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STABILIZING THE GONADOTROPIN ACTIVITY WITH THE USE OF ORGANIC COMPOUNDS

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ABSTRACT

Complex studies of optimum quantitative and qualitative composition of carbohydrates and amino acids necessary for gonadotropines activity stabilization were the aim of our investigations. This was determined by biological active substances ─ amino acids: L- lysine , L- glycine and carbohydrates: sucrose and mannitol, which stabilize gonadotropins activity for longer use while preserving activity. As a result of the studies we found out that the addition 10 mg/sm^3 of L-lysine and 75 mg/sm^3 of sucrose for extended incubation provides the highest gonadotropin activity compared with other experimental and control batches of samples. In the experimental series of samples, where the stabilizer was added at a rate of 10 mg/sm^3 L-lysine and 75 mg/sm^3 mannitol, the activity was stable during 6 weeks of incubation and constituted more than 50% of the initial theoretical activity of hCG.

Keywords: Chorionic Gonadotropin, Stabilization, L-lysine, L-glycine, Immunochemiluminescent assay

INTRODUCTION

Human chorionic gonadotropin (hCG) is a placental glycoprotein hormone that acts through binding to a G-protein-coupled receptor, leading to increased adenylatecyclase activity **(Lapthorn *et al.*, 1994, Erbel, 2002)**. The increase in cAMP level stimulates the *corpus luteum* to produce progesterone until the placenta itself acquires the ability to produce this pregnancy-sustaining steroid. After dissociation of hCG into its subunits, fully bioactive hormone can be regained by the recombination of subunits **(Lapthorn *et al.*, 1994, Erbel, 1999)**. Native hCG has four N-linked carbohydrate chains, two in the a-subunit at Asn-52 and Asn-78 (ahCG[glycan52,78]) and two in the b-subunit at Asn-13 and Asn-30 (bhCG). The mono- and di-antennary glycan structures are mainly sialylated, representing 10% of the total weight of hCG **(Lapthorn *et al.*, 1994, Erbel, 1999)**.
Three-dimensional structure of human chorionic gonadotropin (hCG) shows that each of its two different subunits has a similar topology, with three disulphide bonds forming a cystine knot. This same folding motif is found in some protein growth factors. The heterodimer is stabilized by a segment of the β-subunit which wraps around the α-subunit and is covalently linked like a seat belt by the disulphide Cys 26–Cys 110. This extraordinary feature appears to be essential not only for the association of these heterodimers, but also for the receptor binding by glyco-protein hormones **(Lapthorn *et al.*, 1994).**
Lapthorn et al. (1994) described the crystal structure of hCG (Fig. 1). In Fig. 1 black lines represent the β-subunit of hCG and dark gray lines – the α-subunit of hCG. Light gray shaded areas show pertinent epitopes **(Lapthorn *et al.*, 1994).**
During the recent years, the structure of gonadotropic hormones has been decoded for different species of fishes, animals and human **(Wide and Wide, 1984, Fiddes and Goodman, 1981, Fiddes, 1984, Baenziger *et al.*, 1992, Moyle *et al*, 1994, Zambrano *et al.*, 1995, Howles, 1996, de Leeuw *et al.*, 1996, Nozaki *et al.*, 2013).** The molecules of hCG and of chorionic gonadotropin (CG) of various animal species being considerably homologous are not identical. The α-hCG subunit is identical to α-subunit of luteinizing hormone (LH), follicle-stimulating hormone (FSH), as well as thyroid-stimulating hormone (TSH), and consists of 92 amino acid residues. α-CG contains two oligosaccharide chains attached to the polypeptide chain by a N-glycosidic bond between N-acetylglucosamine and an amid group of two asparagine residues. The β-hCG subunit, the polypeptide chain consisting of 145 amino acid residues, is specific of this hormone, but displays a high level of structural homology of about 80% with the β-subunit of luteinizing hormone, differing from the latter by its C-terminal section prolonged by 24 amino acid residues. β-CG contains 6 oligosaccharide chains, 2 of which are joined by an N-glycosidic bond at asparagine residues, while 4 are linked by an O-glycosidic bond between N-acetylgalactosamine residues and the OH group of serine residues of the C-terminal section of the polypeptide chain.

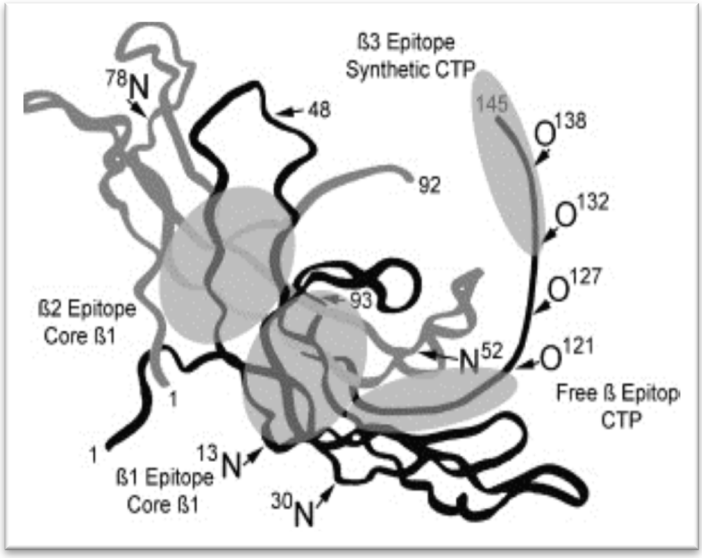

Figure 1 Crystal structure of hCG (according to *Lapthorn A., Harris D., Littlejohn A., Lustbader J., Canfield R., Machin, Morgan, F., Isaacs N., 1994. Crystal structure of human chorionic gonadotropin. Nature 369 (6480), 455-461).*

Specific biological properties of the CG are determined by β-subunit. Structural affinity between β-subunit of the CG and that of the LH is manifested by the similarity of their biological and immunological properties **(Dimitrov, 1979, Pierce, 1981, Fiddes, 1984, Lempiäinen *et al.*, 2012).**
The carbohydrate part, characterized by a considerable heterogeneity, constitutes about 30% of the CG molecular weight. It includes sialic acid, L-fructose, D-galactose, D-mannose, N-acetylglucosamine and N-acetylgalactosamine. Carbohydrate components of the CG are needed for binding subunits, maintaining the conformation of the molecule and protecting polypeptide chains of subunits from degradation under the action of proteolytic enzymes. Elimination of carbohydrate residues causes a considerable decrease of half-life

of the CG in the organism **(Chemistry Encyclopedia 1998)**. The CG molecule is relatively easily dissociated into subunits, for instance, under the effect of urea or propionic acid. Isolated subunits are deprived of biological activity, but can recombine producing a biologically active CG molecule **(Waddell, 2006).**

It has been suggested that CG and its β-subunit are produced not only in the chorion of trophoblast and placenta, but also in the tissues of the fetus (and even to a greater degree than in the placenta), in many tissues of both sexes, i.e., during the entire ontogenesis of mammals; it can also be produced by some tumors related by their origin to placenta trophoblast cells. The synthesis of α- and β-subunits occurs independently. Both dimeric (intact) hormone molecules and free (unbound) CG subunits are released into the blood **(Cole, 2009)**.

High levels of chorionic hormone are synthesized by placenta and extracted with urea, from which it can be isolated and purified. Particularly, purified gonadotropins are obtained by means of lyophilisation and preserved dry. Lyophilized preparations are stable enough at conservation, however, lyophilisation is an expensive and labor-consuming stage in the process of obtaining gonadotropins, while their solutions are unstable, which represents a disadvantage in their use. According to the performed studies, both inorganic ($ZnCl_2$ or $AlCl_3$) and organic (methionine, glycine, mannitol, arginine, lactose, sucrose) compounds are used for stabilizing gonadotropic preparations **(Cameo *et al.*, 2004, Maiti *et al.*, 2011).** Research in this direction may result in considerable advantages consisting in the decrease of costs of preparation production and ensuring the sufficient stability of liquid forms of gonadotropic preparations with their prolonged activity **(American Society for Reproductive Medicine, 2008, Cameo *et al.*, 2004, Maiti *et al.*, 2011)**. Therefore, the aim of our study was a comprehensive search for an optimum quantitative and qualitative composition of organic compounds needed for the stabilization of the hCG.

MATERIAL AND METHODS

According to the general pattern of investigation represented in Tables 1 and 2, two experiments were conducted.

Table 1 Pattern of studies of the dynamics of gonadotropins activity with addition of amino acids to the solvent

Group	Characteristic of groups	Manipulations
Control A	Gonadotropin solved in phosphate buffer (pH 7.34)	Solved preparation of gonadotropin was stored at 40°C, and the activity of hormone was determined every two weeks for two months by immuno-electrochemiluminisc ent methods
Experimental B	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L-lysine10 mg/sm^3+75 mg/sm^3 of saccharose	
Experimental C	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L- lysine10 mg/sm^3+50 mg/sm^3 of saccharose	
Experimental D	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L- lysine10 mg/sm^3+75 mg/sm^3 of mannite	
Experimental E	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L- lysine10 mg/sm^3+50 mg/sm^3 of mannite	

In the first experiment we used L- lysine + saccharose or mannite as primary stabilizer.

Table 2 Pattern of studies of the dynamics of gonadotropins activity with addition of amino acids to the solvent

Group	Characteristic of groups	Manipulations
Control A	Gonadotropin solved in phosphate buffer (pH 7.34)	Solved preparation of gonadotropin was stored at 40°C, and the activity of hormone was determined every two weeks for two months by immuno-electrochemiluminisc ent methods
Experimental B	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L-glycine 0,25 mg/sm^3+75 mg/sm^3 of saccharose	
Experimental C	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L- glycine 0,25 mg/sm^3+50 mg/sm^3 of saccharose	
Experimental D	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L- glycine 0,25 mg/sm^3+75 mg/sm^3 of mannite	
Experimental E	Gonadotropin solved in phosphate buffer (pH 7.34) with adding of L- glycine 0,25 mg/sm^3+50 mg/sm^3 of mannite	

In the second experiment we used L- glycine + saccharose or mannite as primary stabilizer.

The experiments differed between themselves by the amino acids added to the solvent (L-lysine or L-glycine + saharose or mannite) and their concentrations. Each experiment consisted of five series of samples (control A, experimental B, C, D and E (Table 1, 2). The human chorionic gonadotropin (hCG) was obtained in the Institute of Animal Biology, National Academy of Agrarian Science of Ukraine (Director Prof. Vasyl Vlizlo) from the urine of pregnant women (12-16 weeks of gestation) by means of filtering and precipitation by alcohol, acetone and ammonium acetate. The concentration of intact gonadotropin was established by means of electro- and immuno-chemiluminescent method based on difference between total and free hCG (Frimell 1987). The initial concentration of total hCG was 32717.3 mIU/ml (international units), while the concentration of free hCG was 217.3 mIU/ml. Theoretically, the activity of hCG was 32500 mIU/ml (Table 1, 2).

The obtained gonadotropin was solved in phosphate buffer (pH 7.34) and aliquoted by 2500 mIU/ml. L-lysine, L-glycine and L-methionine were added to the aliquoted samples, in the amount of 10 and 0.2 mg/cm^3, according to the pattern of analysis, and the volume was brought to 1 cm^3 with phosphate buffer with pH 7.34. The samples were placed into a thermostat for incubation at the temperature of 40°C. After each 2 weeks during two months, the concentrations of total (hCG+β-hCG) and free (β-hCG) gonadotropin were measured. The hCG concentration was determined by the difference between (hCG+β-hCG) and (β-hCG) (**McPherson and Pincus, 2006**).

RESULTS AND DISCUSSION

The dynamics of gonadotropin activity with addition of L-lysine to the solvent is presented in table 3 and figure 2. Differences in the activity of chorionic hormone preserved in a thermostat at the temperature of 40° C with added L- lysine were studied in all groups (table 3).

Table 3 Dynamics of gonadotropin activity with addition of L-lysine to the solvent

Series of samples	Filler	Quantity of filler	Theoretical activity	Dynamics of gonadotropin activity				
				6 hours % compared to initial theoretical activity	2 weeks % compared to initial theoretical activity	4 weeks % compared to initial theoretical activity	6 weeks % compared to initial theoretical activity	8 weeks % compared to initial theoretical activity
A	-		2500	65.8%	31.4%	18.9%	15.0%	14.1%
B	L-lysine +saharosse	10 + 75	2500	86.9%	78.7%	51.2%	50.4%	39.1%
C	L-lysine+saharosse	10+50	2500	81.6%	71.5%	45.9%	36.2%	24.2%
D	L-lysine+mannite	10+75	2500	64.8%	55.2%	54.3%	52.4%	32.7%
E	L-lysine+mannite	10+50	2500	74.9%	54.2%	42.1%	39.2%	27.4%

After 12 hours, changes in hormone activity in all series as compared to the initial theoretical activity and between experimental groups were demonstrated. After 2 weeks of conservation the concentration of intact hCG decreased by 68.6% in the control group, by 21.3% in the 1st experimental group, by 28.5% in the 2nd experimental group and by 45% and 46% in the D and E experimental group, respectively. The lowest activity of hCG was established in control and experimental groups, where 10 mg/cm^3 of L-lysine + 75 mg/cm^3 mannite was added. The activity of hormone in those series decreased by 35%, whereas in groups B, C, E its activity was higher and ranged from 75 to 85% of the initial theoretical activity.

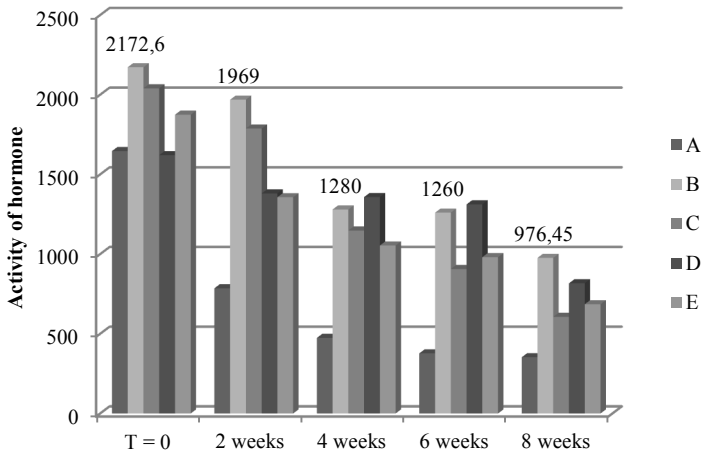

Figure 2 Dynamics of gonadotropin activity with addition of L-glycine to the solvent in absolute values
The X axis represents the time of incubation of hCG with addition of L-lysine to the solvent;
The Y axis represents the changes of hormone activity in the absolute values compared to its initial concentration.

After 4 weeks of incubation, the activity of hormone decreased in all experimental groups, by 51 and 54% in B and D groups respectively, whereas in groups C and E it was 42 and 45%, respectively; in the control group the activity of hormone decreased almost 4 times, as compared to the initial theoretical activity. Such tendency persisted during 6 weeks of incubation.
So, adding 10 mg/sm^3 of L-lysine and 75 mg/sm^3 of sucrose and mannitol revealed the highest activity of gonadotropin, as compared to other experimental and control series of samples over a long incubation. However, in the experimental series of samples, where we used 10 mg/sm^3 L-lysine and 75 mg/sm^3 mannitol as stabilizer, the activity was stable during 6 weeks of incubation and constituted more than 50% of the initial theoretical concentration of hCG.
On the next stage of our research we used glycine as a stabilizer of hormone activity, the obtained results being presented in table 4 and figure 3.
The highest activity of hCG after 6 hours of incubation was observed in the group, where we added 0.25 mg/sm^3 L-glycine + 50 mg/sm^3 saccharose.
Adding of 0.25 mg/sm^3 of L-glycine + 50 mg/sm^3 of saccharose and 0.25 mg/sm^3 of L-glycine + 75 mg/sm^3 of saccharose on the 2nd week of conservation resulted in 61 and 69.8% preservation of the intact hCG concentration, respectively, as compared to the initial level. On the 4th week of conservation, a further decrease of the concentration of intact gonadotropin was observed in the control and D and E experimental series of samples. The lowest activity of hormone was demonstrated in the control group and in the group, where we added 0.25 mg/sm^3 L-glycine + 50 mg/sm^3 mannite during the entire period of incubation. After 8 weeks of incubation, the activity of hormone in the control group decreased by 86% and in the E group – by 81%, as compared with the initial concentration.
As a result of the performed comparative analysis regarding the effects of adding the studied concentrations of amino acids during the conservation of hCG samples, the highest activity of the hormone during the entire period of incubation in the presence of 0.25 mg/sm^3 L-glycine + 50 mg/sm^3 saccharose and 0.25 mg/sm^3 L-glycine + 75 mg/sm^3 saccharose was demonstrated (Fig. 3).

Table 4 Dynamics of gonadotropin activity with addition of L-glycine to the solvent.

Series of samples	Filler	Quantity of filler	Theoretical activity	Dynamics of gonadotropin activity				
				6 hours % compared to initial theoretical activity	2 weeks % compared to initial theoretical activity	4 weeks % compared to initial theoretical activity	6 weeks % compared to initial theoretical activity	8 weeks % compared to initial theoretical activity
A	-		2500	65.8	31.4	18.9	15.0	14.1
B	L-glycine +saharosse	0.25 +75	2500	77.7	69.8	48.0	38.5	21.4
C	L-glycine+saharosse	0.25+50	2500	84.7	60.7	37.9	37.3	22.8
D	L-glycine+mannite	0.25+75	2500	62.3	45.2	27.7	15.4	15.1
E	L-glycine+mannite	0.25+50	2500	53.5	44.7	28.3	27.6	18.6

The extensive analysis of the data from literature testifies to the fact that the research of optimum methods of stabilization of gonadotropic preparations for their use both in human and veterinary medicine is urgent and is being performed by many researchers **(van Zuylen, 1997, Tsivou *et al.*, 2010).** Moreover, the spectrum of gonadotropin-releasing drugs in use is constantly expanding, and they find their application in different areas: the creation of test systems in medicine, sport (doping control), hormone therapy of reproductive abnormalities, etc. **(van Zuylen, 1997, Tsivou *et al.*, 2010).** Given this demand and interest in gonadotropic preparations, researchers continue to search for an optimum stabilizer of hormones activity during their incubation. It has been established that the use, for instance, of mannite as a stabilizing agent does not cause a visible decrease of the activity of the hormone after 24 weeks of conservation **(Geigert, 1999, Sammartiany, 2000)**.
The US patent No 5270057 describes a lyophilized composition containing gonadotropin (for example, LH, TBG, FSH and hCG) stabilized with polybasic carboxylic acid or its salt. The US patent No 5650390 reveals a lyophilized composition containing FSH, LH or hCG stabilized with a combination of sucrose and glycine **(Samaritani and Natale, 1995, Jang and Sung, 2008).** However, lyophilized products are inconvenient due to the fact that they have to be dissolved in water for injection (WFI) before their use. Moreover, the process of production of lyophilized products includes a stage of freezing-drying, and requires, thus, a serious financing. As an alternative for overcoming these limitations, the stability of the protein can be improved by adding a stabilizer to the dissolved protein. Examples of used protein stabilizers include surfactants, plasma albumin, polysaccharides, amino acids, polymers, etc. **(Wang 1999).**
The most suitable stabilizer should be chosen and used taking into consideration unique physical and chemical properties of individual proteins. The combined use of different protein stabilizers may cause unfavorable side reactions leading to an effect opposed to the desirable one, due to a competitive interaction and side reactions between individual stabilizers. Besides, the successful stabilization of proteins in the solution requires a great attention and many efforts, since individual protein stabilizers have a certain range of concentrations required for stabilizing respective proteins **(Wang 1999).** Glycine solution favors the accumulation of a greater number of molecules of water around FSH, making thus more stable the remotest hydrophobous amino acids among the multitude of amino acids constituting hFSH and in this way stabilizing the latter. Some compositions include methionine, which stabilizes the FSH preventing its oxidation in water solution **(Wang 1999).**

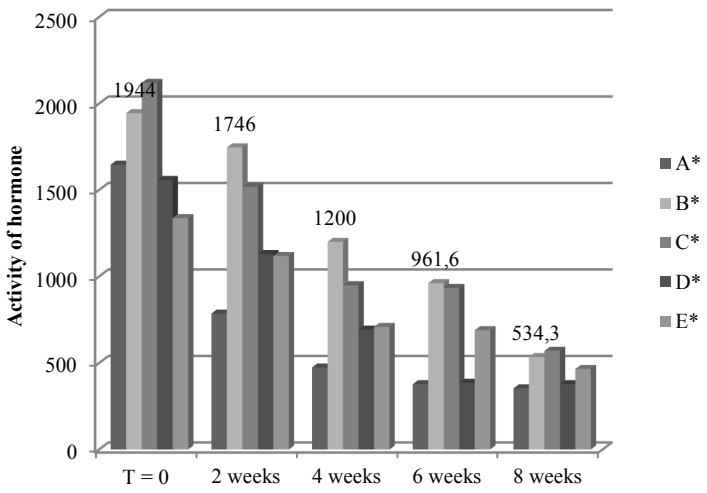

Figure 3 Dynamics of gonadotropin activity with addition of L-glycine to the solvent in absolute values
The X axis represents the time of incubation of hCG with addition of L-lysine to the solvent;
The Y axis represents the changes of hormone activity in absolute values compared to its initial concentration.

CONCLUSION

It has been demonstrated that the addition of various doses of L-lysine, L-glycine and saccharose/mannite into the solvent for gonadotropin causes various levels of stabilizing effects, in particular:

- the conservation of samples of chorionic hormone supplemented with 10 mg/cm^3 of L-lysine + 75 mg/cm^3 saccharose yielded the most stable concentration of gonadotropin: during 6 weeks more the 50% and after 8 weeks – almost 40% of the initial concentration of the hCG;
- in the conditions of a prolonged conservation of the gonadotropin in the samples containing 0.25 mg/sm^3 L-glycine + 75 mg/sm^3 saccharose, the activity of hCG was higher during the entire period of experiment, as compared with control and other experimental series.

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