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Isolation of Human Lutropin from Woman Urine and Comparison of Its Properties with Pituitary Hormone

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Abstract. Lutropin was isolated from woman preovulatory urine by immunoaffinity chromatography using a column with monoclonal antibody (mAb) anti-βhLH(37) coupled to Sepharose CL 4B. As it was demonstrated the isolated hormone, as well as pituitary lutropin, were separated in SDS-PAGE into several well visible fractions with 30–94 kDa molecular mass and scarcely visible fractions with 14–20 kDa. All fractions reacted only with mAb anti-αhCG(99)-HRP but not with mAb anti-βhLH-HRP. Pretreatment of pituitary lutropin with PN-Gase F did not affect its electrophoretic pattern. After boiling the hormone with SDS and βME no fractions in SDS-PAGE were observed. No substantial differences in affinity chromatography on Con A-Sepharose between pituitary and urinary lutropin were noted. Some differences between these two hormone preparations were observed in assays performed with several ELISA variants. Using two pairs of mAbs anti-βhLH for ELISA technique no hormone was assayed in urine samples from women, collected between 12 and 16 days of menstrual cycles.

Key words: human lutropin; human luteinizing hormone; urine lutropin; pituitary lutropin; affinity chromatography; SDS-PAGE; comparative assays.

Introduction

Human lutropin (human luteinizing hormone – hLH) is a heterodimeric glycoprotein synthesized in anterior pituitary cells[1, 19]. Its secretion is controlled by luliberin from hypothalamus[11, 22]. The smaller subunit αhLH is N-glycosylated at two sites: Asn-52 and Asn-78, however, the bigger β subunit contains only one glycosylated site at Asn-30[11, 19]. These N-glycans are bi- and triantenuary and contain: N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, fucose, sialic acid and sulphate residues[5]. The pituitary hLH consists of multiple isoforms in terms of carbohydrate content and molecular size[21, 23]. These structural differences are also reflected in variations in biological and immunological activities[9, 21]. Several genetic variants of lutropin with one or two amino acid residues replaced by other residues in β subunit are associated with some pathological abnormalities[4, 16, 26].

Lutropin after binding to LH/CG receptor in ovary, trophoblast or testis stimulates synthesis of estrogens, progesterone or androgens[1]. The hormone is excreted

Abbreviations used: αMM – α methyl-D-mannoside, βME – β-mercaptoethanol, BSA – bovine serum albumin, Con A – concanavalin A, ELISA – enzyme linked immunosorbent assay, HSA – human serum albumin, hLH – human luteinizing hormone (lutropin), HRP – horse radish peroxidase, mAb – monoclonal antibody, PAGE – polyacrylamide gel electrophoresis, PN-Gase F – peptide N-glycosidase F, SDS – sodium dodecyl sulfate, TBS – 0.05 M Tris/HCl pH 7.5 in 0.15 M NaCl.

to urine expecially plentiful just before ovulation. Identification of lutropin peak in woman urine is a good method of monitoring fertile days[10].

ELISA with two monoclonal antibodies, to detect hLH, was elaborated in our laboratory[24] and the technique was applied for assay of lutropin in urine and for investigations of the hormone[13, 14].

In this paper we describe isolation of hLH from preovulatory urine by immunoaffinity chromatography. Some comparative properties of the hormone derived from urine and pituitary are also presented.

Materials and Methods

Chemicals and biopreparations. Acrylamide, bisacrylamide and 3,3'-diaminobenzidine tetrachloride were from Fluka. Cyanogen bromide and tris(hydroxymethyl) aminomethane (Tris) were purchased from BDH, α-methyl-D-mannoside (αMM), N,N,N',N'-tetramethyl-ethylenediamine, sodium dodecyl sulfate (SDS) and imidazole were from Sigma. Nitrocellulose sheets (0.45 μm) were from Schleicher and Schuell. To concentrate hLH Centricon 10 from Amicon was used. Other chemicals were products of POCh, Gliwice.

Human luteinizing hormone, isolated from pituitary gland, was purchased from Calbiochem (USA). Monoclonal antibodies anti-αhCG(99) and mAbs anti-βhLH as well as their horse radish peroxidase (HRP) conjugates were obtained as described earlier[13, 14, 20]. Peptide-N-glycosidase F (PN-Gase F) from *Flavobacterium meningosepticum* was from Boehringer, Mannheim (Germany).

Dot-spot of hLH. Solution of pituitary lutropin was spotted (0.6–20 ng/2 μl) on nitrocellulose sheet and dried at room temperature. The sheet was blocked overnight with 2% casein in TBS, and after washing with TBS, it was incubated with mAb conjugated to HRP (5 μg/ml) for 1 h. Finally, the sheet was washed and incubated with 0.2% 3,3'-diaminobenzidine tetrachloride and 0.03% hydrogen peroxide with 0.01 M imidazole in 0.05 M Tris/HCl, pH 7.6 for 20 min.

Polyacrylamide gel electrophoresis (PAGE). It was performed in slabs using 12% acrylamide and 1% SDS according to LAEMMLI[15]. For electrotransfer of proteins onto nitrocellulose sheet, 200 mA current for 1.5 h was applied. Further procedure for localization of hormone fractions (Western blot) was as described above for "Dot-spot of hLH" method.

Assay of hLH. The hLH was assayed ussually by standard ELISA technique using mAb anti-αhCG(99) and mAb anti-βhLH(104)-HRP as described earlier[14].

Immunosorbents. No. 1 – 2.5 ml Sepharose 4B, freshly activated with cyanogen bromide[17], after washing with water was shaked overnight at 5°C in 2.5 ml 0.1 M carbonate buffer pH 9.7 with 0.5 M NaCl and 1 mg mAb anti-αhCG(99). Then the gel was incubated with 5 ml 0.5 M Tris/HCl pH 8.5 at room temperature for 4 h. Finally, the gel was washed with 0.1 M glycine/HCl pH 2.5 and then with PBS.

No. 2 – 2 ml Sepharose CL 4B freshly activated with cyanogen bromide[17] washed with water was suspended in 2.5 ml 0.1 M carbonate buffer pH 9.5 with 6.5 mg mAb anti-βhLH(37) and shaken overnight at 5°C. Then the gel was shaken with 25 ml 1M ethanolamine at room temperature for 3 h, and finally washed with PBS.

Affinity chromatography. The chromatography of lutropin on Con A-Sepharose was performed according to general procedure of PAPANDREOU et al.[18], as described earlier[13], using for elution buffer A containing: 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ in 10 mM Tris/HCl pH 8.0 with 0.15 M NaCl and 0.1% BSA.

Digestion with PN-Gase F. To 6 μg (0.26 nmole) of pituitary hLH in 56 μl 0.1 M phosphate buffer, pH 7.4 with 25 mM EDTA (disodium salt) and 0.05% HSA, 4 μl PN-Gase F (0.8 unit) were added. The solution was incubated overnight at 37°C. For SDS-PAGE 9 and 3 μl of the incubation solution were used and for ELISA the solution was diluted 1000-times with 0.2% casein in PBS.

Results

Assays of urinary and pituitary hLH with ELISA method

Four ELISA variants were applied in the assay of pituitary hLH[13] and the hormone in preovulatory urine. Results are presented in Table 1. The statistical differences between the both hormones were noted in ELISA variants 2 and 3.

Table 1. Comparison of hLH determinations for pituitary and preovulatory urine derived hormone using ELISA variants

No.	mAb anti-used for HRP		Relative absorbances ($\bar{A} \pm SD$)	
	coating	conjugate	pituitary[a]	urine[b]
1	-αhCG(99)	-βhLH(104)	100 ± 3.4	100 ± 4.8
2	-αhCG(99)	-βhLH(37)	85 ± 5.6	109 ± 5.7
3	-αhCG(99)	-βhLH(15)	30 ± 9.1	40 ± 7.4
4	-β-hCG(104)	-βhLH(37)	36 ± 7.2	30 ± 8.3

SD was calculated from 6 simultaneous assays.
[a] Pituitary hLH (100 mU/ml).
[b] Preovulatory urine hLH (96 mU/ml).

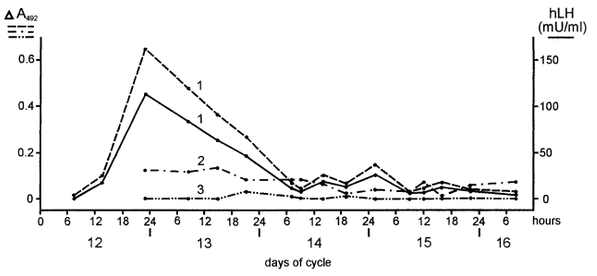

Fig. 1. The use of mAbs anti-βhLH for assay of the hormone in urine portions of one woman. The following mAbs were used for ELISA: 1 – mAb anti-αhCG(99) and mAb anti-βhLH(104)-HRP (variant 1 ELISA); 2 – mAb anti-βhLH(104) and mAb anti-βhLH(37)-HRP; 3 – mAb anti-βhLH(107) and mAb anti-βhLH(15)-HRP

To examine the utility of four mAbs anti-βhLH in the assay of hLH core fragment, they were applied for ELISA for each portion of urine collected from two healthy women between 12 and 16 days of their menstrual cycles. For comparison hLH was also assayed in urine portions with standard ELISA technique (variant 1) described earlier[14]. Apart from distinct hLH peak, obtained by standard ELISA, no other peak was noted when 2 pairs of mAbs anti-βhLH were used for assay. Figure 1 presents determination of hLH in urine samples collected from one woman.

Immunoaffinity chromatography

Lutropin from preovulatory urine was retained on immunosorbent No. 1 columns but elution either with 0.1 M glycine/HCL pH 2.5 or with 3 M potassium thiocyanate was not possible. Therefore, isolation of hLH from urine was performed on Sepharose – mAb anti-βhLH(37) (immunosorbent No. 2) column. Five hundred millilitre of preovulatory urine (105 mU hLH/ml) was filtered through two small (1 ml) immunosorbent columns and then the columns were washed with PBS. As it was assayed in eluted fractions, 90% of the total hormone was retarden on the columns. Lutropin was eluted almost completely using 3 M potassium thiocyanate with human serum albumin (HSA) (20 μg/ml). In combined fractions with the hormone, after dialysis to deionized water 70% of hLH were assayed. Finally, the obtained solution was concentrated using Centricon 10 yielding 1.02 μg hLH.

Dot-spot of pituitary hLH

All spots (0.6–20 ng hLH/2 μl) on nitrocellulose sheet were visualized after treatment with mAb anti-αhCG(99)-HRP and then with 3,3'-diaminobenzidine tetrachloride solution, as described in Materials and Methods. No spot was visible when mAb anti-βhLH(104 or 37)-HRP was applied. This indicated that only mAb anti-αhCG(99)-HRP reacts with hLH spotted on nitrocellulose and that this conjugate can be used for Western blot.

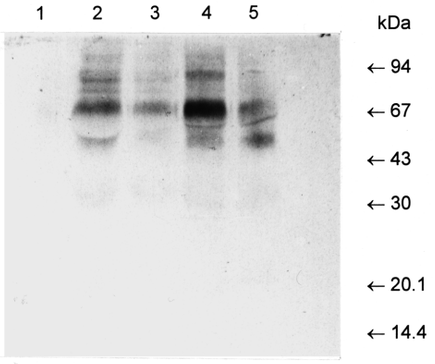

Fig. 2. Western blot of urine and pituitary hLH preparations. The following samples were subjected to SDS-PAGE: 1 – 40 μg HSA; 2 – 0.8 μg hLH isolated from urine; 3 – 0.2 μg hLH isolated from urine; 4 – 1 μg pituitary hLH with 2 μg HSA; 5 – 0.3 μg pituitary hLH with 2 μg HSA. For visualization mAb anti-αhCG(99)-HRP was used

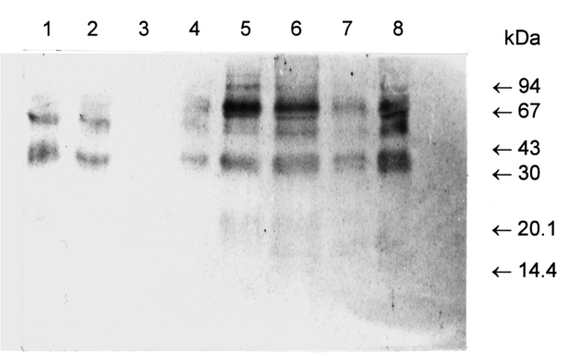

Fig. 3. Western blot of pituitary hLH. Samples subjected to SDS-PAGE were: 1 – 1 μg hLH with 0.4 mg SDS boiled for 5 min; 2 – 1 μg hLH with 0.4 mg SDS and 0.4 mg βME; 3 – as 2, boiled for 5 min; 4 – 0.3 μg hLH; 5 – 0.9 μg hLH; 6 – 0.9 μg hLH; 7 – 0.3 μg hLH incubated with PN-Gase F; 8 – 0.9 μg hLH incubated with PN-Gase F

Western blot

Western blot of pituitary hLH and the hormone isolated from woman urine is presented in Fig. 2. Well visible are 29, 49, 67 and 95 kDa fractions, lower molecular mass fractions are feeble. There are some small differences between patterns of both hormones. In urinary hLH fraction ca 80 kDa and in pituitary hormone

fraction ca 60 kDa were visible as the strongest bands. The hormone fractions were localized only with mAb anti-αhCG(99)-HRP. No fractions were detected with mAb anti-βhLH(104 or 37) conjugated with HRP. Prolonged incubation of lutropin with PN-Gase F had no affect on fraction pattern (Fig. 3). When the hormone sample was boiled with SDS for 5 min. some fractions disappeared, but boiling with SDS and βME destroyed all hormone fractions (Fig. 3). Similar affects of PN-Gase F, SDS and SDS with βME on lutropin were noted for ELISA technique.

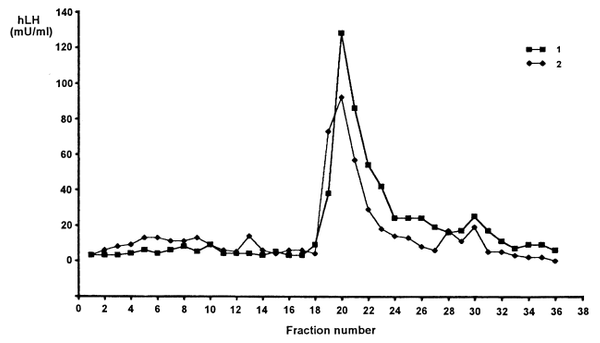

Fig. 4. Chromatography of hLH on Con A-Sepharose. 1 – pituitary lutropin, 2 – lutropin from urine, fractions No. 18–29 were eluted with 10 mM αMM in buffer A, fractions No. 30–36 were eluted with 300 mM αMM in buffer A

Affinity chromatography on Con A-Sepharose

Separations of pituitary hLH and the hormone isolated from preovulatory urine on Con A-Sepharose are presented in Fig. 4. No significant differences were noted between the both hormones, eluted with 10 mM and 300 mM αMM in buffer A.

Discussion

Statistical differences in assays of pituitary and urinary hLH with two ELISA variants (No. 2 and 3; $p \leq 0.05$), presented in Table 1, suggest some immunological as well as structural differences between these two hormones. As it was noted by KOVALEVSKAYA et al.[12] a big peak of βhLH core fragment is excreted one day after hLH peak in woman urine. Results presented in Fig. 1 suggest that four mAbs anti-βhLH, used in our laboratory, are useless to determine of the hLH core fragment.

One can find in literature some short informations on immunoaffinity chromatography of hLH[2, 8, 25], however, shortage of details makes it difficult to apply this method for isolation of hLH from urine. In our experiments immunosorbent No. 1 was useless because we could not elute the hormone bound to sorbent. Useful was immunosorbent No. 2 – Sepharose – mAb anti-βhLH (37) was better and for elution of hLH the best was 3 M potassium thiocyanate. The addition of HSA to the eluting solvent prevents the hormone against inactivating dissociation, as it was demonstrated earlier[14]. Surprising was that hLH adsorbed on nitrocellulose sheets (Dot-spot, Western blot) could be detect only with HRP conjugate with mAb anti-αhCG(99) but not with that of mAb anti-βhLH(104 or 37). On the surface of lutropin molecule many epitopes were identified[2]. It is possible that after adsorption of hLH on cellulose sheet epitopes to mAb(104) and mAb(37) were inaccessible. Other interesting observation is that hLH, run in SDS-PAGE, was separated for several fractions with molecular weight >23 kDa. FURUI et al.[4] observed in Western blot, after SDS-PAGE of pituitary hLH, two fractions of 22 and 28 kDa molecular mass; in a blot of concentrated urine from women after menopause fractions with 30, 35 and 43 kDa were noted by ILES et al.[7] Also agregates were identified. In a blot, obtained after SDS-PAGE of hLH subunits[3], also aggregates were identified. The increase of molecular weight of hLH fractions observed in presented here Western blot can be explained either by higher content of carbohydrates in lutropin molecule (21.4% according to HARA et al.[6]) or by aggregation of the hormone molecules. To verify the first supposition we incubated hLH with PN-Gase F overnight, but no effect on fractions in Western blot was noted. Boiling of hLH with SDS had some effect on number of fractions in Western blot, but boiling with SDS and βME destroyed lutropin completely. Results presented here and results obtained earlier[13, 14] suggest existence of some differences between pituitary and urinary lutropin isohormones.

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References

1. BISHOP W. H., NUREDDIN A. and RYAN R. I. (1976): Pituitary luteinizing and follicle stimulating hormones. In: PARSONS J. A. (ed.): Peptide hormones. The Macmilan Press Ltd., London, 273–298.
2. COSTAGLIOLA S., NICCOLI P., FLORENTINO M. and CARAYON P. (1994): European collaborative study of luteinizing hormone assay: 1. Epitope specificity of luteinizing hormone monoclonal antibodies and surface mapping of pituitary and urinary luteinizing hormone. J. Endocrinol. Invest., **17**, 397–406.

3. FURUHASHI M., SUZUKI S. and SUGANUMA N. (1996): Disulfide bonds 7–13 and 59–97 of the α-subunit play of different role in assembly of human chorionic gonadotropin and lutropin. Endocrinology, **137**, 4196–4200.
4. FURUI K., SUGANUMA N., TSUKAHARA S. I., ASADA Y., KIKKAWA F., TANAKA M., OZAWA T. and TOMODA Y. (1994): Identification of two point mutations in the gene coding luteinizing hormone (LH) β-subunit, associated with immunologically anomalous LH variants. J. Clin. Endocrinol. Metab., **78**, 107–113.
5. GREEN E. D. and BAENZINGER J. U. (1988): Asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. J. Biol. Chem., **263**, 25–35.
6. HARA K., RATHNAM P. and SAXENA B. B. (1978): Structure of the carbohydrate moieties of α subunits of human follitropin, lutropin and thyrotropin. J. Biol. Chem., **253**, 1582–1591.
7. ILES R. K., LEE C. L., HOWES I., DAVIES S., EDWARDS R. and CHARD T. (1992): Immunoreactive β-core like material in normal post – menopausal urine: human chorionic gonadotrophin or LH origin? Evidence for the existence of LH core. J. Endocrinol., **133**, 459–466.
8. JACK G. W., BLAZEK R., JAMES K., BOYD J. E. and MICKLEM L. R. (1987): The automated production of immunoaffinity chromatography of the human pituitary glycoprotein hormones: thyreotropin, follitropin and lutropin. J. Chem. Technol. Biotechnol., **39**, 45–55.
9. KEEL B. A. and GROTJAN H. E. JR. (1989): Luteinizing hormone microheterogeneity. In KEEL B. A. and GROTJAN H. E. JR. (eds.): Microhetrogeneity of the glycoprotein hormones. CRC Press Inc., Boca Raton, 149–184.
10. KESNER J. S., WRIGHT D. M., SCHRADER S. M., CHIN N. W. and KRIEG E. F. JR. (1992): Methods of monitoring menstrual function in field studies. Efficacy of methods. Reprod. Toxicol., **6**, 385–400.
11. KÖNIG W. (1993): Gonadoliberin. thyroliberin, gonadotropins, thyrotropin. In KÖNIG W. (ed.): Peptide and protein hormones. VCH Verlagsgesellschaft GmbH, Weinheim–New York–Basel––Cambridge, 11–36.
12. KOVALEVSKAYA G., BIRKEN S., O'CONNOR J., SCHLATTERER J., MAYDELMAN Y. and CANFIELD R. (1995): HLH beta core fragment imunoreactivity in the urine of ovulating women: a sensitive and specific immunometric assay for its detection. Endocrine, **3**, 881–887.
13. KUROWSKA E., KOCHANOWSKA I. E., BORATYŃSKI J. and SZEWCZUK A. (1997): Investigations on human lutropin from pituitary gland and urine. Arch. Immunol. Ther. Exp., **45**, 329–334.
14. KUROWSKA E., KOCHANOWSKA I. E., KUROPATWA M., BORATYŃSKI J. and SZEWCZUK A. (1995): Stability of human lutropin studied with immunoenzymatic technique. Arch. Immunol. Ther. Exp., **43**, 181–185.
15. LAEMMLI U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685.
16. LIAO W. X., ROY A. C., CHAN C., ARULKUMARAN S. and RATMAN S. S. (1998): A new molecular variant of luteinizing hormone associated with female infertility. Fertil. Steril., **69**, 102–106.
17. MARCH S. G., PARIKH J. and CUATRECASAS P. (1974): A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem., **60**, 149–152.
18. PAPANDREOU M. J., ASTERIA C., PETTERSSON K., ROLIN C. and BECK-PECOZ P. (1993): Concanavalin A affinity chromatography of human serum gonadotropins evidence for changes of structure in different clinical conditions. J. Clin. Endocrinol. Metab., **76**, 1008–1013.
19. PIERCE I. C. and PARSONS T. F. (1981): Glycoprotein hormones structure and function. Ann. Rev. Biochem., **50**, 465–495.
20. RAPAK A. and SZEWCZUK A. (1993): Semiquantitative assay of human chorionic gonadotropin by a simple and fast immunofiltration technique. J. Clin. Chem. Clin. Biochem., **31**, 153–157.
21. STANTON P. G., POZVEK G., BURGON P. G., ROBERTSON D. M. and HEARN M. T. W. (1993): Isolation and characterization of human LH isoforms. J. Endocrinol., **138**, 529–543.
22. SZEWCZUK A., KOCHANOWSKA I. E. and KUROWSKA E. (1994): Ludzki hormon luteinizujący (hLH). Post. Hig. Med. Dośw., **48**, 491–504.
23. SZEWCZUK A., KOCHANOWSKA I. E. and KUROWSKA E. (1996): Izoformy hormonu luteinizującego. Post. Hig. Med. Dośw., **50**, 9–20.
24. SZEWCZUK A., KUROPATWA M., KOCHANOWSKA I. E., MAJEWSKA A., MAĆKIEWICZ Z., KUPRYSZEWSKI G. and RAPAK A. (1994): Search of antibodies for immunoenzymatic assay of human lutropin. Arch. Immunol. Ther. Exp., **42**, 453–458.
25. WEISE H. C., GRAESSLIN D., LICHTENBERG V. and RINNE G. (1983): Polymorphism of human pituitary lutropin (LH). Isolation and partial characterisation of seven isohormones. FEBS Lett., **159**, 93–96.
26. WEISS J., AXELROD L., WHITCOMB R. W., HARRIS P. E., CROWLEY W. F. and JAMESON J. L. (1992): Hypogonadism caused by a single amino acid substitution in the beta subunit of luteinizing hormone. N. Engl. J. Med., **326**, 179–183.

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