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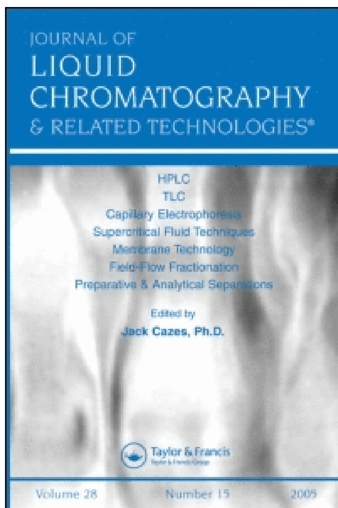

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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To cite this Article Hallin, Per and Madej, Andrzej(1985) 'Fractionation Of Bovine Lutropin With The Aid Of Anion-Exchange High Performance Liquid ChromatographY', Journal of Liquid Chromatography & Related Technologies, 8: 14, 2663 — 2674

To link to this Article: DOI: 10.1080/01483918508076597
URL: http://dx.doi.org/10.1080/01483918508076597

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JOURNAL OF LIQUID CHROMATOGRAPHY, 8(14), 2663-2674 (1985)

FRACTIONATION OF BOVINE LUTROPIN WITH THE AID OF ANION-EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Two bovine lutropin standard preparations (NIH-LH-B9, NIAMDD-bLH4) were applied on a recently developed anion-exchange column (Protein Pak DEAE 5PW,Waters Associates). The content of the eluted fractions was examined by a homologous radioimmunoassay of lutropin. In order to compare this fractionation with other independent methods, the two lutropin preparations were applied to SDS polyacrylamide gel eletrophoresis. The results indicate that the elution with a Tris-hydrochloric acid buffer pH 6.5 allows within 2 minutes a convenient separation of the most LH immuno-reactive components of these preparations. Analysis on gel electrophoresis indicates that after fractionation on this anion-exchange column, some stained bands were removed from NIH-LH-B9 but not from NIAMDD-bLH-4.

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INTRODUCTION

High performance ion exchangers were found to be very useful for protein separation (1-4). Several investigators e.g. Robertson et al.(5), Weise et al.(6) and Storring et al.(7) who characterized the physico-chemical properties of human FSH and LH, found that both hormones existed in multiple molecular forms. These forms have been separated mainly on charge basis, with the aid of isoelectrofocusing. In the present report we describe a fast method for fractionation of bovine lutropin standard preparation at a physiological pH, using an anion-exchange resin.

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MATERIALS

Two standard preparations of lutropin (NIH-LH-B9 and NIAMDD-bLH-4) were analyzed with a high performance ion exchange medium (Protein Pak DEAE 5PW, Waters Associates). HPLC was performed with a Waters Associates chromatographic system, consisting of two M 6000 solvent delivery systems, an U6K injector and a M 660 solvent programmer (Waters Associates,Milford,USA). The eluted material was monitored at 280 nm with a LDC Spectromonitor III (Laboratory Data Control, Riviera Beach, USA) at ambient temperature. Water of HPLC grade was obtained by passing distilled water through a Milli-Q-Water System using an Organex-Q cartridge and a sterile filter attached to its outlet (Millipore Corp.,Bedford, USA). All reagents used were of analytical grade.

METHODS

The solvent system consisted of two buffers, A and B, mixed by the solvent programmer and the pumps to appropiate proportions

of the respective buffers. Composition of buffer A at different pHs was as follows: i) 20 mmol/l acetate buffer pH 5.6 ii) 20 mmol/l Tris-hydochloride pH 6.5 and iii) 20 mmol/l Tris-hydrochloride pH 7.0. Buffer B was in all fractionations prepared like buffer A, except that buffer B also contained 0.5 mol/l of sodiumchloride. The lutropin preparations were diluted in the appropriate starting buffer.

The samples were eluted with a linear gradient running for 20 minutes from 98%/2% of buffers A/B to 100% of buffer B. Material corresponding to all peaks eluted during the run was collected, the protein concentration of each fraction determined and the eluates then frozen.

When subjected to radioimmunoassay the samples were diluted in the appropriate buffer. Samples loaded were in the range 100 - 1000 ug.

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The protein content of the assayed material was estimated from calculations based on UV absorbance of a 0.1% solution of the respective standard preparation measured at 280 nm in a 1 cm quartz cell.

The method for radioimmunological determination of lutropin according to Stupnicki & Madej (8) has been used, separating free and antibody-bound hormone as described by Eisenman & Chew (9). This was carried out by the addition of 0.1 ml of the second antibody (anti-rabbit gamma-globulin), followed by 0.5 ml polyethylene glycol (3.2% final concentration). The tubes were then centrifugated for 30 minutes at + 4° C and 2000 x g, the supernatant was decanted, and the last drops were removed by an absorbent tissue. The amount of radioactivity in the precipitate was counted with an automatic gamma-ray counter (85% efficency). The bovine standard preparations NIAMDD-bLH-4 and LER-1716-2 were used as standards and iodinated LER-1716-2 was used as tracer in the homologous radioimmunoassay. The assays were performed in 0.05 mol/l sodium

phosphate buffer pH 7.5 containing 0.85% sodiumchloride and 0.2% bovine serum albumin (sensitivity 0.1 ng per tube).

Before and after fractionation on HPLC, the two standard preparations and the main peak of the eluted material were analyzed, using 10-15% SDS polyacrylamide gel electrophoresis according to Blobel & Dobberstein (10). The resulting electrophoretic pattern was detected with silver staining method, according to the manufacturer (Bio Rad Laboratories, Richmond, USA - Bulletin 1089, 1982).

RESULTS

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Figure 1a-c depicts the elution profile of bovine lutropin NIH-LH-B9 after fractionation on DEAE column at different pHs. As can be seen, the best resolution was achieved at pH 6.5 using 20 mmol/l Tris-hydrochloride buffer. All subsequent fractionations were therefore performed using this buffer. Fractionation of bovine lutropin NIH-LH-B9, resulted in one main distinct peak followed by three smaller ones (Figure 2). The fractions were collected every 30 seconds during 14 minutes, and the major LH immunoreactive material was found in the first eluted peak. Some LH immunoreactivity (approximately 4 times lower than in the material found in the first eluted peak) was recorded in the fraction eluted 9 minutes after injection (Figure 3).

The chromatography of NIAMDD-bLH-4 preparation resulted in only one peak eluted 2 minutes after injection, and containing material with almost all LH immunoreactivity (Figure 3 and Figure 4). The residual LH immunoreactivity (approximately 10 times lower) was found in the fraction collected 9 minutes after injection (Figure 4). The data from the gel electrophoresis (Figure 5, A vs B) showed that following the fractionation of bovine lutropin NIH-LH-B9, some stained bands were removed. The fractionation of

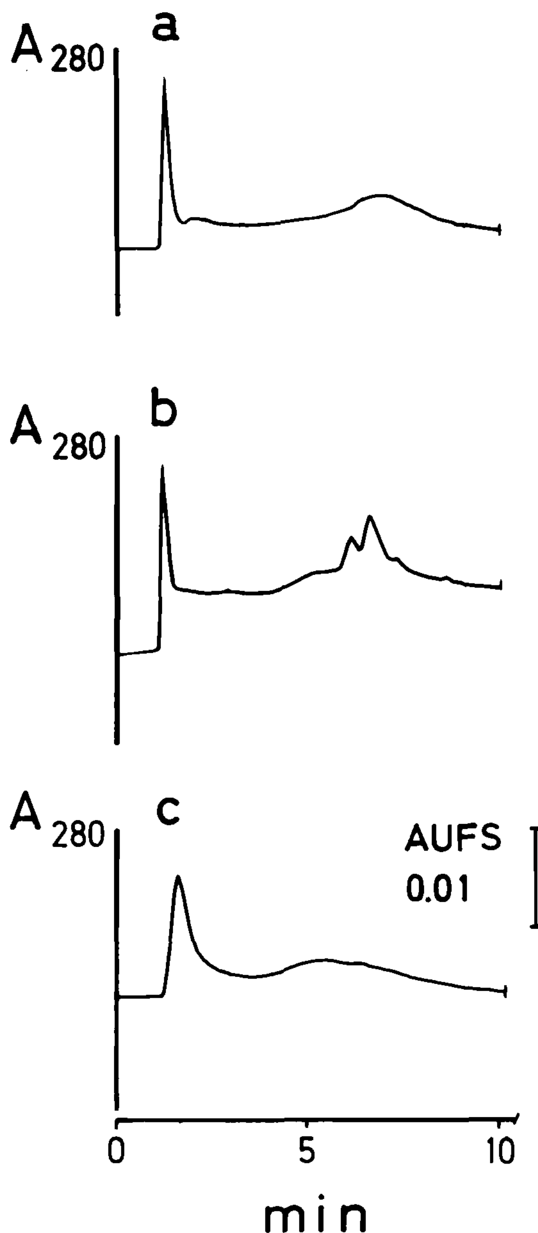

Figure 1
High-performance ion exchange chromatography of LH (NIH-LH-B9) on Waters DEAE 5PW column (75 x 7.5 mm I.D.). Flow rate 2.0 ml/min, room temperature; UV-280 nm; AUFS 0.1 ; sample size approximately 100 ug in 1000 ul buffer; gradient from 98%/2% of buffers A/B to 100% of buffer B during 20 min. a) Buffer A: 20 mmol/l NaAc/20 mmol/l HAc (pH 5.6) Buffer B: 20 mmol/l NaAc/20 mmol/l HAc + 0.5 mol/l NaCl b) Buffer A: 20 mmol/l Tris-HCl (pH 6.5) Buffer B: 20 mmol/l Tris-HCl + 0.5 mol/l NaCl c) Buffer A: 20 mmol/l Tris-HCl (pH7.0) Buffer B: 20 mmol/l Tris-HCl + 0.5 mol/l NaCl

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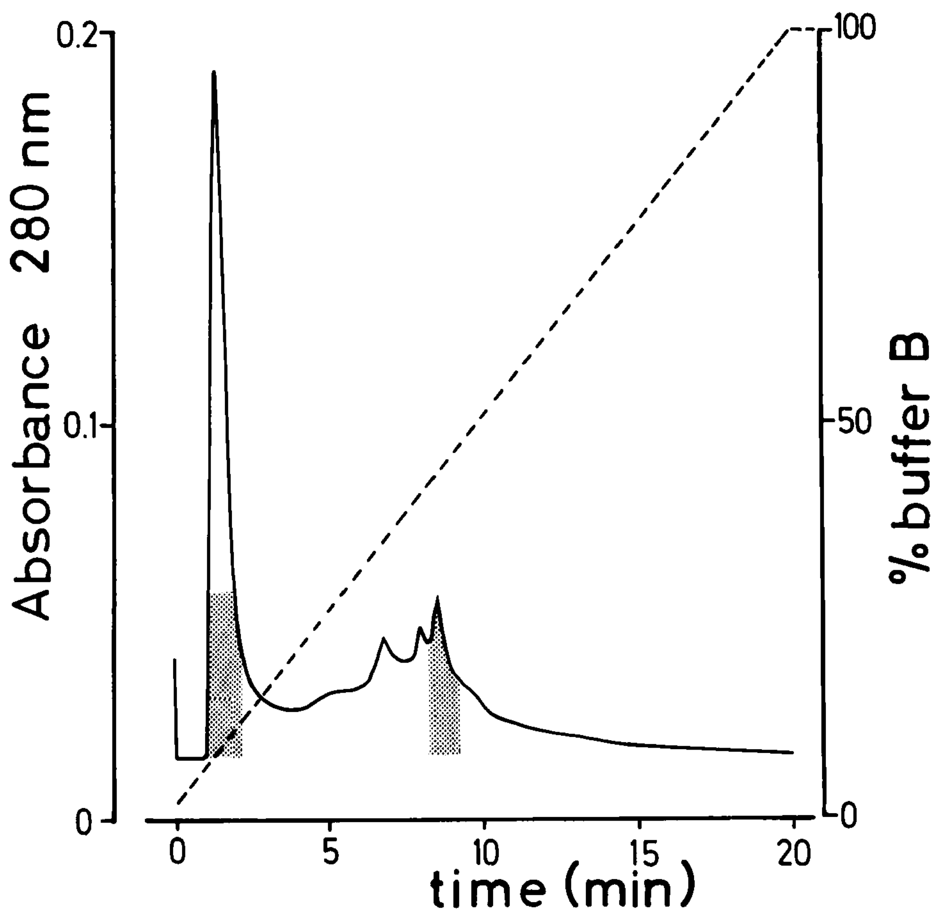

Figure 2.
High-performance ion exchange chromatography of LH (NIH-LH-B9) on Waters DEAE 5PW column (75 x 7.5 mm I.D.) with 20 mmol/l Tris-hydrochloride buffer pH 6.5 as buffer A and buffer B was made as buffer A but containing 0.5 mol/l sodiumchloride. AUFS 0.2; sample size approximately 450 ug in 1000 ul starting buffer; UV 280 nm ; flow rate 2.0 ml/min; gradient from 98% / 2% of buffers A/B to 100% of buffer B during 20 min. The immunological activity was found in the shaded areas (fraction Nos. 1 and 9).

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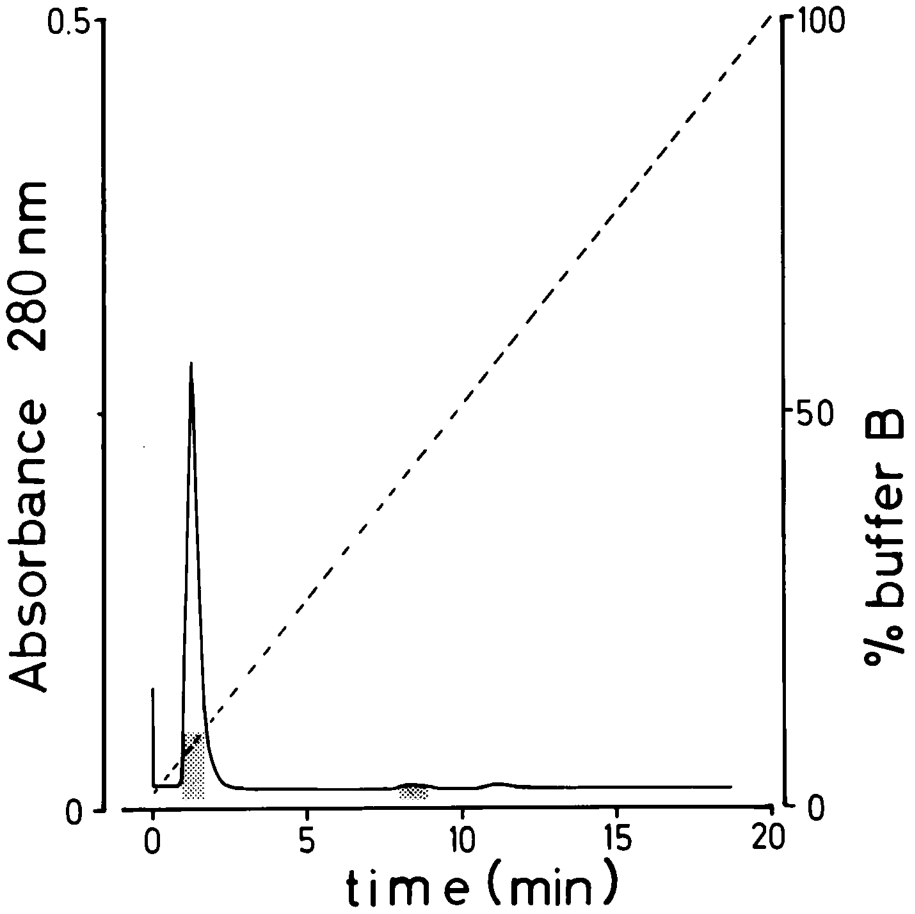

Figure 3
High-performance ion exchange chromatography of LH (NIAMDD-bLH-4) on Waters DEAE 5PW column (75 x 7.5 mm I.D.) with the same conditions as in Figure 1. AUFS 0.5; sample size 1000 ug in 1000 ul of starting buffer. The immunological activity was found in the shaded areas (fraction Nos. 1 and 8).

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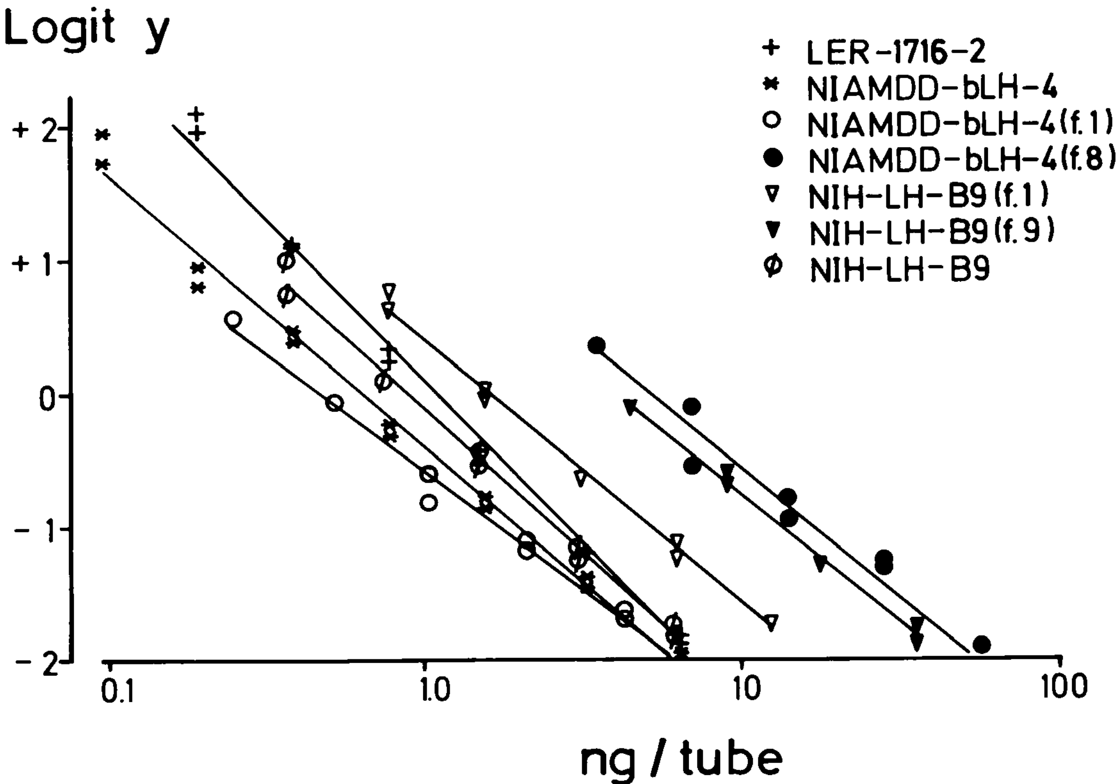

Figure 4
LH immunoreactivity of fractions obtained as described in Figure 1 and 2. The lutropin preparations, NIAMDD-bLH-4 and LER-1716-2 were both used as standards and iodinated LER-1716-2 was used as tracer in the homologous radioimmunoassay. The displacement curves of the bound radioactivity were linearized with the logit transformation of $y = (B-N)/(B_0-N)$ versus log dose of added protein per tube.

bovine lutropin NIAMDD-bLH-4 did not reduced the number of stained bands, as can be seen in the electrophoretic pattern (Figure 5, D vs C).

DISCUSSION

According to the manufacturer, the Water Protein Pak DEAE 5PW column is very useful for rapid purification of glycoprotein hormones or larger proteins in the pH range 2-12.
Separation of the components can take place under very mild conditions, preserving biological activity (e.g. 2). In the gradient elution used here, small changes in buffer pH seem to be of importance. The data from the present study indicates that 20 mmol/l Tris-hydrochloride buffer pH 6.5 would be a good choice of mobile phase for fractionation of bovine lutropin standards. It is interesting to note that gel electrophoresis of material derived from the main peak after fractionation of NIH-LH-B9 resulted in a smaller number of stained bands than gel electrophoresis of starting material. In contrast, the LH immunoreactivity of above-mentioned material was lower than that in NIH-LH-B9 before fractionation.

This accords with findings of Zaidi et al. (11) and Khan et al. (12) who reported that isoelectrofocusing of either hLH or baboon FSH resulted in a considerable loss of their immunoreactivity. These authors suggested that either all preparations tested contained easily dissociable biologically inactive immunoreactive material or that loss of immunoreactive sites may have occurred during the fractionation procedure.

Lack of any improvement in LH immunoreactivity as well as electrophoretic behaviour after fractionation of NIAMDD-bLH-4 on DEAE column may be due to the fact that this is a new and highly purified bovine LH standard preparation.

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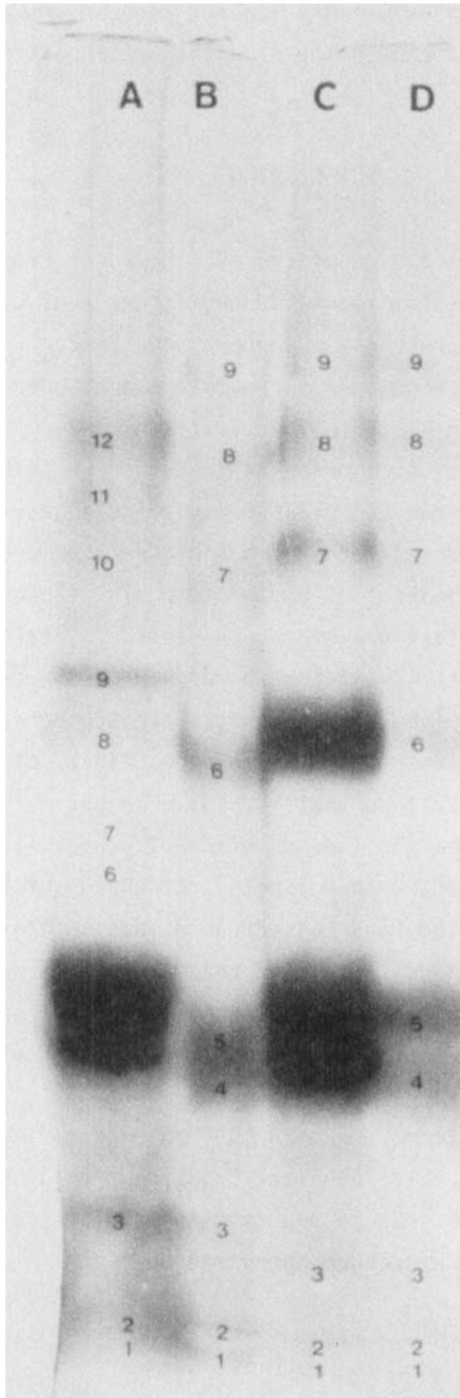

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Several electrophoretic bands found here from lutropin standard preparation accords with findings of Ward et al.(13). However, in present study even more stained bands were shown up due to silver staining method, which is supposed to be 100-times more sensitive than Coomassie Brilliant Blue (14).

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Natural Science Research Council to S.Renlund and the Swedish Council for Forestry and Agricultural Research to JE.Kihlström and A.Madej. The authors are indebted to NIH as well as NIAMDD,USA, for providing NIH-LH-B9, NIAMDD-bLH-4 and LER-1716-2. The advice of Tomas Popoff (Waters Associates, Sundbyberg, Sweden) who also was kind enough to lend the DEAE column and the help of Dr.Gösta Eggersten (Uppsala University,Sweden) with gelectrophoresis is gratefully acknowledged.

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Figure 5
Analysis by polyacrylamide gelectrophoresis (10-15%) of the eluted fractions having the highest LH immunoreactivity. A) bovine lutropin NIH-LH-B9 B) bovine lutropin NIH-LH-B9 (f.1) from DEAE-purification (see Figure 1) C) bovine lutropin NIAMDD-bLH-4 (f.1) from DEAE-purification (see Figure 2) D) bovine lutropin NIAMDD-bLH-4.

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