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STORAGE OF ACTIVITY OF THE HUMAN CHORIONIC GONADOTROPIN HORMONE IN SOLUTION AT ADDITION OF ORGANIC COMPOUNDS TO THE PHARMACOLOGICAL COMPOSITION

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Abstract

The more stable among the tested samples were samples with saccharose in the concentration of 50–75 mg per cm^3. While adding of L- lysine to samples the most stable activity was discovered in the experimental series of samples with the content of lysine of 10 mg per cm^3 – activity increased by 54 % as compared to theoretical initial activity of HCG during 8 weeks. While storing gonadotropin with L-glycine fluctuations of hormone activity in all series of samples were observed. Adding of 0.2 mg per cm^3 of L-glycine had a more expressedstabilizing effect. Adding of 0.2 mg per cm^3 of L-methionine produced relativelyhigh and stableactivity ofgonadotropinduring the 6 weeksstorage. Adding of 0.25 mg/cm^3 of L-glycine and 75.50 mg/cm^3 of saccharose to experimental samples during 2 weeks at 40 °C provided 69.8 % and 60.7 % saving activity of hCG respectively. Activity of gonadotropin in a series of samples with the addition of L- glycine and mannitol was significantly lower and at the end of the study was at an appropriate rate with the control series models. The highest activity of gonadotropin was detected while adding fillers – 10 mg/cm^3 L-lysine and 75 mg/ m^3 saccharose and mannitol – to recipes as a stabilizer.

Keywords: chorionic gonadotropin, saccharose, L – lysine, mannitol, L – glycine; hCG (human chorionic gonadotropin), CG (chorionic gonadotropin).

1. Introduction

The gonadotropins form a family of structurally relatedglycoprotein hormones. Typical members include chorionicgonadotropin (CG), follicle stimulating hormone (FSH; follitropin), luteinizing hormone (LH; lutropin) and thyroidstimulating hormone (TSH; thyrotropin). Human chorionic gonadotropin (hCG) is a placental glycoprotein hormone that acts through binding to a G-protein-coupled receptor, leading to increased adenylatecyclase activity [1–6]. hCG is a heterodimer consisting of two non-covalently associated subunits, α and β, each encoded by a different gene, located on chromosomes 19 and 7, respectively. Biological activity depends on the association of these subunits [7]. Both subunits are glycosylated and glycosylation during biosynthesis is absolutely essential for right folding of each subunit. Both subunits have several disulfide bridges and the crystal structures of hCG and human FSH reveal that three of the disulfide bridges in both subunits form cystine knots.Within a species the α-subunit is essentially identical for each member of the gonadotropin family; it is also highly conserved from species to species. The β-subunits are different for each member, i. e. CG, FSH, TSH and LH, but show considerable homology in structure. In humans the α-subunit consists of 92 amino acid residues, whilst the β subunit varies in size for each member [9]. The β subunit of hCG is substantially larger than the other β subunits in that it contains approximately 34 additional amino acids at the C-terminus referred to herein as the carboxy terminal protein (CTP). Relatively pure gonadotropin preparations are commercially available. The stability of proteins in aqueous formulations is generally a problem in pharmaceutical industry. Likewise the stability of aqueous solutions of the gonadotropins is insufficient to allow storage for longer times [8]. Therefore, usually those preparations are stored in a dry form, as obtained after lyophilization. A stabilized gonadotropin containing lyophilized pharmaceutical formulation is disclosed in European Patent № 448,146 (Akzo N. V.). These preparations contain organic carboxylic acids, particularly citric acid, and optionally a non-reducing sugar such as sucrose.

Liquid formulations containing the gonadotropin recombinant-hCG stabilized with the non-reducing sugar, preferably mannitol, in an aqueous solution in a phosphate buffer at pH 7, are disclosed in WO 96/29095. Solutions comprising gonadotropins and a polycarboxylicacid salt are known from European Patent № 448,146 (Akzo N. V.). These solutions, containing, for instance, citric acid are described for preparing stabilized lyophilised gonadotropin formulations. At the storage of such solutions per se for longer times the gonadotropins are insufficiently stable.

It is well known that highly purified proteins with time loose stability, and to maintain the hormone activity it is necessare to add some concentration of sucrose such as lactose and mannitol or of proteins and aminoacids such as albumin and glycine. However, these means in any case are not suitable for injection because of their lack of solubility and allergity or in some cases because of their potential toxicity or because of all these effects simultaneously. Also, the storage of such compositions for a longer time does not provide sufficient stabilization activity of gonadotropins in solution. However, it is impossible to provide a standard recipe for all proteins and choosing of the best recipe requires considerable effort in selection whichactually becomes the main aim of our work.

2. Materials and methods

The human chorionic gonadotropin (hCG) was obtained in the Institute of Animal Biology, National Academy of Agrarian Science of Ukraine (Director Prof. V. Vlizlo) from the urine of pregnant women (12–16 weeks of gestation). To evaluate the influence of filler on the stability of gonadotropins activity different samples of human chorionic hormone were prepared, with the contents of 2.500 mIU per ml of different fillers: saccharose, mannitol, lysine, together and separate. All samples were prepared using the solution of fillers in a phosphate buffer at pH 7.34 (**Fig. 1**).

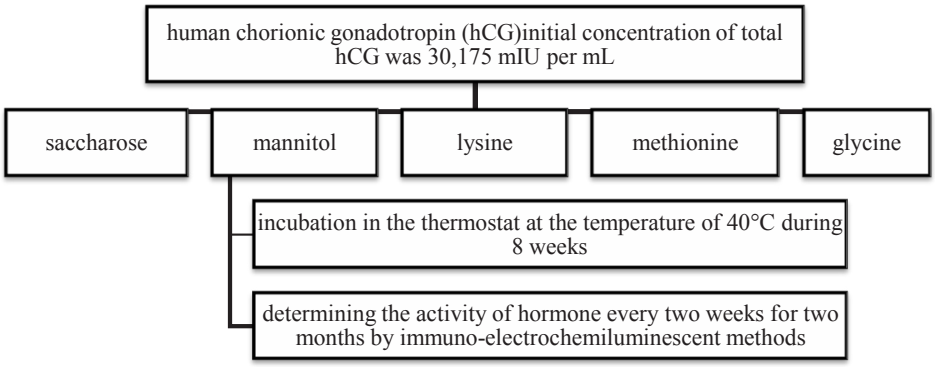

Fig. 1. Scheme of the experiment

The concentration of intact gonadotropin was established by means of electro- and immuno-chemiluminescent method based on the difference between total and free hCG [10]. The initial concentration of total hCG was 30,175 mIU per mL while the concentration of free hCG was 375 mIU per mL. Theoretically, the activity of hCG was 29,800 mIU per mL. The samples were placed into a thermostat 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) [11].

3. Results

The results of activity tests performed on HCG during 2, 4, 6 and 8 weeks storage in the presence of stabilizers described above are shownin **Fig. 2, 3** and **Table 1**. After two weeks in a sample containing 25 mg/cm^3 of saccharose, a 53 % reduction of the initial hCG concentration was found; in the sample containing 75 mg/cm^3 of saccharose, the concentration was higherand amounted to almost 68 % of the initial theoretical activity (**Fig. 2**).

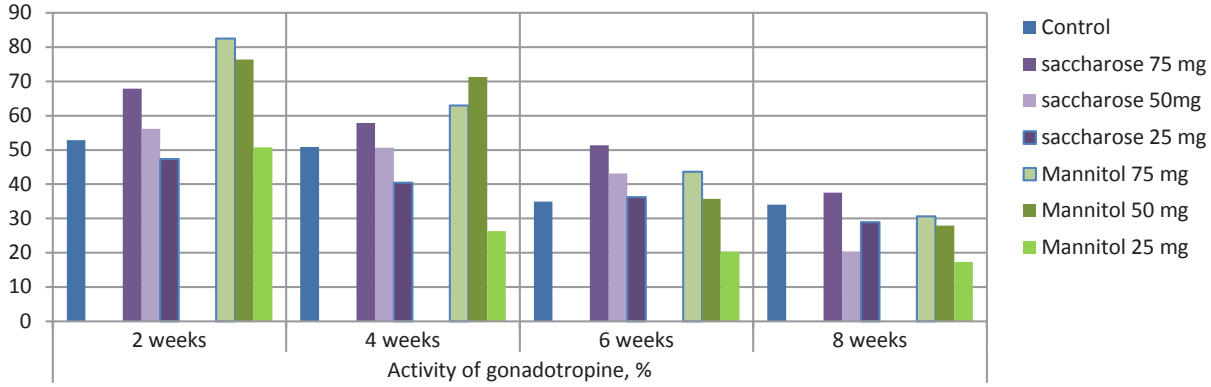

Fig. 2. Dynamics of gonadotropin activity with addition of saccharose and mannitol to the solvent

In the X axi, the time of incubation of hCG with the addition of saccharose and mannitol to the solventare presented; in the Y axis the changes of hormone activity in the percentage from the initial concentrationare presented

Thus, it was found thatsaccharoseis an effectivestabilizingagent againstgonadotropin denaturation (**Fig. 3**).

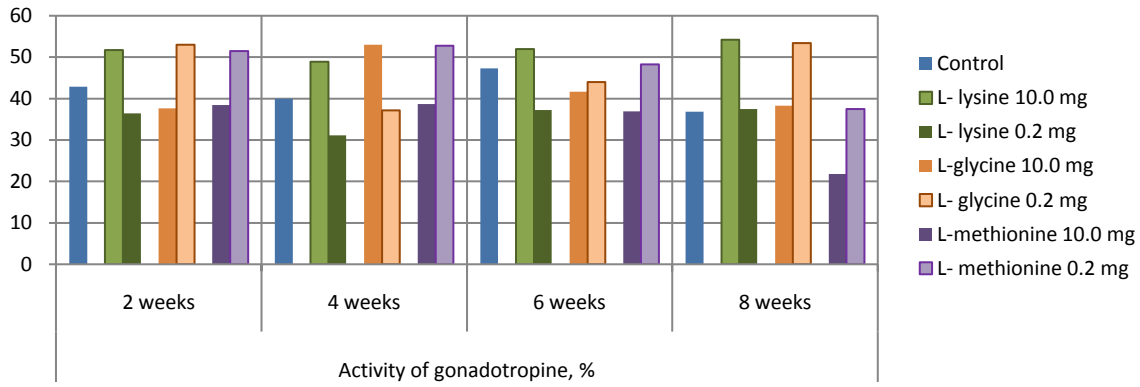

Fig. 3. Dynamics of gonadotropin activity with the addition of aminoacids to the solvent

In the X axis the time of incubation of hCG with the addition of aminoacids to the solventare presented;in the Y axis the changes of hormone activity in the percentage from the initial concentrationare presented

Changes occurring in all series of samples at thehCG storage in a thermostat at 40 °C after adding L-lysine were established in the experiment.The highest activity was found in the first series of experimental samples, 51.93 %, containing 10 mg/cm^3 of L-lysine in the sample. On the 8th week of storage of samples in an incubator, in the 2nd series of experimental samples no changes in hCG activity were observed as compared to a 6-week storage while a tendency to an increase of gonadotropins activity in the 1st series of experimental samples and to its decrease in the control samples was established.

Therefore, the best results in the maintaining ofhCG concentration on the highest level were obtained after adding 10 mg/cm^3 of L-lysine during 8 weeks and 0.2 mg/cm^3 of L-glycine.

Further investigations were performed on the samples with joint action of sacharoses and amino acids in the best concentrations which were obtained before (**Table 1**).

Table 1

Dynamics of gonadotropin activity with the addition of saccharose and aminoacids to the solvent during 2 month of storage at the temperature 40 ºC

Series of samples	Activity of gonadotropin, %			
	2 weeks	4 weeks	6 weeks	8 weeks
Control	31,4	18,9	15,0	14,1
L-glycine+saccharose 0,25+50	60,7	37,9	37,3	22,8
L-glycine+saccharose 0,25+75	69,8	48,0	38,5	21,4
L-glycine+mannitol 0,25+50	44,7	28,3	27,6	18,6
L-glycine+mannitol 0,25+75	45,2	27,7	15,4	15,1
L-lysine+saccharose 10+50	71,5	45,9	36,2	24,2
L- lysine+saccharose 10+75	78,7	51,2	50,4	39,1
L-lysine+mannitol 10+50	54,2	42,1	39,2	27,4
L-lysine+mannitol 10+75	55,2	54,3	52,4	32,7

The best properties during all incubation period were shownin the samples with L-lysine + saccharose 10+75 mg/cm^3. On the 4 and 6 weeks, the highest level of activity was observed in the samples with L-lysine + mannitol 10+75 mg/cm^3.

4. Discussion

An 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 [12, 13]. The interest to the gonadotropic preparations, researchers continue to search the optimal stabilizer of hormones activity during their incubation. It has been established that the use, for instance, of mannitol as a stabilizing agent does not cause a visible decrease in the activity of the hormone after 24 weeks of conservation [14, 15]. The US patent № 5270057 describes a lyophilized composition containing gonadotropin (for example, LH, TBG, FSH and hCG) stabilized with polybasic carboxylic acid or its salt. Within 6 weeks of incubation at 40 °C, a higher activity of hCG in samples containing 50–75 mg/cm^3 saccharose was established in our investigation. 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 [16] found that hCG is stable at + 4 °C for at least 3 weeks and Mc Chesney [17] found that hCG is stable for at least 4 weeks at + 4 °C.

After incubation in a samplecontaining of saccharosethe concentration ofthe hormonein the samples of the 2nd experimental groupwason the same levelwith the controlandconstituted about 51 % we obtained in our investigation. After 8weeks ofresearch the hCGactivity in the samplesof the second serieswas the lowestand amounted to 20 % of the initial concentration,the highestactivity of gonadotropinbeing observed inthe 1st series ofexperimentalsamples – 37 %. The storage of samples with mannitol led to a decreased hormone activity by 49 % in the third series of samples and by 17.5 and 23.6 % in the samples of the first and second series, respectively. Activity of samples was 30.7, 27.5 and 17.27 % in the 1, 2 and 3 series, respectively, after 8 weeks of incubation.

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 [18, 19]. 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 (McChesneyetal., 2005), the effect of added urea on hCG loss was studied. Lempiäinen *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) [20].

The studies have shown that the activity of hCG after 2 weeks of storage in the control series of samples decreased by almost 58 %, in the 2nd series of experimental samples by 63.56 %, while in the 1st experimental series, where 10 mg/cm^3 of L-lysine was introduced, the gonadotropin activity was 51.72 % of the initial theoretical activity of hCG. After 4 weeks of storage of samples, a tendency to a further reduction of the gonadotropin concentration was observed in all series. The highest activity was found in the first series of experimental samples, 51.93 %, containing 10 mg/cm^3 of L-lysine in the sample. On the 8th week of storage of samples in an incubator, in the 2nd series of experimental samples no changes in hCG activity were observed, as compared to a 6-week storage, while a tendency to an increase of gonadotropins activity in the 1st series of experimental samples and to its decrease in the control samples was established.

Adding of 0.2 mg/cm^3 of L-glycine after 2 weeksleads to 53 % preservation of the hCG activity, accordingto the theoreticalinitialactivity, whereas in the control samples and experimental series, where 10 mg/cm^3 of L-glycine was added, the hCG concentration decreased by 57 and 62 %, respectively. After 4-weeks storage an increase of hormone activity by 16 % in the samples with 10 mg/cm^3 of L-glycine were found as compared to 2 weeks of incubation. In the rest of samples the hormone activity continued to decline. However, at the 8th week of storage in the control and first experimentalseriesof samples it was reduced andremained on thesame level, while in the 2nd series of experimental samples it increased by almost 10 % in respect to the previous activity of gonadotropin.

In the 2nd experimental series of samples containing 0.2 mg/cm^3 of L-methionine, the hCG activity was almost 52 % of the theoretical initial activity on the 2nd week of incubation. These indicators in this series of samples were at the same level at the 4th week of storage, however, on the 6th week of storage the hCG concentration decreased by 4 %. The activity of gonadotropin in the 1st experimental series of samples containing 10 mg/cm^3 of L-methionine after two weeks of cultivation was the lowest and amounted to only 38.41 % of the initial theoretical concentration and remained at the same level within 6 weeks of storage. In the samples with 0.25 mg/cm^3 of L-glycine + 75 or 50 mg/cm^3 of saccharose, its activity was the highest and constituted 69.8 % and 60.7 %, respectively, of the initial activity of gonadotropin. After 4 and to 8 weeks of incubation the tendency to decrease in the hormone activity was observed in all series of samples. In the samples with L-lysine + saccharose after 2 weeks the activity of hCG was on the highest level as compared to other groups with added stabilizer – 71.5 and 78.5 % in the samples with L-lysine + saccharose 10+50 mg/cm^3 and L-lysine + saccharose 10+75 mg/cm^3, respectively. In the samples with mannitol + L-lysine, the hCG activity decreased by 45 %. The best properties during all incubation period were shown in the samples with L-lysine + saccharose 10+75 mg/cm^3. On the 4 and 6 weeks of storage, the highest level of activity was observed in the samples with L-lysine + mannitol 10+75 mg/cm^3.

5. Conclusions

The best properties during all incubation period were shown in the samples with L-lysine + saccharose 10+75 mg/cm^3. On the 4 and 6 weeks of storage the highest level of activity was observed in the samples with L-lysine + mannitol 10+75 mg/cm^3. Obtained results can be used in the preparation effective, modern and save medical substances for hormonal therapy in animals.All these literature data and also our investigation confirm the need of find in gorganic substances to en sure optimal conditionsfors to rage of high activity HCG during incubation for future use in human and veterinary medicine.

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THE EARLY SPRING SYNUSIAS IN THE FORESTS OF FAGETO-CARPINETO-QUERCETA ROBORIS SUBFORMATION ON THE TERRITORY OF PRECARPATHIAN REGION (UKRAINE)

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