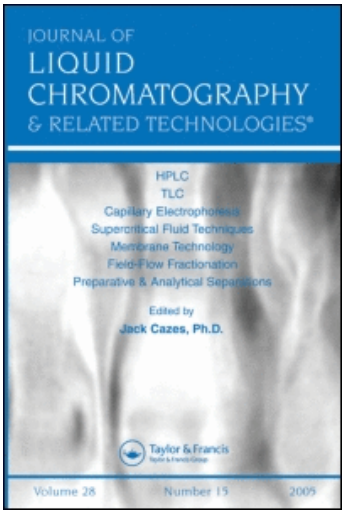

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Preservation of Biological LH and FSH Activity after Application on HPLC. Comparison between Cation-Exchange and Reverse-Phase High Performance Liquid Chromatography

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PRESERVATION OF BIOLOGICAL LH AND FSH ACTIVITY AFTER APPLICATION ON HPLC. COMPARISON BETWEEN CATION-EXCHANGE AND REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A cation-exchange high-performance liquid chromatography procedure is described for the separation of bioactive lutropin from follitropin in a human urinary gonadotropin standard preparation (1st IRP 70/45). The results clearly demonstrate that recovered protein retained most of its biological activity following the chromatography. Due to a lack of efficient and quick methods for purifying pituitary or urinary gonadotropins, this method could prove to be valuable for this purpose. This method has also been shown to be quick (less than 20 min) and efficient for separation of impurities or degradation products from bovine follitropin with 92% preservation of its biological activity. Our results suggest that ion exchange HPLC procedure will play a powerful role in the isolation of gonadotropins and other biologically active compounds.

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INTRODUCTION

Attempts to use the reverse-phase high performance liquid chromatography (HPLC) technique to purify or isolate the glycoprotein hormones and their subunits have been made (1,2,3,4,5 & 6). With aid of reverse-phase HPLC, the procedures for preparing highly purified hormones have been shown to be more rapid than using conventional methods. In an earlier report (6),we have used the same method for purifying bovine lutropin (LH), which resulted in a considerable loss of bioactivity.

In all of the previously mentioned reverse-phase HPLC methods, purification resulted in partial reduction of the biological activity of the glycoprotein hormones. This is not surprising, since the pH must be very acidic for good resolution with hydrophobic solvents in these procedures.

In this report we describe the biologic properties of bovine follitropin (bFSH - NIH-FSH-B1) and human urinary gonadotropin standard preparation (IRP) when they are subjected to either a reverse-phase or cation-exchange HPLC system. Lutropin and follitropin bioactivity recovered in the eluted fractions of the two standard preparations was determined by the use of in vitro bioassays.

MATERIALS

Rats of the Sprague-Dawley strain and mice of NMRI strain were obtained from AB Anticimex, Stockholm, Sweden. Falcon tissue culture plates (Type 3040) containing 96 chambers were purchased from Falcon Plastics, Oxnard,CA.,USA, Eagle's Minimum Essential Medium, Hank's Balanced Salt Solution, Fungizone, Penicillin, Streptomycin, L-Glutamine and chicken serum were all obtained from Flow Laboratories, Irwine, Scotland. Bovine serum albumin (Fraction V), trypsin, soybean trypsin inhibitor, collagenase and deox-

yribonuclease were all obtained from Sigma,St. Louis,MI.,USA. [2,4,6,7] ^{3}H Oestradiol and [2,4,6,7] ^{3}H Testosterone were purchased from Radiochemical Centre, Amersham, U K.

1st International Reference Preparation (IRP) of human pituitary gonadotropin (FSH and LH) for bioassay (code no. 69/104) was used as a standard in both FSH and LH bioassays and the International Reference Preparation of human urinary gonadotropin (FSH and LH 70/45) applied to HPLC were provided by the National Institute of Biological Standards and Control (NIBSC), London, UK. Bovine FSH standard preparation NIH-FSH-B1 , kindly supplied by NIAMDD, Bethesda ,USA , was also applied to HPLC. Androstenedione (19 OH $\Delta^{3,4}$,17 dione) was purchased from Research Plus, Steroid Lab. Inc., Denville,NJ.,USA. The Waters high performance liquid chromatographic system was used, which included two M 6000 pumps, a M 660 solvent programmer and an U6K injector (Waters Associates, Milford, USA).

The samples were either run in the cation-exchange column (Waters Protein Pak SP 5 PW) or in two stainless steel columns (150 x 8 mm) connected in series and packed with reverse-phase coated silica. The latter columns were slurry packed with Spherisorb 5 um ODS silica (Phase Separations Ltd., Queensferry, UK) in our laboratory at 450 bar (45 MPa) pressure. The samples were analyzed at 280 nm with a LDC Spectromonitor III (Laboratory Data Control, Riviera Beach, USA) variable wavelength UV-detector. Eluted fractions were immediately placed in a freezer, until analysis in the bioassays. All reagents were of either HPLC grade or of reagent grade.

Column efficiency

A value for the height equivalent to a theoretical plate (HETP) was measured on the basis of the toluene peak at varying

flow rates with methanol/water (70%/ 30%) as the eluant. The lowest HETP values were found to be 13.2 μm and 15.0 μm at 1.5 ml/min for toluene with the two reverse-phase columns used here.

METHODS

Determination of FSH bioactivity.

An in vitro bioassay procedure based on the FSH stimulated estradiol production by the immature rat Sertoli cells was utilized (8).

Bioassay of LH

An in vitro bioassay method based on the LH dependent testosterone production by the mouse Leydig cell suspension was utilized (9). The standards and the test materials produced response lines parallel to one another in both assays.

Fractionation of bFSH and 1st IRP on reverse-phase HPLC

The solution compositions and their flow rates used in this investigation, were the same as reported previously (6), i.e. A-buffer contains 0.5% trifluoroacetic acid in water and B-buffer contains 80% acetonitrile, 19.5% water and 0.5% trifluoroacetic acid. The two buffers were titrated in the first experiment to pH 3.3 with a concentrated ammonia solution. In the same manner, the pH was adjusted to 4.3 and 5.3 in the second and third experiments, respectively. A flow rate of 1.5 ml/min was used, which was monitored by a pressure of approximately 2000 psi (13 MPa). A

linear gradient was run from (A% / B%) 95/5 to 37/63 over 39 minutes. All separations were performed at room temperature. The eluted fractions were collected, rapidly frozen and stored at -20°C until the bioassays were performed. The bovine FSH was applied at three pH levels but IRP (70/45) was only applied at pH 3.3. Samples were loaded in the range of 250 to 2000 µg for bFSH and approximately 25 IU for FSH/LH (IRP) in their respective experiments.

Fractionation of bFSH and 1st IRP on cation-exchange HPLC.

The solvent system consisted of two buffers, A and B, mixed by the solvent programmer and the pumps to appropriate proportions of the respective buffers. Buffer A was prepared by diluting 20 mmol/l phosphate buffer to the required pH. Buffer B was made as buffer A, with the addition of sodiumchloride to a final concentration of 340 mmol/l. The pH was adjusted with mono-and disodium phosphate to 7.0 , 6.0, 5.0 and finally 5.5 in the experiments when bFSH was applied to the system. The 1st IRP was only applied at pH 5.5.

RESULTS

Fractionation of bFSH and 1st IRP on reverse-phase HPLC.

The profiles of the two standard preparations are shown in Figure 1. Bovine FSH was fractionated into three major components, whereas the elution pattern from the 1st IRP (70/45) was resolved into multiple components with this procedure.

The collection of fractions for bFSH was started 31 minutes after the injection and was continued every 30 seconds up to 47 minutes. The collection of fractions for the 1st IRP was started

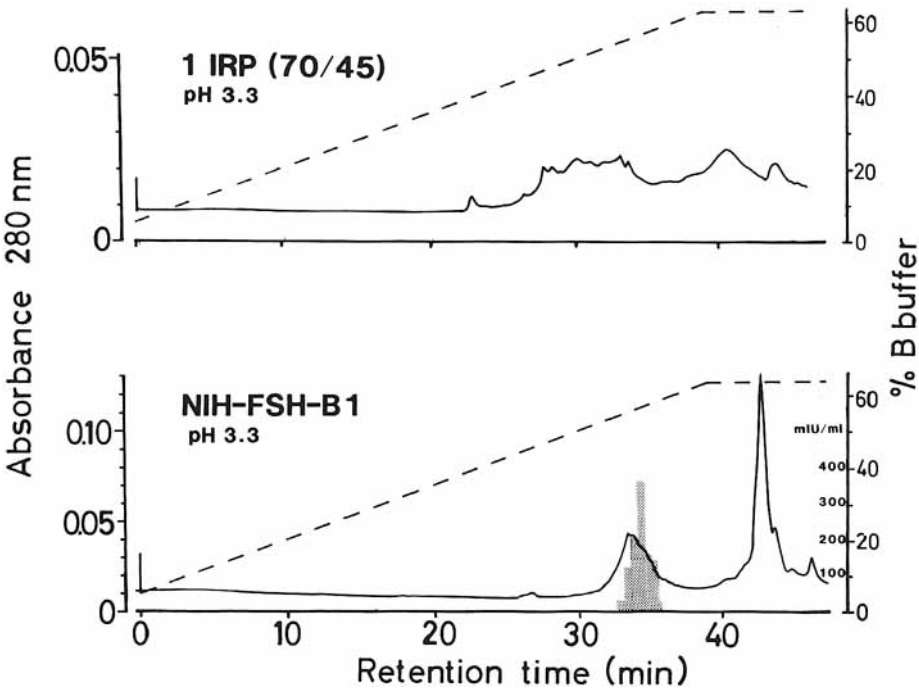

Figure 1.
Reverse-phase high performance liquid chromatography of human urinary gonadotropin (1st IRP 70/45) and bovine follitropin (NIH-FSH-B1). Column: 5 um ODS silica (150 x 8) x 2; Flow rate: 1.5 ml/min, room temperature; AUFS: 0.1 , UV-280 nm; Sample size: 25 IU of 1st IRP, 2 mg of NIH-FSH-B1; Gradient from 95% / 5% of buffers A/B to 37% / 63% during 39 min. Buffer A) 0.5% trifluoroacetic acid in water; Buffer B) 80% acetonitrile - 19.5% water - 0.5% trifluoroacewtic acid . Both buffers adjusted to pH 3.3 with concentrated ammonia solution. FSH bioactivity in hatched area.

25 minutes after injection at an interval of one minute up to 47 minutes. All eluted fractions from the two standards were tested in the in vitro bioassay for bFSH and for both FSH and LH in the case of IRP.

The highest FSH bioactivity was found in fractions 6-11 (see Figure 1) for bFSH. However, the recovered bioactivity was approx-

imately 4 % of that found in native bFSH. In comparison, material from the eluted fractions from the 1st IRP exhibited no FSH bioactivity but had a slight LH bioactivity. Since the pH at the above mentioned reverse-phase separation is very acidic, the pH in the two following experiments was changed to 4.3 and 5.3. The chromatographic pattern for bFSH was very similar to that found at pH 3.3, but the biologic potencies of all fractions were still very low (data not shown).

Fractionation of bFSH and 1st IRP on cation-exchange HPLC.

The observation that bFSH as well as the 1st IRP lost most of their biological activity after fractionation on reverse-phase HPLC, suggested a substitution of reverse-phase with a milder procedure such as using an ion exchange column.

Our initial studies were conducted using the mobile phase described by Kato et al. (7). As shown in Figure 2, the bovine standard preparation NIH-FSH-B1 was resolved in almost one main component at pH 7.0 and 6.0 with this buffer. Bioassay of the material eluted from the column gave a high FSH biological potencies for all the fractions tested.

When pH was adjusted to 5.0, the elution pattern changed and two peaks on the chromatogram were observed. Biological activity was found only in the material eluted in the second peak. However, this material represented only 6% of the protein loaded onto the column.

The pH shift to 5.5 of the mobile phase resulted in the best separation of other components from the original bFSH (see Figure 3a), and improved the relative biologic FSH activity to native FSH. Almost all biologic FSH activity (92%) was retained and was eluted in one fraction (see Figure 3b). No LH bioactivity could be found in any of the fractions obtained from this standard preparation.

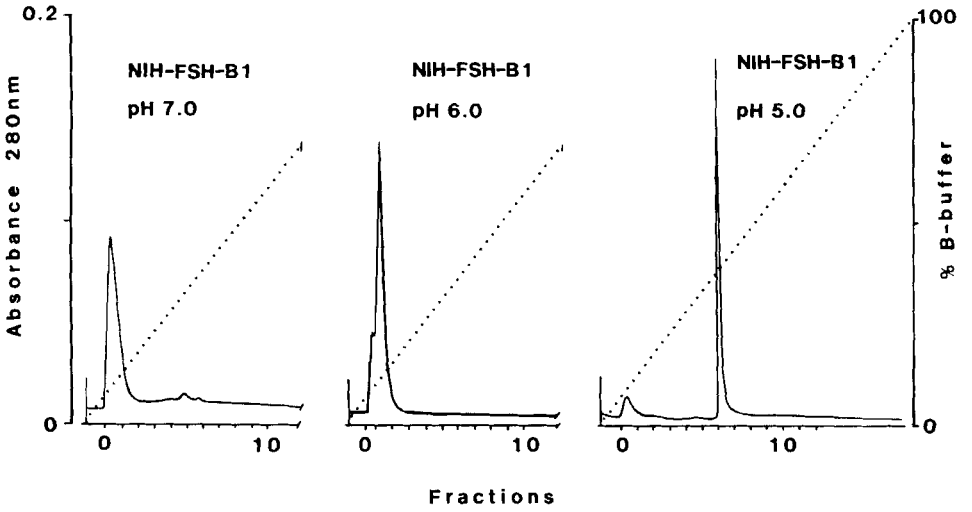

Figure 2.
High performance ion exchange chromatography of bovine follitropin (NIH-FSH-B1) on Waters Protein Pak SP 5 PW column (75 x 7.5 mm I.D.) at different pH. Flow rate: 2.0 ml/min, room temperature; AUFS: 0.2 , UV-280 nm; Sample size: 250 ug in 1 ml buffer; Gradient from 98% / 2% of buffers A/B to 100% of buffer B during 20 min. Buffer A) 20 mmol/l phosphate buffer; Buffer B) 20 mmol/l phosphate buffer + 340 mmol/l sodiumchloride.

Using these chromatographic conditions, the 1st IRP (70/45) was also applied to find similarities or differencies in resolution behavior of this preparation compared to the bFSH standard.

The elution profile obtained from this fractionation revealed the presence of two fractions, very similar to that of bFSH (see Figure 4a). No additional distinct peaks in between these two products were seen. Most of the biological FSH activity for the 1st IRP was like bFSH found in the material eluted at the end of the gradient (see Figure 4b). The distribution of LH bioactivity was also determined for 1st IRP (70/45) and was found in the eluted material which also contained FSH bioactivity . Analysis of the eluted fractions after cation-exchange HPLC revealed a biological FSH distribution reasonably similar to that of bFSH.

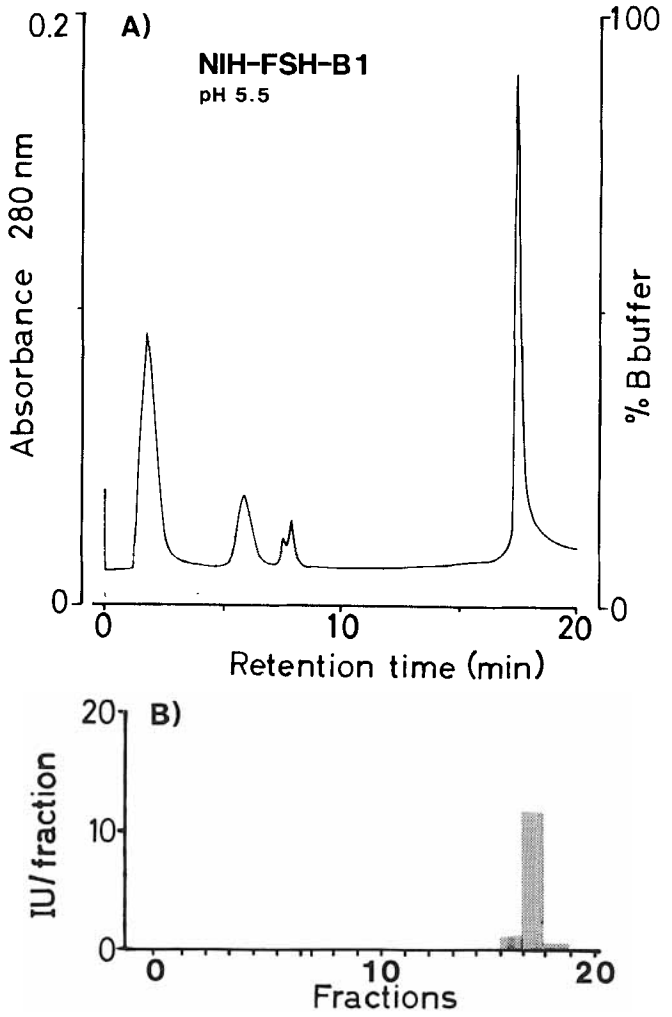

Figure 3.
A) High performance ion exchange chromatography of bovine follitropin (NIH-FSH-B1) on Waters Protein Pak SP 5 PW column (75 x 7.5 mm I.D.) at pH 5.5. Sample size: 400 ug in 1 ml buffer. All other conditions as in Figure 2.
B) FSH bioactivity in eluted material based on estradiol production by the immature rat Sertoli cell culture.

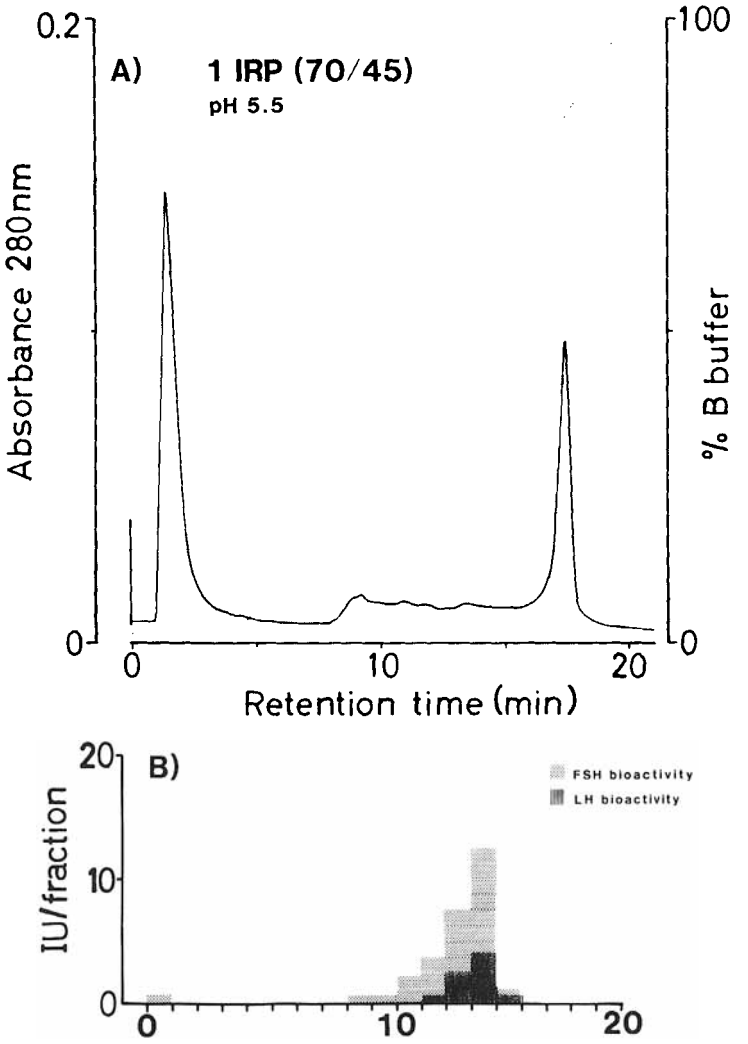

Figure 4.
A) High performance ion exchange chromatography of human urinary gonadotropin (1st IRP 70/45) on Waters Protein Pak SP 5 PW column (75 x 7.5 mm I.D.) at pH 5.5. Sample size: 25 IU of FSH/LH in 1 ml of buffer. All conditions as in Figure 2.
B) FSH bioactivity in eluted material based on estradiol production by the immature rat Sertoli cell culture. LH bioactivity in eluted material based on testosterone production by the mouse Leydig cell suspension.

Only 35% of the original LH bioactivity, while almost all of the FSH bioactivity, was recovered in the eluted fractions.

DISCUSSION

Further purification of standard preparations has been attempted with three main purposes: 1) to remove breakdown products obtained during storage 2) to separate bioactive material from immunoreactive contaminants (such as free subunits) which are devoid of bioactivity, and 3) to separate LH bioactivity from FSH bioactivity in the urinary gonadotropin preparation.

The results from the reverse-phase HPLC application of bFSH and 1st IRP showed resolution of more than one homogenous peak. For bFSH, the biological activity was reduced to only 4 % of native bFSH. Likewise, we were not able to increase this activity following pH adjustment to 4.3 or 5.3. The observation that 1st IRP also lost its biological activity, suggested a denaturation from the low pH or from the relatively high concentrations of the organic solvents. This latter problem could perhaps be resolved if short-alkyl-chain silica are chosen instead of ODS silica.

The ion-exchange HPLC technique according to Kato et al.(7) and described in this report, can be routinely applied without any modification. It has the advantage of using a physiological solvent systems and can provide, as in one of these experiments, a recovery of protein greater than 90% within 10 minutes. This technique also achieved a good separation (see Figure 3a) of biologically active from non-biologically active material.

The use of this procedure resulted in the purification of bFSH which retained more than 92% of its original bioactivity. In the case of the bovine FSH preparation, no LH bioactivity could be recovered in any of the eluted fraction. However, 35% of the total LH bioactivity in the human urinary preparation was eluted in the

same fractions as material having FSH bioactivity. Previous studies with the same standard preparation (10, 11) have indicated the presence of significant amounts of LH (about 30% of the total), which exhibit unusually high acidic pI (pH 4.0 to 5.5) values and remain present in the same fractions as FSH after high resolution isoelectrofocusing procedures. The results of the present study suggest that the LH activity recovered after ion-exchange HPLC most probably represents this acidic LH fraction, as about 60% of the bioactive LH is not eluted by the cation-exchange chromatography. As this acidic LH is a part of circulating and pituitary LH only in postmenopausal women and does not exist in women and men of fertile age (12,13, Ndeto et al., unpublished data), it is reasonable to suggest that this procedure will be useful for normal pituitary and plasma samples.

The pituitary and peripheral gonadotropins exist in multiple molecular forms, which can be separated due to differences in their charge properties by using isoelectrofocusing (see 14 for review). However, the critical studies on the physiological significance of this molecular heterogeneity are hampered by the technical difficulties in the use of isofocusing procedures to fractionate serum samples with low gonadotropin content. The present method provides a very efficient and quick procedure to concentrate and partially purify FSH for such analysis. Whether or not, this method results in a change in the composition of different molecular forms of FSH, should, however, be established before such studies can be started. A similar method using anion-exchange columns, may also be useful for partial purification and concentration of LH from different species.

On the basis of the data presented, it is hoped that the ion-exchange HPLC procedure will become a powerful tool to attain partial purification of gonadotropins without any obvious loss of biological activities. Moreover, this method may be a very useful intermediate step for concentration of plasma and pituitary gonadotropins before they are subjected to high resolution isofocusing procedures to study the structure function relationship in these hormones.

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