STABILIZING THE GONADOTROPIN ACTIVITY WITH THE USE OF DIFFERENT 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: saccharose, L-lysine which stabilizes gonadotropins activity for longer use while preserving activity. As a result of the studies we found out that during 6 weeks of incubation at 40°C, the activity of hCG was the highest in the samples with 50 and 75 mg/sm³ of saccharose. Concentration of the hormone was high at 8 week of the incubation in the group of samples with 75 mg/sm³ of saccharose. In the series of hCG samples with 10 mg/sm³ of L-lysine, the activity of hormone was 54% during 8 weeks. Compatible adding of 10 mg/cm³ of L-lysine + 75 mg/sm³ of saccharose provided the best stabilization gonadotropin activity.

Key words: chorionic gonadotropin, stabilization, saccharose, L-lysine, immune-chemiluminescent assay.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a placental glycoprotein hormone that acts through binding to a G-protein-coupled receptor, leading to the increased adenylatecyclase activity (Lapthorn et al., 1994; Erbel et al., 1999). 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, the fully bioactive hormone can be regained by recombination of the subunits (Lapthorn et al., 1994; Erbel et al., 2002). 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).

The three-dimensional structure of hCG shows that each of its two different subunits has a similar topology, with three disulphide bonds forming a cystine knot. The 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 receptor binding by glyco-protein hormones. Lapthorn et al. (1994) described the crystal structure of hCG. During the recent years, the structure of gonadotropic hormones has been decoded for different species of fishes, animals and human (Fiddes et al., 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 consists 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-acetylglucosamine residues and the OH group of serine residues of the C-terminal section of the polypeptide chain. Specific biological properties of the hCG are determined by the terminal section of the polypeptide chain.

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-acetylgalactosamine and N-acetylglucosamine. 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 the half-life of CG in the organism. 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 and Smith, 2006). It has been suggested that CG and its β-subunit are produced not only in the 136 chorion of trophoblast and placenta, but also in the tissues of the fetus, 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 level of hCG 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 researchers’ study, both inorganic (ZnCl₂ or AlCl₃) and organic (methionine, glycine, mannitol, arginine, lactose, sucrose) compounds are used for stabilizing gonadotropic preparations (Cameo et al., 2004; Maïtì et al., 2011). Therefore, the aim of our study was a comprehensive search of an optimum quantitative and qualitative composition of organic compounds needed for the stabilization of the hCG.

**MATERIALS AND METHODS**

According to the general pattern of investigation presented in Tables 1, 2, 3 three experiments were conducted. The experiments differed between themselves by the stabilizers added to the solvent (L-lysine, saccharose) and their concentrations. Each experiment consisted of three series of samples (control, experimental 1 and experimental 2 (Table 1). The human choriionic gonadotropin (hCG) was obtained in the Institute of Animal Biology, N AAS 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 immunochemiluminescent methods based on difference between total and free hCG. The initial concentration of total hCG was 30,175 mIU per mL (international units), while the concentration of free hCG was 375 mIU per mL. Theoretically, the activity of hCG was 29,800 mIU per mL (Tables 1, 2, 3). The obtained gonadotropin was solved in phosphate buffer (pH 7.34) and aliquoted by 2.500 mIU per mL. The samples were placed for incubation at the temperature of 40°C. After each 2 weeks during two months, the concentration of total (hCG+β-hCG) and free (β-hCG) gonadotropin was measured. The hCG concentration was determined by the difference between (hCG+β-hCG) and (β-hCG) (Frimel, 1987; McPherson et al., 2006).
Table 1. Pattern of studies of the dynamics of gonadotropins activity with addition of different quantities of saccharose to the solvent

<table>
<thead>
<tr>
<th>Characteristic of groups</th>
<th>Manipulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - Gonadotropin solved in phosphate buffer (pH 7.34)</td>
<td>Solved preparation of gonadotropin was stored at 40°C, and the activity of hormone was determined every two weeks for two months by immuno-electrochemiluminescent methods</td>
</tr>
<tr>
<td>Experimental 1 - Gonadotropin solved in phosphate buffer (pH 7.34) with adding 75 mg/sm³ of saccharose</td>
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<tr>
<td>Experimental 2 - Gonadotropin solved in phosphate buffer (pH 7.34) with adding 50 mg/sm³ of saccharose</td>
<td></td>
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<tr>
<td>Experimental 3 - Gonadotropin solved in phosphate buffer (pH 7.34) with adding 25 mg/sm³ of saccharose</td>
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</tbody>
</table>

Table 2. Pattern of studies of the dynamics of gonadotropins activity with addition of different quantities of L-lysine to the solvent

<table>
<thead>
<tr>
<th>Characteristic of groups</th>
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</tr>
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<tr>
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</tr>
<tr>
<td>Experimental 1 - Gonadotropin solved in phosphate buffer (pH 7.34) with adding 10 mg/cm³ of L-lysine</td>
<td></td>
</tr>
<tr>
<td>Experimental 2 -Gonadotropin solved in phosphate buffer (pH 7.34) with adding 0.2 mg/cm³ of L-lysine</td>
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</table>

Table 3. Pattern of studies of the dynamics of gonadotropins activity with addition of amino acids to the solvent

<table>
<thead>
<tr>
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<th>Manipulations</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>Experimental 1 - Gonadotropin solved in phosphate buffer (pH 7.34) with adding 10 mg/cm³ of L-lysine + 75 mg/sm³ of saccharose</td>
<td></td>
</tr>
<tr>
<td>Experimental 2- Gonadotropin solved in phosphate buffer (pH 7.34) with adding 10 mg/cm³ of L-lysine + 50 mg/sm³ of saccharose</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSIONS

Dynamics of gonadotropin activity with addition of saccharose to the solvent is present in Table 4 and Figure 1.

Table 4. Dynamics of gonadotropin activity with addition of saccharose to the solvent

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Control</th>
<th>Experimental 1</th>
<th>Experimental 2</th>
<th>Experimental 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>1321.1</td>
<td>1696.2</td>
<td>1404.1</td>
<td>1183.0</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1272.0</td>
<td>1447.0</td>
<td>1267.0</td>
<td>1011.0</td>
</tr>
<tr>
<td>6 weeks</td>
<td>874.6</td>
<td>1284.5</td>
<td>1078.0</td>
<td>906.0</td>
</tr>
<tr>
<td>8 weeks</td>
<td>850.6</td>
<td>938.0</td>
<td>509.0</td>
<td>723.55</td>
</tr>
</tbody>
</table>

Within 6 weeks of incubation at 40°C, the activity of hCG in the samples containing 50-75 mg/sm³ saccharose was higher as compared to the control samples and the third experimental group. The concentration of hCG after 6 weeks of incubation in the samples of the first and second experimental groups decreased by 2.3 and 1.9 times with respect to the initial concentration. Whereas in the first experimental and control groups the rate was much lower. The highest concentration of the hormone was founding the group of samples containing 75 mg/sm³ at 8 weeks of incubation, as compared with the samples of the control, second and third experimental group.

Dynamics of gonadotropin activity with addition of L-lysine to the solvent is present in Table 5 and Figure 2.

After 2 weeks of conservation in the sample with 25 mg/sm³ of saccharose, a decrease of HCG activity by 53% from the initial activity was noted. In the sample with 75 mg/sm³ of saccharose, the concentration of the hormone was the highest constituting almost 68% of initial concentration. The HCG content after in the second experimental group was on the same level with the control one and equaled to 51%. After 8 weeks from the start of the research, the concentration of HCG in the sample of the second experimental group was the lowest and constituted 20% of the initial concentration, the highest concentration of hormone was in the sample of the first experimental group - 37% (Figure 1).
In the 1st experimental series, where 10 mg/cm³ of L-lysine were inserted, the concentration of gonadotropin was 51.72% of the initial concentration of hCG (Table 5, Figure 2).

![Figure 2](image)

In the X axis, the time of incubation of hCG with addition of saccharose to the solvent are presented in the Y axis, the changes of hormone activity in the % from initial concentration are presented.

By the 4th week of samples conservation, a tendency to the further decrease of gonadotropin concentration in all series of samples was demonstrated. However, by the 6th week of conservation, the hCG concentration in all experimental and control series increased and exceeded the respective values obtained at the 4th week of incubation (Figure 1). The highest concentration (51.93% of the initial one) in the 1st experimental series with 10 mg/cm³ of L-lysine in the sample was noted. By the 8th week of samples conservation in the thermostat, no changes of concentration of intact hCG were found, as compared with the 6th week conservation in the control and 2nd experimental series. A tendency to the increase of concentration of intact gonadotropin in the 1st experimental group was demonstrated (Figure 3).

Prolonged storage of hCG at 40°C with addition of L-lysine + saccharose revealed changes in all experimental groups and in the control one (Table 6).

Already at the 6th hour after the dissolution of gonadotropin, differences in its activity in all series of samples with respect to the initial theoretical activity and between experimental batches of samples were determined. The lowest activity of hCG was found in the control and experimental series of samples, where 10 mg/sm³ L-lysine and 50 mg/sm³ saccharose was added as stabilizer (Figure 3).

![Figure 3](image)

In the X axis, the time of incubation of hCG with addition of saccharose to the solvent are presented in the Y axis, the changes of hormone activity in the % from initial concentration are presented.

After the 2nd week of conservation, the hCG concentration in the control series of samples decreased to almost 32%. In the experimental series of samples, the hormone activity was 78.7 and 71.5% in the first and second group, respectively, as compared with the initial concentration. On the 4th week of conservation, a tendency to the decrease of gonadotropin concentration in the control and 2nd experimental group was observed. In the samples of the 1st experimental group, the activity of hCG was on the highest level and amounted to 51.2%. The same tendency was also preserved on the 6th week of incubation. After 8 weeks of incubation still the highest activity of hCG in the 1 experimental group of samples was noted. The extensive analysis of
the data from literature testifies to the fact that the research of optimum methods of stabilization of gonadotrophic preparations for their use both in human and veterinary medicine is urgent and is being performed by many researchers (van Zuyleen et al., 1997; Tsivou et al., 2010). With the spectrum of gonadotropin-releasing drugs in use constantly expanding, they find their application in different areas: the creation of test systems in medicine, sport (doping control), hormone therapy of reproductive abnormalities, etc. (van Zuyleen et al., 1997; Tsivou, 2010). Given this demand and interest to the gonadotrophic preparations, researchers continue to search the optimal 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, 1989; Samaritani et al., 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 saccharose and glycine (Samaritani et al., 2000; Jang and Sung, 2006). Within 6 weeks of incubation at 40°C, a higher activity of hCG in samples containing 50-75 mg/sm³ saccharose was established. These results are confirmed by the research of scientists.

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).

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).

In our investigation we used different organic compounds for stabilizing hormone activity for storage during 8 weeks at the temperature +40°C. Some authors used other temperature regimes and organic components. So, they used a competitive radioimmunoassay measuring hCG, hCGβ and hCGβcf together. De Medeiros et al. (1991) found that hCG is stable at +4°C for at least 3 weeks and Mc Chesney et al. (2005) found that hCG is stable for at least 4 weeks at +4°C. Glycerol provided protection against urea-induced hCG degradation at -20°C, and it has earlier been shown to protect urinary FSH and LH against degradation at -20°C (Kesner et al., 1995; Saketos et al., 1994).

Glycerol is thought to increase the proportion of liquid solution in partially frozen urine and hence prevent the formation of liquid regions with very high solute concentrations. Glycerol also stabilizes protein conformation and can thereby reduce the reaction with cyanate. Addition of glycerol can be used to reduce the loss of hCG at -20°C but it cannot be used in doping control because it can be used as a plasma expander and is therefore included in the list of prohibited substances (Kesner et al., 1995; Saketos et al., 1994).

Our aim was to evaluate the stability of hCG, hCGβ, hCGα and hCGβcf in urine during storage at various temperatures. Since urine contains fairly high concentrations of urea (0.2-0.8 mol/L) that has been suggested to cause degradation of LH at -20°C, the effect of added urea on hCG loss was studied. Lempääläinen et al. evaluated the protective effect of different additives; glycerol has been
shown to prevent the degradation of other gonadotropins, ethylene diamine (EDA), which protects proteins against carbamylation caused by urea-derived cyanate, EDTA, and bovine serum albumin (BSA) (Lempiäinen et al., 2012).

CONCLUSIONS

In our investigation we used different organic compounds for stabilizing hormone activity for storage during 8 weeks at the temperature +40°C. The most higher activity of HGh in the samples with adding of 10 mg/cm^3 of L-lysine + 75 mg/sm^3 saccharose we established. In the sample with 75 mg/sm^3 of saccharose, the concentration of the hormone was the highest and constituted almost 68% of initial concentration in compare with the sample where we added 50 and 25 mg/sm^3 of saccharose.

REFERENCES


