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DEVELOPMENT AND EVALUATION OF MULTIPLE EMULSION SYSTEMS CONTAINING CHOLESTEROL AND SQUALENE

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Abstract

The aim of this study was to prepare a stable multiple emulsion system containing cholesterol and squalene and to investigate the skin hydrating effect of the multiple emulsion formulations on normal and acetone induced dry skin.

Multiple emulsions were prepared via two step emulsification method. Formulations were stored at different accelerated conditions for three months and physical changes, viscosity, pH and droplet size were tested to characterize the emulsion systems. Formulations F4, F5 and F6 with the primary: secondary surfactant ratio 2:1 was found to be stable and selected for further in vitro occlusion and skin hydration tests. In vitro occlusion test results showed that the volume of the oily phase affects occlusive properties of the multiple emulsion formulations. The gain in moisturizing was performed measuring the skin electrical capacity using a Corneometer. The multiple emulsion formulation F4 showed the best skin hydrating effect on normal and acetone induced dry skin.

Keywords: *multiple emulsions, squalene, skin hydrating effect*

Kolesterol ve Skualen İçeren Çoklu Emülsiyon Sistemlerinin Geliştirilmesi ve Değerlendirilmesi

Bu çalışmanın amacı, kolesterol ve skualen içeren, kararlı yapıda çoklu emülsiyon sistemlerinin hazırlanması ve hazırlanan çoklu emülsiyon formülasyonlarının normal ve aseton etkisi ile kurumuş deri üzerindeki nemlendirici etkinliğinin araştırılmasıdır.

Çoklu emülsiyon formülasyonlarının hazırlanmasında iki basamaklı emülsifikasyon yöntemi kullanılmıştır. Formülasyonlar farklı ve hızlandırılmış koşullarda 3 ay süre ile saklanmış, fiziksel değişiklikler, viskozite, pH ve damlacık büyüklüğü test edilmiştir. Primer:sekonder sürfaktan oranı 2:1 olan F4, F5 ve F6 kodlu formülasyonlar kararlı bulunarak daha sonraki in vitro oklüzyon ve deri nemlendirme testleri için seçilmişlerdir. In vitro oklüzyon test sonuçları, çoklu emülsiyon formülasyonlarının yağlı faz hacminin, oklüzif özellikleri üzerine etkili olduğunu göstermiştir. Deri nemindeki artış, derinin elektriksel kapasitesinin Corneometre kullanılarak ölçülmesi ile saptanmıştır. F4 kodlu çoklu emülsiyon formülasyonu normal ve aseton etkisi ile kurumuş deri üzerinde en iyi nemlendirme etkisini sağlamıştır.

Anahtar Kelimeler: *çoklu emülsiyon, skualen, deri nemlendirme etkisi*

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INTRODUCTION

The stratum corneum (SC), which is the outermost layer of the skin, functions as an important barrier to maintain biological homeostasis (1). It consists of terminally differentiated keratinocytes, referred to as corneocytes, embedded in lamellar lipid bilayers consisting of cholesterol, free fatty acids and ceramides. Changes in the lipid content and organization of these lipids influence the barrier function of skin (2,3).

It has been reported that several skin diseases might be correlated with the impaired barrier function. Skin protection or barrier recovery has been claimed by showing increased SC hydration after topical application of barrier lipids on damaged skin. The only treatments that allowed normal barrier recovery were found as the applications of complete mixtures of ceramide, fatty acid and cholesterol, or pure cholesterol (4,5). Cholesterol is the only sterol found in human SC and is crucial for promoting the intermixing of different lipid species (6).

Squalene is a minor constituent of oils and fats and it finds applications in nutraceuticals, cosmetics, and pharmaceutical industries. As squalene occurs naturally at about 10% in human sebum the use of squalene as an emollient in skin care products is considered to be naturally and highly relevant (7).

Although multiple emulsions are still infrequently used, their potential applications are numerous and the investigation of these systems is now an active field of research, especially in such product areas as pharmaceutical drug delivery systems, cosmetics and foods (8). In practice, they are thermodynamically unstable systems with a strong tendency for coalescence, flocculation and creaming. The composition and method of preparation are of significant importance. Reducing the droplet size of the inner emulsion is one of the several approaches to overcome instability problems (9,10).

The aim of this study is to formulate stable multiple emulsion systems with occlusive and skin hydrating properties. After the preparation of the multiple emulsions, characterization and accelerated stability studies were performed for three months by keeping formulation samples at different conditions. During this period, physical changes, changes in pH and viscosity were periodically determinated, microscopic analysis and droplet size analysis were performed. The most stable formulations were selected for in vitro occlusion and skin hydration tests performed on normal and acetone induced dry skin.

EXPERIMENTAL

Materials

Paraffin Oil and Span 80® were purchased from Fluka (Switzerland). Tween 80® and Cholesterol were obtained from Merck (Germany). Squalene was provided from Mayumi (Japan). Plurol Oleique® was a kind gift of Gattefosse (France). All other materials were of analytical grade.

Preparation of Multiple Emulsions

After determination of required HLB and optimum surfactant blend W/O/W multiple emulsions (F1-F9) (Table 1) were prepared via a two step emulsification process (2) (Figure1).

Table 1. Multiple emulsion formulations prepared via two step emulsification

W/O/W PHASE RATIO	PRIMARY-SECONDARY SURFACTANT RATIO	FORMULATIONS
30:20:50	1:1	F1
20:30:50	1:1	F2
30:40:30	1:1	F3
30:20:50	2:1	F4
20:30:50	2:1	F5
30:40:30	2:1	F6
30:20:50	3:1	F7
20:30:50	3:1	F8
30:40:30	3:1	F9

In the first step, a primary W/O emulsion was prepared by adding the water phase to the oil phase (paraffin oil, cetyl alcohol, cholesterol and squalene) containing Span 80® and Plurol Oleique® as lipophilic surfactant mixture. Both phases were heated separately, mixed together after reaching the production temperature and then emulsified using a homogenizator (CAT X 620). Salt (NaCl) is added to the dispersed phase of formulations to avoid the coarsening phenomena. In the second step, the primary W/O emulsion was re-emulsified in the outer water phase containing Tween 80® as hydrophilic surfactant by mechanical stirring (IKA RW 20). The stirring rates and times were determined following pre-formulation studies both for primary and multiple emulsions. Multiple emulsion formulations containing 1 and 2% gelatin in the outer phase of the emulsion were also prepared and the effect of gelatin on the emulsion stability was investigated.

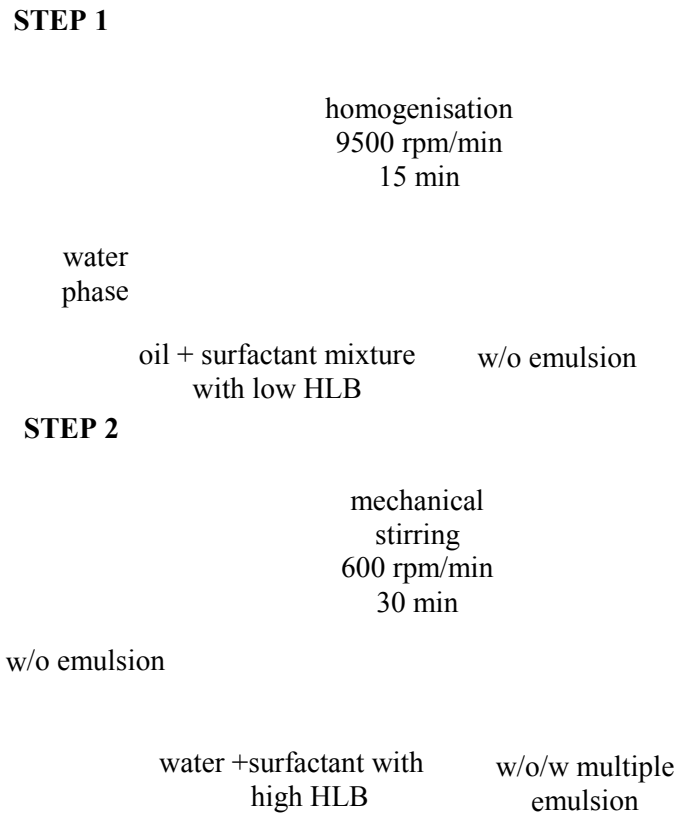

Figure 1. Preparation of W/O/W type multiple emulsion via the two step emulsification process.

Characterization of Multiple Emulsions

Multiple emulsions were analyzed to assure the formation and the stability of the emulsion system.

Macroscopic Analysis

Organoleptic characteristics (color, consistency, appearance), homogeneity (creaming, phase separation) were investigated visually.

pH Determination

pH values of the freshly prepared multiple emulsion formulations and of the formulations stored at different conditions were determined by a digital pH-Meter (Schott CG-809). Every experiment was carried out in triplicate.

Viscosity Determination

Changes in viscosity were examined using Brookfield Model DV-II Viscometer at 50 and 100 rpm/min for freshly prepared multiple emulsion formulations and for formulations stored at different conditions, respectively. Every experiment was carried out in triplicate.

Microscopic Analysis

Microscopic analysis was carried out using an optical microscope (SOIF H-ZSX) combined with a computer imaging system (Samsung), and observations were made at 100X magnification after diluting in the appropriate amount of external phase of the emulsion. The shape and homogeneity of the multiple droplets is followed immediately after the preparation of the multiple emulsion formulations. Formulations stored at different conditions were investigated under microscope after 1 day, 1 week and 1, 2 and 3 months of preparation.

To evaluate the type of multiple emulsion formulations, a drop of a water soluble dye (methylene blue) solution was added to the system and the colored mixture examined by optical microscopy.

Droplet Size Analysis

The mean droplet size of the multiple emulsion formulations F4, F5 and F6 was determined using particle size analyzer (Malvern Mastersizer) for the freshly prepared formulations and for the formulations stored at different conditions after 1, 2 and 3 months of preparation. Every measurement was the average of 5 measurements at 1750 and 2000 rpm.

Stability Tests on Multiple Emulsions
Centrifugation Test

Freshly prepared multiple emulsion formulations and formulations stored at different conditions were centrifuged at 3000 rpm for 15 minutes (Hettich Microliter) by placing 10 mg of samples in the centrifugal tubes.

Thermal Stability Test

Multiple emulsion samples were stored in tightly closed glas vessels at 4 ±1^{o}C (refrigerator), 25 ±0.5^{o}C (room temperature) and 40 ±0.1^{o}C (oven) and examined periodically to test the physical characteristics mentioned above.

In vitro Occlusion Test

50 mL beakers were filled with 25 mL distilled water and sealed with cellulose acetate filter (Sigma, England) for the in vitro occlusion test. Only the most stable multiple emulsion

formulations (F4, F5 and F6) has been chosen for this analysis. Samples were spread on the filter (20 mg/cm2) and stored at 32 °C for 48 h. The occlusion factor F was calculated according to Eq. 1 after 6, 24 and 48 h (11). Every experiment was carried out in triplicate.

$$F = 100 \times [(A-B)/A] \qquad \text{(Eq.1) (11)}$$

A: water loss without the sample (reference)
B: water loss with sample

Skin Hydration Tests

A Corneometer CM 820 (Courage Khazaka) was used to determine the stratum corneum hydration by capacitance measurements. 12 volunteers of both genders aged between 19 and 57, healthy, without any sign of skin pathologies on the volar forearms were selected. All volunteers signed a written consent paper. Experiments were carried out during April and volunteers were not allowed to use skin care products on their forearms before one week of the experiments. The measurements have been performed in an environment having a constant relative humidity (40% RH) and a constant temperature (25 °C) in order to avoid the influence of the cutaneous parameters by external agents.

The effects of the multiple emulsions F4, F5 and F6 were tested on normal skin and on acetone treated skin of volunteers, respectively (Figure 2). Volunteers take place 30 minutes before the experiment and first measurement was taken which used as reference value.

Left Forearm	Right Forearm
Reference	Reference
	Acetone treated reference
F4	Acetone treated+ F4
F5	Acetone treated+ F5
F6	Acetone treated+ F6

Figure 2. Schematic representation of the test sites on both forearms.

For normal skin test, efficacy measurements were carried out 1, 3, 6 and 24 hrs after a single application of multiple emulsions on volar forearm within a delimited area of 2x2 cm piece. Measurements collected from 5 points and the mean value was determined. 5 sec pause was given between each measurement to avoid the occlusive effect of the probe of the Corneometer.

For acetone treated skin test, the test spots on the volar forearm were brought into contact twice with 5 mL acetone pro analysis for 1 min. Skin contact was made by fixing Pyrex tubes filled with acetone on the skin while the forearm was gently moving up and down. The acetone was then discarded. Efficacy measurements were carried out 1, 3, 6 and 24 hrs after a single application multiple emulsions. The excess of the formulations was removed with a paper towel from the skin. One test spot treated only with acetone and was used to check the duration of physiological repair process of the skin.

To compare the effect of the formulations on SC hydration statistically, two way ANOVA and paired samples t-test was applied on computer. Statistical significance was set at $P< 0.05$.

RESULTS AND DISCUSSION

Multiple emulsions are often stabilized using a combination of hydrophilic and hydrophobic surfactants. The ratio of these surfactants is important in achieving stable multiple emulsions and needs to be determined during formulation development (10,12). In our study, multiple emulsion formulations F4, F5 and F6 with the primary:secondary surfactant ratio 2:1 showed the best long term stability.

The preparation method of multiple emulsions is based on the two step emulsification process. The amount of cholesterol and squalene kept constant in the primary emulsion, whereas cetyl alcohol and paraffin oil amounts were modified relevant to the phase ratio of the ending formulation. Cooling the primary emulsion before adding to the outer water phase had undesirable effect on the stability of the resulting multiple emulsion system. Therefore this step was carried out immediately after the preparation of the primary emulsion by heating the outer water phase to the same temperature. The primary emulsion was added in small pieces.

It was shown that the stability of the primary W/O emulsion requires the presence of an electrolyte in the aqueous phase (13). In this respect we decided to use NaCl at a concentration of %0.4 in the inner aqueous phase of multiple emulsions. Microscopic observations showed that the emulsion formulations containing NaCl protected their homogeneous multiple globule structure after 1 month storage at room temperature.

The formation of multiple emulsion systems with improved stability owing to the formation of interfacial complex film between polymers (acacia, gelatin, PVP) and surfactants have been described (10). However, we observed phase separation in all our formulations including 1 and 2% gelatin after 24 hours of preparation.

In the development of W/O/W multiple emulsions, it is necessary to estimate their physical stability experimentally. The results of the physical analysis of the emulsion systems prepared are summarized in Table 2.

Table 2. The results of the physical analysis of the multiple emulsion formulations (F1-F9) S: Stabil PS: Phase Separation

	F1	F2	F3	F4	F5	F6	F7	F8	F9
4±1 °C									
1 Day	S	S	S	S	S	S	S	S	S
1 Week	S	S	S	S	S	S	S	S	S
1 Month	S	S	S	S	S	S	S	S	S
2 Months	S	S	S	S	S	S	S	S	S
3 Months	S	S	S	S	S	S	PS	PS	PS
25±1 °C									
1 Day	S	S	S	S	S	S	S	S	S
1 Week	S	S	S	S	S	S	S	PS	S
1 Month	S	S	S	S	S	S	S	-	S
2 Months	S	S	S	S	S	S	S	-	S
3 Months	PS	PS	S	S	S	S	PS	-	PS
40±1 °C									
1 Day	S	S	S	S	S	S	S	PS	S
1 Week	S	S	S	S	S	S	S	-	S
1 Month	S	S	S	S	S	S	S	-	PS
2 Months	PS	S	S	S	S	S	S	-	-
3 Months	-	PS	PS	S	S	S	PS	-	

There was no change in the color of the multiple emulsion formulations F4, F5 and F6 up to 3 months at all conditions. The white color of the formulations F1, F2 and F9 turned to yellowish white in 2 months at room temperature. The same color change was observed in the formulation F8 within one week. There was no change in the color of multiple emulsion formulations stored at 4±1 °C throughout the period of the analysis.

pH values of the freshly prepared formulations were in the range 5.51 and 5.91 which is close to the pH range of the human skin (Table 3). pH values of multiple emulsion formulations kept at different conditions have decreased with time.

Table 3. pH values and ±standart errors of multiple emusion formulations (F4, F5, F6) stored at different conditions

	F4	F5	F6
4±1 °C			
1 Day	5.38±0.01	4.70±0.03	5.88±0.01
1 Week	5.37±0.02	4.70±0.02	5.86±0.02
1 Month	5.30±0.01	4.61±0.01	5.70±0.04
2 Months	5.30±0.02	4.59±0.02	5.60±0.04
3 Months	5.30±0.02	4.56±0.01	5.60±0.03
25±1 °C			
1 Day	5.80±0.02	5.51±0.02	5.91±0.01
1 Week	5.76±0.02	5.12±0.01	5.90±0.02
1 Month	5.60±0.01	4.95±0.03	5.85±0.01
2 Months	5.60±0.02	4.80±0.02	5.70±0.05
3 Months	5.57±0.02	4.70±0.04	5.70±0.05
40±1 °C			
1 Day	5.81±0.01	4.83±0.02	5.82±0.01
1 Week	5.70±0.02	4.80±0.02	5.80±0.01
1 Month	5.56±0.03	4.55±0.02	5.70±0.03
2 Months	5.50±0.01	4.50±0.04	5.70±0.02
3 Months	5.50±0.03	4.12±0.05	5.68±0.04

The viscosity of the multiple emulsions was measured at 50 rpm and 100 rpm to follow the time and temperature dependence of viscosity. The viscosity values at 100 rpm were selected for Table 4. Viscosities of all the multiple emulsion formulations decreased during storage with time.

Table 4. Viscosities (cPs) of multiple emulsion formulations (F1-F9) stored at different conditions

	F1	F2	F3	F4	F5	F6	F7	F8	F9
4±1 °C									
1 Week	8456	9623	9010	8940	8220	9092	8010	8321	8974
1 Month	7789	9500	8500	7880	7927	7122	6890	6420	6300
2 Months	6540	8647	8001	7370	6853	7010	6000	5100	5001
3 Months	5880	6500	7300	6756	5690	6560	PS	PS	PS
25±1 °C									
1 Day	9045	10163	10560	9840	9146	10642	10556	10231	10880
1 Week	8253	9200	9564	9045	7510	8800	7984	PS	7550
1 Month	7990	8965	8400	9012	6530	8007	5114	PS	4005
2 Months	7000	7100	6553	8923	5112	7103	4660	PS	PS
3 Months	PS	PS	PS	8289	4540	6200	PS	PS	PS
40±1 °C									
1 Week	8000	8600	7882	8850	7310	8010	6500	PS	5770
1 Month	7140	7400	6300	7600	6221	7920	5800	PS	PS
2 Months	6230	6500	5000	6845	5103	6670	4115	PS	PS
3 Months	PS	PS	PS	6800	4070	6000	PS	PS	PS

PS: Phase Separation

Upon microscopic analysis, the structure of W/O/W multiple emulsion formulations was checked. The existence of multiple droplets was determined microscopically during the experiments until the phase separation was observed. It was also revealed that water was the continuous phase of the formulations. The microscopic view of the multiple emulsion formulation F4 is shown in Figure 3.

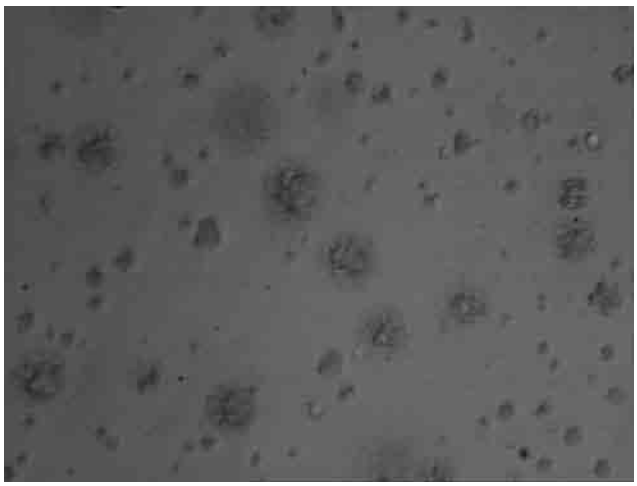

Figure 3. Microscopic view of multiple emulsion formulation F4 after preparation.

The size of droplets is an important parameter in physical stability of an emulsion (14). The average droplet size of the multiple emulsions F4, F5 and F6 kept at different conditions determined at 2000 rpm is given in Table 5.

Table 5. Droplet sizes ±standart errors of multiple emulsion formulations F4, F5 and F6.

	F4	**F5**	**F6**
4±1 °C	(μm)	(μm)	(μm)
1 Month	2.112±0.07	9.164±0.45	16.700±0.22
2 Months	2.113±0.65	9.350±0.20	15.031±0.14
3 Months	2.540±0.03	8.206±0.50	13.618±0.79
25±1 °C			
1 Day	2.005±0.02	9.628±0.05	16.407±0.06
1 Month	2.075±0.04	9.063±0.34	16.856±0.12
2 Months	2.050±0.82	9.500±0.55	17.107±0.61
3 Months	3.100±0.80	9.367±0.51	21.505±0.50
40±1 °C			
1 Month	2.114±0.14	9.030±0.35	16.946±0.15
2 Months	3.441±0.28	8.350±0.10	19.610±0.15
3 Months	4.987±0.76	7.154±0.58	22.359±0.90

The mean droplet size of the multiple emulsions did not change significantly and no visual sign of physical destabilization was observed during storage time at 4±1 °C, 25±1 °C and 40±1 °C. When the effect of the oily phase volume on droplet size of multiple emulsions was investigated, an increase in droplet size with increasing oily phase volume was observed.

Resistance of an emulsion to centrifugation depends on the strength of the interfacial film between the aqueous and oily phases (13, 15). The results of the centrifugation test performed on W/O/W multiple emulsions (F1-F9) has been given in Table 6.

Table 6. The results of the centrifugation test

	F1	F2	F3	F4	F5	F6	F7	F8	F9
4±1 °C									
1 Day	S	S	S	S	S	S	S	S	S
1 Week	S	S	S	S	S	S	S	S	S
1 Month	S	S	S	S	S	S	S	PS	S
2 Months	S	S	S	S	S	S	PS	-	PS
3 Months	PS	S	PS	S	S	S	-	-	-
25±1 °C									
1 Day	S	S	S	S	S	S	S	PS	S
1 Week	S	S	S	S	S	S	S	-	S
1 Month	S	S	S	S	S	S	S	-	PS
2 Months	PS	S	S	S	S	S	PS	-	-
3 Months	-	PS	PS	S	S	S	-	-	-
40±1 °C									
1 Day	S	S	S	S	S	S	S	PS	S
1 Week	S	S	S	S	S	S	S	-	S
1 Month	S	S	S	S	S	S	S	-	PS
2 Months	PS	PS	S	S	S	S	PS	-	-
3 Months	-	-	PS	S	S	S	-	-	-

S: Stabil PS: Phase Separation

The data we obtained from our investigations lead us to reveal that the multiple emulsion formulations F4, F5 and F6 were the most stable formulations. These formulations exhibited appropriate consistency and homogeneity for application to the skin and homogeneous multiple droplets were observed via microscopic analysis even after 3 months of storage at room conditions.

Occlusion of the skin surface leads to the entrapment of water which normally would lose to the environment. This results in an increase in skin hydration, particularly in stratum corneum (16). The occlusive character of a formulation is based on film formation after application to the skin (11). In Figure 4, the results of the in vitro occlusion test are showed.

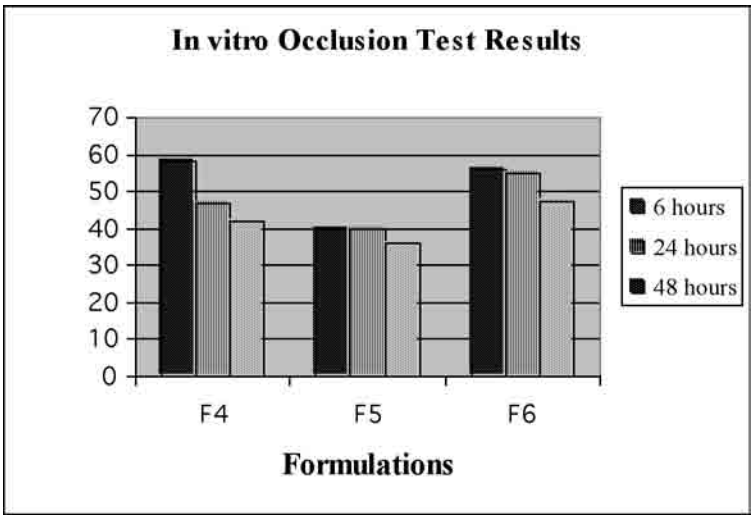

Figure 4. The results of the in vitro occlusion test

After 6 hours the occlusive effect of F4 and F6 was significantly different when compared with that of F5 ($P<0.05$). Formulation F6, which has the highest oily phase volume, maintained its occlusive effect after 48 hours. This result is in accordance with the finding that the occlusion factor F depends on the volume of the oily phase (11).

The Corneometer is generally the most commonly used instrument to evaluate the skin water content. It measures the change in skin capacitance of the superficial SC and is influenced by the water content and lipid content of the skin. A good contact between the measuring probe and the skin surface are key factors to ensure the necessary stability during testing with Corneometer (17).

The results of the skin water content measurements after single application of 3 multiple emulsion formulations (F4, F5, F6) averaged overall 12 volunteers are represented in Figure 5 and Figure 6, for normal and acetone induced dry skin, respectively. The results obtained by the Corneometer are expressed in Corneometric Units.

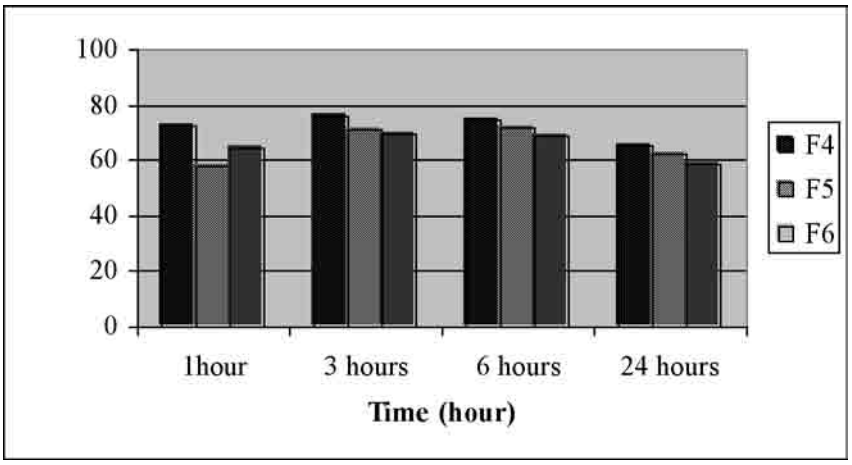

Figure 5. A comparison of the skin hydrating effect of formulations F4, F5 and F6 on normal skin.

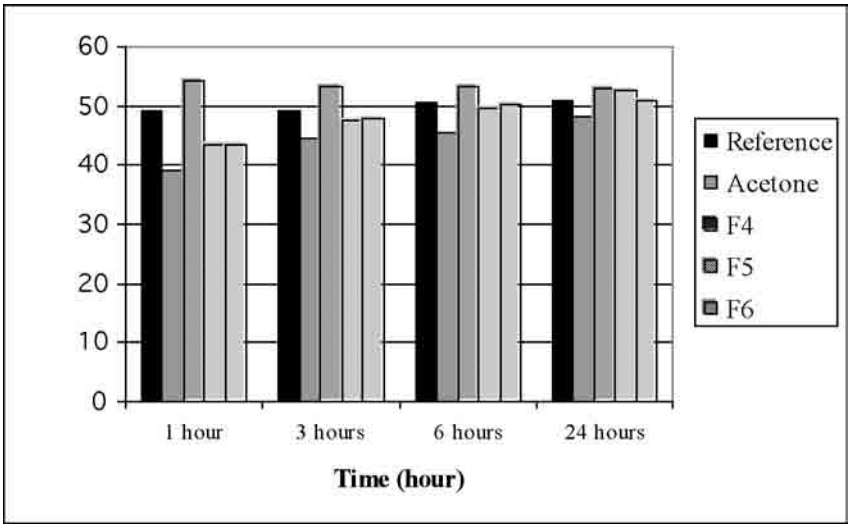

Figure 6. A comparison of the skin hydrating effect of formulations F4, F5 and F6 on acetone induced dry skin.

The mean reference value detected as 58.51±1.04 Corneometric Units showed that the application area on the volar forearm of volunteers was between low and normal humidity (18). On normal skin, multiple emulsion formulation F4 improved SC hydration to a greater extent than formulations F5 and F6. Skin moisture values after application of the three formulations did not lead to any statistical difference between the formulations.

Dry and scaly skin can be experimentally induced by exposure to the organic solvent acetone. It has been concluded that acetone irritated skin had no effect on TEWL. Capacitance measurements showing the drying effect of acetone on the skin are more useful. It has been also reported that the superficial damage induced by acetone could be corrected by short term treatment (18).

In our study, acetone treatment resulted in a 22% decrease in SC hydration. Figure 6 also indicates that after 24 hours the physiological barrier repair was not able to restore SC hydration completely. This result is in accordance with the literature (18). After 1 hour of a single application of multiple emulsions there was a significant difference between the effects of formulation F4 and other formulations ($P<0.05$). Our results confirmed that the higher quantity of lipids within emulsion formulation does not necessarily bring better moisturisation of normal skin. Formulations F5 and F6 lead to an increase in skin moisture level almost equally. Treatment with multiple emulsion formulation F4 significantly enhanced the hydration level compared to the values of irritated skin after 1, 3, 6 and 24 hours ($P<0.05$).

It has been recognized that SC lipids such as cholesterol, free fatty acids and ceramides play a major role in the maintenance of the skin barrier (19). We have seen that cholesterol and squalene containing multiple emulsion formulations were useful to improve the skin humidity both on normal and on acetone induced dry skin.

CONCLUSION

The stability and release characteristics of multiple emulsions are influenced by different factors, such as surfactant type, surfactant ratio and physical properties of the system, i.e., droplet size, viscosity, phase volume ratio (10). In our study the formation of multiple W/O/W emulsions with improved stability has been achieved via reducing the droplet size of the primary emulsion and determination of the optimum surfactant blend.

The hydration of the skin is considered to be a marker of its state of health whereas the dryness of the skin is a sign of functional disorder (19). This study shows clearly that, related to the methodological strategies applied, the multiple emulsion formulations containing cholesterol and squalene exert positive results both on the moisture level of normal skin and on skin repair after damage. The mechanism behind this effect needs further investigation.

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