Effect of L-Ascorbic Acid on the Formulation and Characterization of a Multiple Emulsion from Paraffin Oil

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Summary: Multiple emulsions contain at least three phases. The material entrapped in internal phase is released slowly and thus its effects can be prolonged. The other benefit of multiple emulsions is protection of material entrapped in internal phase. The purpose of the study was to prepare a stable multiple emulsion containing a skin antiaging agent L-Ascorbic acid (vitamin C) using paraffin oil. L-Ascorbic acid, which is a very unstable ingredient and is decomposed in the presence of oxygen, in a concentration of 2% was entrapped in the inner most aqueous phase of w/o/w multiple emulsion. Formulation of multiple emulsion was stored at different accelrated conditions *i.e.* 8 °C, 25 °C, 40 °C and 40 °C+75% RH (Relativ Humidity) for 28 days to predict the stability of formulation. Formulation was found to be stable at lower temperatures for a given period of time and no phase sepration was observed in any sample. Different parameters like pH, globule size, electrical conductivity and effects of centrifugation were studied for a period of 28 days to confirm the stability of multiple emulsion. It was found that there was no significant change in globule sizes in any sample kept at most of the conditions. Significant changes in both pH and conductivity were observed in the samples of formulation kept at 8 °C. Insignificant changes both in pH and electrical conductivity were observed in samples of formulation kept at 40 °C and 40 °C + 75%RH conditions throughout study period. The data obtained was evaluated statistically using two way ANOVA (Analysis of Variance) and LSD (Least Significant Difference) tests.

Introduction

Multiple emulsions are complex dispersion systems, known also as 'emulsion in emulsion'. The most common multiple emulsions are of W/O/W but in some specific applications O/W/O emulsions can also be prepared. These emulsion systems, at least in theory have significant potential in pharmacy and cosmetics (prolonged release of active substances, the possibility of combining incompatible substances in one product, the protection of fragile substances). In practice, multiple emulsions are thermodynamically unstable with strong tendency for coalescence, flocculation and creaming [1]. Multiple emulsions were also investigated for cosmetics for their potential advantages of prolong release of active agent , incorporation of incompatible materials and protection of active ingredients by dispersion in internal phase [2].

Both hydrophilic and lipophilic emulsifiers are used for the formation of multiple emulsions. Multiple emulsions have shown promise in many technologies, particularly in pharmaceutics and in separation sciences. Their potential biopharmaceutical applications [3], include the use as adjuvant vaccines, as prolonged drug delivery systems [4, 5], as sorbent reservoirs in drug over dose treatments [6] and in mobilization of enzymes [7]. The use of multiple emulsions in separation has included the separation of hydrocarbons [8]. Multiple emulsions have also been formulated as cosmetics [9]. In addition, they have potential advantages of prolonged release of drug, incorporation of incompatible materials and protection of active ingredients as they are dispersed in internal phase [10].

The preparation of multiple emulsions with synthetic oils like paraffin oil is preferred due to long term stability. This oil has also been preferred because of its cosmetics benefits for skin. Paraffin oil is a mixture of refined liquid saturated aliphatic (C14-C18) and cyclic hydrocarbons obtained from petroleum [11].

L-Ascorbic acid is a white or almost white, crystalline powder or colorless crystals; discolor on exposure to air and moisture; freely soluble in water,

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soluble in alcohol, and practically insoluble in ether. Its chemical structure is given in Fig. 1.

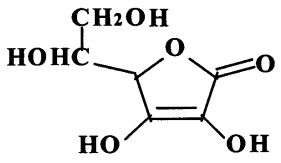

Fig. 1: Chemical structure of L-ascorbic acid.

It is found in quite high concentrations in the aqueous fractions of many animal tissues including the spinal cord, lung and eye. In plants, some fruits may contain more than 1%. It normally occurs at a level of about 0.1mM in human blood plasma Because of its enediol structure; it displays a rather low first pKa (about 4.2) and accordingly exists almost entirely as a monoanion in most tissues. The role of L-Ascorbic acid, as an antioxidant and in the protection of skin against the deleterious effects of UVB light, has been recognized by many workers [12-14]. In addition to this, L-Ascorbic acid also improves the synthesis of collagens increasing the suppleness of the skin [15]. But this vitamin is very unstable due to oxidation problems.

Results and Discussion

Stability of Primary Emulsion

Stability of samples of primary emulsion kept at different storage conditions was studied and organoleptic parameters regarding stability (color, liquefaction, phase separation) and centrifugation tests are presented in Table-1.

Stability of Formulation

Stability of samples of formulation kept at different storage conditions was studied and organoleptic parameters regarding stability (color, liquefaction, phase separation) and centrifugation tests are presented in the Table-2.

Globule Size

Globules sizes of formulation kept at different storage conditions up to 28 days have been examined and represented in Fig. 2.

pH Tests

pH values of formulation kept at different storage conditions up to 28 days have been determined. pH values of samples of formulation at different storage conditions have been shown in Fig. 3.

Electrical Conductivity Tests

Electrical conductivity values of formulation kept at different storage conditions for 28 days have been determined. Electrical conductivity values have been shown in Fig. 4.

Table-1: Organoleptic Parameters and Centrifugation Tests for Primary Emulsion.

Time	Liquefaction				Color				Phase Separation				Centrifugation			
Hrs/Wks	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0 hr	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
1 hr	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
24 hrs	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
72 hrs	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
7 days	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
14 days	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
21 days	-	-	-	-	W	W	W	W	-	-	-	-	-	-	+	+
28 days	-	-	-	-	W	W	W	W	-	-	-	-	-	-	+	+

- =No Change; + =Slight Change
A= 8 °C; B= 25 °C; C=40 °C; D=40 °C+75% Relative Humidity; W= White

Table-2: Organoleptic parameters and centrifugation tests for formulation.

Time	Liquefaction				Color				Phase Separation				Centrifugation			
Hrs/Wks	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0 hr	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
1 hr	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
24 hrs	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
72 hrs	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
7 days	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
14 days	-	-	-	-	W	W	W	W	-	-	-	-	-	-	-	-
21 days	-	-	+	+	W	W	YW	YW	-	-	+	+	+	+	+	+
28 days	-	-	+	+	W	W	YW	YW	-	-	+	+	+	+	++	++

- =No Change; + =Slight Change; W= White; YW= Yellowish White; ++ = More Change; A= 8 °C; B= 25 °C; C=40 °C; D=40 °C+75% Relative Humidity

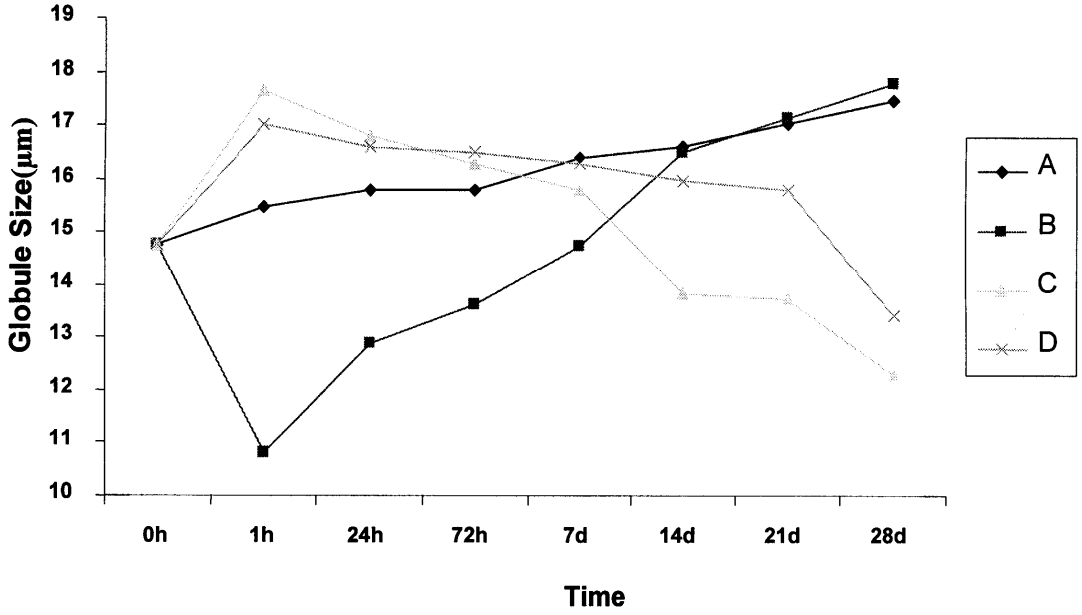

Fig. 2: Globule Sizes of Samples of Formulation Kept at Different Conditions A= 8 °C, B = 25 °C, C = 40 °C and D = 40 °C + 75% RH.

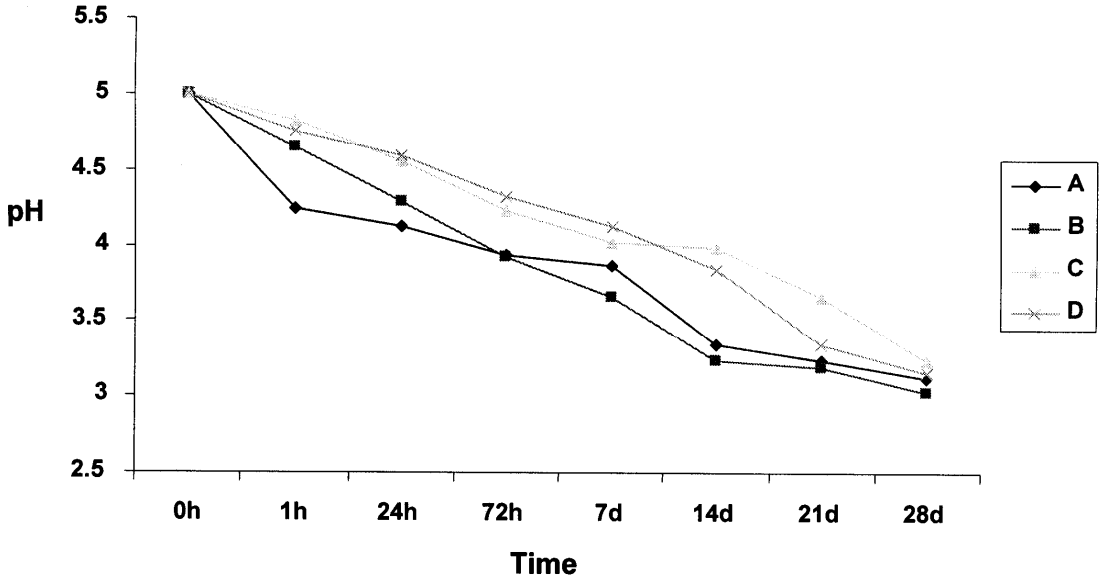

Fig. 3: Comparisons of pH values of formulation kept at 8 °C, 25 °C, 40 °C and 40 °C +75%RH.

Discussion

Organoleptic Evaluations

Color

Freshly prepared primary emulsion was white in color. There was no change in color of primary emulsion at different storage condition *i.e.* 8 °C, 25 °C, 40 °C and at 40 °C + 75% relative humidity up to the observation period of 28 days. This showed that primary emulsion was stable at different storage conditions up to 28 days.

The freshly prepared formulation was also white in color. There was little change in color of

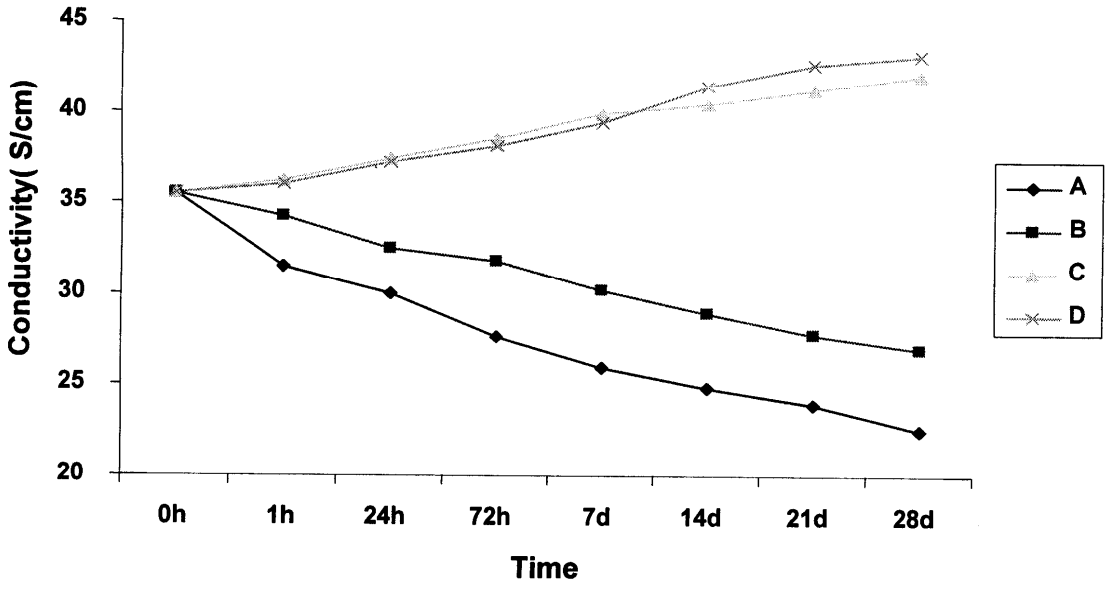

Fig. 4: Comparison of Conductivity Values of Formulation Kept at 8 °C, 25 °C, 40 °C and 40 °C +75%RH.

samples kept at 40 °C and at 40 °C + 75% relative humidity. The color of formulation in these samples became yellowish white. The change in color appeared from 21st day and persisted up to 28 days of analyses period. The change in color at the end of observation periods was might be due to the oily phase separation which is promoted at higher temperature or due to degradation of ascorbic acid.

Liquefaction

No liquefaction was observed in the primary emulsion at any storage condition *i.e.* at 8 °C, 25 °C, 40 °C and at 40 °C + 75% RH up to 28 days.

While in the case of formulation no liquefaction was observed in the samples kept at 8^0C and 25 °C during whole of the observation period *i.e.*, 28 days but slight liquefaction was observed in samples kept at 40 °C and 40 °C + 75% RH from 21st day of observation but there was no increase in liquefaction till the end of study period. Liquefaction is a sign of instability.

Phase Separation

No phase separation was observed in any of samples of primary emulsion kept at 8 °C, 25 °C, 40 °C and 40 °C+ 75% relative humidity up to observation period of 28 days with naked eye. This indicated that primary emulsion was stable at all the storage conditions for 28 days.

In case of formulation, no phase separation was seen in any of the samples kept at 8 °C and 25 °C up to the period of 28 days. But at the temperature of 40 °C and 40 °C +75% relative humidity samples of formulation showed slight phase separation from 21st day of observation.

Centrifugation Test

In this study centrifugation test was performed for both primary and multiple emulsions at different storage conditions up to a period of 28 days at different time intervals. In case of primary emulsion no phase separation after centrifugation was seen in any of the samples kept at different storage conditions up to 14th day but slight phase separation on centrifugation was seen from 21st day to 28th day of observation in the samples kept at 40 °C and 40 °C+ 75%RH and no more phase separation was observed till the end of study period.

In the case of formulation no phase separation after centrifugation was seen in any of the sample kept at different storage condition up to 14th day. Slight phase separation on centrifugation was seen in the samples kept at different storage condition after 21st day of observation and there was no further

increase in phase separation in the samples kept at 8 °C and 25 °C till the end of 28th day but in case of samples kept at 40 °C and 40 °C + 75% RH conditions more phase separation on centrifugation was observed at 28th day.

Globule Size

The increase or decrease in the globule size indicates the process of instability (2). The multiple droplets may coalesce with the other oil drops, or the internal aqueous droplets may be expelled individually; or more than one drop may be expelled; or the internal globules may coalesce before being expelled out resulting in the shrinkage of internal droplets; or water may pass from the external phase to the internal aqueous phase resulting in the swelling of internal droplets and then complete rupture of droplets. The globule size can be determined by light microscope or by electron microscope. Light scattering and diffraction methods are also used for the determination of globule size. Coulter counter is also a practical method for the determination of particle size (2).

In this study, light microscope fitted with digital camera was used for the determination of globule sizes. The globule sizes of samples of formulation stored at different conditions changed with time. The globule sizes of formulation stored at 8 °C, 25 °C and were found to increase with the passage of time. This was due to the swelling of globules which was due to the transfer of external aqueous droplets to internal water phase as electrical conductivity of samples at most of conditions was decreased with the passage of time.

There was first increase in the globule sizes of samples of formulation stored at 40 °C and 40 °C + 75% relative humidity (RH) and then there was a decrease in size. This was due to the shrinkage of globules which was due to the expulsion of internal aqueous droplets to external water phase as electrical conductivity of samples at most of conditions was increased with the passage of time.

The globule size of the freshly prepared formulation was 14.75 µm. The globule size of sample kept at 8 °C increased gradually from first 1st hour to last day. On 28th the globule size was 17.42 µm. Tha globule size of sample kept at 25 °C decreased first and the there was increase in globule size *i.e.*, 17.73 µm at 28th day. Globule sizes of samples kept at 40 °C and 40 °C + 75%RH decreased gradually and continuously from 1st hour up to 28th day observation. Globule sizes of samples kept at 40 °C and 40 °C + 75%RH were 12.26 µm 13.40 µm, respectively on 28th day.

By using two way analysis of variance (ANOVA) technique at 5% level of significance, it was found that results of change of globule sizes are insignificant at different levels of time and temperature. As no significant change in globule sizes of samples kept at different storage conditions was observed for a period of 28 days so there was no need to perform individual comparison test *i.e.* LSD.

pH

In this work pH of freshly prepared formulation was 5.00 which was measured without stirring and it is nearest to the pH of skin. pH of samples of formulation kept at different storage conditions *i.e.* 8 °C, 25 °C, 40 °C and 40 °C + 75% RH were found to decrease continuously up to the 28th day of observation. The pH of sample of formulation kept at 8 °C decreased continuously from the first day to the last day. On 28th day pH was 3.12. The pH of sample kept at 25 °C also decreased continuously and on 28th day pH was found to be 3.03. pH of samples kept at 40 °C and 40 °C + 75% RH also showed continuous decrease and on 28th day pH were 3.24 and 3.15, respectively.

The decrease in pH was might be due to the production of highly acidic by-product from any of the acidic ingredients of the paraffin oil such as carbon containing compounds or due to degradation of ascorbic acid. By using two way analysis of variance (ANOVA) technique at 5% level of significance, it was found that the change in pH of different samples was significant at different levels of time and temperature.

By using LSD technique and taking the average pH of formulation at 0 hour as a standard and comparing it with average pH of samples of formulation at other time levels it was found that most significant change in pH was observed from 1st hour up to the total observation period of 28th day. By taking the average pH of sample kept at 25 °C as a standard and comparing it with pH of other temperature levels, it was found that most significant

change in pH was observed in the sample kept at 8 °C. So it can be concluded that most prominent change in pH was seen in sample kept at 8 °C just after preparation of formulation. But in the samples kept at 40 °C and 40 °C + 75%RH insignificant changes occurred.

Electrical Conductivity

The increase in electrical conductivity of formulation is due to the transfer of electrolytes entrapped in the inner aqueous phase of multiple emulsions from inner aqueous phase to external aqueous phase and decrease in electrical conductivity is due to the transfer of electrolytes which are lost in to external aqueous phase during the process of manufacturing towards internal aqueous phase.

In this work electrical conductivity of freshly prepared formulation was 35.5 µS/cm. Electrical conductivity of samples of formulation kept at 8 °C and 25 °C was found to decrease with the passage of time but the electrical conductivity of samples of formulation kept at 40 °C and 40 °C + 75% RH was found to increase at the end of the 28th day.

The electrical conductivity of sample of formulation kept at 8 °C and 25 °C decreased continuously from first hour to the last day. On 28th day electrical conductivity was 22.5 µS/cm and 26.9 µS/cm, respectively. Electrical conductivity of samples kept at 40 °C and 40 °C + 75%RH also increased continuously and on 28th day were 42.0 µS/cm and 43.1 µS/cm, respectively.

By using two way analysis of variance (ANOVA) technique at 5% of level of significance, it was found that change in electrical conductivity was significant at different levels of temperature and insignificant at different levels of time.

By taking the average electrical conductivity of sample kept at 25 °C as a standard and comparing it with the electrical conductivity of samples kept at other storage conditions, it was found that most significant change in electrical conductivity was observed in samples kept at 8 °C and 25 °C.

Experimental

Materials

Abil-EM 90 was purchased from Franken chemicals (Gebindc), Tween 80 and magnesium sulfate were purchased from Merck (Germany), Double distilled water prepared by using distillation plant (Germany) at university lab., digital pH meter WTW and digital conductivity meter of WTW (Germany), Stability chambers were of Sanyo (Japan) . Water bath (China), Electrical balance (Precisa, Switzerland), Digital humidity meter (TES Electronic Corp, UK), Centrifuge machine (Hettich, Germany), Mechanical mixer (IKA, Germany), Refrigerator (Dalwance, Pakistan), Microscope (Nikon, Japan), Microscopic camera (Germany), Microscopic software (MiniSee, Japan) and SPSS version 10.0 were used.

Methods

Preparation of Multiple Emulsion

Primary emulsion was prepared by heating oil phase consisting of paraffin oil and lipophilic surfactant (Abil EM 90) to 75 °C ± 1 °C. Aqueous phase consisting of water and magnesium sulfate was also heated to the same temperature. Aqueous phase was added to the oil phase drop by drop while stirring at 2000 rpm. Agitation was continued until cooling to room temperature of 25 °C. For the multiple emulsion, aqueous phase consisted of water and hydrophilic surfactant (Tween 80). Primary emulsion was added little by little to the aqueous phase at 1000 rpm for 10 minutes. Emulsion was then homogenized at 800 rpm for 5 minutes and at 500 rpm for 5 minutes more.

Formula of Formulation

Primary Emulsion (PE)

Paraffin oil	16%
Abil EM 90	5%
Magnesium sulfate	0.7%
Ascorbic acid	3.0%
Distilled water q.s.	100%

Multiple Emulsion

PE	90%
Tween 80	2.0%
Distilled water q.s.	100%

Properties of Primary and Multiple Emulsions

Both primary and multiple emulsions were analyzed to assure the formulation of desired emulsions.

Physical Analysis

Primary and multiple emulsions were analyzed organoleptically (color, thickness, look, feel) and physically (creaming and phase separation).

Types of Emulsions

Types of emulsions were analyzed by diluting the emulsion with oil and water separately on the glass slide.

Microscopic Tests

Multiple emulsions were analyzed under microscope to confirm the multiple characters. A drop of multiple emulsion was placed on the glass slide and diluted with water and covered by glass cover. A drop of immersion oil was placed on the cover slide and observed under the microscope.

Globule Size

Globule size of the multiple emulsions were determined for the freshly prepared emulsions and for the emulsions kept at different conditions, *i.e.* 8 °C ± 0.1 °C, 25 °C ± 0.1 °C, 40 °C ± 0.1 °C and, 40 °C ± 0.1 °C with 75% relative humidity (RH) conditions. Analysis was performed after 1 hour to 28 days on different time intervals. Average globule sizes were determined by microscope fitted with camera.

pH Determination

pH value of freshly prepared formulation and samples of formulation kept at different conditions were determined by a digital pH-Meter at different time intervals.

Centrifugation Tests

Centrifugal tests were performed on both for primary emulsion and for formulation immediately after preparation. The centrifugal tests were repeated for multiple emulsions after 24 hours, 3 days, 1st week, 2nd, 3rd and 4th week of preparation. The centrifugal tests were performed at 25 °C and at 5000 rpm by placing 10 g of sample in centrifugal tubes.

Stability Tests

Stability tests were performed at different conditions for formulation to note the effect of these conditions on the storage of formulation. These tests were performed on samples kept at 8 °C ± 0.1 °C (in refrigerator), 25 °C ± 0.1 °C (in oven), 40 °C ± 0.1 °C (in oven) and 40 °C ± 0.1 °C (in oven) with 75% relative humidity (RH). Physical characteristics of formulation, *i.e.* color, creaming and liquefaction, were noted at various intervals for 28 days.

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