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## **Preparation and stability of cosmetic formulations with an anti-aging peptide**

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### **Synopsis**

Wrinkling of the skin is the most obvious sign of deterioration of the human body with age. This process involves a number of genetic, constitutional, hormonal, nutritional, and environmental factors, in addition to the influence of frequently repeated facial movements during laughing, smoking, etc. This article reviews the physiological basis and mechanism of action of the active cosmetic ingredient acetyl hexapeptide-8 (Argireline®). We prepared two formulations: an emulsion with an external aqueous phase for normal to dry skin, and a gel for oily skin. Laboratory analyses, rheology tests and *in vitro* release assays were used to evaluate the stability of these formulations for cosmetic treatment.

### **INTRODUCTION**

The search for new compounds to prevent or attenuate skin aging and enhance self-image (1) is a priority of current research on active cosmetics. Given the social implications surrounding physical appearance, we have undertaken work to investigate the treatment of facial expression wrinkles. Favorable results with botulin toxin infiltration led to the development of a new active principle with effects similar to the botox effect, named Argireline®, as an alternative to botulinum toxin.

Unlike other creams developed to treat aging wrinkles, the formulas tested in this study are intended to treat expression wrinkles. Substances with a botox-like effect act upon the same phenomenon as botulin toxin, but via a different mechanism of action. To understand the mechanism of action of the formulas we tested, a brief review of how expression wrinkles are formed may be helpful.

Expression wrinkles (2) form as a result of repeated muscle contraction caused by dermal atrophy and the appearance of hypodermal fibrosis (3). Facial movements cause cells of the dermis to contract and relax, and subject fibroblasts anchored by the network of

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collagen and elastin fibers to similar stresses. As a result, the skin becomes contracted into a permanent expression wrinkle, where the extracellular matrix of collagen and elastin has been found to break down (4).

Several processes are vulnerable to alteration from skin wrinkling in cosmetic terms (5):

- Neuronal exocytosis involves neurotransmitter release from synaptic vesicles into the synaptic space. Synaptic vesicles bearing neurotransmitters are taken up by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and fused with the cell membrane, releasing neurotransmitters in the process. The receptor complex consists of three proteins: synaptobrevin (VAMP), syntaxin, and synaptosomal-associated protein (SNAP-25) (6).
- Contraction and relaxation of fibroblasts, the cells that produce collagen and elastin and are responsible for maintaining the extracellular matrix, are transmitted to the connective tissue, where these forces successively stretch and relax the skin.

Specifically, type A botulin toxin produced by *Clostridium botulinum* acts by irreversibly destroying SNAP-25 protein in the SNARE complex, thus preventing the release of acetylcholine and paralyzing the involved muscle (7). Between 15 and 20 days after infiltration, new nerve endings are formed, and these endings become active within two or three months. After three to six months, nerve signals to the muscle are completely restored (8).

A novel aspect of Argireline<sup>®</sup> is its ability to act via topical application, which offers multiple advantages in comparison to formulations based on botulin toxin. The main advantage of Argireline<sup>®</sup> lies in its lower toxicity per unit weight. One gram of botulin toxin is enough to kill one million persons, whereas Argireline<sup>®</sup> is about 4000 times less potent, and thus constitutes a safer alternative for treating wrinkles (9). With the latter, injections to the face, which are potentially uncomfortable or painful, are unnecessary. Moreover, Argireline<sup>®</sup> can be used as an interim treatment between botulin toxin injections, since it prolongs the effects of Botox and reduces the frequency of microinjections. The synthetic peptide is cheaper, and is indicated for persons who have developed immunity to botulin toxin after prolonged use.

Argireline<sup>®</sup> (acetyl hexapeptide-8), the active principle in the formulations studied here, is a hexapeptide formed from a chain of six amino acids linked via a synthetic process. This peptide has two main actions. One is muscle relaxation by inhibiting the SNARE complex, but unlike botulin toxin, Argireline<sup>®</sup> does not irreversibly destroy the SNAP-25 protein, but modifies its conformation and competes with it for sites in the SNARE complex. The hexapeptide, an analog of the N-terminal end of the SNAP-25 protein, does not completely destroy the SNARE complex but only destabilizes it slightly, such that the synaptic vesicles cannot bind and release acetylcholine efficiently (10). As a result, a degree of neurotransmission is preserved in equilibrium with muscle relaxation, muscle contraction is attenuated, and wrinkle formation is prevented (11). Argireline<sup>®</sup> is also able to relax fibroblasts that relax the collagen and elastin matrix, through a mechanism involving calcium ion uptake.

The main objective of this study was to develop a formulation that ensured transformation of the active principle (acetyl hexapeptide-8) into a cosmetically active product. We therefore studied stability, defined as the ability of the formulation to maintain its initial characteristics. The parameters we measured as relevant to structural changes that can occur in cosmetic formulations were changes in organoleptic characteristics (a fun-

damental consideration for user acceptability), heat stability, and rheological characteristics over time and at different temperatures.

## MATERIAL AND METHODS

### MATERIALS

The products used as components in our formulations were:

- Argireline® (acetyl hexapeptide-8), Batch F1460/04, supplied by Lipotec (Barcelona, Spain).
- Neo PCL o/w Autoemulsionable® (cera alba, stearyl heptanoate, cetearyl octanoate, cetyl palmitate, stearyl alcohol, steareth-7, steareth-10, stearyl caprylate, isopropyl myristate, myristyl alcohol, dimethicone, paraffinum liquidum), Batch 0512651, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Tefose 2561 (PEG-6 stearate, Ceteth-20, glyceryl stearate, Steareth-20), Batch 0503697, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Cyclomethicone pentamer (cyclopentasiloxane), purity 99.26%, Batch 0509565, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Sorbitol 70%, Ph. Eur., purity 70.1%, Batch 0405020, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Glycerol, Ph. Eur., purity 99.8%, Batch 05F0204, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Hispagel 200®, Batch 0409242, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Propylene glycol, Ph. Eur., water content <0.1%, Batch 04K23FP, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Phenonip® (phenoxyethanol, methylparaben, butylparaben, ethylparaben, propylparaben), Batch 0510592, supplied by Roig Farma-Fagron (Terrasa, Spain).
- Kathon CG® (methylchloroisothiazolinone 1.5%, methylisothiazolinone 0.37%), purity 75.2%, Batch 0504885, pH 2.6.
- Deionized distilled water, supplied by Interapothek (Murcia, Spain).

### METHODS

*Preparation of formulations.* We prepared an oil/water emulsion for normal-to-dry skin and a cream for oily skin. Argireline® is sold under the brand name Lipotec® as a transparent aqueous solution that contains 0.5 g/l acetyl hexapeptide-8, 0.5% Phenonip®, and 99.45% water. It was refrigerated upon arrival at the laboratory and kept at 4°C, and added cold to all formulations at a concentration of 5% Argireline® solution. The compositions of the cream and gel formulations are listed in Table I.

The cream was prepared by weighing the ingredients of the oil and water phases, heating the oil phase until all components had melted, heating the water phase to the same temperature under gentle shaking to ensure homogeneity, and obtaining the emulsion by adding the water phase to the oil phase. The system was stabilized by gentle shaking while the formulation cooled to room temperature.

The gel was prepared by weighing all ingredients, slowly adding water, and shaking gently (to avoid the formation of bubbles) until a gel had formed. The formulations were

Table I  
Formulas for the Gel and Cream Formulations with Argireline®

Formula	Composition
Gel	Hispagel 200 25% Propylene glycol 3% + Argireline® 5% Phenonip 0.3% Water to a volume of 100 ml
Cream	Oil phase Neo PCL O/W 23% Tefose 1.5% Cyclomethicone pentamere 2% Water phase Sorbitol 4% Glycerine 4% Kathon 0.1% Water to a volume of 100 ml + Argireline® 5%

stored at 4°C and room temperature (25°C). To prepare cream and gel we used a propeller Heidolph RZR 1.

*Organoleptic characteristics.* Organoleptic characteristics were classified with descriptive terms (12) as thick, hard, creamy, smooth, soft, dry, thin, spreadable, cool, or warm. The cream and gel were scored for color, odor, texture, consistency, and appearance (exudates) 24 h after preparation and after storage at both temperatures for 30, 60 and 90 days, six months, and 12 months.

*pH.* Chemical stability was evaluated as pH during storage for three months to predict the behavior of the formulations in contact with human skin. To measure pH we used a Crison 501 digital pH/mV-meter with the electrode for viscous samples.

*Rheological characteristics.* The rheological properties of the formulations were studied as viscosity, a parameter closely related with stability (13). Assays were run at increasing shear rates in a Brookfield DV II+ viscosimeter (Brookfield Engineering Laboratories, Stoughton, MA) connected to a PC with the appropriate software. Rheological data were recorded periodically during a maximum period of 30 days.

*Stability.* The activity of Argireline® peptide was studied as the effect of temperature on the stability of the active principle. Samples of the commercially available Argireline® solution were stored at 25°C, 40°C, and 60°C in an incubator for 24 h, and activity was then determined with high-performance liquid chromatography.

#### *In vitro release*

Assays with no membrane. To avoid manipulations and vehicles that might interfere with the cutaneous release of Argireline®, we studied release from the gel and cream formulations *in vitro*. In this study we tested diffusion in a system with no membrane, in which the excipient and the receptor phase were in direct contact (14,15). Both formulations were also studied in an *in vitro* release system that simulated the physiological conditions of drug desorption (16). To simulate these conditions, the formulations were placed in a 32°C bath at 60 rpm in the release media phosphate-buffered solution (PBS) at pH 5.6. Release was measured by spectrophotometry over time and at

a wavelength of 260 nm, at which absorption of the active principle is maximal. The same formulation with no active principle was assayed as a control.

Diffusion across a membrane. Most published studies involve Franz-type cells (17–19). The FDC-400 cell (Vidra-Foc, Barcelona, Spain) consists of two compartments with a membrane clamped between the donor and receiver chambers. As the receptor phase we used a phosphate-buffered solution at pH 5.6 (normal skin pH). Three types of membrane (all 47 mm in diameter with 0.45- $\mu$ m pore size) were tested: methylcellulose, nylon, and polysulfone (supplied by Millipore, Madrid, Spain).

The concentration of Argireline® in the receptor cell was measured by UV-spectrophotometry at 260 nm ( $\lambda_{max}$ ). The method was previously validated and verified for accuracy, precision, and linearity (20). A Perkin Elmer UV/Vis Lambda 40 UV-spectrophotometer was used for all measurements.

## RESULTS AND DISCUSSION

The data are given as the mean and standard deviation of six determinations made with samples of each formulation at each temperature and after each storage period. All results were compared by analysis of variance (ANOVA) for a 95% confidence level to identify significant differences.

### ORGANOLEPTIC CHARACTERISTICS

Tables II and III show the changes in organoleptic properties with time in the gel and cream formulations, respectively. The temperature or duration of storage did not significantly affect the external appearance or texture of either formulation after 12 months. After 30 days, refrigerated samples showed better consistency than samples stored at room temperature. Consistency tended to decrease in the gel formulation after 12 months, with no differences between samples stored under refrigeration or at room

Table II  
Changes in Organoleptic Characteristics of the Gel Formulation During Storage

Storage conditions		Organoleptic characteristics				
Time	Temp. (°C)	Color	Texture	Odor	Consistency	Exudate
0 days	4	Transparent	Smooth, thin, cool	Noticeable	Viscous, easy to spread	No
	25	Transparent	Smooth, thin	Noticeable	Viscous, easy to spread	No
30 days	4	Unchanged	Unchanged	Unchanged	Unchanged	No
	25	Unchanged	Unchanged	Unchanged	Thinner	No
60 days	4	Unchanged	Unchanged	Unchanged	Unchanged	No
	25	Unchanged	Unchanged	Unchanged	Unchanged	No
90 days	4	Unchanged	Unchanged	Unchanged	Unchanged	No
	25	Unchanged	Unchanged	Unchanged	Unchanged	No
6 months	4	Unchanged	Unchanged	Unchanged	Unchanged	No
	25	Unchanged	Unchanged	Unchanged	Unchanged	No
12 months	4	Unchanged	Unchanged	Change	Decrease	Yes
	25	Unchanged	Unchanged	Change	Decrease	Yes

Table III  
Changes in Organoleptic Characteristics of the Cream Formulation During Storage

Storage conditions		Organoleptic characteristics				
Time	Temp. (°C)	Color	Texture	Odor	Consistency	Exudate
0 days	4	White	Smooth, creamy	Noticeable	Viscous, easy to spread	No
	25	White	Smooth, creamy	Noticeable	Viscous, easy to spread	No
30 days	4	Unchanged	Unchanged	Unchanged	Viscous, harder	No
	25	Unchanged	Unchanged	Unchanged	Viscous, softer	No
60 days	4	Unchanged	Unchanged	Unchanged	Unchanged	No
	25	Unchanged	Unchanged	Unchanged	Unchanged	No
90 days	4	Unchanged	Unchanged	Unchanged	Unchanged	No
	25	Unchanged	Unchanged	Unchanged	Unchanged	No
6 months	4	White	Smooth, creamy	Unchanged	Viscous, harder	No
	25	White	Smooth, creamy	Unchanged	Viscous, harder	No
12 months	4	White	Creamy, hard	Unchanged	Viscous, harder, crust formation	Yes
	25	White	Smooth, creamy	Unchanged	Viscous, harder	No

temperature. In the cream formulation, consistency increased after six months, and a crust had formed after storage for 12 months at 4°C.

#### pH

The data in Table IV show that pH was acidic in both of the freshly made up formulations, but was higher in the gel than in the cream. No significant changes over time were seen in either formulation regardless of storage temperature, a finding that makes these formulations suitable for topical application (only in regard to pH value).

#### RHEOLOGICAL CHARACTERISTICS

Rheological assays to measure viscosity under different storage conditions and at different times indicated that both formulations showed pseudoplastic behavior. Figures 1 and 2 plot the mean values for the cream formulation after 24 h, seven days, and 30 days of storage at 4°C and 25°C, respectively. Storage temperature had no significant effect on

Table IV  
Changes in pH During Storage

pH		Time (days)			
		0	30	60	90
Gel	4°C	6.18	6.1	6.18	6.12
	25°C	6.36	6	6.25	6
Cream	4°C	4.1	4	4.2	4.1
	25°C	3.92	4	3.95	4.05

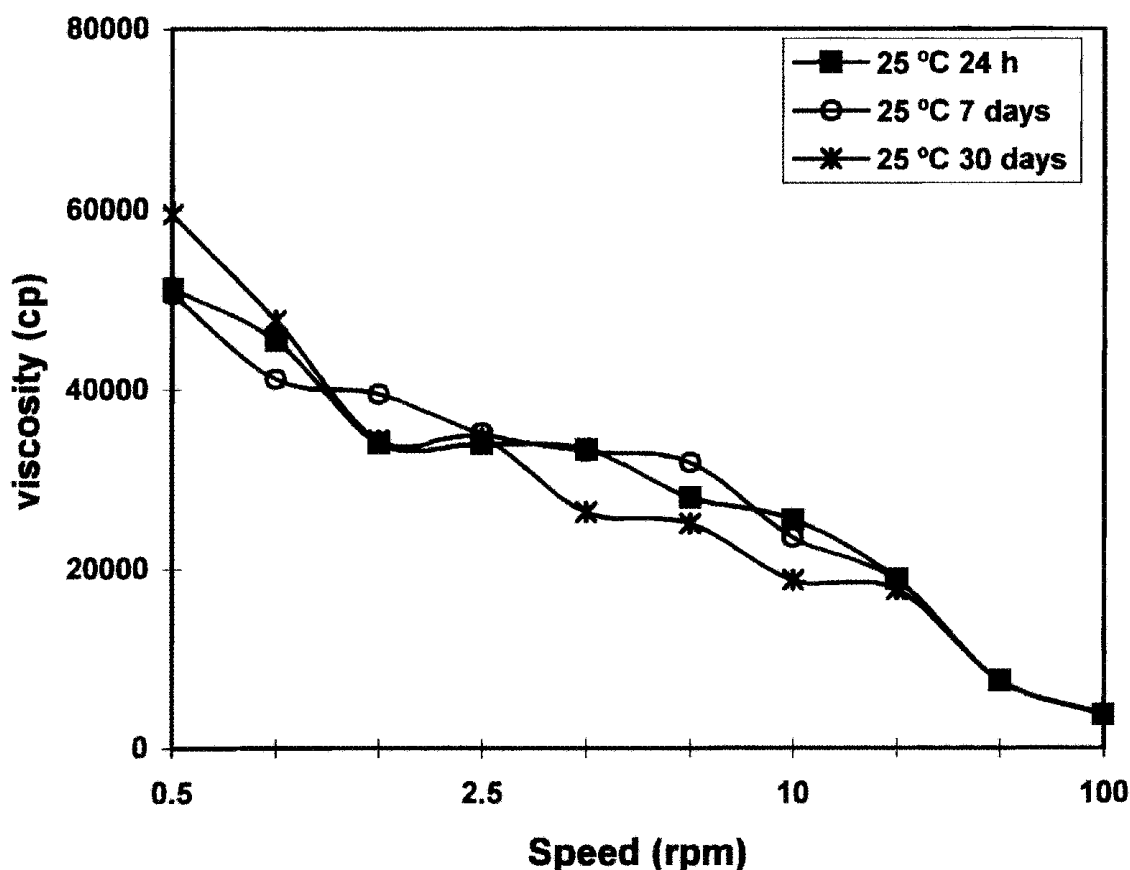


Figure 1. Viscosity vs speed of Argireline® creams maintained at 25°C as a function of storage time.

viscosity, and shear rates were the same in both samples at all assay times, a result that suggests that the cream formulation can be safely stored at room temperature.

Figures 3 and 4 show the findings for the gel formulation after storage for up to 30 days at the two temperatures. Viscosity was slightly lower in refrigerated samples than in samples kept at room temperature, as a result of thermal gelling (seen at low shear rate) (21). In samples tested after 30 days of storage, viscosity was the same at both temperatures.

At both storage temperatures, viscosity was lower in the gel than in the cream formulation. However, in general, temperature did not affect either formulation under our study conditions. No significant changes in rheological characteristics were seen in either formulation during the 30-day period in which viscosity was studied.

#### STABILITY

The chromatographic data are shown in Table V. Figures 5 to 7 are chromatograms of acetyl hexapeptide-8 at room temperature (25°C) and after being heated to 40°C and 60°C for 24 h. The presence of the active principle decreased to 58.8% and 41%, respectively, making extreme temperatures a factor to take into consideration in efforts to improve the stability of the active ingredient during storage and during heating, if this is required in the process of formulation.



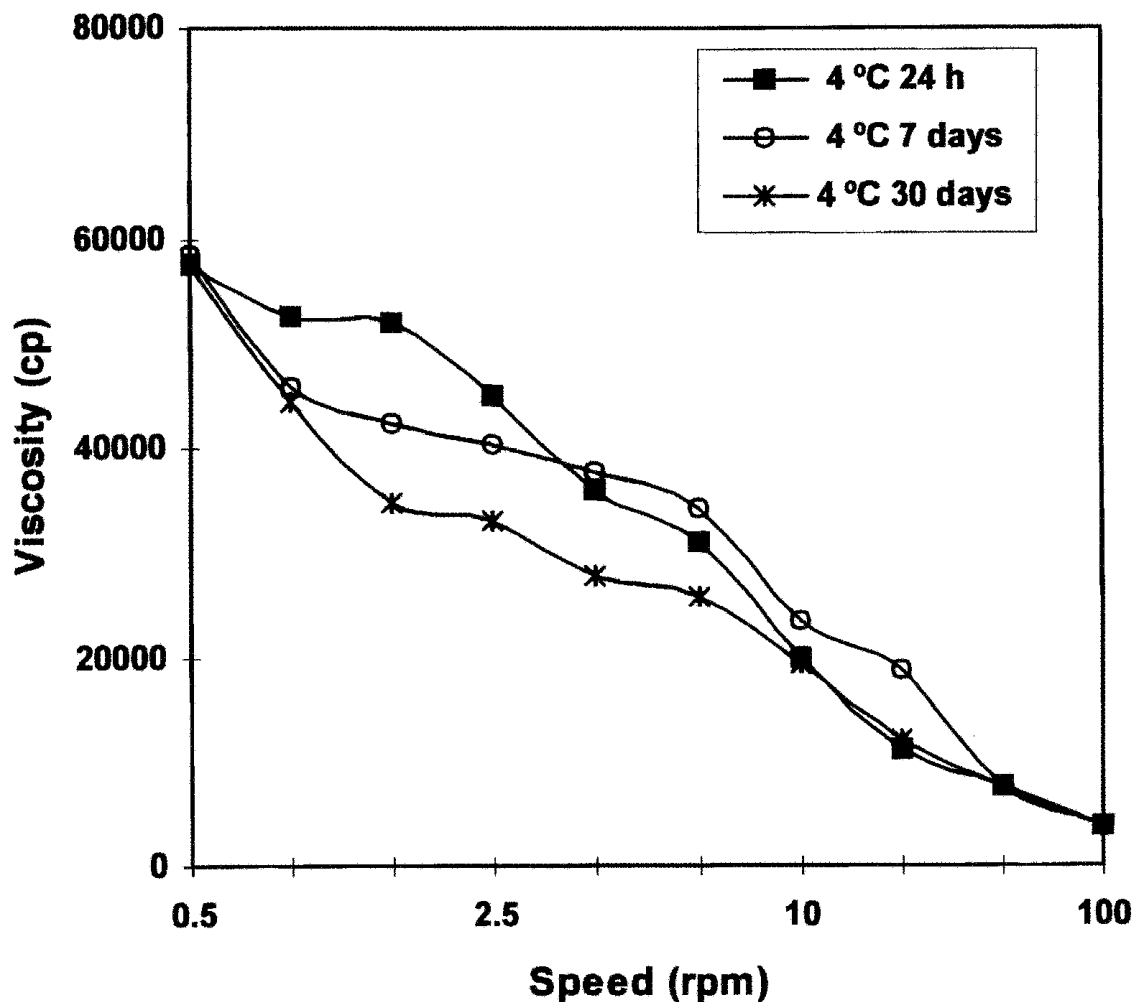


Figure 2. Viscosity vs speed of Argireline® creams maintained at 4°C as a function of storage time.

#### IN VITRO RELEASE

*Release assays with no membrane.* Figure 8 shows the percentage of acetyl hexapeptide-8 released from the cream and gel excipient into the medium with time in samples stored at 4°C and 25°C. In the cream formulation, release was greater from samples stored at room temperature than from refrigerated samples. The viscosity of the cream formulation at 25°C was lower than at 4°C; hence the faster release of the active principle. However, in the gel formulation, percentage release was lower from samples stored at 25°C than from refrigerated samples, because of gelling as noted above in the rheological assays (22).

The data showed an increase in release from both excipients with time at both temperatures, with maximal release after 90 min. The rate of release of the active principle was considered suitable for use in topical preparations since it did not interfere with other processes that take place when the active principle is placed in contact with the skin.

*Diffusion across the membrane.* We selected as the most suitable membrane that which offered the least resistance to diffusion of the active principle, in order to minimize the

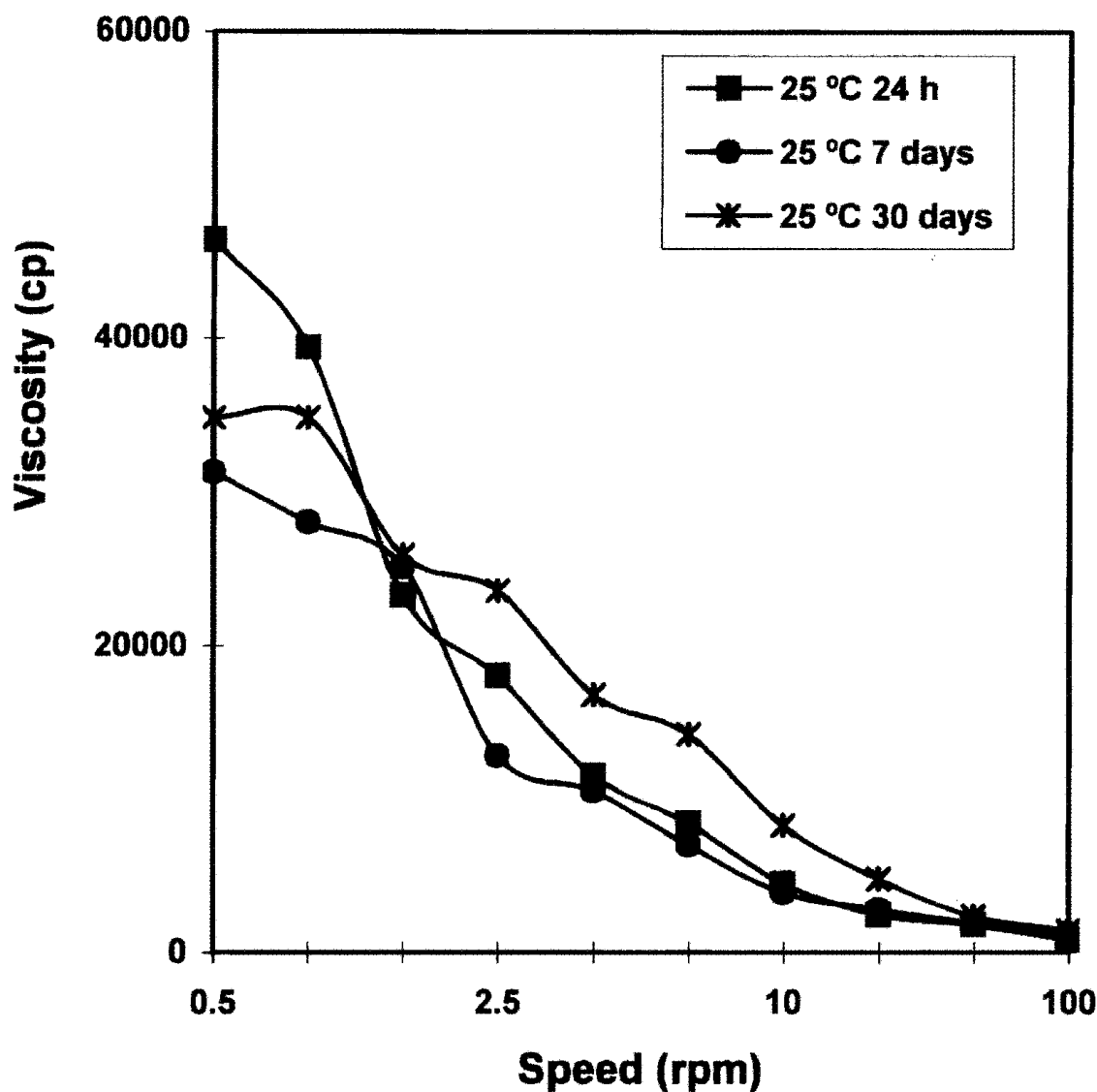


Figure 3. Viscosity vs speed of Argireline® gel maintained at 25°C as a function of storage time.

influence of the membrane on the results. For this study, a  $5 \text{ mg ml}^{-1}$  solution of Argireline® was used as the donor phase, and PBS (pH 5.6) was used as the solvent to prepare the drug in the donor phase. This buffer was also used as the receptor phase. We previously verified that sink conditions were maintained. Diffusion was slightly more rapid with the nylon membrane, and we therefore used this membrane for subsequent studies. Both preparations were subjected to *in vitro* diffusion assays in a Franz cell. All assays were performed under the same conditions as described above in the section on membrane selection.

Release assays to measure the amount and percentage of active principle in the receptor cell (Figures 9 and 10) showed that release was greater from the cream formulation (50% after five hours) than from the gel (20% after five hours). The difference may reflect thermal gelling of the latter formulation upon contact with the dispersion medium *in vitro*, an effect that would be expected to increase viscosity. Diffusion of the peptide was first detected ten minutes after the assay was started.

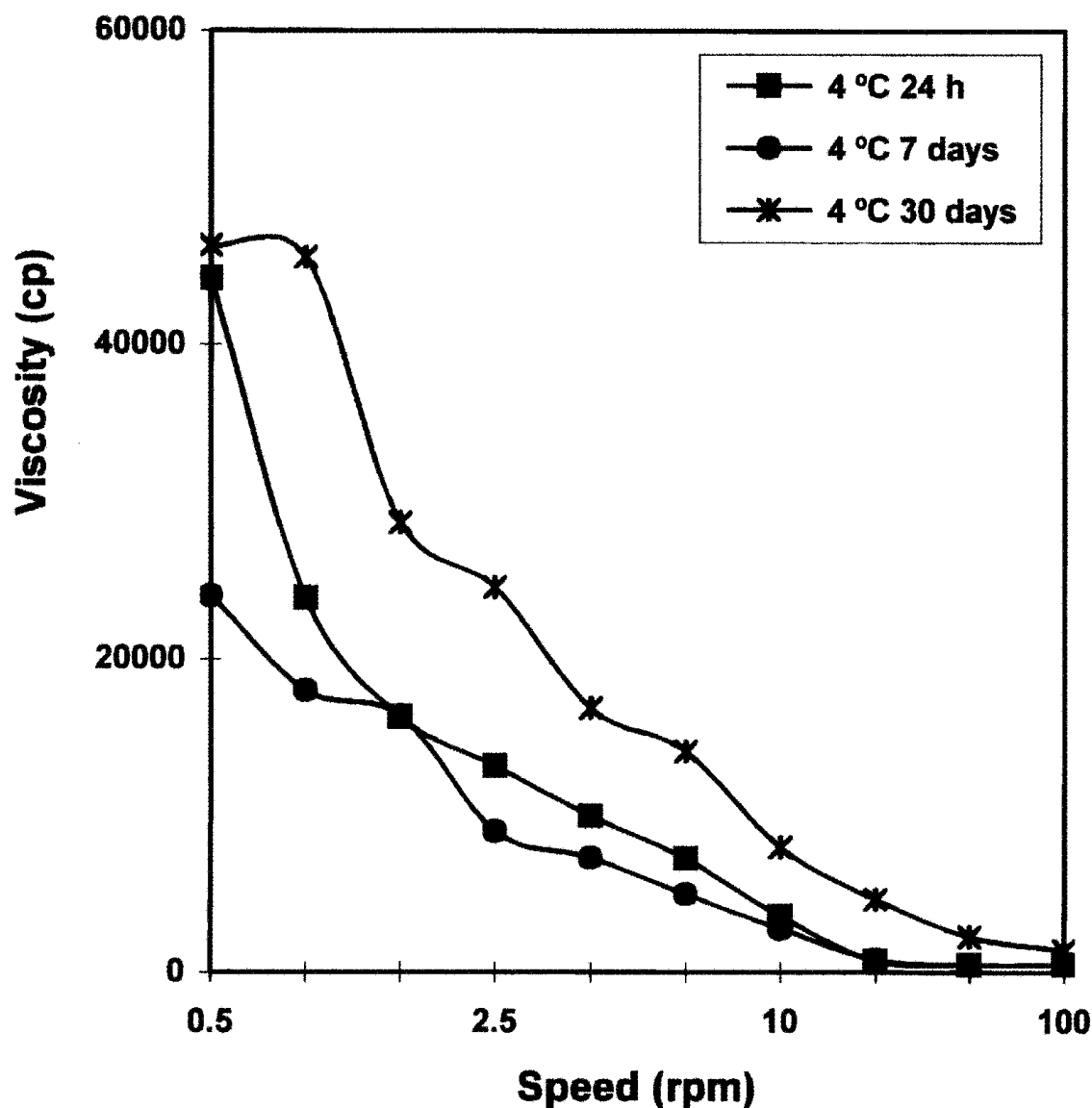


Figure 4. Viscosity vs speed of Argireline® gel maintained at 4°C as a function of storage time.

Table V  
Chromatographic Parameters of Stability at Different Temperatures

Temperature (°C)	RT	AUC	µm/ml	%
25	18.5	1899098	500	100
40	18.5	1119000	294	58.80
60	18.5	781954	205	41

Although the rate of absorption below 50% with both formulations may appear low, the results in general show that both the cream and the gel formulations satisfied the requirements for cosmetic products intended for topical application, since the cosmetically active substance, 8-acetyl hexapeptide, is targeted to treat the most superficial layers of the skin (23).

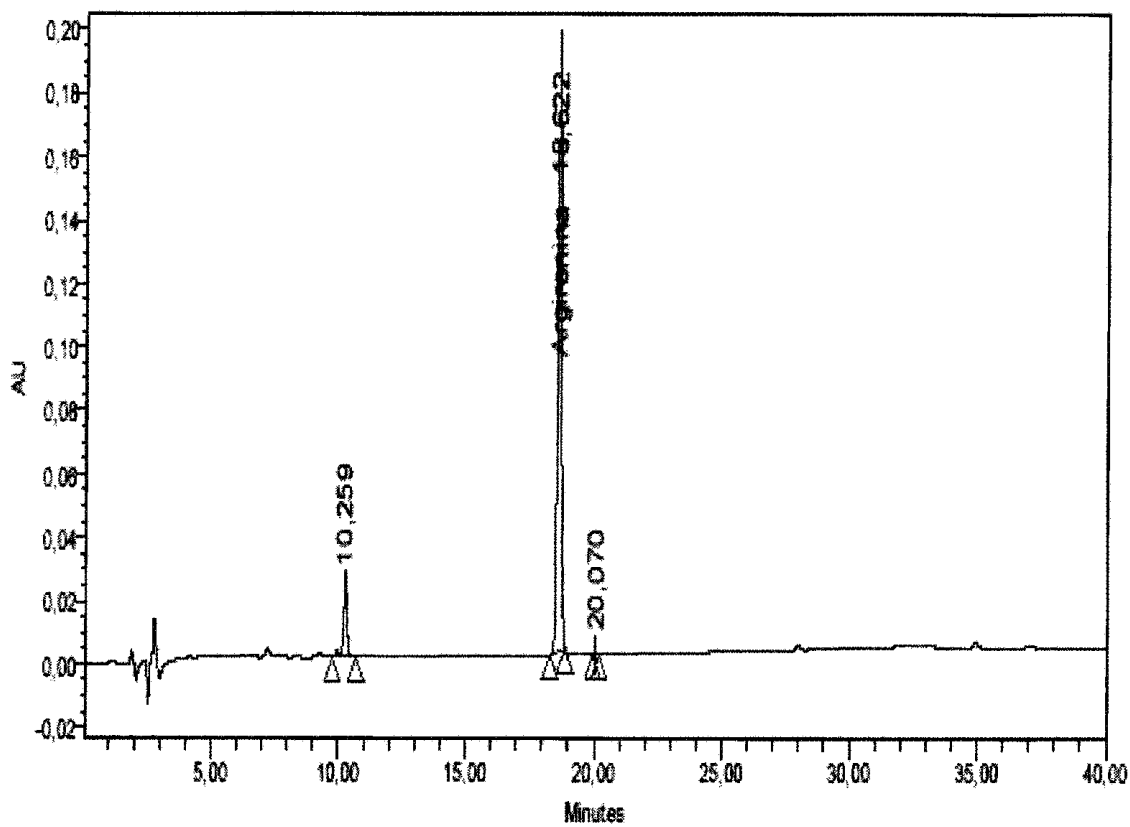


Figure 5. Chromatogram of Argireline® at initial time.

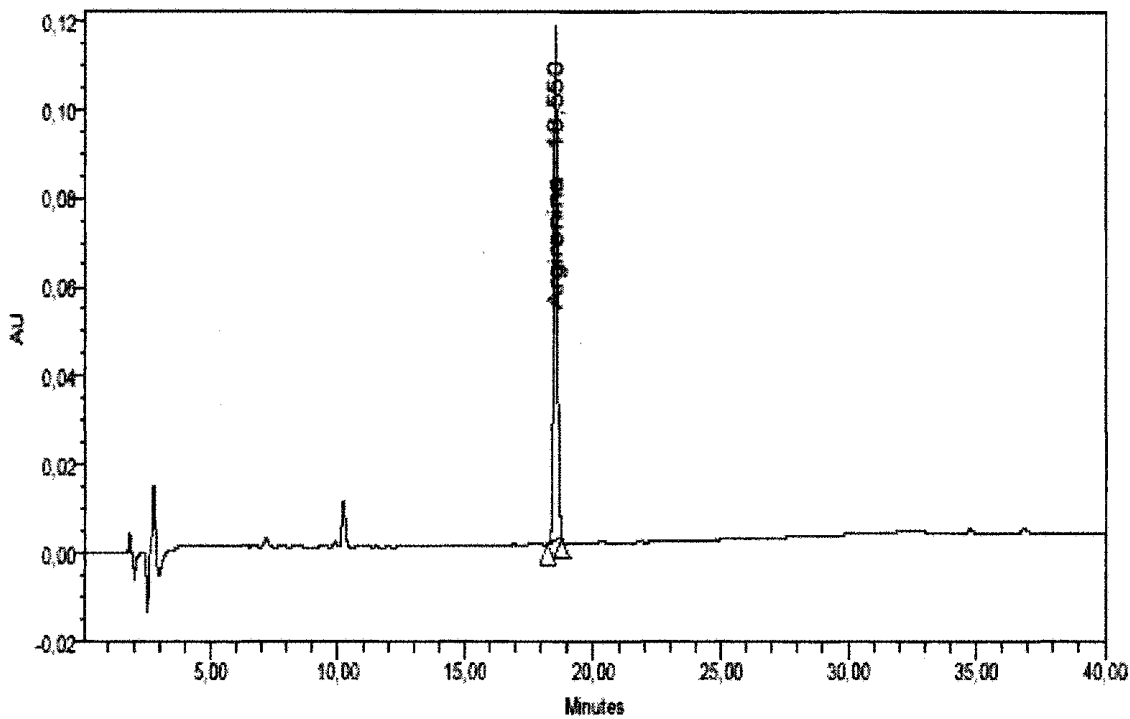


Figure 6. Chromatogram of Argireline® 24 hours after being subjected to temperature of 40°C.

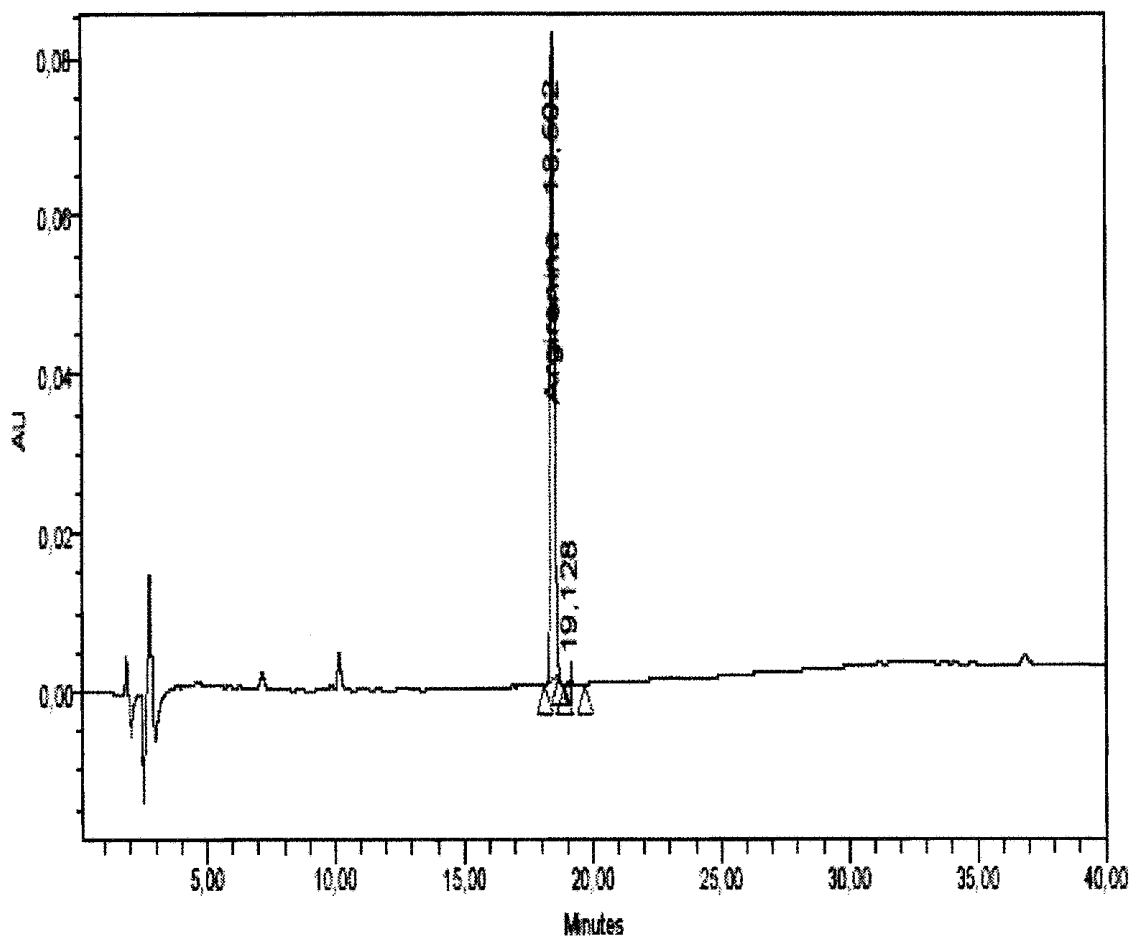


Figure 7. Chromatogram of Argireline® 24 hours after being subjected to temperature of 60°C.

Its mechanism of action differs from that of botulinum toxin (24). It penetrates the stratum corneum but does not penetrate the dermis (5). Its sites of action are the nociceptors, thermoreceptors, and mechanoreceptors connected to the nervous system via afferent fibers, which in turn are connected to the underlying musculature. This enables 8-acetyl hexapeptide to act upon muscle fibers without penetrating the muscle tissue (25).

## CONCLUSIONS

The formulations we tested showed good thixotropy and a slightly acid pH, and their rheological behavior and organoleptic properties were stable for the most part under the temperature and storage conditions reported here (4°C and 25°C). Interestingly, we found evidence of activity of the active principle under extreme temperature conditions (40°C and 60°C).

The excipients did not impede the release of 8-acetyl hexapeptide or contact with the skin, and facilitated release throughout the 90-min assay period. Release was greater from samples stored at room temperature than from refrigerated samples.

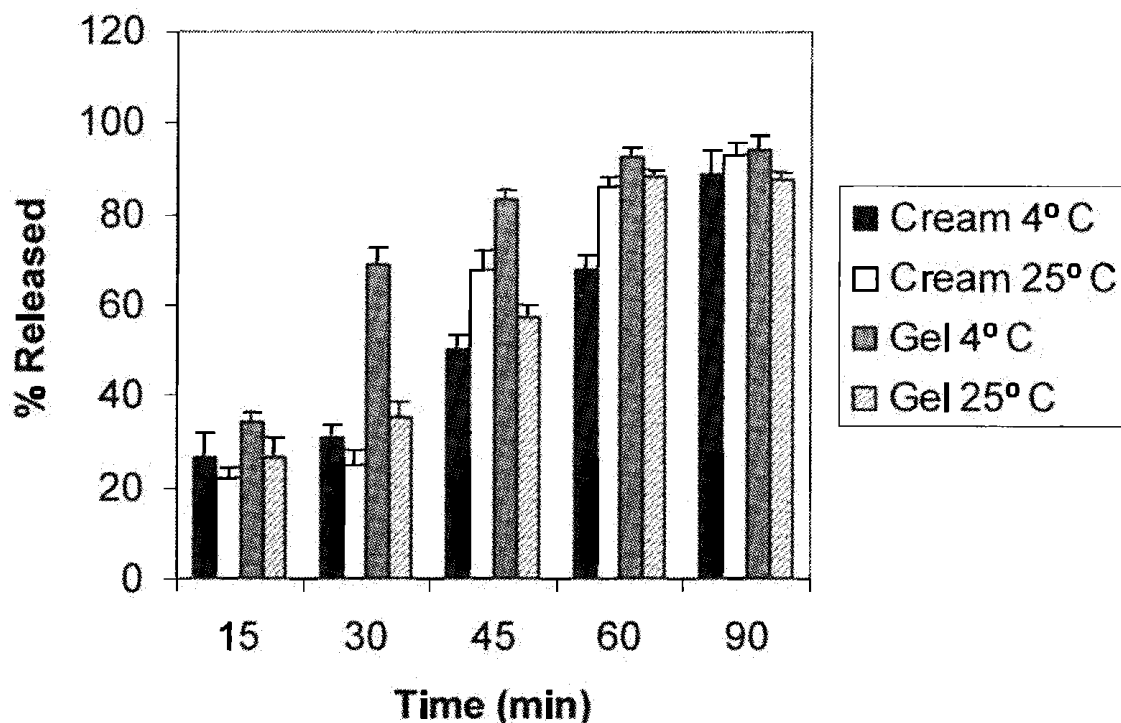


Figure 8. Release without membrane of the Argireline® samples maintained at 25° and 4°C.

The gel formulation showed evidence of thermal gelling during the first 15 days of storage after preparation, and this reduced diffusion of the peptide from samples stored at 25°C. The results of *in vitro* assays confirmed that the active principle penetrated the artificial membrane and that it is a suitable delivery from both excipients.

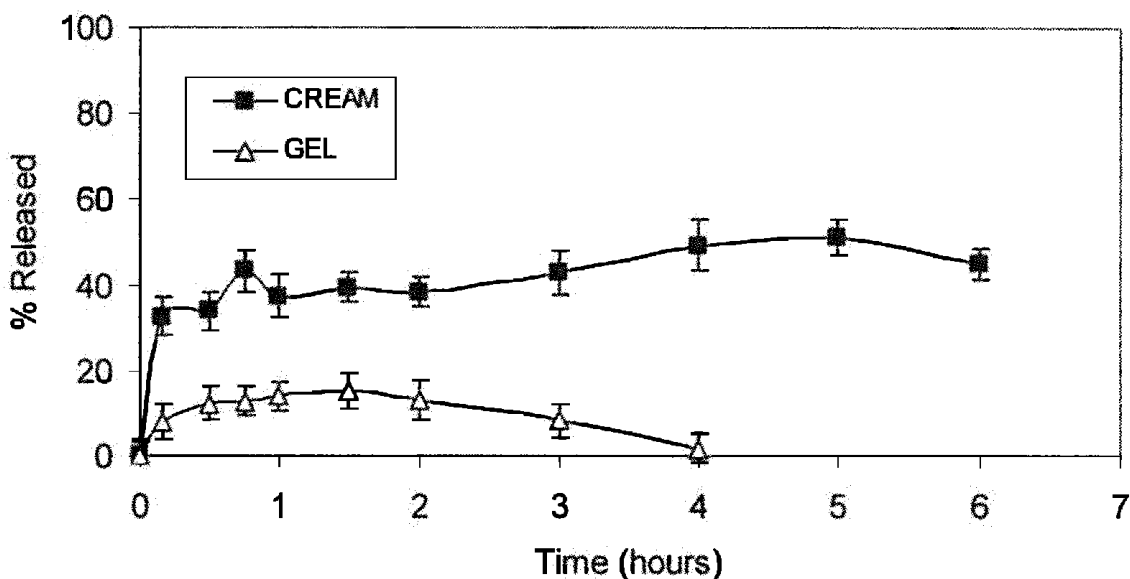


Figure 9. Percentage of Argireline® released from the gel and cream as a function of the time.

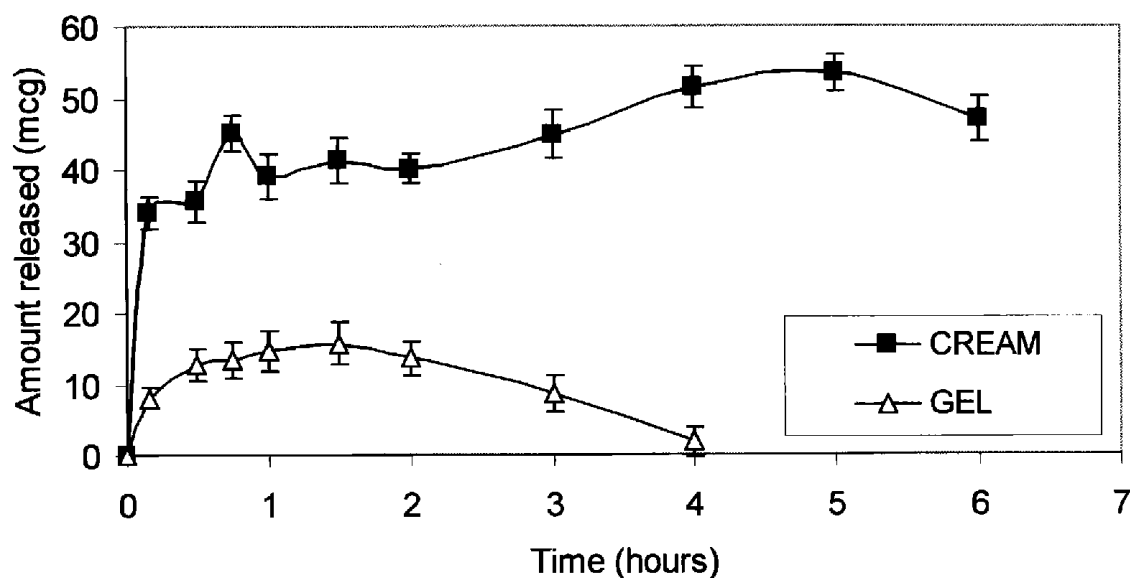


Figure 10. Amount of Argireline® released as a function of the time for the two tested samples.

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