
The Oral hCG
RESEARCH CENTER

Pharmacology and pharmacodynamics of hCG solutions to be administered by the oral sublingual approach.

The influence of different excipients and storage procedures.

- Authors:

1.Dr. Sergio Ariel Vaney
PhD-Pharmacist

2. Daniel Oscar Belluscio
MD

The oral hCG Research Center
Guido1953- 1119 Buenos Aires
Argentina
Email: hcgobesity@fibertel.com.ar

Oral hCG formulation

Introduction

- Currently Human Chorionic Gonadotropin (hCG), both the version obtained from urine of pregnant women or from recombinant DNA, can be obtained in the market in the form of liquid vials or lyophilized powder for injectable administration.
- Our current research provided us an insight to advance in the development for a new formulation, or pharmaceutical presentation for hCG, to be administered by the oral-sublingual route.
- Our objective is to develop a novel formulation or pharmaceutical liquid form of hCG with sufficient stability to grant a therapeutically effective presentation of hCG apt to be used by the oral/sublingual approach

Oral hCG formulation

- Theoretical Aspects:
  - Analysis and study of pharmacotechnical aspects to assess the ideal conditions for obtaining stability in the proposed Oral hCG formulation.

hCG stability study: analytical method and results.

HCG Stability Study: analytical method and results

Human Chorionic Gonadotropin in liquid pharmaceutical form

- Objective:
  - The following study aims to establish the conditions that will provide a stable pharmaceutical preparation of a liquid formulation of hCG for oral administration.

- Summary:
  - We propose an accelerated stability study in order to evaluate variables that could affect the chemical stability of hCG in a liquid solution.

HCG Stability Study: analytical method and results

Study Design: Objectives

- Gather information as regards the stability of hCG in a liquid environment.
- Need to establish variables or parameters evaluating the biological, physical and chemical properties of hCG under different conditions.
- These considerations will determine the analytical methods that will allow us to formulate those stability environments to quantifiable parameters.
- Perform preliminary studies to assess four study parameters or variables that in our opinion can affect the chemical stability of hCG in a liquid solution.

HCG Stability Study: analytical method and results

Defined Study Parameters:

1. PH.
2. Ionic Force (influence of electrolytes.)
3. Influence of Excipients.
4. Temperature.

HCG Stability Study: analytical method and results

Materials

- 5000 IU Human Chorionic Gonadotropin– lyophilized (GONACOR 5000 – Massone Institute)
- Phosphoric Acid 85% (Carlo Erba)
- Sodium Hydroxide (Merck – Analytical Grade)
- Sodium Chloride (Merck – Analytical Grade)
- Injectable quality distilled water (Roux Ocefa)

HCG Stability Study: analytical method and results

Method

- The samples under study were submitted to accelerated stability conditions: they are maintained at temperatures from 40ºC to 50ºC during approximately 12 weeks.
- We will define as quantifiable parameter the purity (hCG concentration) according to time.
- The quantification of the concentration parameter will be established through an analytical method called HPSEC or High Performance Size-Exclusion (Molecular exclusion chromatography.)
- This chromatographic method allows the separation of substances according to their molecular weight and is used for the separation of proteins and substances with high molecular weight.

HCG Stability Study: analytical method and results

Standard working conditions

Phase A	0.1M phosphate ph 6,7 + 0.1M Sodium sulfate
Isocratic conditions	100% phase A.
Column	TSK G 2000 SWXL
Flow Rate	0.5 ml/min
UV Detector	214 nm
Injection Volume	40 microlite (5000 IU)

HCG Stability Study: analytical method and results

Sample Preparation

- All samples subject to the following study were elaborated according to operative conditions established in the following order:
  1. The hCG was diluted in the adequate solvent (injectable quality distilled water) and homogenized.
  2. The pH of the obtained solution was adjusted with Phosphoric Acid at 85% and Sodium Hydroxide 1M solution to reach pH7.
  3. The obtained solution was filtered through sterile syringe filters of 0.22 microns (Minisart 16534 K – cellulose acetate) to guarantee solution sterility.
  4. The obtained solution was bottled in 10 cc. vials and the vials were tapped with rubber tops and aluminum security precincts.
  5. All described operations were performed under laminar flux conditions.

HCG Stability Study: analytical method and results

Graphs results to evaluate the chemical stability of hCG according to the four established parameters or variables.

HCG Stability Study: analytical method and results

1 – PH

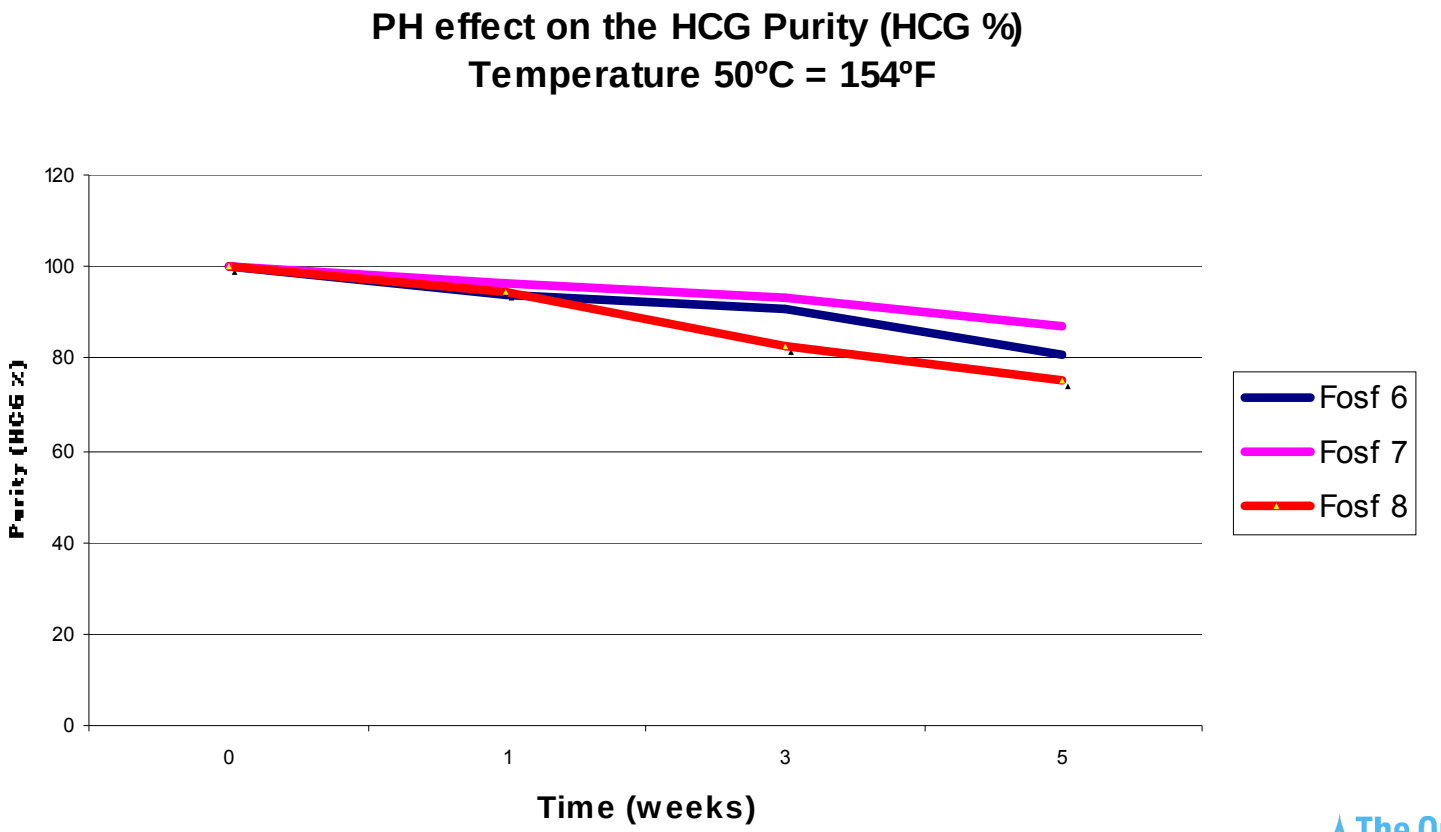
- To evaluate the effect of pH on the chemical stability of hCG in liquid solution, three samples were prepared. Their composition can be observed in Table 1:

Table 1

Samples	Composition
Fosf 6	HCG 5000 IU Phosphoric acid 85% 0.98mg Sodium Hydroxide solution 1M q.s to PH 6 Water injection q.s to 1 ml
Fosf 7	HCG 5000 IU Phosphoric acid 85% 0.98mg Sodium Hydroxide solution 1M q.s to PH 7 Water injection q.s to 1ml
Fosf 8	HCG 5000 IU Phosphoric acid 85% 0.98mg Sodium Hydroxide solution 1M q.s to PH 8 Water injection q.s to 1ml

HCG Stability Study: analytical method and results

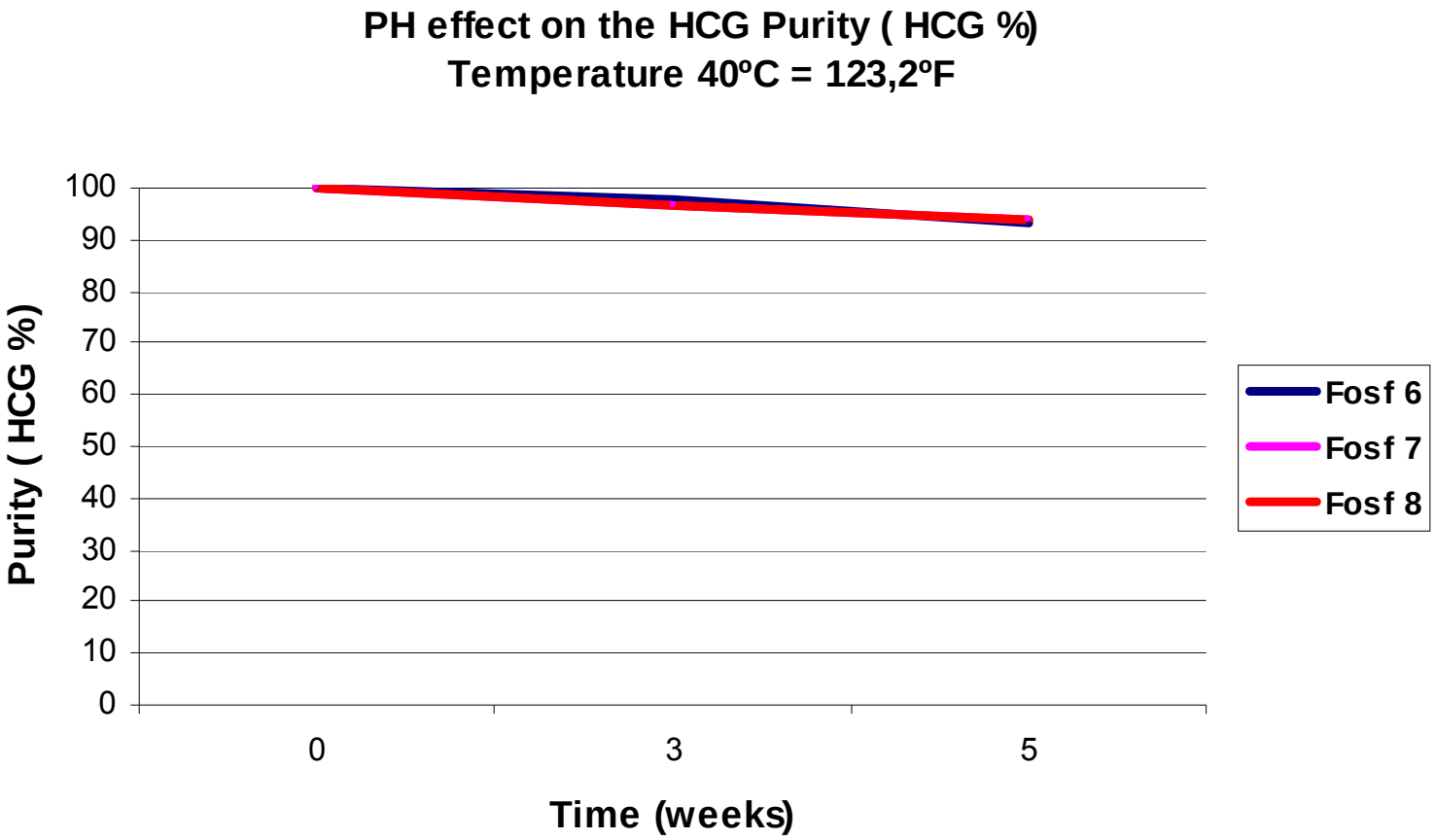
- Here in Graphs 1 and 2 we display the stability curves obtained for samples under experimental conditions:



Graph 1

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results



Graph 2

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

- Influence of pH on Purity (% hCG)

Temperature 50ºC = 154ºF				
Samples	Time 0 week	Time 1 week	Time 3 week	Time 5 week
Fosf 6	100	94.1	90.76	81
Fosf 7	100	96.09	93.12	86.93
Fosf 8	100	94.21	82.50	74.96

Temperature 40ºC = 123,2ºF			
Samples	Time 0 week	Time 3 week	Time 5 week
Fosf 6	100	97.5	93
Fosf 7	100	96.72	93.74
Fosf 8	100	96.77	93.55

HCG Stability Study: analytical method and results

2 – Ionic Force (influence of electrolytes)

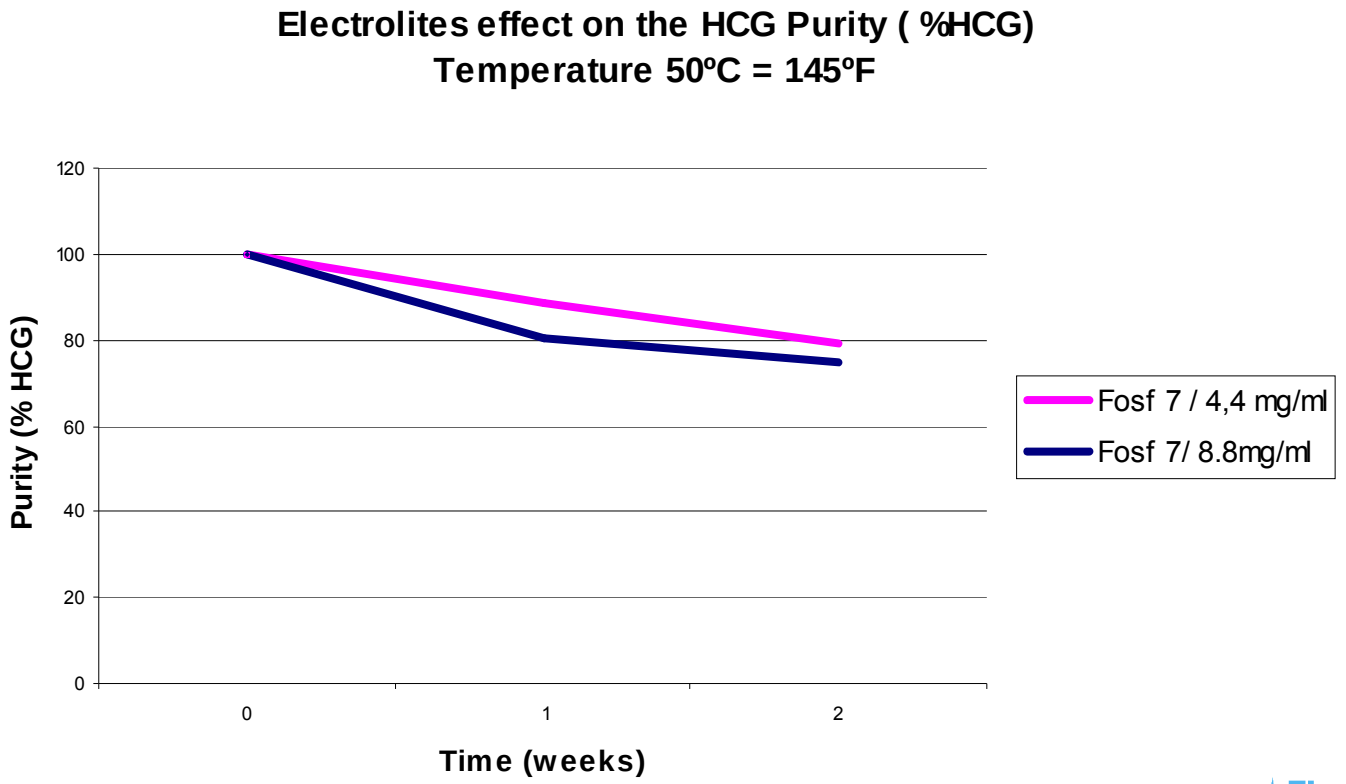
- To assess the influence of electrolytes on the chemical stability of hCG in liquid solution, 2 samples were prepared, their composition can be observed in Table 2:

Table 2

Samples	Composition
Fosf 7 / 4,4 mg/ml (150 mOsm)	HCG 5000 IU / ml Sodium chloride 4,4 mg/ml Phosphoric acid 85% 0.98mg Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1 ml
Fosf 7 / 8,8 mg/ml (300 mOsm)	HCG 5000 IU / ml Sodium chloride 8,8 mg/ml Phosphoric acid 85% 0.98mg Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1ml

HCG Stability Study: analytical method and results

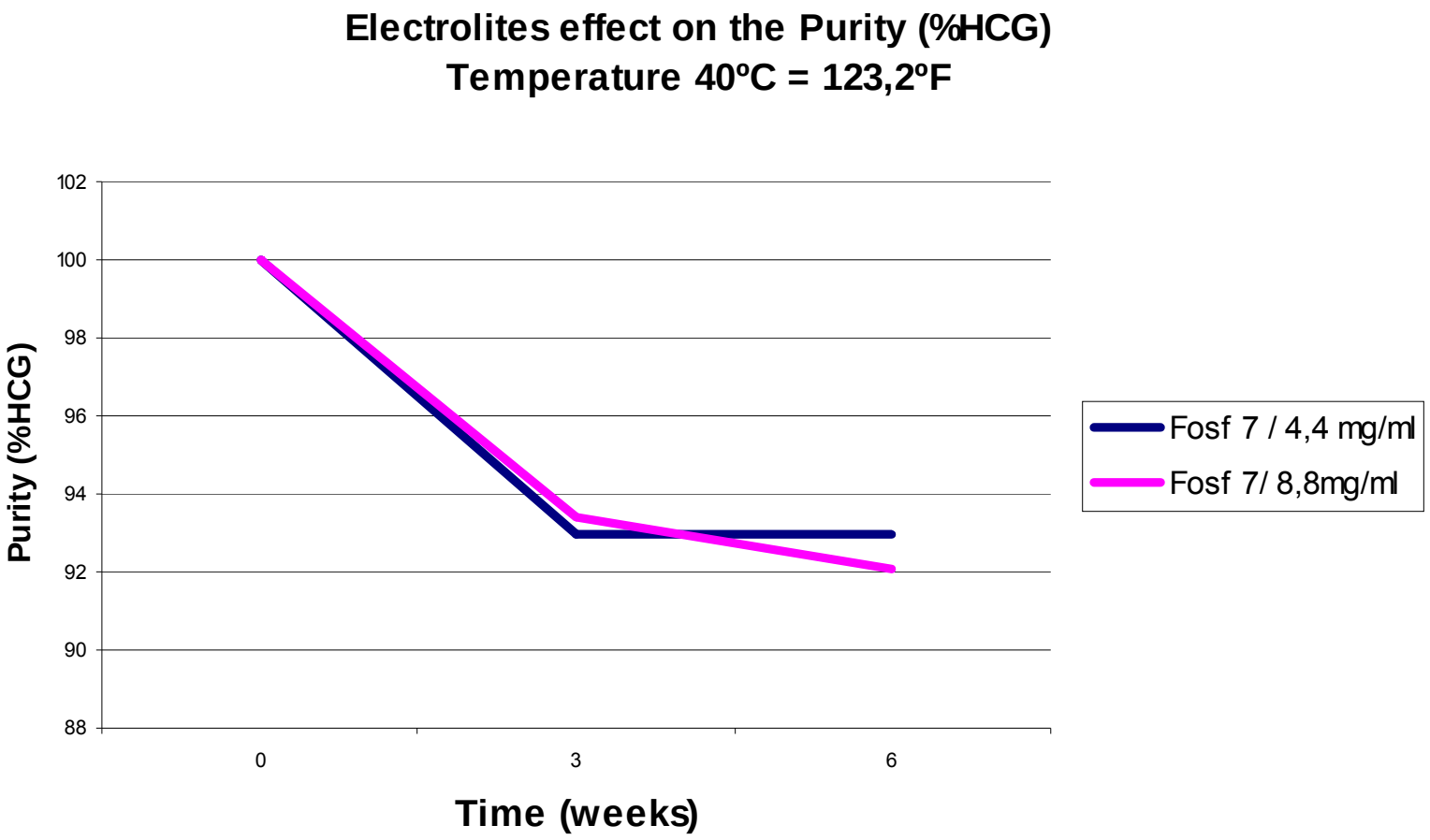
- In Graph 3 and Graph 4 we can observe the stability curves obtained for both samples.
- The samples have been evaluated at temperatures of 25ºC and 4ºC, additionally to the proposed experimental conditions.



Graph 3

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results



Graph 4

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

- Influence of Electrolytes (Ionic Force) on Purity (%HCG)

Temperature 50ºC = 154ºF				
Samples	Time 0 week	Time 1 week	Time 2 week	Time 4 week
Fosf 7 / 4,4mg/ml	100	88.5	79.2	72.2
Fosf 7/ 8,8mg/ml	100	80.5	75	67.9

Temperature 40ºC = 123,2ºF			
Samples	Time 0 week	Time 3 week	Time 6 week
Fosf 7 / 4,4mg/ml	100	93	93
Fosf 7/ 8,8mg/ml	100	93,4	92,1

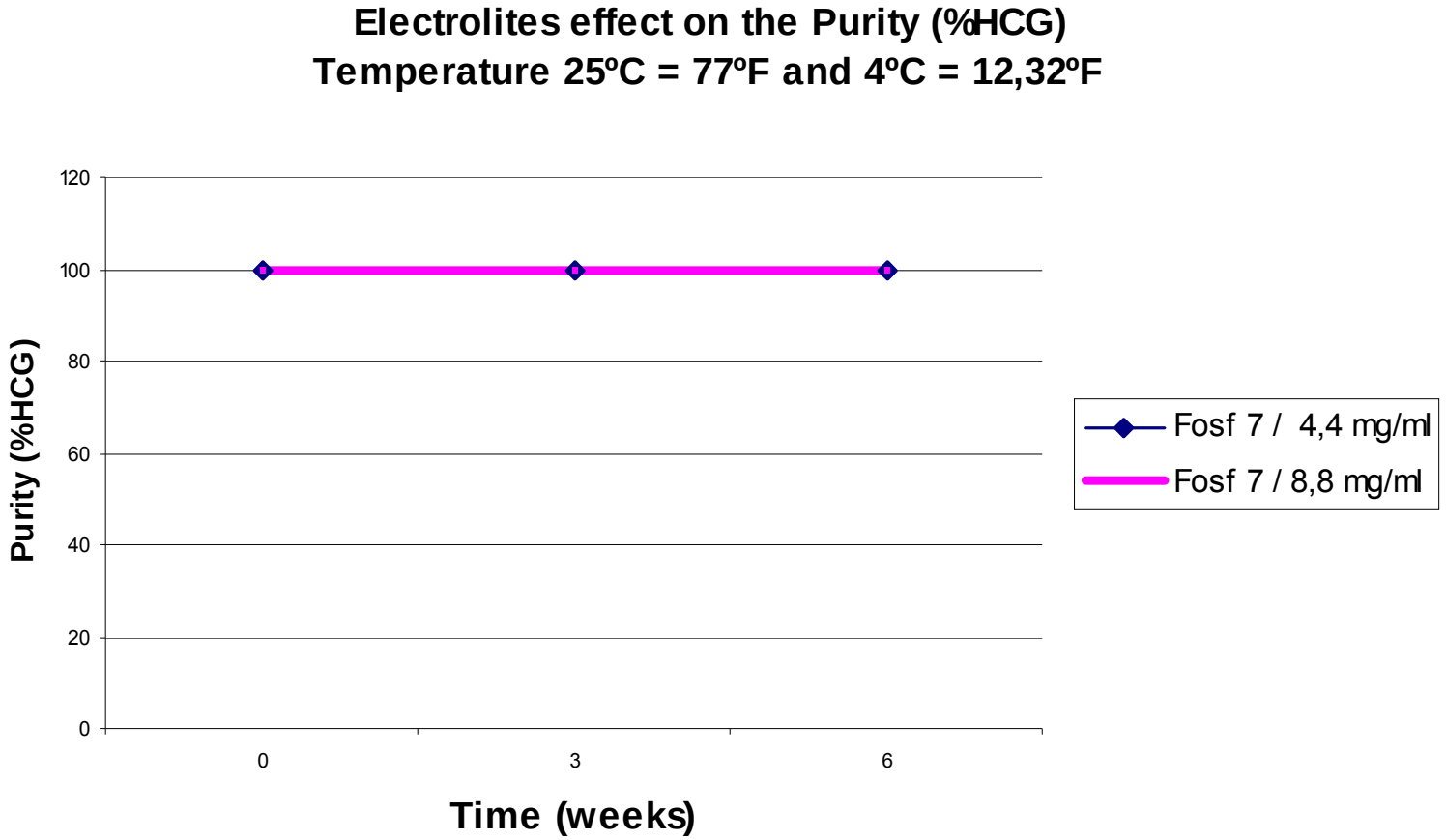
HCG Stability Study: analytical method and results

- Influence of Electrolytes (Ionic Force) on Purity (%HCG) (2)

Temperature 25ºC = 77ºF			
Samples	Time 0 week	Time 3 week	Time 6 week
Fosf 7 / 4,4mg/ml	100	100	100
Fosf 7/ 8,8mg/ml	100	100	100

Temperature 4ºC = 12.32ºF			
Samples	Time 0 week	Time 2 week	Time 4 week
Fosf 7 / 4,4mg/ml	100	100	100
Fosf 7/ 8,8mg/ml	100	100	100

HCG Stability Study: analytical method and results



Graph 5

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

Conclusion

- In proposed experimental conditions (temperatures of 50ºC and 40ºC) we observe that an increase in concentration of electrolytes negatively affects purity (%hCG) of Human Chorionic Gonadotropin in liquid solution.

- No changes are observed at temperatures between 25ºC and 4ºC.

HCG Stability Study: analytical method and results

Comparative Study- Effect of different Electrolytes: Silver

- In order to qualitatively evaluate the influence of electrolytes on the chemical stability of hCG in liquid solutions, two samples were analyzed by ultraviolet spectrophotometry. Their composition is observed in Table 3:

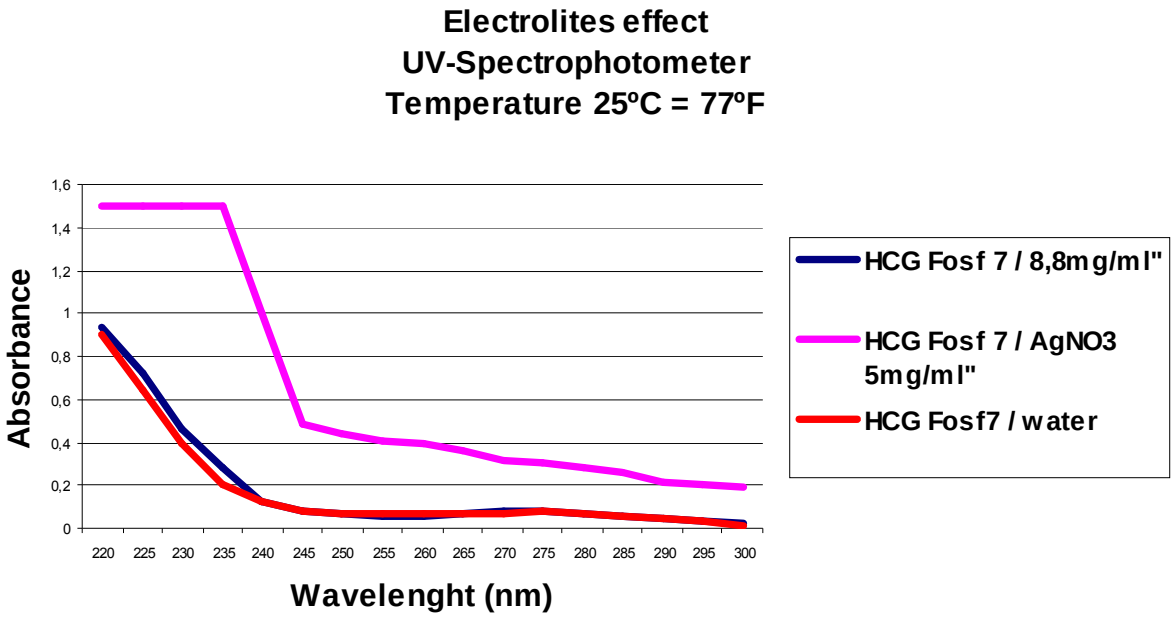
Table 3

Samples	Composition
Fosf 7 / Nacl	HCG 5000 IU / 10ml Sodium chloride 8,8 mg/ml Phosphoric acid 85% 0.98mg Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 10 ml
Fosf 7 / $AgNO_3$	HCG 5000 IU / ml Silver nitrate 5 mg/ml Phosphoric acid 85% 0.98mg Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 10ml

HCG Stability Study: analytical method and results

Working Conditions

- The samples under study were analyzed by a Beckman 25 spectrophotometer, scanning between 300nm–220nm, using quartz buckets of 10 mm of width and maintaining temperature between 22ºC–25ºC in a thermostatic bath.
- The obtained specters of both samples are displayed in Graph 5:



Graphic 5

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

Conclusions

- The spectrophotometric comparative study of both samples evidences that the use of sodium chloride as electrolyte favors the chemical stability of hCG in liquid solution.

- By contrast, the use of silver salts in liquid solution degradates the hCG molecule.

HCG Stability Study: analytical method and results

3 – Influence of Excipients

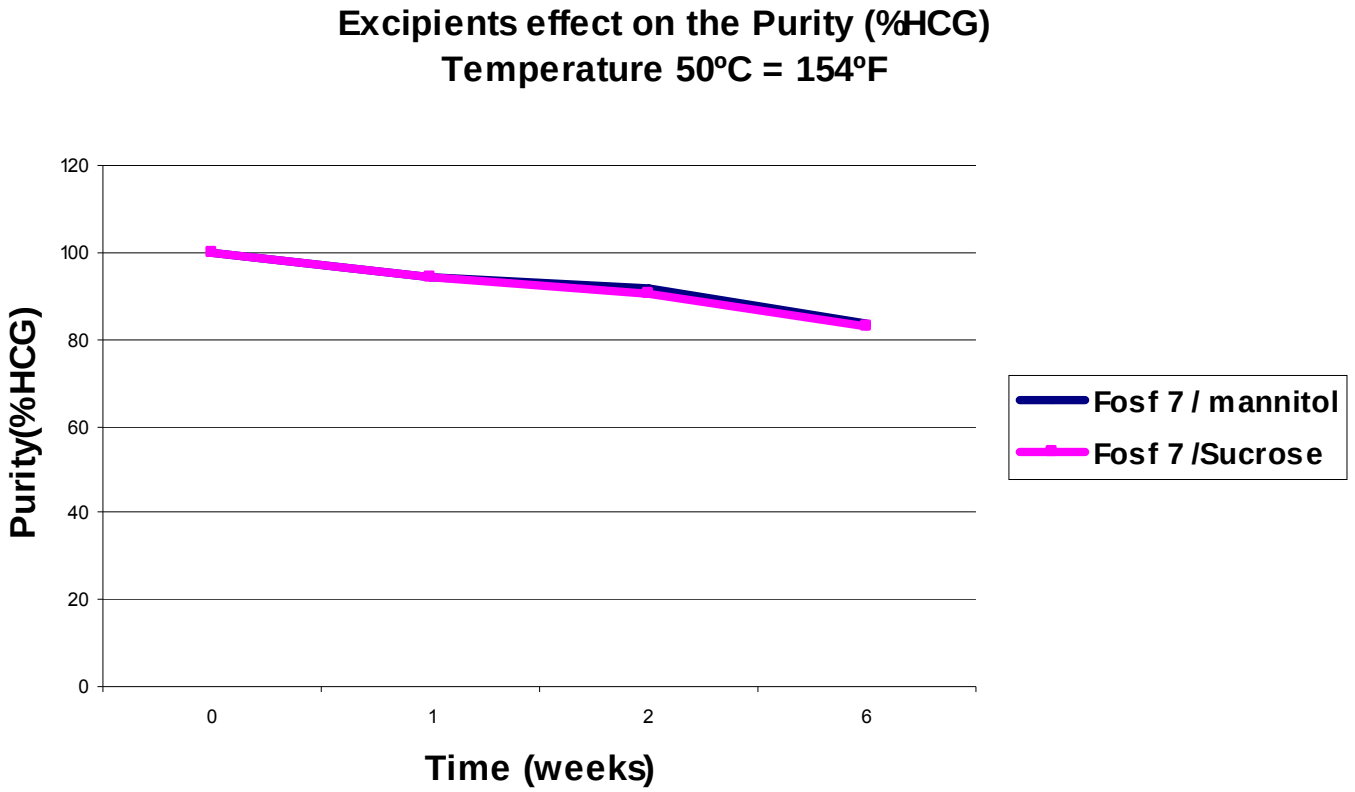
- To compare the effect of excipients on the chemical stability of hCG in liquid solution, two samples were prepared. Their composition can be observed in Table 4.

Table 4

Samples	Composition
Fosf 7 / mannitol	HCG 5000 IU / ml Mannitol 54 mg/ml Phosphoric acid 85% 0.98mg/ml Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1 ml
Fosf 7 / Sucrose	HCG 5000 IU / ml Sucrose 102 mg/ml Phosphoric acid 85% 0.98mg/ml Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1ml

HCG Stability Study: analytical method and results

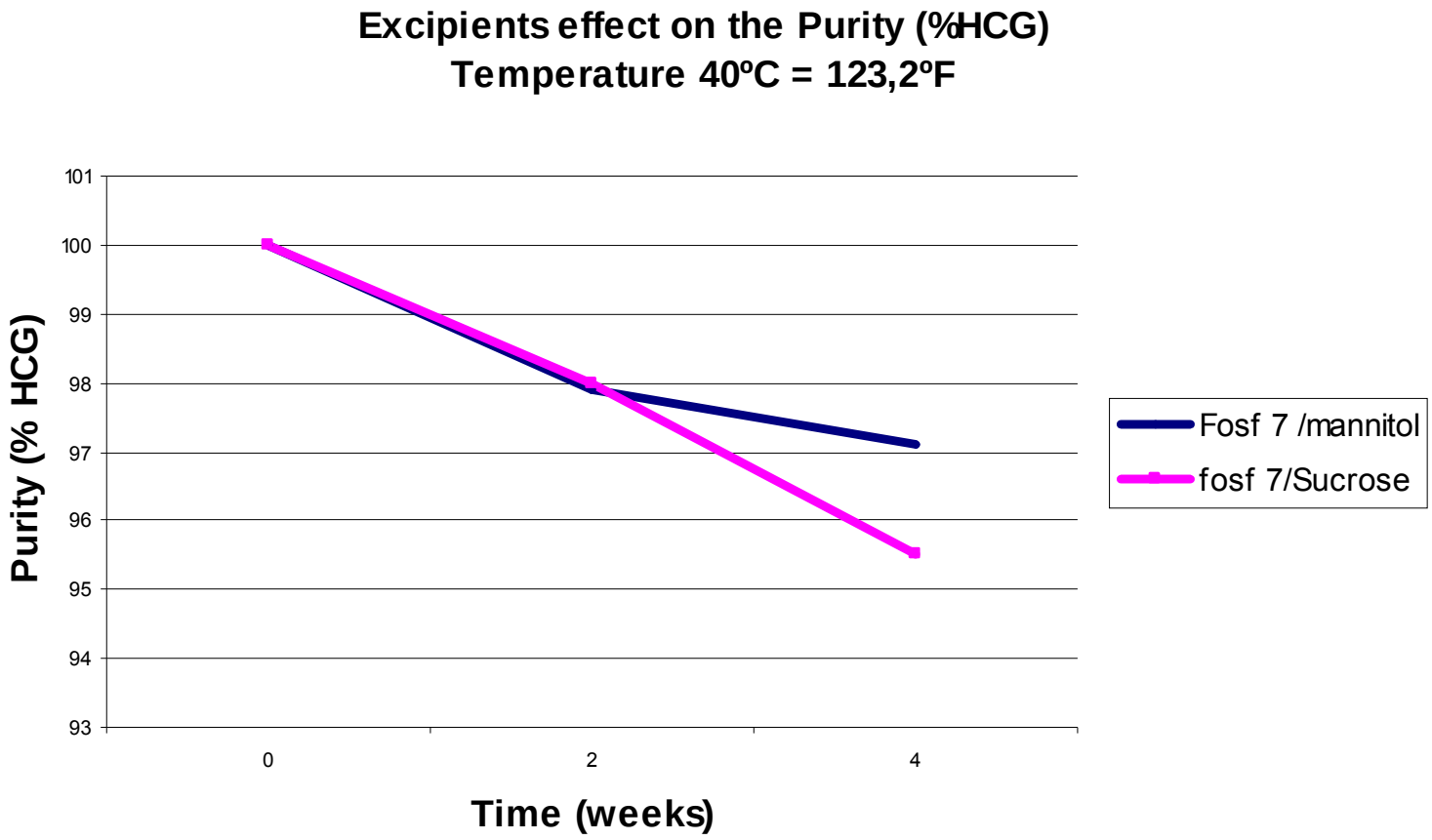
- We can observe the stability data obtained for both samples in Graph 7 and Graph 8.
- Samples were also subjected to temperatures of 25ºC and 4ºC.



Graph 7

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results



Graph 8

HCG Stability Study: analytical method and results

- Influence of excipients on Purity (%hCG)

Temperature 50ºC = 154ºF				
Samples	Time 0 (weeks)	Time 1 (weeks)	Time 2 (weeks)	Time 6 (weeks)
Fosf 7 / mannitol	100	94	91.7	83.5
Fosf 7 / Sucrose	100	94.1	90.3	83

Temperature 40ºC = 123,2ºF			
Samples	Time 0 (weeks)	Time 2 (weeks)	Time 4 (weeks)
Fosf 7 / mannitol	100	97.9	97.1
Fosf 7 / Sucrose	100	98	95.5

HCG Stability Study: analytical method and results

- Influence of excipients on Purity (%hCG) (2)

Temperature 25ºC = 77ºF			
Samples	Time 0 (weeks)	Time 3 (weeks)	Time 6 (weeks)
Fosf 7 / mannitol	100	100	100
Fosf 7 / Sucrose	100	100	100

Temperature 4ºC = 12,32ºF			
Samples	Time 0 (weeks)	Time 2 (weeks)	Time 4 (weeks)
Fosf 7 / mannitol	100	100	100
Fosf 7 / Sucrose	100	100	100

HCG Stability Study: analytical method and results

Comparative Study – Excipients

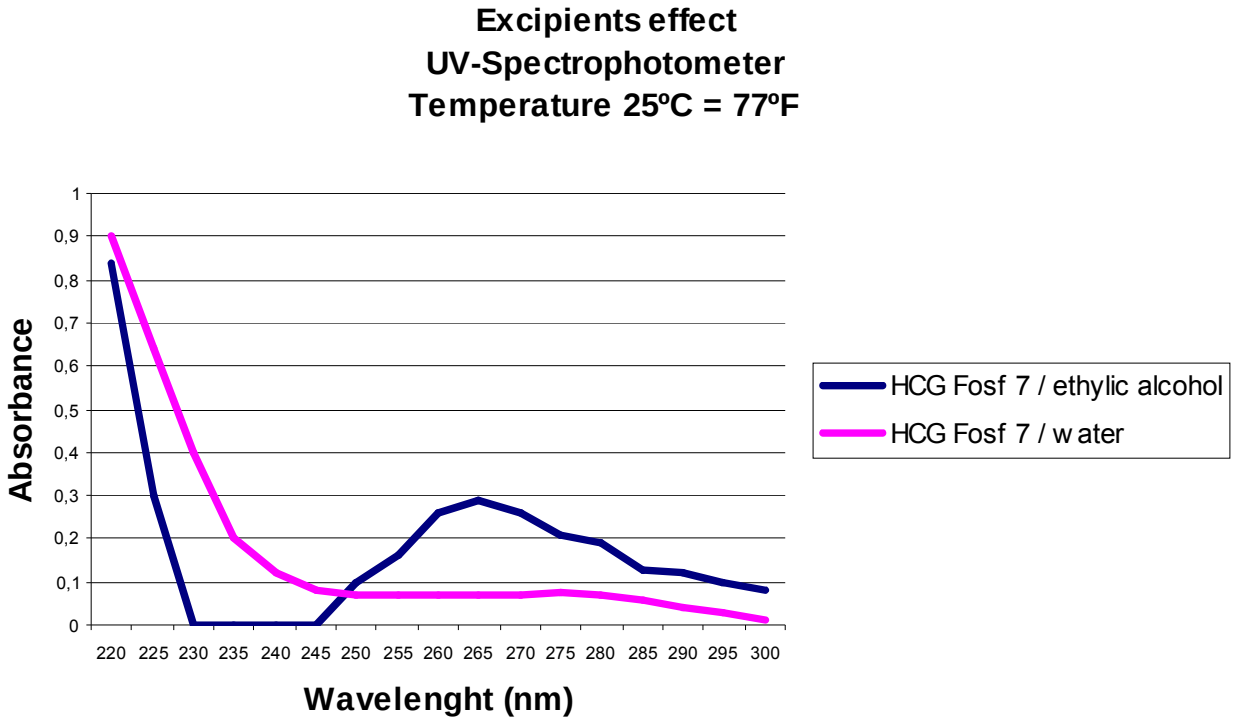
- To qualitatively evaluate the influence of excipients on the chemical stability of hCG in liquid solutions, two samples were analyzed by ultraviolet spectrophotometry (400nm–200nm) Their composition is observed in Table 5:

Samples	Composition
HCG Fosf 7 / Water	HCG 5000 IU / 10ml Phosphoric acid 85% 0.98mg Sodium hydroxyde solution 1M q.s to PH 7 Water injection q.s to 10 ml
HCG Fosf 7 / Ethylic alcohol	HCG 5000 IU / ml Ethylic alcohol 5% v/v Phosphoric acid 85% 0.98mg Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 10ml

HCG Stability Study: analytical method and results

Working Conditions:

- The samples under study were analyzed by a Beckman 25 spectrophotometer, scanning between 300nm–220nm, using quartz buckets of 10 mm of width and maintaining temperature between 22ºC–25ºC in a thermostatic bath.
- The obtained specters of both samples are observed in Graph 9:



Graph 9

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

Conclusion:

- The spectrophotometric comparative study of both samples evidences that the use of ethyl alcohol as excipient has a negative effect of the chemical stability of hCG in liquid solution.

HCG Stability Study: analytical method and results

4 – Temperature

- To assess the effect of temperature on the chemical stability of hCG in liquid solution, two groups of samples were prepared as observed in Table 6.
- Both groups were submitted to temperatures of 25ºC, 55ºC, 65ºC, 80ºC and then analyzed by HPLC or High Performance Liquid Chromatograph

Table 6

Samples	Composition
A, C, E, G.	HCG 15000 IU / ml Buffer Phosphate 0.01M Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1 ml
B, D, F, H.	HCG 15000 IU / ml Sodium chloride 0.15M Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1ml

HCG Stability Study: analytical method and results

Working Conditions

Elution	Solution 0.1M potassium phosphate, PH 7 + solution 0.15M sodium chloride
Column	TSK – G 3000 SW
Flow Rate	1 ml/min
UV Detector	280 nm
Injection Volume	10 microliter

HCG Stability Study: analytical method and results

Stability of Human Chorionic Gonadotropin (HCG)

Temperature effect

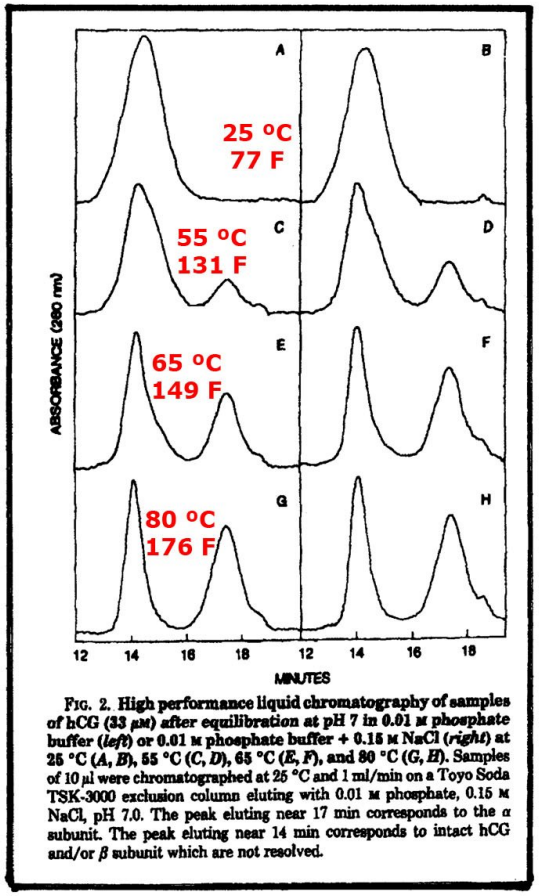


FIG. 2. **High performance liquid chromatography of samples of hCG (33 μM) after equilibration at pH 7 in 0.01 M phosphate buffer (*left*) or 0.01 M phosphate buffer + 0.15 M NaCl (*right*) at 25 °C (*A, B*), 55 °C (*C, D*), 65 °C (*E, F*), and 80 °C (*G, H*).** Samples of 10 μl were chromatographed at 25 °C and 1 ml/min on a Toyo Soda TSK-3000 exclusion column eluting with 0.01 M phosphate, 0.15 M NaCl, pH 7.0. The peak eluting near 17 min corresponds to the α subunit. The peak eluting near 14 min corresponds to intact hCG and/or β subunit which are not resolved.

Graph 10

- Graph 10 shows chromatographic results obtained for both sample groups.
- In both cases we can observe that temperatures above 25ºC initiate the process of pharmacologic modifications of the hCG molecule.
- These changes are evidenced as signs or peaks that appear in different elusion times (14 and 17 minutes) and correspond to the dissociation of the hCG molecule into its alpha and beta subunits.

HCG Stability Study: analytical method and results

Stability of Human Chorionic Gonadotropin (hCG) Kinetic Analysis

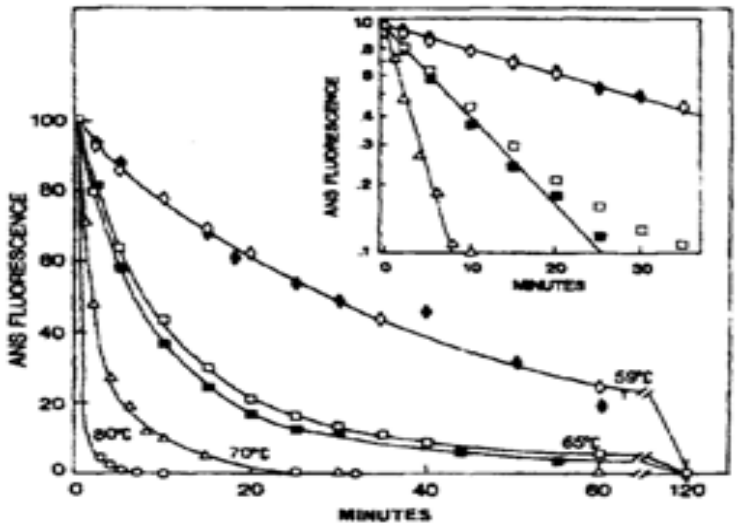


FIG. 5. The time course for the dissociation of 3.3 μM hCG at pH 7 in the presence (*filled symbols*) and absence of 0.15 M NaCl at 59 °C (◇), 65 °C (□), 70 °C (△), and 80 °C (○). The *inset* shows the first order kinetic plots for the data. Each point corresponds to a separate 250-μl sample of 3.3 μM hCG which was incubated at the desired temperature in a 0.5 cm diameter cuvette. The sample was removed at the indicated time and place on ice to quench the reaction. A small volume of concentrated ANS was subsequently added to give a final concentration of 250 μM and the fluorescence measured at 25 °C.

Graph 11

- According to this we can propose the following expression:

$$HCG \overset{Kd}{\leftrightarrow} \alpha + \beta$$

(Kd = dissociation constant)

- This constant can be calculated by studying the kinetic reaction of hCG at different temperatures.

- In Graph 11 and 12 we visualize the curves obtained through fluorescence (analytical method that allows us to measure light emissions- fluorescence) produced by certain substances in solution.

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

- The capacity of substances in solution to produce fluorescence will depend on: concentration, PH, temperature, presence of electrolytes, presence of other fluorescent substances.

- The sample under study (hCG) is treated with ANS (1,8 anilinonaphthalene sulphonate) fluorescent substance, and its fluorescence is measured given time at a determined temperature. Composition of samples is shown in Table 7:

Table 7

Samples	Composition
A (black dots)	HCG 1500 IU / ml Buffer Phosphate 0.01M Sodium hydroxide solution 1M qs to PH 7 Sodium chloride 0.15M Water injection q.s to 1 ml
B (white dots)	HCG 15000 IU / ml Buffer Phosphate 0.01M Sodium hydroxide solution 1M q.s to PH 7 Water injection q.s to 1ml

HCG Stability Study: analytical method and results

- On the curves obtained we can see that the dissociation reaction corresponds to a kinetic curve of 1st order represented in the following equations:

$$\text{Log } C = \text{Log } C0 - kd \cdot t / 2.303$$

$$t\ ½ = 0.693 / kd \qquad t\ 90 = 0.105 / kd$$

- Applying the Arrhenius equation to results obtained in Graph 9, we obtain Graph 10:

$$\text{Log } K2 / K1 = - Ea / 2 \cdot 303\ R\ (1/T2 - 1/T1)$$

K2 = dissociation constant at T2

K1 = dissociation constant at T1

HCG Stability Study: analytical method and results

Observations

- According to the straight line obtained in Graph 12 it is possible to infere (by extrapolation) that the value of the dissociation constant at a temperature of 37ºC has a value of 0.003 (minutes -1).

- Our conclusion is that in physiological conditions of PH and Temperature, the half life of hCG is of approximately 40 hours.

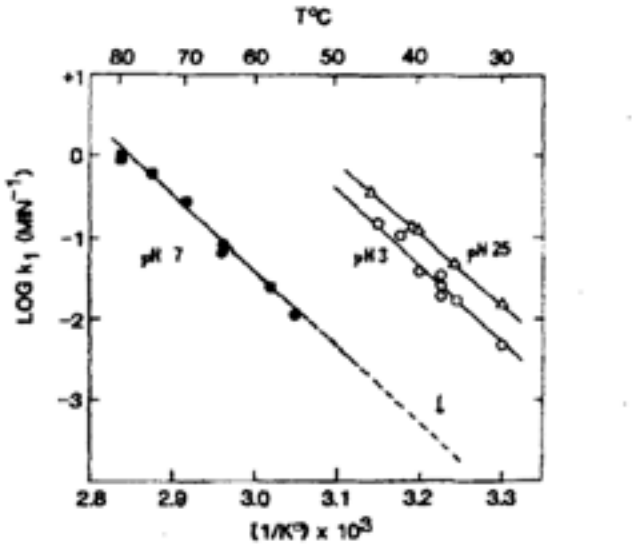


FIG. 6. **Arrhenius plots of first order rate constants for dissociation of hCG subunits at pH 7 (●), pH 3 (○), and pH 2.5 (△). The values at pH 7 were determined from the data in Fig. 5. The values at pH 2.5 and 3.0 were determined in separate experiments not shown. The *dashed line* shows extrapolation of the pH 7 data to 37 °C (*arrow*) where a $t_{1/2}$ of 40 h is predicted.**

Graph 12

The Oral hCG RESEARCH CENTER

HCG Stability Study: analytical method and results

Conclusions

✓ 1) PH

- hCG resulted more stable in a liquid solution at a pH of 7, over pH 6 and pH 8.

✓ 2)Temperature

- The hCG molecule is thermolabile. Thus, at a temperature above 25ºC its alpha and beta units are dissociated.

✓ 3)Electrolytes

- The presence of electrolytes or salts such as Sodium Chloride synergize the dissociation of the hCG molecule.

✓ 4)Half-life

- In physiological conditions of PH and temperature, the half life of hCG is of approximately 40 hours.

- Conclusions (II):

✓ 5) Ionic Force (influence of electrolytes)

- The chemical stability of hCG is negatively affected by the presence of electrolytes at temperatures of 50ºC and 40ºC. However, at temperatures between 4ºC and 25ºC the chemical stability depends on the electrolyte present in the solution.

- The comparative spectrophotometric study between two electrolytes (sodium chloride and silver nitrate) demonstrated that the use of sodium chloride favors the chemical stability of hCG in liquid solutions.

Conclusions (III):

✓ 6)Excipients

- The chemical stability of hCG is not modified by the actions of reduction sugars or poly alcohols at temperatures between 4ºC and 25ºC.

✓ 7)Alcohol- Silver

- The use of ethyl alcohol and/or silver as excipients have a negative effect of the chemical stability of hCG in liquid solution.