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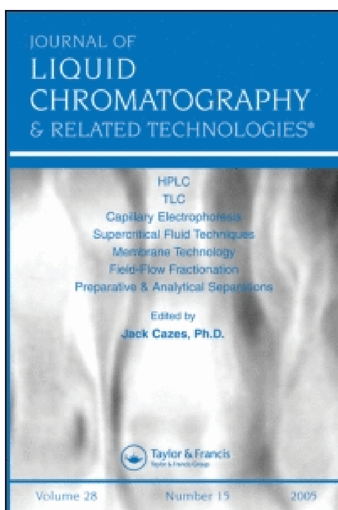

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Per Hallin[a]; Malgorzata Madej[b]; Andrzej Madej[b]
[a] Department of Zoophysiology, Uppsala University, Uppsala, Sweden [b] Department of Clinical Chemistry Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden

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SEPARABLE FORMS OF RADIOIODINATED OVINE LUTROPIN (oLH) SUBUNITS, FRACTIONATED BY ANION EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ANALYZED BY RADIOIMMUNOASSAY

Per Hallin[1], Malgorzata Madej[2], and Andrzej Madej[2]

[1]Department of Zoophysiology
Uppsala University
P.O. Box 560
S-751 22 Uppsala, Sweden
[2]Department of Clinical Chemistry
Faculty of Veterinary Medicine
Swedish University of Agricultural Sciences
S-750 07 Uppsala, Sweden

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ABSTRACT

Radioiodinated oLHα and oLHβ subunits were fractionated with the aid of high performance liquid chromatography (HPLC) using a Waters Protein Pak DEAE 5PW anion exchange column. The content of these subfractions differed in their binding maxima to their respective subunit antisera. An increase of the pH from 6.5 to 7.5 and 8.5 affected the chromatographic profile of 8-week-old radioiodinated β-subunit. Overall, material from the various radioactive peaks exhibited binding to β-subunit antiserum in the range of 32.0% - 81.0%, depending on the storage time of the tracer and the pH. Shifting strategies, we either applied the labelled subunits to a Pharmacia gel filtration column or subjected them to cellulose adsorption prior to HPLC. The radioiodinated α- and β-subunits subjected to HPLC after gel filtration were both eluted in only one peak with respective immunoreactivities of 46.6% and 73.2%.

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When radioiodinated β-subunit was applied first to a cellulose column and then to HPLC, the chromatographic profile showed two radioactive peaks with retention times of 5 min (73.2% immunoreactivity) and 7.5 min (43.0% immunoreactivity), respectively.
It was concluded that an 8-week-old-tracer is useful in such studies, owing to its highly stable immunoreactivity after repurification on an anion exchange HPLC.

INTRODUCTION

Recently, liquid chromatography has been used to study the microheterogeneity of glycoprotein hormones (1-3). It has even been shown by using isoelectrofocusing that subunits of human lutropin (hLH) exist in multiple forms, each of them with a different pI (4).

In an earlier study (5), a fractionated radioiodinated α-subunit of ovine LH on reverse-phase high performance liquid chromatography (C_{18} column) showed a chromatogram with a symmetric peak of radioactivity. However, in the same study a radioiodinated oLHβ subunit yielded an asymmetric peak. Similar asymmetry was also reported by Stanton et al. (6), who analyzed radioiodinated β-subunit of bTSH with a C_{18} reverse-phase system.

The aim of present work was to study the behaviour of the radioiodinated lutropin α- and β-subunits subjected to high performance anion exchange chromatography.

MATERIALS

Standard preparations of ovine lutropin α-subunit (NIAMDD-WRR-1-α) and ovine lutropin β-subunit (NIAMDD-WRR-2-β), provided by the National Hormone and Pituitary Program, NIADDK, Bethesda, Maryland, USA, were radioiodinated with ^{125}I-Na (The Radiochemical Centre, Amersham, UK) and analyzed with high performance ion exchange medium (Protein Pak DEAE 5PW, Waters Assoc., Milford, USA). HPLC was performed with a Waters Associates

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chromatographic system according to the method described by Hallin and Madej (7). The eluted materials were collected every 30 s and the radioactivity in an aliquot of 0.05 ml from each fraction was measured in a gamma-ray counter (Searle Analytic Inc. Illinois, USA).
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.

In some experiments the subunits were fractionated by gel filtration on a Pharmacia PD10 column (see gel filtration section) or applied on cellulose CF-11 medium (see cellulose adsorption section).

Antisera to ovine LHα and β-subunits were also obtained from the National Hormone and Pituitary Program, USA (NIAMDD-anti-oLH-α-1 and NIAMDD-anti-oLH-β-1).

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METHODS

Iodination of oLHα (WRR-1-α) and oLHβ (WRR-2-β) was performed by a modified Chloramine-T method (8) using 8 ug of this reagent, 2 ug of protein and an exposure time of 40 s. The specific activity of oLHα was 58 uCi/ug and that of oLHβ was 30 uCi/ug. After iodination the material was subjected to either gel filtration followed by HPLC, cellulose adsorption followed by HPLC or HPLC only. The tracer was stored at + 4 ^{o}C for 2 days, or up to 8 weeks after iodination before using it in the different chromatographic treatments.

Gel filtration on PD10 (Sephadex G-25M, Pharmacia AB, Uppsala, Sweden) was employed on a prepacked column (dimensions 0.9 x 10 cm) and eluted with a 50 mmol/l phosphate buffer, pH 7.5, containing 0.2% bovine serum albumin (BSA).

Cellulose adsorption on Whatman CF-11 was conducted according to Jeffcoat (9). The radioiodinated lutropin subunits were applied to a column (dimensions 0.4 x 4.0 cm) and eluted from the cellulose with 10 mmol/l phosphate buffer, pH 7.5, containing 6% BSA.

The HPLC solvent system consisted of two buffers, A and B. Buffer A consisted of 20 mmol/l Tris-hydrochloride. The composition of buffer B was similar to that of buffer A in all fractionations, except that the former also contained 0.5 mol/l of sodium chloride. The samples were eluted (2.0 ml/min) using a linear gradient (12.5 mmol/ml) running directly after injection for 20 min (98%/2% of buffers A/B to 100% of buffer B). Fractions were collected every 30 s during the experiment at ambient temperature.

To avoid losses of radioactivity due to adsorption, each fraction tube was prefilled with 0.5 ml of a 50 mmol/l sodium phosphate buffer, pH 7.5, containing 0.45 % sodium chloride, 10 mmol/l EDTA, 0.001% merthiolate, and 0.2% BSA.

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Radioimmunoassays of oLHα were performed using the reference standard oLHα (WRR-1-α) and oLHα antiserum (NIAMDD-anti-oLH-α-1) at a final dilution of 1:90,000. The reference standard oLHβ (WRR-2-β) and antiserum (NIAMDD-anti-oLH-β-1) at a final dilution of 1:90,000 were used in the radioimmunoassay of oLHβ . After separation of radioiodinated α- and β-subunits (ca. 10 uCi/ subunit or 370 kBq), the amount of radioactivity was measured in 0.05 ml of each fraction. The eluted fractions were diluted with phosphate buffer to obtain 8000 cpm/ 0.1 ml. These solutions were incubated overnight at room temperature with antiserum against either α- or β-subunits of oLH, both at a final dilution of 1:90,000. The separation of free and bound hormone was carried out using the new system, consisting of second-antibody coupled to micro-Sepharose according to the manufacturer (Technical note, Pharmacia AB, Sweden). All assays were performed in 50 mmol/l sodium phosphate buffer, pH 7.5, containing 0.45% sodium chloride, 10 mmol/l EDTA, 0.001% merthiolate, and 0.2% BSA.

RESULTS

The chromatograms resulting from the application of the radioiodinated ovine LHα subunit either directly to an anion exchange HPLC or indirectly via a Pharmacia PD10 prior to anion exchange HPLC, using a buffer system at pH 6.5, are shown in Fig.1. The first peak of radioactive material appeared 1.5 min after injection, regardless the treatment. Direct injection of the radioiodinated α-subunit using HPLC resulted in at least two peaks of radioactivity with a retention times (r.t) of 1.5 and 6.5 min. The immunoreactivity of the material from these two peaks was 46.6% and 51.0%, respectively.

The material applied to HPLC after gel filtration on PD10 eluted in only one main peak, having a retention time of 1.5 min and with an immunoreactivity of 53.0%.

The radioiodinated tracer (WRR-1-α) was in this experiment stored at + 4 ^{o}C for 4 weeks before use in the assay.

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Figure 2 shows the chromatographic profiles of the radioiodinated β-subunit with and without gel filtration prior to HPLC at pH 6.5. As for the α-subunit the β-subunit subjected to gel filtration showed one main peak, eluting 1.5 min after injection and containing 73.2% of the immunoreactivity.

Moreover, the material not applied to gel filtration prior to HPLC showed a pattern with three different peaks containing radioactivity. The immunoreactive material in these peaks were 65.0%, 81.0% and 75.4%, respectively.

The radioiodinated tracer (WRR-2-β) was in this experiment stored at + 4 ^{o}C for 4 weeks before use in the assay.

The chromatographic profiles of the radioiodinated oLHβ subunit fractionated at pH 6.5, 7.5 and 8.5 are presented in Fig.3. At pH 6.5 the first non adsorbed material containing radioactivity

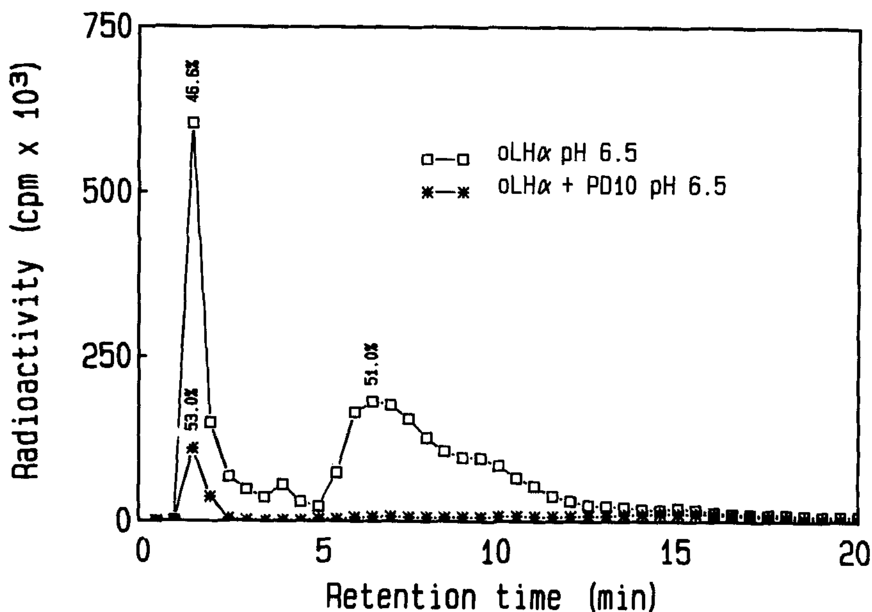

Figure 1.
High performance ion exchange chromatography of radioiodinated oLHα at pH 6.5 (□) on a Waters Protein Pak DEAE 5PW column (75 x 7.5 mm I.D.), and gel filtration on PD10 (✱) prior to DEAE.
Buffer A: 20 mmol/l Tris-HCl
Buffer B: 20 mmol/l Tris-HCl + 0.5 mol/l NaCl
Flow rate: 2.0 ml/min; gradient 2% - 100% of buffer B/ 20 min
The binding of tested fractions to oLHα antiserum is expressed as a percentage of the total radioactivity added.

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eluted in a broad peak (r.t 2.5 min) followed by another peak with a retention time of 5.5 min. The level of immunoreactivity was only 40.0% in the former material, but reached 75.4% in the latter. When using the eluant buffer adjusted to pH 7.5, the oLHβ subunit was fractionated into three main radioactive peaks; the first eluting after 1.5 min, the second after 3 min (72.2% immunoreactivity) and the third after 5.5 min (70.0% immunoreactivity).

With the eluant buffer at pH 8.5, the chromatographed oLHβ subunit resulted in an elution pattern with three distinct peaks of radioactivity, followed by a very small fourth peak. The first

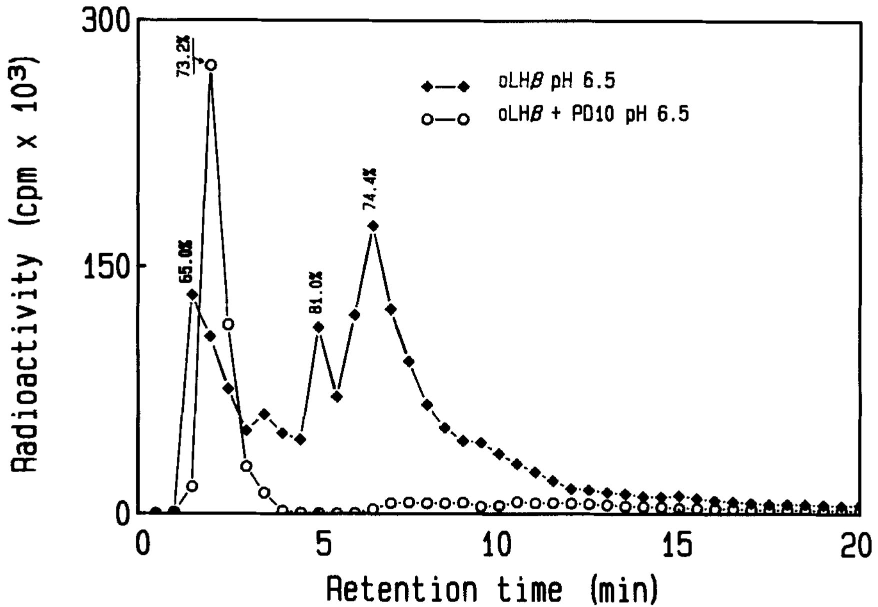

Figure 2.
High performance ion exchange chromatography of radioiodinated oLHβ at pH 6.5 (◆) on a Waters Protein Pak DEAE 5PW column (75 x 7.5 mm I.D.), and gel filtration on PD10 (O) prior to DEAE. (see description of Fig.1)
The binding of tested fractions to oLHβ antiserum is expressed as a percentage of the total radioactivity added.

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material eluted after 1.5 min retention time and exhibited 68.0% immunoreactivity to its antiserum, while the second (r.t 3.5 min) and third (r.t 5.5 min) peaks both exhibited 70.0% immunoreactivity. The material in the fourth peak (r.t 11.5 min) showed 32.0% immunoreactivity to the antiserum.

The radioiodinated tracer (WRR-2-β) in this experiment was stored at + 4°C for 8 weeks before use in the assay.

The chromatograms of the radioiodinated β-subunit when subjected to either gel filtration or cellulose adsorption before

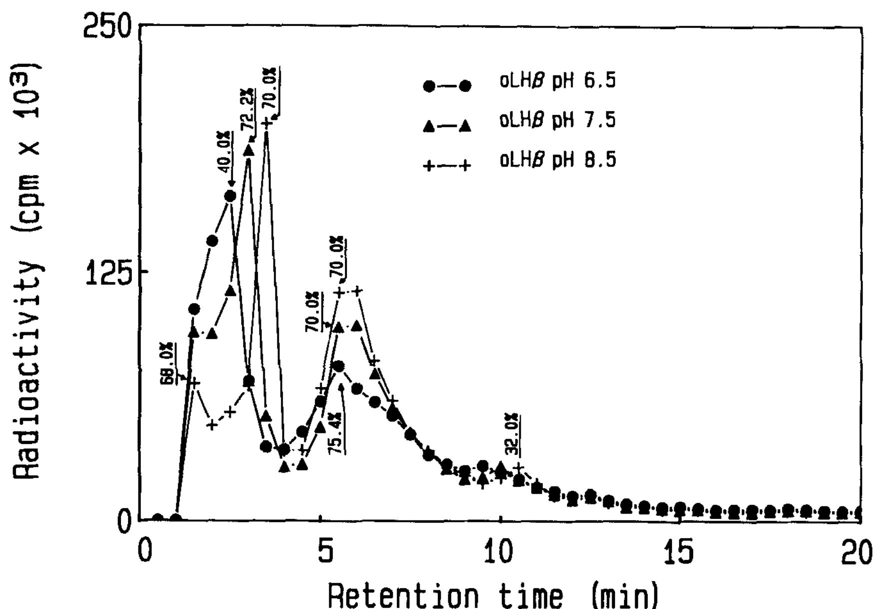

Figure 3.
High performance ion exchange chromatography of radioiodinated oLHβ at pH 6.5 (●), pH 7.5 (▲), and pH 8.5 (+) on a Waters Protein Pak DEAE 5PW column (75 x 7.5 mm I.D.).
(see description of Fig.1)
The binding of tested fractions to oLHβ antiserum is expressed as a percentage of the total radioactivity added.

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ion exchange HPLC are shown in Fig. 4. Gel filtration prior to HPLC resulted in one peak with a retention time of 2 min and with an immunoreactivity of 73.2%.

The retention times for the two main radioactive peaks in the material, eluted using cellulose adsorption followed by HPLC were 5.0 and 7.5 min and the immunoreactivity of the material was 73.2% and 43.0%, respectively.

The radioiodinated tracer (WRR-2-β) in this experiment was stored at + 4 ^{o}C for 2 days before use in the assay.

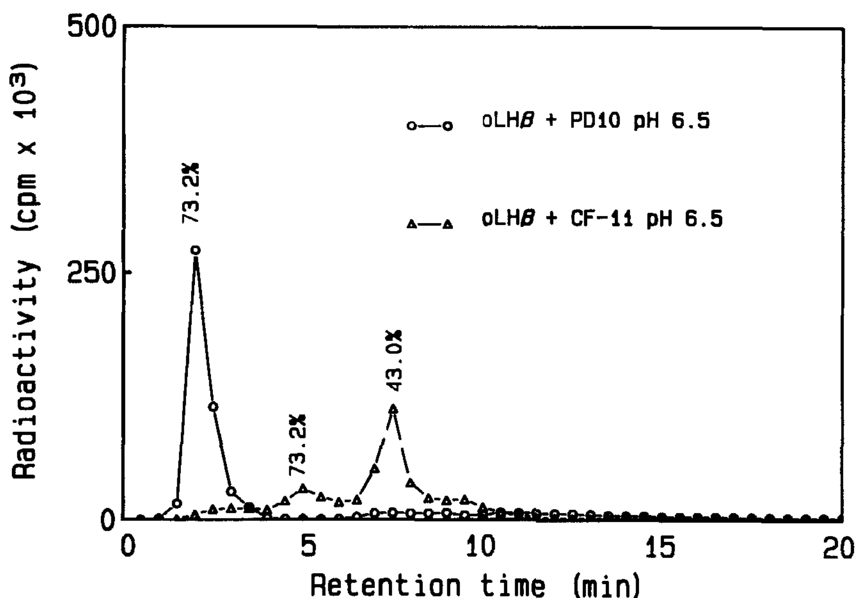

Figure 4.
High performance ion exchange chromatography of radioiodinated oLHβ at pH 6.5 with gel filtration (O) and cellulose adsorption on Whatman CF-11 (Δ) prior application on a Waters Protein Pak DEAE 5PW column (75 x 7.5 mm I.D.)
(see description of Fig.1)
The binding of tested fractions to oLHβ antiserum is expressed as a percentage of the total radioactivity added.

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Chromatography of radioiodinated oLHα and β-subunits led to a mean recovery of 48%. When the radioiodinated oLHβ subunit was first fractionated on a Pharmacia PD10 column and thereafter subjected to HPLC, the mean recovery increased to 60%. When oLHβ was finally chromatographed after cellulose adsorption on CF-11,the recovered radioactivity increased to almost 100%.

DISCUSSION

The results of the present study indicate that ion exchange HPLC is more suitable than the reverse-phase system (5),

for eluting individual forms of radioiodinated oLHα and oLHβ subunits. By direct applying the iodinated material to the above-described ion exchange HPLC system, we were able to detect at least two forms of radioiodinated oLHα and four forms of radioiodinated oLHβ.

With the aid of isoelectrofocusing, van Ginkel and Loeber (4) reported the presence of nine different α-subunit subfractions (pI 4.76 to 9.32) and four different β-subunit subfractions of hLH (pI: 7.60 to 9.61). It is tempting to speculate that forms of radioiodinated oLHβ subunit reported here are similar to those reported by van Ginkel and Loeber (4). For the radioiodinated α-subunit of oLH, we used a pH that could not possibly have been optimal for all existing α-subunit forms.

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As was reported by Stanton et al. (10) that the optimal separation on a Pharmacia Mono-Q anion exchange column (linear gradient: 17.5 mmol/ml) occurs when the pH of the eluent is 0.5 - 1.0 pH unit below the pI of the most alkaline component of the protein in question.

However, because of the Donnan effect, the pH of the microenvironment in an anion exchange column can be up to 1 pH unit above that of the eluant buffer (11). Based on the chromatographic profile for the oLHβ subunit (Fig.2), the best resolution with the highest restored immunoreactivity was achieved at pH 6.5 (maximum 81.0%). Consequensly, a buffer of pH 6.5 may actually increase to about 7.5 in the column matrix, and may thereby fall within the β-subunit pIs. It would therefore perhaps be better to use a cation exchanger for the fractionation of the most acid forms of the α-subunit, need to be elucidated.

In conclusion, it seems that the 8-week-old radioiodinated β-subunit tracer can be used for radioimmunoassay studies after repurification with the anion exchange procedure described here, owing to the tracer's stable immunoreactivity over time.

Imaoka and Funae (12) reported an 86-92% recovery of the various forms of cytochrome P-450 on a Waters Protein Pak DEAE 5PW column. In the present study we found that the recovery of radio-labelled hormone increased as the amount of bovine serum albumin added was increased (i.e. almost 100% recovery when oLHβ radioactivity was dissolved in 3% BSA).

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