RESEARCH

Quantification and determination of topical delivery of epidermal growth factor from semisolid formulations

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ABSTRACT

In order to obtain a procedure to quantify EGF by ELISA from semisolid formulations different buffers and organic solvents were used, having different polarities and miscibility with water. We studied it from oil-in-water (o/w) cream, water-in-oil (w/o), polyethylene glycol (PEG) ointment, and a jelly. Precision, accuracy and selectivity were determined and variation coefficients were less than 10% after different extraction method with a recovery of 95.54 -108.98%. According to results, it is possible to estimate the EGF concentration with high precision and reproducibility. No statistically significant differences were detected, when compared a placebo extraction solution with the ELISA standard solution demonstrating that placebo does not produce interferences in the quantification of the molecule. To determine the delivery of the active ingredient from different preparations we used a static diffusion cell system to evaluate the release and penetrability of the active ingredient throughout abdominal pig skin. Results showed that release and penetrability of EGF increased proportionally with the hydrosolubility of the vehicle. Therefore, the rate was obtained in jelly, PEG > o/w > w/o. Additionally, PEG ointment and o/w cream allowed the highest distribution of labeled 125I EGF in the epidermis and dermis and receptor medium.

Keywords: Epidermal growth factor, release, quantification

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RESUMEN

Cuantificación del EGF contenido en formulaciones semisólidas. Determinación de la liberación tópica del Factor de crecimiento epidérmico. Con el objetivo de obtener un procedimiento para cuantificar el EGF mediante ELISA a partir de formulaciones semisólidas, diferentes tampones y solventes orgánicos con diferentes polaridades y miscibilidad en agua fueron usados. En este estudio las formulaciones utilizadas fueron una crema de aceite-en-agua (o/w), una agua-en-aceite (w/o), un ungüento de polietilenglicol (PEG) y una jalea. La precisión, la exactitud y la selectividad fueron determinadas y los coeficientes de variación fueron menores del 10 % luego de emplear diferentes sistemas de extracción, con una recuperación de 95.54-108.98 %. De acuerdo con estos resultados, es posible calcular la concentración de EGF con alta precisión y reproducibilidad. No se observaron diferencias significativas en la cuantificación del placebo comparada con la solución estándar del ELISA, por lo que se demostró que el placebo no produce interferencias en la cuantificación de la molécula. Para determinar la liberación y la penetrabilidad del ingrediente farmacéutico activo a partir de diferentes formulaciones se utilizó un sistema de celda de difusión estática y piel abdominal de cerdo. Los resultados mostraron que la liberación y la penetrabilidad del EGF aumentaron proporcionalmente con la hidrosolubilidad del vehículo. Por lo tanto, se obtuvo el siguiente orden de liberación jalea, PEG > o/w > w/o. Además, el ungüento PEG y la crema o/w permitieron una distribución mayor del EGF125I en la epidermis, la dermis y el medio receptor.

Palabras clave: Factor de crecimiento epidérmico, liberación, cuantificación

*I*ntroduction

Wound healing is a complex, integrated and critically orchestrated cascade of events to restore the skin barrier function [1]. Growth factors and their receptors regulate key events in this process. Therefore, an effective exogenous delivery of peptides and proteins would be crucial to enhance wound healing [2]. One of these factors, the epidermal growth factor (EGF) is a single 53 aminoacid polypeptide, originally isolated from submaxillary glands in mice [3]. Topical administration of this macromolecule accelerates regeneration of skin in partial thickness wounds, second-degree burns in pigs or dermatome wounds in humans [4]. Successful re-epithelialization was obtained in patients with chronic wounds [5].

However, the EGF administration should meet some pre-requisites to achieve therapeutic efficacy and significantly enhance wound healing. Some of these pre-requisites are prolonged local bioavailability of the active growth factor [6], treatment regimen [7], effective dose [8] and use of a protease inhibitor [9], among others. In this sense, Bukley *et al*. demonstrated that prolonged exposure to EGF enhances wound healing by its sustained release from pellets implanted in subcutaneous sponges, increasing the formation of granulation tissue when compared to daily injections of EGF into sponges or its topical application to surgical incisions in saline [6]. Additionally, formulating EGF in creams also provides a method to release this growth factor to partial thickness wounds for prolonged periods of time. Clinical trials using skin-graft-donor sites have been conducted to determine whether EGF would accelerate the rate of epidermal regenera-

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tion in humans. These studies have confirmed that EGF accelerates wound healing [5].

The aim of this study was to evaluate the release profile and the *in vitro* penetrability of EGF for four semisolid formulations containing it as an active pharmaceutical ingredient.

Materials and methods

Materials

Human recombinant EGF (hrEGF) with more than 95% of purity, monoclonal antibodies CB-EGF-1 mouse and CB-EGF-2 peroxidase-conjugated antibody were purchased by the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

Carbopol 940 was purchased from Noveon, Cleveland, USA.

EDTA, PEG 350 and 400, Cetyl alcohol, gelatin, white wax, cholesterol, stearyl alcohol, Chloroform, Carbon tetrachloride, Diethyl ether were purchased from Merk, Darmstadt, Germany.

Composition of the semisolid formulations

Hydrophilic jelly was elaborated using Carbopol 940, EDTA like enzyme inhibitor, water and preservatives.

A polyethylene PEG ointment was elaborated using PEG 350 and 4000 MW.

Emulsion o/w was elaborated using cetyl alcohol, EDTA, ionic emulsifiers, humectation agent, stabilizer, preservatives and water.

Emulsion w/o was elaborated with petrolatum, white wax, cholesterol, stearyl alcohol and water.

Manufacturing processes for semisolid are conventional method for PEG ointment and jelly. In the case of emulsions, fusion method was used [10].

EGF quantification in semisolid formulations

Different organic solvents and buffer solutions were used to extract the active ingredient, depending on the features of the semisolid preparations (Table 1).

Water or PBS were selected as extraction solvents, since the jelly and PEG ointment are water-soluble formulations. Tween 20-0.02% was also used for protein stabilization [11]. The w/o and o/w formulations required breaking the emulsion to separate the oil phase, and was achieved by adding chloroform, carbon tetrachloride or diethylether.

EGF was quantified by using an ELISA assay for jelly and PEG ointment after the total dissolution of 0.5 g of these semisolid formulations in either buffer or water.

The extraction of EGF from the other two formulations was accomplished by dissolving 0.5 g of each formulation in the organic solvent given in table 1, followed by adding a buffer solution or water to this suspension.

Each sample was centrifuged for 20 min at 1000 g using a Hitachi, HIMAC SCT5BA centrifuge. The aqueous phase was collected to quantify EGF by ELISA. Accuracy, precision and selectivity of the extraction method were also determined, as described below.

Accuracy determination

Accuracy was determined using semisolid formulations at different concentrations of EGF: 5 mg/g, 10 mg/g, 15 µg/g and 20 mg/g. Six replicas of each concentration were prepared, and the EGF was extracted, as explained above. Linear regression and the null hypothesis slope were equal to 1, and the intercept equal to 0 was tested. Accuracy was determined as the recovery of EGF after its extraction and quantification by ELISA, as compared with the optimal value of 100%.

Table 1. Solvents and buffers used to extract EGF from semisolid formulations.

Formulation	PBS 1X - Tween 20 - 0.02%	Water	Carbon tetrachloride	Chloroform	Diethyl ether
Jelly	5 mL	-			
	-	5 mL	-	-	-
PEG	5 mL	-			
		5 mL	2 mL	-	-
Cream o/w and ointment w/o	-	5 mL		2 mL	-
		5 mL	-	-	2 mL

Precision determination

The intra-assay variability was determined by a repeated measurement of the sample values in the same plate. Inter-assay precision was determined in six replicated extractions and quantification assays by two analysts for each assay at different times. For both precision assays as described above, the acceptance criteria were less than 10 and 20%, of the variation coefficient (CV) [12-14].

Specificity analysis

Specificity is the ability to asses unequivocally the analyte in the presence of components which may be expected to be present. Statistical comparison, using a Student's *t* test, between the solution obtained after achieving the extraction of placebo and ELISA standard solution (PBS and 0.5% w/v casein) was performed. These samples were analyzed by ELISA.

EGF release profile measurement

To determine the release profile of EGF, we used a diffusion cell described according to Estévez *et al.* [15]. A small teflon-covered magnet, rotating at 600 rpm was used to stir the receptor solution. The receptor chamber was kept at 37 ºC during the experiment. A cellulose acetate membrane with pore diameter of 0.2 mm was used to place the semisolid formulation. It was previously soaked in the receptor solution (phosphate-buffered saline 1X, PBS 1X) during 24 h at 4 ºC to diminish the lag time period.

One gram of the semisolid formulation containing 10 mg of EGF was placed in the receptor compartment, and 150 µL of the buffer solution were sampled at the initial time and after 0.5, 1, 2, 4, 6, 8 and 24 h. After sampling, the same volume of the buffer solution was replaced in the receptor compartment to maintain the total volume. Each sample was further analyzed in duplicates by ELISA.

Penetrability assay

A diffusion cell, assembled with pig abdominal skin punches, was used instead of cellulose acetate membranes, to determine the penetrability on EGF. The skin punches were incubated on receptor medium containing 0.025% gentamicine and PBS 1X during 4 h, at 4 ºC. The skin was placed inside the cell with the epidermis facing the donor chamber. The EGF used

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in this experiment was labeled with ^{125}I following essentially the chloramines-T method [16].

The percentage of EGF in the receptor medium, epidermis, dermis and semisolid was determined by measuring the remaining radioactivity in a gamma counter Wizard, Model 1470, Wallac.

ELISA

To quantify the hrEGF a sandwich-type ELISA was used. Polystyrene 96-well flat-bottom immuno-plate (Nunc, Denmark) were coated with 100 mL of the CB-EGF1 monoclonal antibody against the recombinant EGF, at 10 mg in 1 mL of 0.1 M Na_2CO_3/ $NaHCO_3$, pH 9.6-10.8, for 3 h at 37 ºC. Plates were washed twice with Tween 20 solution at 0.05%.

After washing, 100 mL of a standard EGF, control and unknown samples previously diluted in PBS, and 0.5% w/v casein were added to each well. Plates were incubated for 1 h at 37 ºC and then washed.

Eighty microliters of a peroxidase-conjugated antibody against the recombinant human EGF (CB-EGF2) were added to the plates and incubated for 1h at room temperature. After that time, plates were washed six times. The reaction was developed for 10 min by adding 100 mL of the substrate solution containing 5.5 µL 30% H_2O_2; 5.5 µg O- phenylendiamine and diluted in 0.1 M sodium citrate, pH 5.0. The reaction was stopped by adding 50 µL of 2 M sulfuric acid. Absorbance was measured by using a microtiter plate analyzer Multiscan, Merck-Sensident Scan, Darmstadt, Germany at 492 nm. Positive control and unknown samples concentrations were calculated by interpolating absorbances values in a standard curve generated, when absorbance was plotted against EGF concentration [17].

Statistical analysis

All measurements were expressed as mean ± standard error. Statistical differences were tested using the Student's t test with a significance of $p < 0.05$.

Results and discussion

EGF determination in semisolid formulations

EGF has been widely used in the treatment of damaged skin in semisolid formulation [6], because of that it is necessary to establish a method to extract EGF for quality control in pharmaceutical research and it is mandatory by many regulatory agencies [18]. One of the most commonly used methods to estimate the concentration of EGF is the enzyme-linked immunosorbent assay. In order to obtain a procedure to quantify EGF from semisolid formulations different buffers and organic solvents having different polarities and miscibility with water were used and then EGF was quantified by ELISA.

Figure 1 shows the recovery percent for each semisolid formulation after different extraction procedures described in table 1. The EGF recovery percent was over 90% for PBS 1X-Tween 20- 0.02% solution for jelly and PEG; water/chloroform and water/tetrachloride solutions for o/w; and water/chloroform, water/tetrachloride, water/diethylether solutions for w/o.

The obtained results were previous step for further experiment.

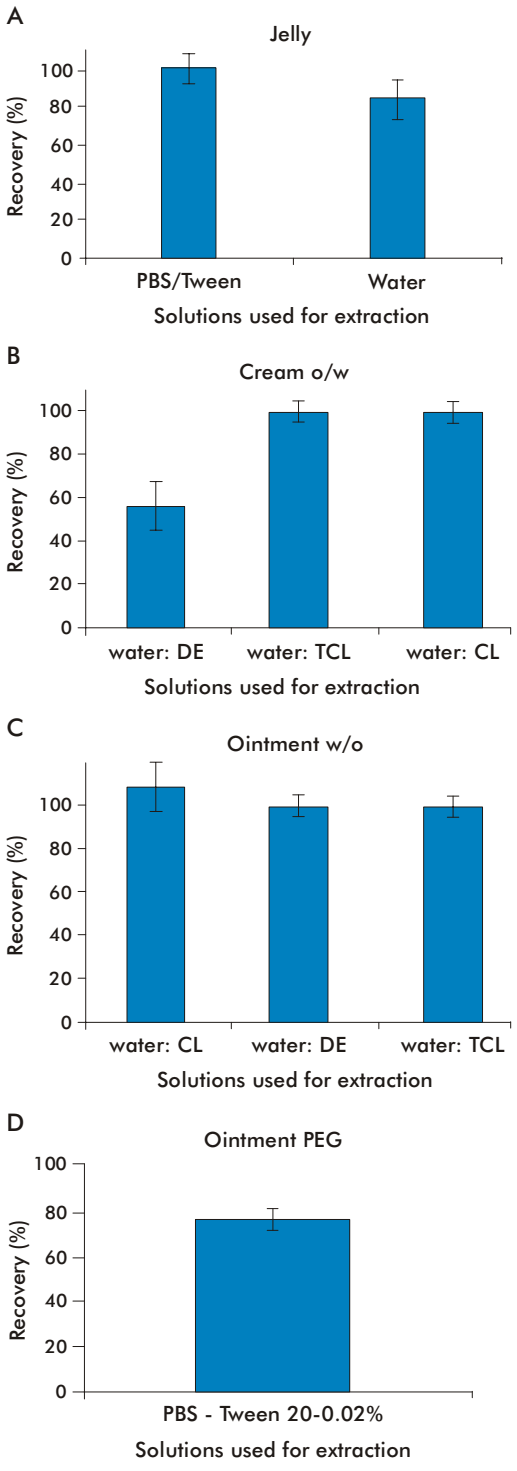

Figure 1. Recovery percent of each semisolid formulation after different extraction. Depicted the recovery percents for A) jelly, B) oil-in-water (o/w), C) water-in-oil (w/o) and D) ointment (PEG). CL- Chloroform, DE- Diethyl ether, TCL- Carbon tetrachloride.

Accuracy determinations

Accuracy was calculated as the percent of EGF recovery from the selected semisolid formulations, irrespective of the extraction method used. Results

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Table 2. Results of recovery percent and coefficient variation for accuracy assay for the EGF extraction methods.

Accuracy assay								
	jelly PBS - tween 0.02%		o/w Water-carbon tetrachloride		PEG PBS- tween 0.02%		w/o Water-Chloroform	
Theoretical Concentration (%)	Average Recovery (%)	CV (%)	Average Recovery (%)	CV (%)	Average Recovery (%)	CV (%)	Average Recovery (%)	CV (%)
50	108.9	9.8	107.3	8.3	95.8	7.1	95.5	6.6
100	98.0	7.8	99.4	9.0	106.9	9.9	100.5	8.8
150	104.2	8.2	97.2	9.2	99.9	7.7	102.7	8.2
200	107.1	9.9	107.2	7.9	100.7	8.1	103.1	5.9

obtained are showed in table 2. Extraction methods for PEG and Jelly in PBS 1X-Tween 20-0.02%, o/w in carbon tetrachloride and w/o in chloroform didn't show statistically significant differences ($p > 0.05$) in slopes and intercepts respect to values 1 and 0, respectively. Their respective R^2 values were 0.9893, 0.9908, 0.9845 and 0.9973. Each recovery percent was compared (n = 6) to the total amount of EGF formulated by a Student´s t test, and no significant differences were detected. The recovery range was from 95.5 to 108.9 which confirmed the reliability of the assay. All these indicated that both the extraction and quantitative determination methods were accurate. Similar results were not obtained for the rest of the formulations tested. Our results indicated that ELISA assay and the selective method for EGF extraction fulfill the criterion for measuring it in a semisolid formulation.

Precision determinations

Intra- and interassay variability data were used to determine the precision of the assay. Interassay and intrassay precision ranged from 4.6 to 9.9% and from 4.2 to 9.9%, respectively. Table 3 shows the repeatability and reproducibility of the assay under the experimental conditions used to demonstrate low intra and inter- plate variation. The coefficient of variation (CV) was calculated for each EGF semisolid formulation at 10 mg/g. Six quantifications were carried out. The CV was lower than 10% for protein quantification by ELISA, indicative of a precise method. This CV demonstrated that the assay is precise according to ICH requirements [13, 14].

Specificity analysis

In order to confirm that ELISA only detects EGF, specificity assay was performed. The specificity of assay was assessed by comparing a placebo formulation extraction with the standard solution (PBS and 0.5% w/v casein) used in ELISA quantification. This assay rules out any positive signal from the excipients of the formulation. No statistically significant differences were detected, by comparing a placebo extraction solution with the standard solution ($p > 0.05$), indicating that the assay was selective for EGF.

EGF release profile measurement

The overall effectiveness of a topical formulation depends on the release characteristics of the vehicle and the pharmacokinetics of the drug as it diffuses through the skin [10]. In this study the *in vitro* releases for four semisolid formulations containing EGF at 10 µg/g were evaluated. To achieve this, a range of semisolids with various flow characteristics [19] were formulated and the *in vitro* releases and penetrability were evaluated. In the jelly, PEG and o/w cream the compound forms a continuous structure in direct contact with the skin. On the other hand in the w/o ointment the active compound is isolated in individual compartments (droplets) and has to diffuse through the aqueous phase to reach the skin [20]. These characteristics allow different EGF release profile which in turn affects the subsequent percutaneous absorption [21]. Figure 2 shows the release of EGF from the investigated semisolids bases. All the evaluated semisolids showed different release profiles, an effect also resulting from the particular viscosity and hydrophilicity of these preparations [22]. The diffusion coefficients of the EGF from the aqueous solution and the jelly formulation were similar. In the case of jelly, a high percentage of EGF is found in the receptor fluid in a short period of time (about 72% in 8 h), whereas a 75% of EGF is released from the PEG ointment in the same period of time. Both semisolids had hydrophilic properties and low viscosity and thus the release was pronounced.

On the other hand, the presence of cetyl alcohol decreased the EGF release profile from o/w formulation due to the hydrophobic properties of the o/w cream and increased consistency of this base [21]. In this case 38% of EGF was found in receptor fluid of the system after 8h.

The oil phase would also modify the rate of protein release by increasing viscosity in the w/o formulation. This effect is more pronounced in the w/o variant, where the protein is emulsified as small aqueous par-

Table 3. Results of recovery percent and coefficient variation for precision assay for the EGF extraction methods.

CV(%) n=6				
	jelly PBS - tween 0.02%	o/w PBS 1X - Tween 20-0.02% carbon tetrachloride	PEG PBS- tween 0.02%	w/o Water -Chloroform
In tra-assay Analy st 1	7.8	9.0	9.9	8.8
In ter-assay Analy st 1	8.3	7.5	5.4	4.6
In tra-assay Analy st 2	9.9	5.2	6.5	9.5
In ter-assay Analy st 2	4.2	5.6	9.9	8.3

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ticles that have to migrate from inside the oleagirous phase also the higher viscosity resulting in a decrease in the molecular and the droplet diffusion. A comparison of release values of these formulations reveals the importance of the droplet diffusion on the release rate of EGF [22-24]. Therefore the same amount of EGF is released in a longer period of time than in the o/w formulation. Therefore, the profiles of o/w and w/o formulations were typical of systems with prolonged released profiles compared with hydrophilic bases, suggesting them as more efficient presentations for wound healing because the need of prolonged release of EGF in wound healing.

Penetrability assay

Considering the previous results, we further evaluated penetrability by substituting the cellulose acetate membrane by abdominal pig skin.

The release cell was loaded with 0.5 g of PEG, o/w or w/o formulations charged with EGF-^{125}I at 10 mg/g each. Figure 3 shows the distribution of EGF-^{125}I among both cell compartments and the epidermis and dermis. PEG and o/w formulations allowed a wider distribution of labeled protein into the skin layers (21 and 8% in the epidermis, and 41 and 36% in the dermis, respectively) and in the receptor medium. The behavior of the w/o formulation was lower (3% in the epidermis and 2% in the dermis). According to the bases nature and composition, hydrophilic bases would allow a higher delivery and penetrability of the active principle to the deepest skin zones than the oleaginous ones.

Conventional topical vehicles (*e.g.*, ointments, creams or jellys) predominantly exert their effect by releasing the drug onto the skin, which has to diffuse through the different skin layers. Among them, the stratum corneous is the major permeability barrier due to its organized structure, being regarded as the rate-limiting factor for drug penetration at therapeutic levels [25, 26]. Therefore, absorption and penetration are highly influenced by the nature of the topical vehicle. Nonetheless, the extent of diffusion depends on the physicochemical properties of the drug itself.

In general, the modification of drug absorption kinetics by these vehicles is the result of their ability to provide increased hydration by occlusion or some other mechanisms. If the size and solubility properties of the drug are not appropriate, only limited uptake by the skin will occur.

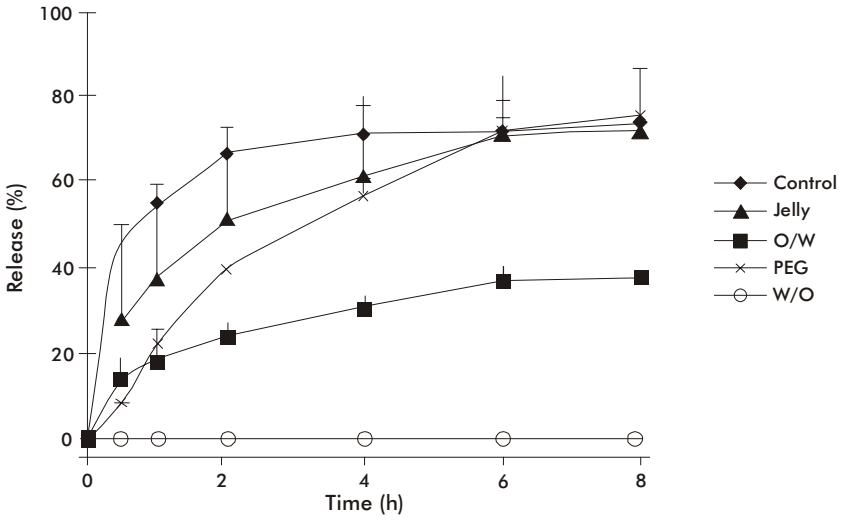

Figure 2. Percent of EGF released from different formulations. Each point represents the mean ± the standard deviation of three determinations.

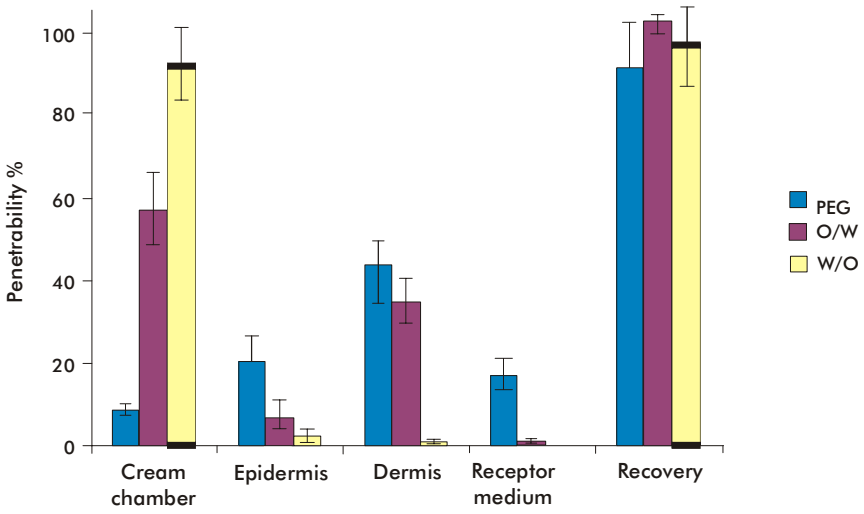

Figure 3. Penetrability of EGF trhought abdominal pigskin of variants PEG, o/w and w/o. Each point represents the mean ± the standard deviation of three determinations.

The release rate and penetrability coefficients of EGF from semisolid increased with the hydrosolubility of the semisolid formulation.

Conclusions

The methods developed for EGF extraction were simple, easy to use, and precise. They are currently in use for routine analysis and quality control of EGF in different semisolid formulations.

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