformed with a hPRL expression vector

An example of RP-HPLC chromatogram of osmotic shock fluids from an E. coli strain, either transformed or not transformed with a hPRL expression vector, is presented in Fig. 1. We can observe that the non-transformed strain does not present any interfering immunoactivity and that the hPRL peak is well resolved from the bulk of bacterial proteins.

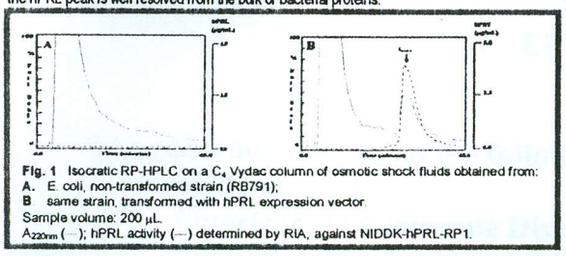

Fig. 1 Isocratic RP-HPLC on a C_4 Vydac column of osmotic shock fluids obtained from:
A. E. coli, non-transformed strain (RB791);
B same strain, transformed with hPRL expression vector.
Sample volume: 200 µL.
A_{220nm} (—); hPRL activity (—) determined by RIA, against NIDDK-hPRL-RP1.

2. RP-HPLC retention times (t_R) of different prolactin preparations

The t_R of different prolactin preparations, either native or biosynthetic, glycosylated or non-glycosylated, derived from different species, were determined in comparison with well known International Standards of pituitary and recombinant human growth hormone (hGH) (Table 1).

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 rec-hGH.

Sample	rec-hGH	pit-hGH	pit-hPRL	pit-hPRL	pit-hPRL	rec-hPRL	rec-hPRL	rec-G-hPRL	oPRL	G-oPRL	pPRL	G-pPRL
Source	(WHO-88/624)	(WHO-88/624)	(NIDDK)	(RUS)	(NOR)	(E coli)	(CHO)	(CHO)	(Sigma)	(NIDDK)	(RUS)	(RUS)
t_R (min)	32.2	32.3	27.6	28.2	28.3	30.7	30.6	19.8	24.0	19.8	23.1	14.3
t_{RR}	-	1.00	0.86	0.88	0.88	0.95	0.95	0.62	0.74	0.62	0.84	0.57

NIDDK, kindly donated by Dr. A.F. Parlow (National Hormone and Pituitary Program, Torrance, CA, USA)
RUS, kindly donated by DR. A. Bulatov (National Reserch Center for Endocrindogy, Moscow, Russia)
NOR, kindly donated by DR. P. Torjesen (Aker University Hospital, Colo, Norway)

- 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;
- There is a good agreement between the t_R of human PRL of different origins, with the exception of the NIDDK preparation.

Typical chromatograms for rec-hGH, pit-hPRL, rec-hPRL and oPRL are reported in Fig. 2

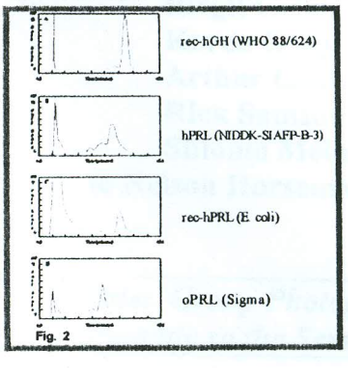

Fig. 2

3. Calibration of a lyophilized periplasmic extract against a highly purified preparation of pituitary hPRL (NIDDK-SIAFP-B-3)

A lyophilized periplasmic extract obtained from a hPRL-secreting E. coli strain, was used as an internal reference preparation (irp), being calibrated against the highly purified preparation of hPRL (NIDDK-SIAFP-B-3).

Dose-response curve for hPRL-NIDDK

$Y_A = 392X_W - 39$ (n = 5; r = 0.9982; P < 0.001)
A = peak area
W = dose (µg of hPRL)

Dose-response curve for the "irp" of rec-hPRL

$Y_A = 51.4X_V - 8.3$ (n = 6; r = 0.9995; P < 0.001)
v = volume (µL) withdrawn from a 100 µL solution of the whole ampoule content

With basis on these two equations, an ampoule content of 13.1 µg of hPRL was determined for this specific "irp".

An analogous dose-response curve was also constructed using the International Standard of rec-hGH (WHO 88/624).

$Y_A = 690X_W - 84.7$ (n =6; r =0 9991; P < 0.001)
W = dose (µg of hGH)

We can observe that purified hGH (WHO) is presenting a much higher slope of the dose-response curve (~70%) when compared to that of purified hPRL (NIDDK). Specific absorbance was therefore determined by spectrophotometric reading at 220 nm, 276 nm, 279 nm and 280 nm, for two purified preparations of hPRL and for the International Standard of rec-hGH (Table 2).

Table 2
Specific absorbance ($A^{0.1\%}$) determination at different wavelengths (λ) on two purified preparations of hPRL, in comparison with the International Standard of rec-hGH (WHO 88/624).

λ (nm)	hPRL-NIDDK[a] ($A^{0.1\%}$)	hPRL-NOR[b] ($A^{0.1\%}$)	rec-hGH-WHO[c] ($A^{0.1\%}$)
220	10.4	14.9	19.6
276	0.78	0.91	0.87
279	0.79	0.90	0.85
280	0.78	0.91	0.83

a. hPRL concentration set up by sample weighing
b. hPRL concentration set up by protein determination (Lowry)
c. hGH ampoule content determined and stated by a WHO-NIBSC International Collaborative Study

4. Accuracy of the method: recovery test

A recovery test was carried out running on RP-HPLC known amounts of highly purified hPRL-NOR and quantifying the eluted peak against our "irp".
Recoveries averaged 91%, while the correlation between added and recovered hPRL was highly significant according to the relation: $Y_{rec} = 0.937X_{add} - 0.211$ (n = 5; r =0.9953; P < 0.001)

5. Precision and sensitivity of the method

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

Shock Number	hPRL (µg/200 µL) single day (n=3)[a]	hPRL (µg/200 µL) inter-day (n=3)[a]
1	4.95 ± 3.1[b]	5.13 ± 3.3[b]
2	4.87 ± 2.5	4.73 ± 5.2
3	3.15 ± 2.1	3.19 ± 17.6
4	1.15 ± 7.5	1.04 ± 10.3
5	0.74 ± 6.7	0.78 ± 24.1

[a] Data collected over a 3-month time period and determined against our "irp" of hPRL
[b] Coefficient of variation (%)

Sensitivity. The value of 0.8 µg/200 µL, obtained with an inter-day CV = 24%, was considered the "working sensitivity" of the method.