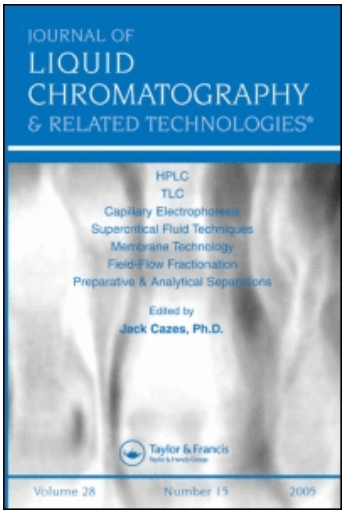

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Biological Characterization of a Pituitary Monkey Lutropin Preparation after Chromatofocusing or after High Performance Anion Exchange Chromatography

Per Hallin[ab]; Shafiq A. Khan[c]

[a] Department of Zoology, Uppsala University, Uppsala, Sweden [b] KabiVitrum AB Peptide Hormones, Stockholm, Sweden [c] Max-Planck Clinical Research Unit for Reproductive Medicine Steinfurter Strasse 107, Münster, Federal Republic of Germany

To cite this Article Hallin, Per and Khan, Shafiq A.(1988) 'Biological Characterization of a Pituitary Monkey Lutropin Preparation after Chromatofocusing or after High Performance Anion Exchange Chromatography', Journal of Liquid Chromatography & Related Technologies, 11: 6, 1261 — 1271

To link to this Article: DOI: 10.1080/01483918808067171

URL: http://dx.doi.org/10.1080/01483918808067171

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(6), 1261-1271 (1988)

BIOLOGICAL CHARACTERIZATION OF A PITUITARY MONKEY LUTROPIN PREPARATION AFTER CHROMATOFOCUSING OR AFTER HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY

Per Hallin* and Shafiq A. Khan

Department of Zoology
Uppsala University
P. O. Box 560
S-751 22 Uppsala, Sweden
and
Max-Planck Clinical Research Unit
for Reproductive Medicine
Steinfurter Strasse 107
D-4400 Münster, Federal Republic of Germany

ABSTRACT

The Pharmacia Fast Protein Liquid Chromatography System equiped with a Mono P HR 5/10 chromatofocusing column was used to characterize the distribution of different bioactive forms of a cynomolgus (*Macaca fascicularis*) pituitary lutropin preparation. The results were compared with the profiles obtained after running the preparation on a Waters DEAE anion exchange column using eluants in the pH range from 6.0 to 8.0. The distribution of preserved biological activity were studied in respective eluted fractions from the different experiments of the applied preparation. Seventy-six percent of the bioactivity was recovered after chromatofocusing whereas preserved bioactivity after ion exchange chromatography ranged between 61-78 % at pH 6.5 to 7.5. No bioactivity was restored after elution at pH 6.0 or 8.0.

Present adress : * KabiVitrum AB Peptide Hormones,
S-112 87 Stockholm Sweden

INTRODUCTION

Many protein and polypeptide hormones display size and charge heterogeneity, occuring as multiple forms with different molecular masses and different pI's (1-6). For the most part, these forms have been detected by fractionation using isoelectrofocusing (IEF) and subsequent quantification by bioassay and radioimmunoassay. A number of studies have been reported for human lutropin (hLH) (1-4) as well as for monkey LH (5-6).

Using specific radioimmunoassay systems, Van Ginkel & Loeber (7) detected nine LHα subunit components and four LHβ subunit components in a highly purified standard hLH preparation. By running another highly purified hLH standard preparation on a Pharmacia anion exchange column (Mono Q), Stockell Hartree et al.(8) reported the elution of this standard into 14 subfractions, all containing different levels of sialic acid and receptor binding activity. However, after SDS gel electrophoresis, these subfractions showed at least four different molecular masses, including the two subunits. Moreover, when a highly purified bovine LH preparation was applied to a high performance liquid chromatography (HPLC) column,the material eluted in almost a single peak at the pH used. When this material was subjected to polyacrylamide gel electrophoresis, nine molecular forms were detected (9).

Based on studies with highly purified human follitropin (hFSH) (10) and monkey FSH (11) it has been suggested, however, that the purification procedures employed by different investigators represent a significant source of variation with regard to the electrofocusing profiles of FSH molecular species.

In this study, the material has been handled, very mildly, and neither denaturation nor precipitation was used to prepare LH from the pituitaries. Moreover, this work was performed to critically assess two different media for further isolation and for the biological characterization of monkey LH. The pituitary preparation was subjected to either chromatofocusing on a Mono P column or HPLC using an anion exchange resin.

METHODS

Pituitary preparation

The pituitary glands from adult female cynomolgus (*Macaca fascicularis*) were removed at autopsy. The anterior lobe was homogenized in 3 x 1 ml ice-cold, 250 mmol/l sucrose solution containing 140 mmol/l saline and then centrifuged for 1 h at 100,000 x g. The supernatant was immediately frozen and stored at -70°C (6). An aliquot, hereafter abbreviated cpf 1, of this supernatant was used.

Standard preparation

The partially purified rhesus pituitary gonadotropin standard LER 1909-2 was provided by NICHD, Bethesda, USA.

Determination of LH bioactivity

An in vitro bioassay method based on the LH-dependent testosterone production by mouse Leydig cell suspensions was utilized (12). The standard and the test materials produced response lines parallel to one another in the assay. The mean index of precision (λ) was found to be 0.04.

Fast protein liquid chromatography - Mono P

The Pharmacia FPLC system (Uppsala, Sweden) was used, which included two P-500 pumps, an LCC-500 liquid chromatography controller, an MV-7 motor valve, a UV-M monitor, a 6 ml-mixer, a pH monitor and a flow-through pH electrode, a FRAC-100 fraction collector and an REC-482 two channel recorder. The samples were run in a HR 5/10 chromatofocusing column equilibrated in a 25 mmol/l triethanolamine - HCl buffer, pH 9.4.

The column was pre-equilibrated in 10 ml of buffer A, and the samples were loaded in 20-200 ul of the same buffer.
The gradient was prepared using Pharmacia Polybuffer 96 - HCl diluted 1/10 with distilled water, adjusted to pH 7.0 and applied to the column, generating a gradient between 9.4 and 7.0. FPLC was carried out with the technical parameters as follows: Mono P HR 5/10 prepacked, buffer A) 25 mmol/l triethanolamine - HCl pH 9.4 buffer B) 10% Polybuffer 96 - HCl pH 7.0, gradient 0 - 100% B during 30 min, detection UV-280 nm, Aufs 0.05, flow rate 0.5 ml/min.

High performance ion exchange chromatography - Waters DEAE

Anion exchange chromatography was carried out according to the method described by Hallin & Madej (9). Briefly, the HPLC solvent system consisted of buffer A) 20 mmol/l Tris - HCl pH 6.0-8.0 and buffer B) 20 mmol/l Tris - HCl containing 500 mmol/l sodium chloride pH 6.0-8.0. Normally we used 10 ul samples diluted in buffer A and loaded into the DEAE column (Waters Protein Pak DEAE 5PW, Waters Associates, Milford MA, USA). The loaded material was mostly eluted with a linear gradient running for 20 min from 2 -100% of B (12.5 mmol/ml) directly after injection at a flow rate of 2.0 ml/min. Detection of the eluant was performed at UV-210 nm, aufs 0.5. All chromatography equipment was from Waters (M-6000 pumps, M-660 solvent programmer, U6K injector) but the samples were monitored with a LDC Spectromonitor III (Laboratory Data Control, Riviera Beach CA, USA). All experiments were carried out at room temperature (22^{o} C). All chemicals were of analytical grade and the water was HPLC grade.

RESULTS

Fractionation of cpf 1 on Mono P

A typical elution profile of the pituitary preparation from the adult female cynomolgus is shown in Fig.1. The protein content

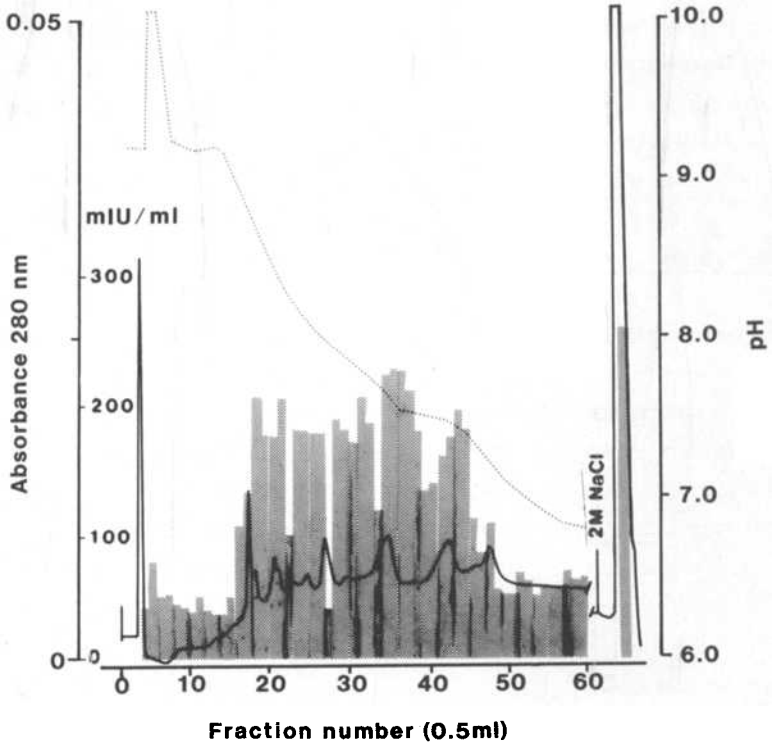

Figure 1.
Elution profile of a monkey pituitary lutropin (LH) preparation on a prepacked Mono P electrofocusing column (HR 5/10) equilibrated in buffer A): 25 mmol/l triethanolamine-HCl pH 9.4. The pH gradient (dotted line) was run for 30 min from 0 % of buffer B to 100 % of buffer B. Buffer B): 10 % Polybuffer 96 -HCl pH 7.0. Flow rate 0.5 ml/min, UV-280 nm, Aufs 0.05. Sample approx. 9.5 IU. LH bioactivity (hatched area) in eluted material based on testosterone production by the mouse Leydig cell suspension.

as well as the LH bioactivity were resolved into multiple components with this procedure.

Fractions were collected every min for 30 min starting immediately after injection. The majority of the applied material (approx. 9.5 IU) was recovered (76 %) during the chromatofocusing while retained bioactivity was eluted after a salt wash with 2 mol/l sodium chloride at the end of the gradient. The highest LH bioac-

tivity in a single fraction was found after this wash. A small part of the material, containing weak bioactivity, was eluted in the void volume, representing pI´s above 9.4. Moreover, LH bioactivity was still retained in the column at pH 7.0, because the pituitary preparation contained materials with acidic pI forms.

Fractionation of cpf 1 on a Waters DEAE ion exchanger

A previous investigation of a cynomolgus LH standard preparation (5), showed that more than 80% of the LH bioactivity is contained within the pH range 7 to 9. Therefore, we started the fractionation at pH 6.5. The fractions were collected every 30 s for 20 min starting immediately after injection (not shown). The bioactivity was distributed over a wide range. As the pH is up to 1 pH unit higher in the microenvironment of an anion exchanger,

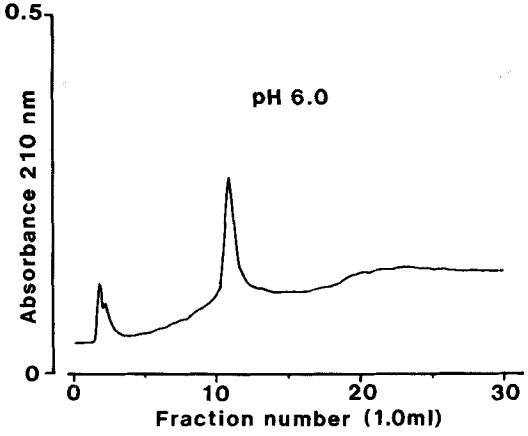

Figure 2.
High performance ion exchange chromatography of a monkey pituitary lutropin (LH) preparation on a prepacked Waters DEAE 5PW column (75 x 7.5 mm) with 20 mmol/l Tris-HCl buffer pH 6.0 as buffer A. Buffer B was identical as buffer A but contained 0.5 mol/l sodium chloride. The salt gradient was run for 20 min from 2 % to 100 % of buffer B. Sample size approximately 9.5 IU in starting buffer. Flow rate 2.0 ml/min, UV-210 nm, Aufs 0.5. No biological activity was found.

than in the surrounding buffer (13) , it was lowered to 6.0 in the second experiment with the anion exchanger. The flow rate was increased to 1.0 ml from 0.5 ml/min. The material from the eluted fractions exhibited no LH bioactivity (see Fig.2).

By increasing the pH of the elution buffer from 6.0 to 7.0,the same amount of material was applied to the column (9.5 IU). All conditions were the same as in the previous experiments. The elution pattern of this third experiment was changed because more protein peaks were observed on the chromatogram (see Fig.3a). Biological activity (78 %) was found in almost all fractions. As shown in Figure 3b, the chromatographic profile at pH 7.5 was different from those of all previous runs. Biological activity (61 %) was, however, observed over a wide range.
Finally, at pH 8.0 (not shown) more material was adsorbed to the column, and the protein peaks on the chromatogram were smaller. No LH bioactivity was detected in the eluted material.

DISCUSSION

As has been reported previously (5), the highly purified cynomolgus LH preparation (i.e. WP-XV-63-2429) exhibited a high degree of charge heterogeneity in terms of bioactivity and immunoreactivity when fractionated by isoelectrofocusing.

The studies presented in this report were performed to assess two chromatographic methods with respect to their ability to isolate different bioforms of a crude monkey pituitary preparation without any prior purification.

Initial efforts to purify this pituitary preparation relied mostly on the anion exchange procedure. Later, the chromatofocusing method developed by Pharmacia was employed as an alternative to ion exchange chromatography. This latter procedure is based on the same principle as isoelectricfocusing, but is much more rapid.

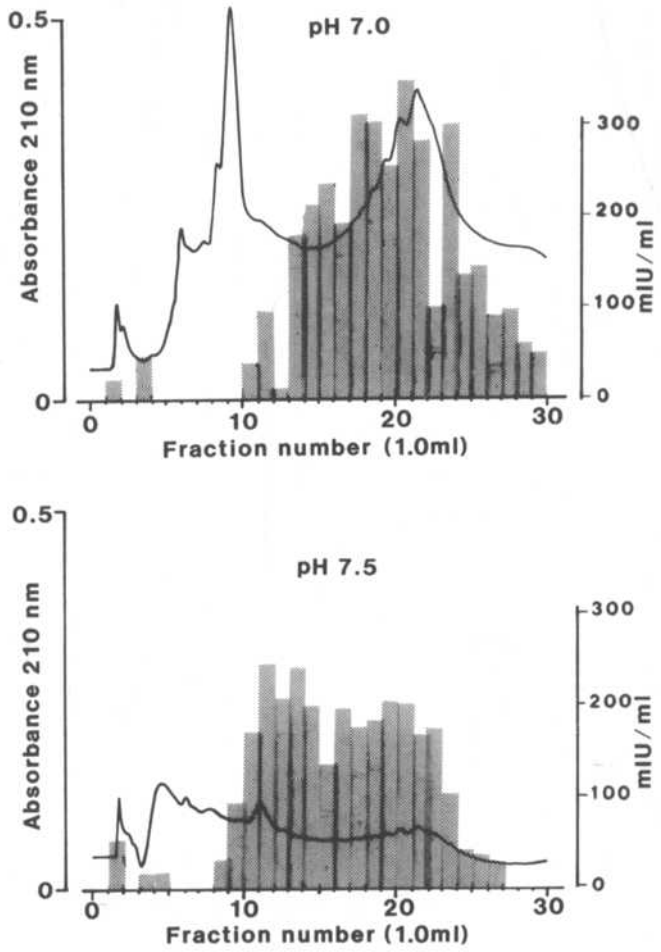

Figure 3. (upper panel)
High performance ion exchange chromatography of a monkey pituitary lutropin (LH) preparation on a prepacked Waters DEAE 5PW column (75 X 7.5 mm) with 20 mmol/l Tris-HCl buffer pH 7.0 as buffer A. Buffer B was identical as buffer A but contained 0.5 mol/l sodium chloride. Otherwise with the same conditions as in Fig.2. LH bioactivity (hatched area) in eluted material based on testosterone production by the mouse Leydig cell suspension.

(lower panel)
Elution with a 20 mmol/l Tris-HCl buffer pH 7.5 as buffer A. Buffer B was identical as buffer A but contained 0.5 mol/l sodium chloride. Otherwise with the same conditions as in Fig.2. LH bioactivity (hatched area) in eluted material based on testosterone production by the mouse Leydig cell suspension.

Since the majority of LH-bioactive species in the cynomolgus standard LH contain bio- and immunoreactivity in the pI range between 7.0 and 9.5 (5), a buffer system in the same pH range was used to elute the different LH forms from the chromatofocusing (tertiary and quarternary amines) resins according to their pI (Technical Booklet Series, Pharmacia, Uppsala, Sweden,1980). The "fingerprint pattern" produced by UV-absorbing material, presented in Fig.1, seems to be associated with the LH-bioactive profile. Note that the molecular species with pI values between 7.5 and 8.5 exhibited highest LH potencies. This confirms the previous finding of Khan et al. (5,6,14) with monkey and baboon LH.

By using the anion exchanger, a strong interaction between the charged biomolecule and the amines may take place until the salt concentration reduce this interaction, and the components elutes from the column. This method is also very effective at separating impurities or degradation products from hormones (15) or to isolate different molecular forms of the radioiodinated oLH subunits (16), since the appropriate buffer composition and buffer pH is established. Our anion exchange results (see Figs.2, 3a-b) indicate that the recovery of LH was maximum when fractionated at pH 7.0, compared to all other pH´s.
It takes also more time to determined the most suitable buffer system for the ion exchange resins compared to chromatofocusing. Moreover, there was no baseline separation, visualized as a sharp "fingerprint pattern", when anion exchange chromatography was used under any of the various pH conditions employed, as was observed with the chromatofocusing method.

Stockell Hartree et al. (8) fractionated various gonadotropins (e.g. hLH, hFSH, hCG) using a Pharmacia Mono Q anion exchanger at pH 7.8 and 9.2. In their study, about 11 % of the applied material was not adsorbed to the column, as compared with ca. 3 % found in our study. The cpf 1 preparation itself also contained much less acidic LH, compared with the hLH in the aforementioned study (8).

As in other heterogeneity studies (5,14) on monkey LH preparations, we found a biological LH distribution similar in pH segments as well as in recovery by using the chromatofocusing technique. Using the high resolution IEF procedure (14) with saline extracts of individual pituitary glands of male and female rhesus monkeys, we also found that the LH molecule could be separated up into nine different molecular species,that the IEF profiles were very similar to those from baboon pituitaries (6) and that no sex-related differences in pI values existed. No differences were found in the chromatograms, of male and female monkey pituitary extract when subjected to chromatofocusing under identical conditions.

Our results further emphasize that it is possible to purify and detect different bioforms of a pituitary preparation using a mild and simple handling procedure before fractionation on a chromatofocusing column.

ACKNOWLEDGEMENTS

We are grateful to the National Institute of Child Health & Human Development, Bethesda, MD , for supplying us with the LER 1909-2 standard. The technical assistance of Ms. H.Westhoven (Münster, F.R.G) is gratefully acknowledged. We also wish to thank H.Lindblom and his staff at the Pharmacia AB, Biotechnology (Uppsala, Sweden) for placing the Departments facilities of our disposal. This work was supported by grants from the Swedish Council for Forestry and Agricultural Research to JE.Kihlström (580/85D 103:4).

REFERENCES

1. Robertson,D.M., van Damme,M-P., Diczfalusy,E. Mol. Cell. Endocrinol. 9, 45-56 (1977)

2. Robertson,D.M., Diczfalusy,E.
Mol. Cell. Endocrinol. 9, 57-67 (1977)

3. van Damme,M-P., Robertson,D.M., Diczfalusy,E.
Mol. Cell. Endocrinol. 9, 69-79 (1977)

4. Storring,P.L., Zaidi,A.A., Mistry,Y.G., Lindberg,M., Stenning, B.E.,Diczfalusy,E.
Acta Endocrinol.(Copenhagen) 101, 339-347 (1982)

5. Khan,S.A., Lindberg,M., Diczfalusy,E.
J. Med. Primatol. 13, 191-204 (1984)

6. Khan,S.A., Katzija,G., Lindberg,M., Diczfalusy,E.
J. Med. Primatol. 14, 143-158 (1985)

7. van Ginkel,L.A, Loeber,J.G.
Acta Endocrinol.(Copenhagen) 114, 572-579 (1987)

8. Stockell Hartree,A., Lester,J.B.,Shownkeen,R.C.
J. Endocrinol. 105, 405-413 (1985)

9. Hallin,P., Madej,A.
J. Liq. Chromatogr. 8, 2663-2670 (1985)

10. Zaidi,A.A., Fröysa,B., Diczfalusy,E.
J. Endocrinol. 92, 195-204 (1982)

11. Khan,S.A., Fröysa,B., Diczfalusy,E.
J. Med. Primatol. 13, 269-282 (1984)

12. van Damme,M-P., Robertson,D.M., Diczfalusy,E.
Acta Endocrinol. (Copenhagen) 77, 655-671 (1974)

13. Scopes,R.K. (Ed.) in: Protein purification: Principles and practice. Springer-Verlag, New York p.84 (1986)

14. Khan,S.A., Syed,V., Fröysa,B., Lindberg,M., Diczfalusy,E.
J. Med. Primatol. 14, 177-194 (1985)

15. Hallin,P., Khan,S.A.
J. Liq. Chromatogr. 9, 2855-2868 (1986)

16. Hallin,P., Madej,M., Madej,A.
J. Liq. Chromatogr. 10, 2965-2976 (1987)