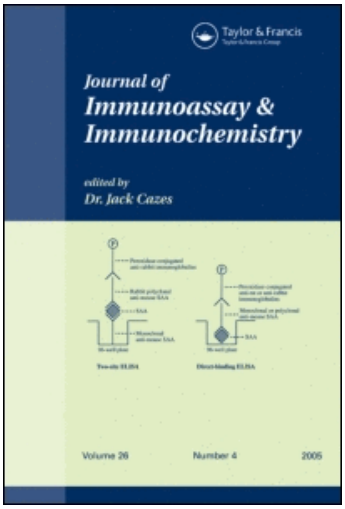


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

# Pattern of Immunoreactivity After Reverse-Phase High Performance Liquid Chromatography of Ovine Lutropin at Different pH

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**To cite this Article** Madej, Andrzej and Hallin, Per(1986) 'Pattern of Immunoreactivity After Reverse-Phase High Performance Liquid Chromatography of Ovine Lutropin at Different pH', Journal of Immunoassay and Immunochemistry, 7: 3, 181 — 197
**To link to this Article: DOI:** 10.1080/01971528608060465
**URL:** http://dx.doi.org/10.1080/01971528608060465

JOURNAL OF IMMUNOASSAY, 7(3), 181-197 (1986)

# PATTERN OF IMMUNOREACTIVITY AFTER REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF OVINE LUTROPIN AT DIFFERENT pH. (*)

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## ABSTRACT

Unlabelled and radioiodinated ovine lutropin (oLH) was fractionated using reverse-phase high pressure liquid chromatography, employing a 5 μm Spherisorb $C_{18}$ column. Following chromatography of unlabelled oLH at pH 3.3, 4.3 and 5.3, all eluted fractions were tested by radioimmunoassay of oLH, oLH α and oLH β. Regardless of pH, the material corresponding to the first peak contained only α-subunit immunoreactivity. With increasing pH, the oLH α, oLH and oLH β immunoreactivity separated into two groups of fractions. The retention times of the radioiodinated α-and β-subunits of oLH corresponded to those of the first (descending part) and last peaks from unlabelled oLH, respectively.

Following chromatography of radioiodinated oLH at pH 3.3, 5.3 and 6.5, all eluted fractions were analyzed using antiserum against either α- or β-subunits of oLH. At pH 3.3 two radioactive peaks were detected: the first corresponded to the fraction with maximum binding to α-subunit antiserum, and the second corresponded to the fraction with maximum binding to β-subunit antiserum. At pH 5.3 and 6.5, maximum radioactivity occurred in conjunction with maximum binding to both antisera. A free α-subunit in the radioiodinated oLH was even detected after chromatography at pH 6.5.

(KEY WORDS: lutropin, HPLC, subunit, radioimmunoassay)

## INTRODUCTION

In recent years reverse-phase high performance liquid chromatography has been used to either purify or separate lutropin sub-



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units (1,2,3). Bristow *et al.* (4) have shown that an increase in the pH of the eluant had a significant effect on the degree of dissociation of hTSH, fractionated by high pressure liquid chromatography (HPLC). In an earlier report (5) we demonstrated that fractionation of bovine lutropin on a reverse-phase HPLC at pH 3.3 without any previous incubation separated it into distinct α- and β-subunits. Here, we report on the influence of different pHs of the eluant on the behaviour of unlabelled and radioiodinated ovine lutropin (NIH-LH-S18) in a reverse-phase HPLC system.



## MATERIALS AND METHODS

Reverse-phase HPLC was performed according to the method described by Hallin *et al.* (5). Briefly, a Waters Associates system with two 5 μm Spherisorb $C_{18}$ columns and a solvent system consisting of two buffers, A and B, was used. Buffer A consisted of 0.5% trifluoroacetic acid in water while buffer B contained 80% acetonitrile, 19.5% water and 0.5% trifluoroacetic acid. Both buffers were adjusted to appropriate pH levels using concentrated ammonia solution. The material tested, injected at 5% of buffer B, was eluted along a linear gradient from 5% to 63% of buffer B for 39 min. The flow rate during all experiments was 1.5 ml/min. Following chromatography of unlabelled ovine lutropin (ca. 1 mg), the eluted fractions were evaporated and the remaining solids were dissolved in 1 ml of redistilled water to determine the protein concentration. The

protein content of the fraction was estimated by its absorbance at 280 nm in a 1 cm quartz cell; measurements were based on the absorbance of 1 mg/ml of ovine lutropin (oLH) solution (0.496). First, each collected fraction at six different dilutions was tested in duplicate by means of a radioimunoassay of oLH using anti-bovine LH serum. The same diluted fractions were then tested by means of homologous radioimmunoassay of oLH$\alpha$ and oLH$\beta$ subunits.

Iodination of oLH (NIH-LH-S18), the $\alpha$-subunit of oLH (WRR-1-$\alpha$) and the $\beta$-subunit of oLH (WRR-2-$\beta$) was performed by a modified Chloramine-T method (6) using 8 µg of this reagent per 2 µg of protein and an exposure time of 40 sec.



Radioimmunoassay of oLH was performed as described by Stupnicki and Madej (7), except for the separation of free and antibody-bound hormone, which was carried out according to Eisenman and Chew (8). The reference standard oLH$\alpha$ (WRR-1-$\alpha$) and oLH$\alpha$ antiserum (NIAMDD-anti-oLH-$\alpha$-1) at a final dilution of 1:90,000 were used in the radioimmunoassay of oLH$\alpha$. The reference standard oLH$\beta$ (WRR-2-$\beta$) and oLH$\beta$ antiserum (NIAMDD-anti-oLH-$\beta$-1) at a final dilution of 1:90,000 were used in the radioimmunoassay of oLH$\beta$. In both radioimmunoassays the log-logit standard curves were linear over the range of 25 pg to 800 pg/tube.

After separation of radioiodinated oLH (ca. 2 µCi or 74 kBq), the amount of radioactivity was measured in 0.05 ml of each fraction. The eluted fractions were then concentrated and diluted with phosphate buffer to obtain 5000 cpm/0.1 ml. These solutions were incubated overnight at room temperature with antiserum against either $\alpha$-

or β-subunits of oLH (NIAMDD-anti-oLH-α-1 and NIAMDD-anti-oLH-β-1, respectively), both at a final dilution of 1:45,000. At this dilution level the iodinated β-subunit did not bind to the α-subunit antiserum and the iodinated α-subunit did not bind to the β-subunit antiserum.

All assays were performed in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.45% sodium chloride, 0.01 M EDTA, 0.001% merthiolate, and 0.2% bovine serum albumin.



## RESULTS

Fractionation of unlabelled ovine lutropin (oLH) at pH 3.3 resulted in two major peaks with retention times of 32.1 and 39.3 min, respectively (Fig. 1). The eluted fractions forming the first peak (I) did not contain any LH-immunoreactive material as assessed by radioimmunoassay of oLH (Fig. 2). Instead, these fractions contained α-subunit immunoreactivity and displayed linear dilution curves. No β-subunit immunoreactivity was detected. The fractions derived from the last distinct peak (II) revealed a nonlinear displacement curve parallel and similar to that of the oLHβ subunit (Fig. 2). Additional tests incorporating a homologous radioimmunoassay of oLHβ confirmed that the material in question was similar to oLHβ. The content of α-subunit immunoreactivity in this material did not exceed 3%. The slopes of the log-logit curve of the α- and β-subunit immunoreactivity from oLH fractionated at pH 3.3 were very similar to their respective standards (-1.060 *vs.* - 1.136 and -1.212 *vs.* -1.266, respectively).

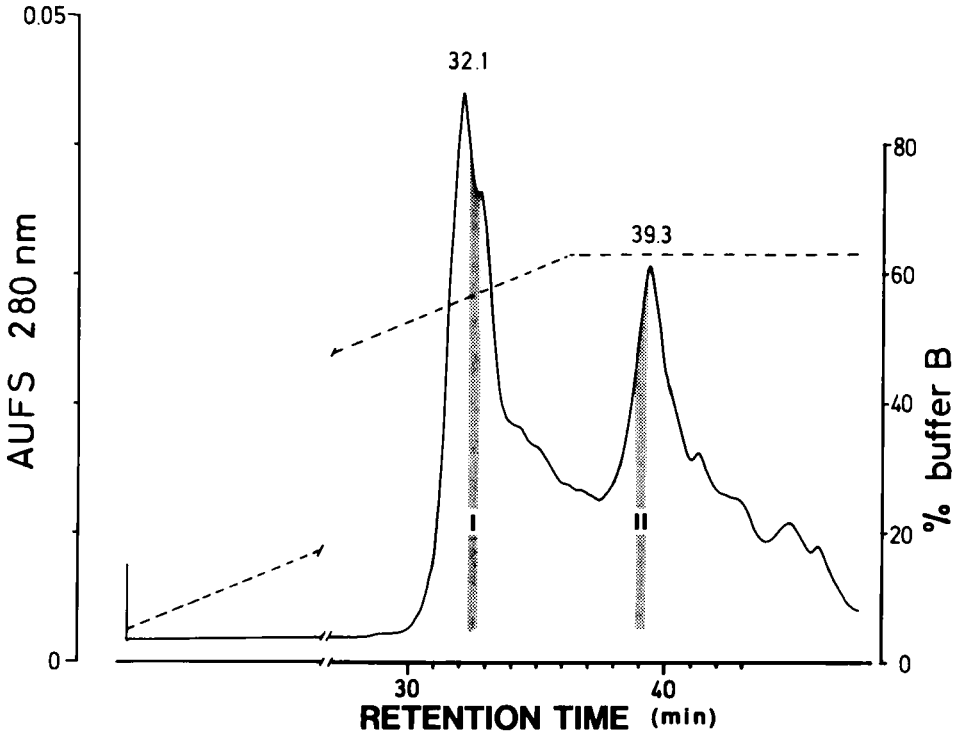


Figure 1
RP-HPLC profile of ovine lutropin standard preparation (NIH-LH-S18) at pH 3.3 (solid line). Conditions in all experiments: 5 µm Spherisorb C-18 column (25 x 0.8 cm) x 2; Flow-rate 1.5 ml/min.: Sample size 1 mg; Linear gradient 5% - 63% of buffer B during 39 min.(broken line). Dotted areas are collected fractions tested in a radioimmunoassay (Fig. 2)
Buffer A: 0.5% trifluoroacetic acid in water (pH 3.3)
Buffer B: 80% acetonitrile, 19.5% water, 0.5% trifluoroacetic acid (pH 3.3)

When pH was increased to 4.3 or 5.3, chromatography resulted in a pattern with three distinct peaks (Figs. 3 and 4, respectively). It was found that regardless of pH, the material corresponding to the first peak occurred at 31.5-31.7 min after injection and contained only α-subunit-immunoreactive material. The absorbance of this ma-

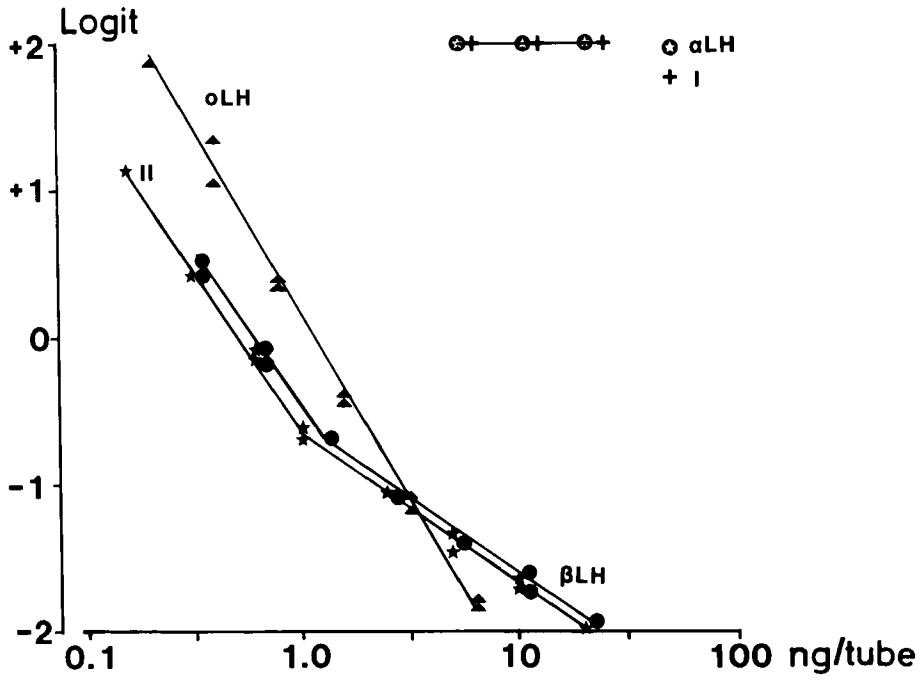


Figure 2
LH immunoreactivity of the two fractions obtained after HPLC at pH 3.3 of the ovine lutropin standard preparation NIH-LH-S18 (see Fig. 1) and the α-(WRR-1-α) and β-subunits (WRR-2-β) of oLH in radioimmunoassay of oLH using anti-bovine LH serum. The displacement curves of the bound radioactivity have been linearized by the logit transformation of $(B-N)/(B_o-N) = y$ *vs* log dose of added protein per tube.

terial at 280 nm tended to decrease with increasing pH (see Figs. 1, 3 and 4). At pH 4.3 and 5.3 a second distinct peak appeared, 34.6-34.8 min after injection, which consisted of fractions possessing oLHα subunit , oLH and oLHβ subunit immunoreactivities. A corresponding peak was never recorded at pH 3.3, although some LH immunoreactivity was found in the respective fractions. The retention time of the last distinct peak ranged between 38.6 and 39.3 min. The fractions

derived from that peak contained both oLH and oLHβ subunit immunoreactivities. When pH was increased from 4.3 to 5.3 the content of α-subunit immunoreactivity in the fraction with both maximum LH and β-immunoreactivity increased concomitantly from 3.8% to 6.3%.

In short, as shown in Figs. 3 and 4, there are two peaks containing LH-immunoreactive material following oLH (NIH-LH-S18) fractionation at pH 4.3 and 5.3. There are also two peaks containing α- and β-subunit-immunoreactive material, respectively (data not shown).

Although the fractions derived from the above-mentioned peaks exhibited linear dilution curves in radioimmunoassays of native LH, the slopes of their log-logit curves were lower than that of the curve for native oLH (-0.770 *vs* -0.926).



When employing the same chromatographic system as mentioned above for fractionating iodinated α- and β-subunits of oLH at pH 3.3 (Fig. 5), the retention time of the β-subunit was identical to that of the second peak obtained when fractionating native LH ( 39.0 *vs* 39.3 min ). The retention time for the α-subunit (Fig. 5) corresponded to that of the descending part of the first peak from unlabelled oLH (cf. Fig. 1). Fractionation of iodinated oLH standard at pH 3.3 resulted in two peaks with retention times of 34.8 and 39.3 min (Fig. 6a). The results obtained when each fraction from the separation was analyzed with antiserum against either oLHα or oLHβ subunits are shown in Figs. 6b and 6c, respectively. About 33% of the fraction derived from the first peak and containing the highest radioactivity was bound to antiserum against oLHα (Fig. 6b). This fraction showed no binding to the antiserum against oLHβ (Fig. 6c).

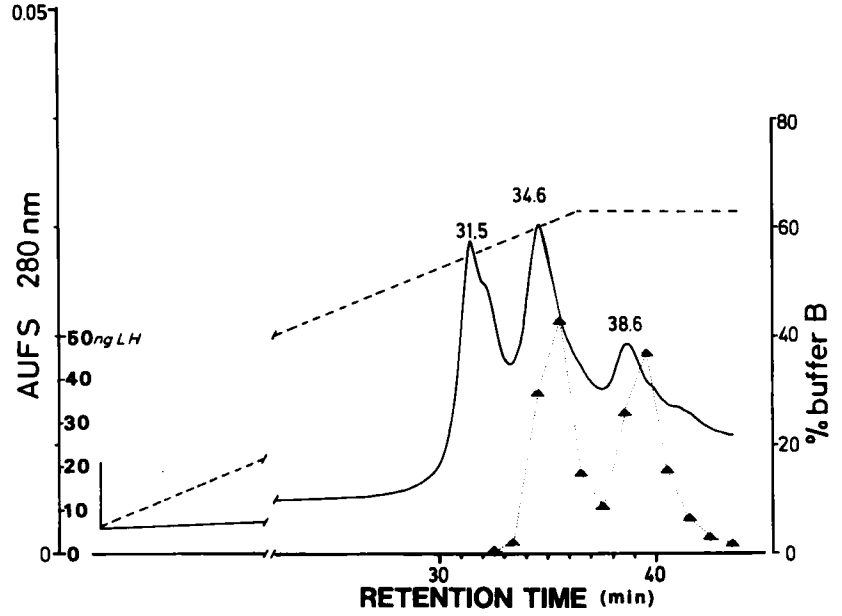


Figure 3
RP-HPLC profile of ovine lutropin standard preparation (NIH-LH-S18) at pH 4.3 (see explanation for Fig. 1). The content of LH in each fraction was measured by radioimmunoassay of ovine LH using anti-bovine LH serum (dotted line).



Conversely, the second peak of radioactivity coincided with maximum binding to antibodies against oLHβ, and this peak showed only 2% binding to oLHα antiserum (Figs. 6b, 6c). When the pH of the eluant was 5.3, only one asymmetric peak of radioactivity, which eluted after about 35 min, was recorded (Fig. 7a). Maximum binding of radioactivity occurred in conjunction with the maximum binding to antisera against both α- and β-subunits (Figs. 7b and 7c). The radioactive fractions containing only α-subunit immunoreactivity appeared up to 33 min after injection (Fig. 7b).

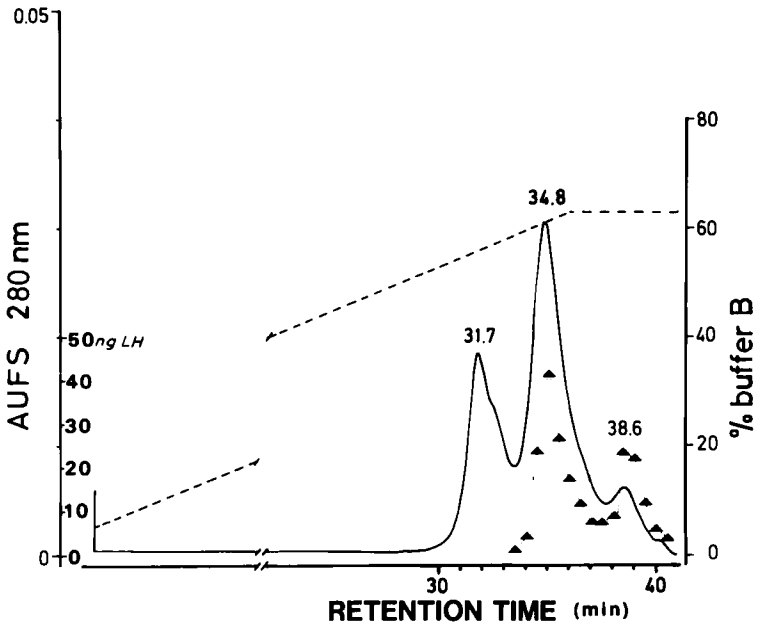


Figure 4
RP-HPLC profile of ovine lutropin standard preparation (NIH-LH-S18) at pH 5.3 (see explanation for Fig. 1). The content of LH in each fraction was measured by radioimmunoassay of ovine LH using anti-bovine LH serum (dotted line).



Fractionation of iodinated oLH at pH 6.5 resulted in a broad peak with highest radioactivity at 36 min (Fig. 8a). Again, the highest radioactive fraction possessed the highest α- and β-subunit immunoreactivity (Figs. 8b and 8c). Fractions with α-subunit immunoreactivity were also detected.

Based on the distribution of radioactivity and on the specificity of each antiserum, we estimated that 74.9%, 20.7% and 20.9% of the α-subunit could be found after fractionation of iodinated oLH (NIH-LH-S18) at pH 3.3, 5.3 and 6.5, respectively.

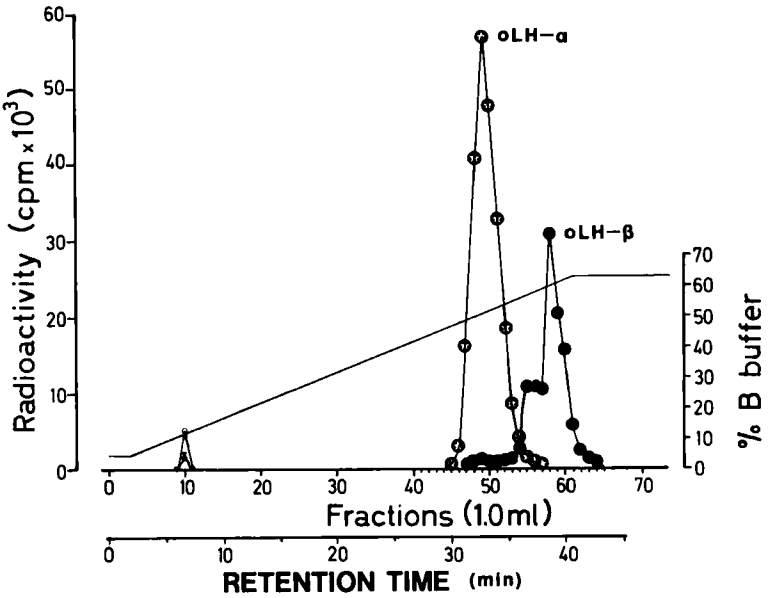


Figure 5
Retention times for radioiodinated α- (WRR-1-α) and β-subunits (WRR-2-β) of ovine lutropin standard preparations using the same conditions as for Fig. 1.



Chromatography of radioiodinated oLHα at pH 3.3 recovered more that 95% of the radioactivity. For radiolabelled oLHβ and oLH, about 57% of the radioactivity was recovered.

## DISCUSSION

The results of this study demonstrate that an increase of pH from 3.3 to 5.3 without any pre-incubation clearly changes the distribution of LH-immunoreactive material and its chromatographic pat-

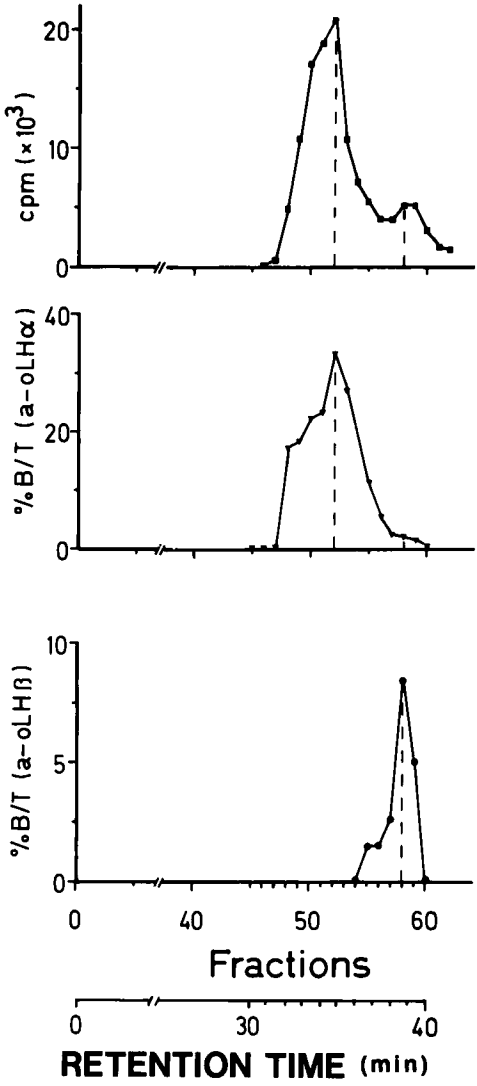


Figure 6
a- upper panel, RP-HPLC profile of radioiodinated ovine lutropin (NIH-LH-S18) at pH 3.3 (conditions as for Fig. 1)
b- middle panel, each fraction was evaluated as to its ability to bind to antiserum against the oLHα subunit an a final dilution of 1:45,000 and expressed as a percentage of the total radioactivity added
c- lower panel, each fraction was evaluated as to its ability to bind to antiserum against the oLHβ subunit at a final dilution of 1:45,000 and expressed as a percentage of the total radioactivity added.

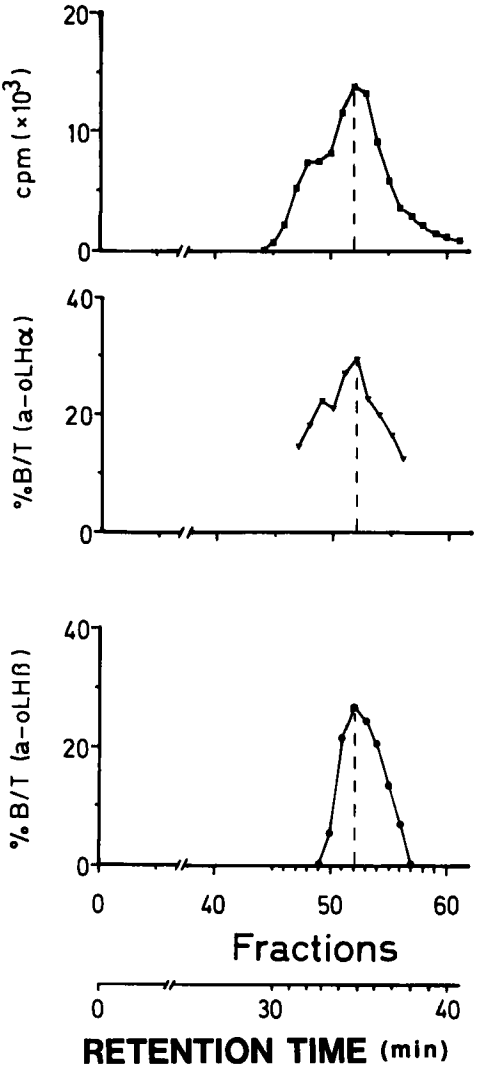


Figure 7
a- upper panel, RP-HPLC profile of radioiodinated ovine lutropin (NIH-LH-S18) at pH 5.3 (conditions as for Fig. 1)
b- middle panel, each fraction was evaluated as to its ability to bind to antiserum against the oLHα subunit at a final dilution of 1:45,000, and expressed as a percentage of the total radioactivity added
c- lower panel, each fraction was evaluated as to its ability to bind to antiserum against the oLHß subunit at a final dilution of 1:45,000 and expressed as a percentage of the total radioactivity added.

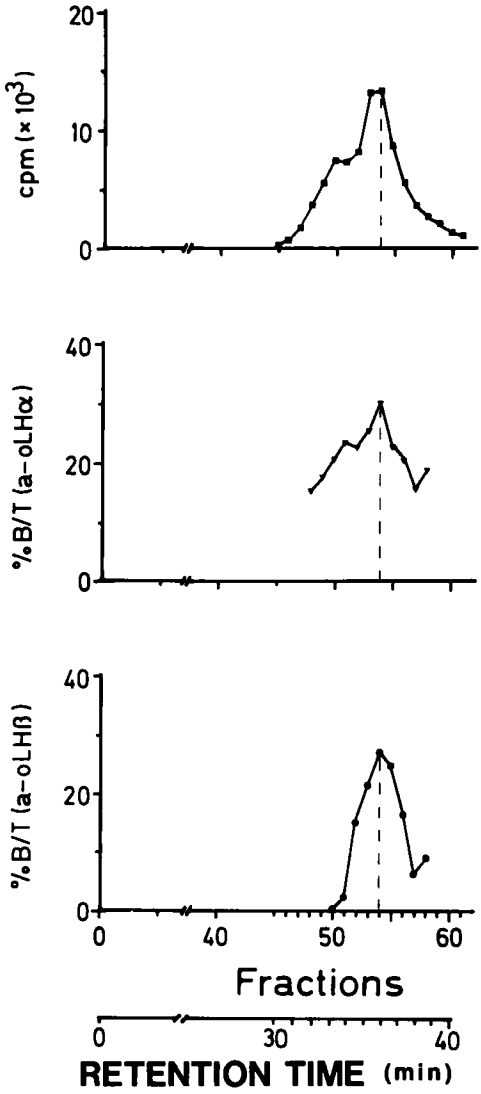


Figure 8
a- upper panel, RP-HPLC profile of radioiodinated ovine lutropin (NIH-LH-S18) at pH 6.5 (conditions as for Fig. 1)
b- middle panel, each fraction was evaluated as to its ability to bind to antiserum against the oLHα subunit at a final dilution of 1:45,000 and expressed as a percentage of the total radioactivity added
c- lower panel, each fraction was evaluated as to its ability to bind to antiserum against the oLHβ subunit at a final dilution of 1:45,000 and expressed as a percentage of the total radioactivity added.

tern after reverse-phase HPLC fractionation of unlabelled oLH. This agrees well with the findings of Bristow *et al.* (4), who reported that the reverse-phase chromatographic pattern of hTSH varies with the pH of the eluant. However, their results demonstrated that the resolution between β- and α-subunits decreased as the pH increased.

In this study the difference in retention time between the two major peaks was independent of pH and averaged 7.1 min. Moreover, we found that the LH-immunoreactive material appeared in-between these peaks when the pH of the eluant was increased from 3.3 to 5.3 Bewley *et al.* (9) found that dissociation of oLH at pH 3.6 is essentially complete in 3-4 h. Furthermore, Salesse *et al.* (10) reported that oLH is entirely dissociated at pH 2.8 and completely re-associated at pH 5.3. Their results also suggest that within the pH range 2.8 to 5.3, a thermodynamically reversible equilibrium exists between an associated, native state and a dissociated, partially unfolded state with a half-transition pH of 4.3 at 26°C.



It has been suggested that non-parallel dilution curves signify immunologically similar but non-identical antigenic components (11, 12,13). In our study the slope of the log-logit curve of the LH-immunoreactive fractions was lower than that of ovine LH, which might tally with the above-mentioned suggestion.

The very small peak in radioactivity corresponding to maximum binding to β-subunit antiserum found here after chromatography of labelled oLH at pH 3.3 agrees with studies by Yang and Ward (14). These authors reported that the relative iodination of the α-subunit in the native lutropin was 12.5 times greater than that of the β-subunit.

The present results might suggest that about 21% of α-subunit in iodinated NIH-LH-S18 represents either contaminated unlabelled hormone or products of iodination. It seems, however, that the latter alternative is the more probable, particularly in light of the findings of Benveniste *et al.* (15) who reported that amounts of α-subunit increased by up to 20% after iodination of hLH (IRC-2). Moreover, Sairam (16) found that NIH-LH-S19 contained only 0.5% of the free α-subunit.



## ACKNOWLEDGEMENTS

Financial support for this project was provided by a grant from the Swedish Council for Forestry and Agricultural Research to J.E. Kihlström (550/83D 103:1) and A. Madej.

The authors are indebted to NIH, NIAMDD and NICHHD, USA, for providing ovine lutropin, ovine LHα, ovine LHβ, and the antisera anti-oLH-β-1 and anti-oLH-α-1.

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(*) This work appeared in part as an abstract at the 7th International Congress of Endocrinology, Quebec City, Canada, 1984.