CHAPTER III

MATERIALS AND METHODS

Materials

1. Cresmer® 1000, Cetomacrogol 1000 (Uniqema, Belgium)
2. Laurex® CS, Cetostearyl alcohol (The East Asiatic Co., Ltd, Thailand), Lot no. CE 25258
3. Tween® 20, Polyoxyethylene-20-sorbitan monolaurate (Acros Organics, USA), Lot no. A0204618001
4. Tween® 60, polyoxyethylene-20-sorbitan monostearate (Acros Organics, USA), Lot no. A0101201001
5. Tween® 80, polyoxyethylene-20-sorbitan monooleate (Acros Organics, USA), Lot no. A0206384001
6. Brij® 78, Polyoxyl-20-stearyl ether (Uniqema, Belgium)
7. Brij® 72, Polyoxyl-2-stearyl ether (Uniqema, Belgium)
8. 20% Mannitol solution (ANB Laboratories Co., Ltd., Thailand), Lot no.484558
9. 50% Dextrose monohydrate solution (Euromem Laboratories, Philippines)
10. Co-enzyme Q10, Ubidecarenone (Eisai Co., Ltd., Japan), Lot no. 55A44K
11. Methanol HPLC grade (Lab-Scan Co., Ltd., Thailand)
12. Ethanol anhydrous RS HPLC grade (Carlo Erba, France)
13. 1,4 Dioxane AR grade (Fisher Scientific, UK)
14. Cream base (Siriraj hospital, Thailand)

Equipment

1. Analytical balance (Satorius, Germany)

2. Differential scanning calorimeter (Netzsch DSC 204F1, Germany)
3. Centrifuge or Minispin (Eppendorf®, Germany)
4. Freeze-dryer (LyoLab w/PC, Lyophilization Systems Inc., USA)
5. High-Performance Liquid Chromatography (HPLC) instrument (Shimadzu, Japan) equipped with the following
   - Column: HiQsil C_{18}, 5 μm, 4.6 mm x 150 mm, (KYA TECH Corporation, Japan)
   - Liquid Chromatography: LC-10ADVP
   - UV-VIS Detector: SPD-10AVP
   - Auto injector: Sil-10ADVP
   - Degasser: DGU-14A
   - System Controller: SCL-10AVP
6. Hot air oven (Binder)
7. Heating magnetic stirrer (VELP® Scientifica, Italy)
8. Photon correlation spectrometer (Autosizer 4700, Malvern Instruments Ltd., UK)
9. Transmission Electron Microscope (TEM) (JEOL Co., Ltd., Japan)
10. Vortex mixer (Vortex-Geniez, USA)
11. Ultrapure water equipped with filter system (Bason Inc., USA), Boost pump, Option 3 water purified, Maximum ultrapure water, and Reservoir (ELGA, USA)
12. Ultrasonic bath (Elma, Germany)

Glassware and Miscellaneous

1. 0.1 μm membrane filter (Millipore, USA)
2. Beaker (Pyrex, USA)
3. Cylinder (Pyrex, USA)
4. Disposable syringe and needle (Terumo, Thailand)

5. Filter device 0.45 μm (Corning, USA)
6. Microcentrifuge tube 1.5 mL (Sarstedt, Germany)
7. Micropipette and disposable pipette tip (Pipet-lite™, USA)
8. Microscope slide and cover slide (Sail brand, China)
9. Nanosep® Centrifugal devices with 300 K molecular weight cut offs membrane (modified polyethersulfone on polyethylene substrate), 35 nm membrane nominal pore size, 0.28 cm^2 effective filtration area, 500 μL-maximum sample volume (Pall Life Sciences, USA)
10. Parafilm (Parafilm "M", USA)
11. Polarizing lens CXL 3126059
12. Screwed-cap tube (Pyrex, USA)
13. Volumetric flask 10-mL and 25-mL (Pyrex, USA)

Methods

1. Preparation of nanoparticles and Coenzyme Q_{10}-loaded nanoparticles from microemulsion system

1.1 Preparation of nanoparticles from microemulsion system

The nanoparticles were prepared by cooling the warm oil-in-water (o/w) microemulsion to room temperature. The formulations of microemulsion were prepared by varying the oil phase (matrix material) and the surfactant phase. The matrix material was the non-ionic emulsifying wax or Brij® 72 (polyoxyl 2 stearyl ether; HLB=4.9) at the concentration of 2 mg/mL. The composition of emulsifying wax was a combination of cetostearyl alcohol and one of the surfactant Tween® 20 (Polyoxyethylene 20 sorbitan monolaurate; HLB=16.7), Tween® 60 (polyoxyethylene-20-sorbitan monostearate; HLB=14.9), or Cetomacrogol 1000

(polyoxyethylene glycol 1000; HLB=15.8) at a weight ratio of 4:1. The non-ionic surfactants used to form microemulsion were Brij® 78 (polyoxyl-20-stearyl ether; HLB=15.3) or Tween® 80 (polyoxylethylene-20-sorbitan monooleate; HLB=15). To study the effect of types and amounts of matrix material and surfactant on the microemulsion formation and nanoparticles size, the ingredients concentration used in the formulation are listed in Table 3-1 (see details in Tables B1-B8 in appendix B).

Table 3-1 The composition of microemulsion.

Chemical	Concentration
Matrix material (oil phase) - Brij® 72 or - Non ionic emulsifying wax (Cetostearyl alcohol with Tween® 20 or Tween® 60 or Cetomacrogol 1000 of weight ratio of 4:1)	2 mg/mL (0.2% w/w)
Surfactant phase - 100 mM Brij® 78 solution or - 10% Tween® 80 solution	4 - 12 mM 20 – 30 mM
Filtered and de-ionized water (water phase)	q.s.

The microemulsion was prepared by accurately weighing the matrix material into a 10-mL screwed-capped glass vial and melting it at 60±2°C. Then, various amounts of surfactant stock solution (10% Tween® 80 and 100 mM Brij® 78 stock solutions) were added to the melted mixture under stirring for 10 min. The water was added lastly to make a required final volume and the sample was then stirred for another 10 minutes. The sample, which appeared clear and non-birefrigent at 60±2°C through cross polarized lenses, was classified as a microemulsion. The warm microemulsion (60°C) was normal cooled down to room temperature (25°C) in 10 minutes of mild magnetic stirring to produce the nanoparticles. To investigate the

effect of cooling method on the nanoparticle size, another cooling method, rapid cooling the warm microemulsion (60°C) in an ice-bath (5°C) under mild magnetic stirring within 10 minutes, was also studied. Each preparation was evaluated for the appearance and particle size. The appropriate formulation, in terms of nanoparticles size and stability, was selected for the further study.

1.2 Preparation of Coenzyme Q_{10}-loaded nanoparticles from microemulsion system

The microemulsion systems which required the low amounts of surfactants to form the small (below 100 nm) and stable nanoparticles (more than 24 hours) were selected to incorporate the Coenzyme Q_{10}. The preparation process of Coenzyme Q_{10}-loaded nanoparticle was the same method as described in the topic of 1.1.; while Coenzyme Q_{10} was added in the oil phase (matrix material).

1.2.1 Effect of non-ionic surfactant concentration on the size of Coenzyme Q_{10}-loaded nanoparticles

In this study, the amount of matrix material (wax) was fixed at 2 mg/mL (0.2%) and the concentration of non-ionic surfactant (Tween® 80) was varied from 20-60 mM (2.62% - 7.86%) in form of 20% Tween® 80 stock solution. It was noted that the concentration of Tween® 80 at 20 mM was around the phase boundary of microemulsion formation and it was increased to 60 mM to observe the effect of surfactant on the size of Coenzyme Q_{10}-loaded nanoparticle. The concentration of Coenzyme Q_{10} used in the experiment was 1 and 2 mg/mL with respect to the amount of matrix material (wax). The total volume of formulation was 2 mL. The details of each formulation are summarized in Table 3-2 (1 mg/mL Coenzyme Q_{10}) and Table 3-3 (2 mg/mL Coenzyme Q_{10}). The obtained Coenzyme Q_{10}-loaded nanoparticle dispersion was subsequently determined on the appearance and particle sizes.

Table 3-2 The composition of 2 mL of Coenzyme Q_{10}-loaded nanoparticles consisting of 2 mg/mL wax and 1 mg/mL Coenzyme Q_{10}.

Rx	Wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	% w/w	mg	% w/w	mM	mL	% w/w	mL	% w/w
C11	4	0.2	2	0.1	20	0.262	13.10	1.732	86.60
C12	4	0.2	2	0.1	24	0.314	15.72	1.680	83.98
C13	4	0.2	2	0.1	30	0.393	19.65	1.601	80.05
C14	4	0.2	2	0.1	35	0.459	22.93	1.536	76.78
C15	4	0.2	2	0.1	40	0.524	26.20	1.470	73.50
C16	4	0.2	2	0.1	45	0.590	29.48	1.405	70.23
C17	4	0.2	2	0.1	50	0.655	32.75	1.339	66.95
C18	4	0.2	2	0.1	60	0.786	39.30	1.208	60.40

Table 3-3 The composition of 2 mL of Coenzyme Q_{10}-loaded nanoparticles consisting of 2 mg/mL wax and 2 mg/mL Coenzyme Q_{10}.

Rx	Wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	%w/w	mg	%w/w	mM	mL	%w/w	mL	%w/w
C21	4	0.2	4	0.2	20	0.262	13.1	1.730	86.50
C22	4	0.2	4	0.2	24	0.314	15.72	1.678	83.88
C23	4	0.2	4	0.2	30	0.393	19.65	1.599	79.95
C24	4	0.2	4	0.2	35	0.459	22.93	1.534	76.68
C25	4	0.2	4	0.2	40	0.524	26.20	1.468	73.40
C26	4	0.2	4	0.2	45	0.590	29.48	1.403	70.13
C27	4	0.2	4	0.2	50	0.655	32.75	1.337	66.85
C28	4	0.2	4	0.2	60	0.786	39.30	1.206	60.30

1.2.2 Effect of matrix material (wax) concentration on the size of Coenzyme Q_{10}-loaded nanoparticles

In this study, the amounts of wax were increasing from 2 mg/mL to 4 and 6 mg/mL to investigate the effect of wax concentration on the Coenzyme Q_{10} nanoparticle preparation. The concentration of non-ionic surfactant (Tween® 80) was varied from 35-70 mM (4.585% - 9.17%) and 35-90 mM (4.585% – 11.79%) for the wax concentration of 4 mg/mL and 6 mg/mL, respectively. The values of surfactant concentration used were around the phase boundary of microemulsion formation and increased up to observe the effect of surfactant. The concentration of Coenzyme Q_{10} used in the experiment was 1 and 2 mg/mL. The total volume of formulation was still equal to 2 mL. The details of each formulation are summarized in Tables 3-4 and 3-5 for the concentration of 4 mg/mL wax (1 and 2 mg/mL Coenzyme Q_{10}) and Tables 3-6 and 3-7 (1 and 2 mg/mL Coenzyme Q_{10}) for the wax concentration of 6 mg/mL. The obtained Coenzyme Q_{10}-loaded nanoparticles dispersion was subsequently determined on the appearance and particles size analysis.

Table 3-4 The composition of 2 mL of Coenzyme Q_{10}-loaded nanoparticles consisting of 4 mg/mL wax and 1 mg/mL Coenzyme Q_{10}.

Rx	Wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	% w/w	mg	% w/w	mM	mL	% w/w	mL	% w/w
D11	8	0.4	2	0.1	35	0.459	22.93	1.532	76.58
D12	8	0.4	2	0.1	40	0.524	26.20	1.466	73.30
D13	8	0.4	2	0.1	45	0.590	29.48	1.401	70.03
D14	8	0.4	2	0.1	48	0.629	31.44	1.361	68.06
D15	8	0.4	2	0.1	50	0.655	32.75	1.335	66.75
D16	8	0.4	2	0.1	55	0.721	36.03	1.270	63.48
D17	8	0.4	2	0.1	60	0.786	39.30	1.204	60.20
D18	8	0.4	2	0.1	70	0.917	45.85	1.073	53.65

Table 3-5 The composition of 2 mL of Coenzyme Q_{10}-loaded nanoparticles consisting of 4 mg/mL wax and 2 mg/mL Coenzyme Q_{10}.

Rx	Wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	% w/w	mg	% w/w	mM	mL	% w/w	mL	% w/w
D21	8	0.4	4	0.2	35	0.459	22.93	1.530	76.48
D22	8	0.4	4	0.2	40	0.524	26.20	1.464	73.20
D23	8	0.4	4	0.2	45	0.590	29.48	1.399	69.93
D24	8	0.4	4	0.2	48	0.629	31.44	1.359	67.96
D25	8	0.4	4	0.2	50	0.655	32.75	1.333	66.65
D26	8	0.4	4	0.2	55	0.721	36.03	1.268	63.38
D27	8	0.4	4	0.2	60	0.786	39.30	1.202	60.10
D28	8	0.4	4	0.2	70	0.917	45.85	1.071	53.55

Table 3-6 The composition of 2 mL of Coenzyme Q_{10}-loaded nanoparticles consisting of 6 mg/mL wax and 1 mg/mL Coenzyme Q_{10}.

Rx	Wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	% w/w	mg	% w/w	mM	mL	% w/w	mL	% w/w
E11	12	0.6	2	0.1	35	0.459	22.93	1.528	76.38
E12	12	0.6	2	0.1	40	0.524	26.20	1.462	73.10
E13	12	0.6	2	0.1	45	0.590	29.48	1.397	69.83
E14	12	0.6	2	0.1	50	0.655	32.75	1.331	66.55
E15	12	0.6	2	0.1	60	0.786	39.30	1.200	60.00
E16	12	0.6	2	0.1	72	0.943	47.16	1.043	52.14
E17	12	0.6	2	0.1	80	1.048	52.40	0.938	46.90
E18	12	0.6	2	0.1	90	1.179	58.95	0.807	40.35

Table 3-7 The composition of 2 mL of Coenzyme Q_{10}-loaded nanoparticles consisting of 6 mg/mL wax and 2 mg/mL Coenzyme Q_{10}.

Rx	Wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	% w/w	mg	% w/w	mM	mL	% w/w	mL	% w/w
E21	12	0.6	4	0.2	35	0.459	22.93	1.526	76.28
E22	12	0.6	4	0.2	40	0.524	26.20	1.460	73.00
E23	12	0.6	4	0.2	45	0.590	29.48	1.395	69.73
E24	12	0.6	4	0.2	50	0.655	32.75	1.329	66.45
E25	12	0.6	4	0.2	60	0.786	39.30	1.198	59.90
E26	12	0.6	4	0.2	72	0.943	47.16	1.041	52.04
E27	12	0.6	4	0.2	80	1.048	52.40	0.936	46.80
E28	12	0.6	4	0.2	90	1.179	58.95	0.805	40.25

1.2.3 Effect of Coenzyme Q_{10} concentration on the size of Coenzyme Q_{10}-loaded nanoparticles

From the previous studies, Coenzyme Q_{10}- loaded nanoparticles, which used the amount of Tween® 80 around the phase boundary of microemulsion formation, were selected for further investigation on the effect of Coenzyme Q_{10} concentration on the nanoparticles preparation. The amounts of Coenzyme Q_{10} were varied from 1 to 4 mg/mL (0.1-0.4%). The amount of Tween® 80 used was 24 mM (3.144%), 48 mM (6.288) and 72 mM (9.432%) for the system containing 2, 4 and 6 mg/mL wax, respectively. The details of each formulation are summarized in Table 3-8.

The nanoparticles were then evaluated for the entrapment efficiency as described in 3. The formulation of Coenzyme Q_{10}- loaded nanoparticles, which

showed small particles, uniformity of particle size distribution, high amount of entrapment efficiency and low amount of Tween® 80, was subjected to the further characterization process including morphology (TEM) and thermal analysis (DSC) as described in the 4.2 and 4.3. In addition, the *in vitro* release study of the selected formulation was also investigated with an aid of centrifugal ultra-filtration technique as described in 5. Moreover, the selected formulation was studied in their stability and undergone in freeze-dry process to increase the stability. Finally, the freeze-drided product was mixed with commercial cream base for investigate the appearance and short term stability of Coenzyme Q_{10}-loaded nanoparticles in a cream base.

Table 3-8 Composition of 2-mL Coenzyme Q_{10}-loaded nanoparticles consisting of various amout of Tween® 80, wax and Coenzyme Q_{10}.

Rx	wax		Coenzyme Q_{10}		20% Tween® 80			Water	
	mg	%w/w	mg	%w/w	mM	mL	%w/w	mL	%w/w
C12	4	0.2	2	0.1	24	0.314	15.72	1.680	83.98
C22	4	0.2	4	0.2	24	0.314	15.72	1.678	83.88
C32	4	0.2	6	0.3	24	0.314	15.72	1.676	83.78
C42	4	0.2	8	0.4	24	0.314	15.72	1.674	83.68
D14	8	0.4	2	0.1	48	0.629	31.44	1.361	68.06
D24	8	0.4	4	0.2	48	0.629	31.44	1.359	67.96
D34	8	0.4	6	0.3	48	0.629	31.44	1.357	67.86
D44	8	0.4	8	0.4	48	0.629	31.44	1.355	67.76
E16	12	0.6	2	0.1	72	0.943	47.16	1.043	52.14
E26	12	0.6	4	0.2	72	0.943	47.16	1.041	52.04
E36	12	0.6	6	0.3	72	0.943	47.16	1.039	51.94
E46	12	0.6	8	0.4	72	0.943	47.16	1.038	51.84

2. Quantitative analysis of Coenzyme Q_{10} by HPLC method

2.1 HPLC condition

A reversed – phase HPLC assay was performed and validated to determine the amount of Coenzyme Q_{10}. The HPLC condition used in this research was modified from the method of Rungsimakarn (2001). The condition is shown as following.

Column	: HiQ-sil® C18, 150 x 4.6 mm, 5 μm Particle size
Mobile phase	: Methanol and ethanol (15:85 v/v)
Flow rate	: 1.2 mL/min
Flow system	: Isocratic elution
Injection volume	: 20 μL
Temperature	: Ambient
Detector	: UV-Visible at a wavelength of 275 nm
Run time	: 10 min

The mobile phase solution, methanol and ethanol, was filtered through 0.1 cellulose acetate membrane filter and degassed before use.

2.2 Preparation of standard solution

Standard stock solution of Coenzyme Q_{10} solution (1 mg/mL) was prepared by dissolving an accurate weight of Coenzyme Q_{10}, 10 mg, with dioxane in 10-mL volumetric flask. The certain volume of 0.05, 0.1, 0.2, 0.4 and 0.8 mL of 1 mg/mL stock standard Coenzyme Q_{10} solution was placed in 10-mL volumetric

flasks, and adjusted to volume with 1,4 dioxane. The final concentrations of working standard solutions were 5, 10, 20, 40 and 80 μg/mL, respectively.

2.3 Validation of HPLC method

Analytical parameters validated were specificity, precision, accuracy and linearity

2.3.1 Specificity

Specificity of the analytical method was determine by injecting 20 μL of the following solution; dioxane (solvent), nanoparticles placebo dispersion (without coenzyme Q_{10}), and Coenzyme Q_{10} in dioxane. This was to be ensuring that the peak of Coenzyme Q10 had been completely separated from the peak of dioxane and other components in the sample (emulsifying wax and Tween® 80).

2.3.2 Accuracy

Five concentrations of Coenzyme Q_{10} working standard solution, 5, 10, 20, 40 and 80 μg/mL, were prepared and injected into HPLC column. Three determinations per concentration were done and the percentage of analytical recovery of each concentration was calculated as the following equation:

$$\% \text{ recovery} = \frac{\text{Measured concentration}}{\text{Actual concentration}} \times 100$$

2.3.3 Precision

Precision of the analytical method was assessed from the percentage of coefficient of variation (%CV) of analyzed Coenzyme Q_{10} concentration, which was obtained by the following equation:

$$\% \text{ CV} = \frac{\text{Standard deviation}}{\text{Means}} \times 100$$

a) Within-run precision

The within run precision was determined by analyzing three sets of the five concentrations of Coenzyme Q_{10} working standard solution, 5, 10, 20, 40 and 80 μg/mL, in the same day. The percentage coefficient of variation (%CV) of the measured concentration of Coenzyme Q_{10} for each concentration was determined.

b) Between-run precision

The between run precision was determined by comparing each concentration of Coenzyme Q_{10} working standard solution, 5, 10, 20, 40 and 80 μg/mL, prepared and injected on different three days. The percentage coefficient of variation (%CV) of the measured concentration of Coenzyme Q_{10} for each concentration in different three days was determined.

2.3.4 Linearity

The standard curve was performed by plotting peak area of Coenzyme Q_{10} versus working standard concentrations, 5, 10, 20, 40 and 80 μg/mL. The standard curve was fitted with a straight line using the least square linear regression analysis.

2.3.5 LOD and LOQ

The LOD and LOQ were calculated from the results of analysis of standard solution for validation by plotting the concentration against peak area. The equation for LOD is 3.3 (SD/S) where, SD is the residual standard deviation of regression line and S is the slope. The equation for LOQ is 10(SD/S) where, SD is the residual standard deviation of regression line and S is the slope.

3. Determination of the entrapment efficiency

Coenzyme Q_{10}-loaded nanoparticles were separated from aqueous dispersion medium before a determination of entrapment by centrifugal ultrafiltration technique (Klang and Benita, 1998). The nanosep® centrifugal device contains a sample reservoir part, an ultrafiltration membrane with a molecular weight cut-off of 300 kDa, and a filtrate receiver part. Firstly, prior to use, the device was filtered with 70% ethanol and ultrapure water for sanitization purpose. The 400 µL of Coenzyme Q_{10}-loaded nanoparticles dispersion was placed in sample reservoir part of the centrifugal device by pipetting, and then firmly placing the sample reservoir into the filtrate receiver and the centrifugal device was tightly capped. The centrifugal tube was then placed into a fixed-angle centrifuge rotor and was centrifuged at 10000 × g for 5 minute. The amount of Coenzyme Q_{10} in sample reservoir part (retentate) and receiver part (filtrate) were analyzed by HPLC. The amounts found were considered as the entrapped and the un-entrapped Coenzyme Q_{10}, respectively. Before HPLC analysis, the retentate and filtrate were dissolved in dioxane in 10-mL volumetric flask for 1 and 2 mg/mL Coenzyme Q_{10} and in 25-mL volumetric flask for 3 and 4 mg/mL Coenzyme Q_{10}. The entrapment efficiency (%) of Coenzyme Q_{10}-loaded nanoparticles was calculated using the following equation:

$$\% \text{ entrapment efficiency} = 100 \times \frac{\text{entrapped Coenzyme } Q_{10}}{\text{total Coenzyme } Q_{10}}$$

When the entrapped Coenzyme Q_{10} is the retenate part (liquid mass) and the total Coenzyme Q_{10} is retentate part plus the filtrate part. The %recovery of Coenzyme Q_{10} was also calculated as the ratio of sum of un-entrapped and entrapped Coenzyme Q_{10} against total Coenzyme Q_{10} in the prepared formulation.

4. Characterization of nanoparticles and Coenzyme Q_{10}-loaded nanoparticles

4.1 Determination of particle size and size distribution

The size and size distribution of the nanoparticles and Coenzyme Q_{10}-loaded nanopaticles were measured by photon correlation spectrometer or PCS (Malvern 4700, Malvern Instruments Ltd., UK) at the scattering angle of 90°.

A photon correlation spectrometer uses He-Ne laser at a fixed wavelength of 632.8 nm. About 3-5 drops of sample nanoparticles were dispersed in 5 mL of triple distilled water, which was put in the quartz cuvette, to ensure that the light scattering signal as indicated by the particle counts per second (CPS) is within the sensitivity range of the instrument (40-200 CPS or medium range). The triple distilled water was filtered through 0.45 μm filter before use to reduce the effect of any impurites. The sample was then placed in the instrument and allowed for temperature equilibrium between samples and sampling holder at 30°C. From PCS measurements, the data are reported as both the intensity weighted mean diameter (z – average diameter), which is an estimate value for the size of particles, and polydispersity index (PI), which is a measure of the width of the distribution or size

distribution. The mean particles size values are the mean of three measurements of 8 minute each divided into 10 subruns.

4.2 Determination of the morphology of Coenzyme Q_{10} nanoparticles

The morphology of the Coenzyme Q_{10}-loaded nanoparticles was investigated by Transmission Electron Microscopy (TEM). Prior to the TEM measurement, the sample was prepared by negative staining method. A drop of Coenzyme Q_{10}-loaded nanoparticles was placed on a grid and left on for 1-3 minutes, then dropped 2% of negative stain solution, phosphotungstic acid (PTA), on a grid and left for 1 minute. After sample dried, it was transferred into TEM for investigating the particles shape.

4.3 Thermal analysis by Differential Scanning Calorimetry (DSC)

The DSC analysis was used to investigate the physical state of the obtained Coenzyme Q_{10}-loaded nanoparticles using a differential scanning calorimeter (Netzsch DSC 204F1, Germany). Approximately 4 mg of samples were weighed into a crimped aluminum pan. An empty pan was used as a reference. The samples were heated at a rate of 5 °C/min from 10 °C to 90 °C. The analysis was performed under nitrogen purge by use of nitrogen as flush gas (60 mL/min). Melting point corresponds to the maxima of DSC curves. The samples were composed of emulsifying wax, Coenzyme Q_{10}, physical mixing of Coenzyme Q_{10} and emulsifying wax, and the obtained CoenzymeQ_{10} nanoparticles.

5. *In vitro* release study

The Coenzyme Q_{10}-loaded nanoparticles were determined on the *in vitro* release pattern by using centrifugal ultrafiltration technique (Klang and Benita, 1998).

One-mL of Coenzyme Q_{10}-loaded nanoparticles dispersion was suspended in 50 ml of 30 % ethanol (Bronaugh, *et al.*, 1999; Shah, Elkins and Williams, 1999) under stirring sink condition at 37 °C ± 2°C. At given time intervals, 400 µL of the release solution, where the Coenzyme Q_{10}-loaded nanoparticles dispersion was suspended, were collected in nanosep® centrifugal device and subjected to centrifugation at 10000 x g for 5 minutes. The fresh fluid, 30% ethanol, was replaced after each collection. The filtrate or free drug solutions were adjusted to volume of 1-mL with dioxane and assayed for Coenzyme Q_{10} content by HPLC method as described previously. The percentage release of Coenzyme Q_{10} was calculated from the ratio of Coenzyme Q_{10} concentration in the filtrate versus the total concentration of Coenzyme Q_{10} in the release solution at each collection time. A plot of percentage release of Coenzyme Q_{10} versus time was performed to investigate the release profile of Coenzyme Q_{10} from nanoparticles.

6. Stability studies of Coenzyme Q_{10}-loaded nanoparticles

6.1 Non freeze-dried Coenzyme Q_{10}-loaded nanoparticles stability (dispersion form)

The stability of Coenzyme Q_{10}-loaded nanoparticles dispersion was observed after storage in light-resistant container at room temperature (25 °C) and in refrigerator (4°C). The samples were measured for the change particles size by PCS after 24 hours, 1, 4 and 8 weeks and for the remaining amount of Coenzyme after storage compared to freshly prepared preparation by HPLC after 4 and 8 weeks.

6.2 Freeze-dried Coenzyme Q_{10}-loaded nanoparticles and short term stability

Coenzyme Q_{10}-loaded nanoparticles dispersions were freeze-dried to obtain a dry product which would be used for further studies such as thermal analysis and stability in the cream base preparation. The freeze-dried product is known to increase the stability of Coenzyme Q_{10}-loaded nanoparticles after long term storage. The freeze-dried Coenzyme Q_{10}-loaded nanoparticles were obtained by freezing the sample at –20°C for 48 hours and drying for 48 hours using Freeze-dryer (LyoLab w/PC., USA). However, from the preliminary study, when only Coenzyme Q_{10}-loaded nanoparticles dispersions were subjected to freeze-dry process, the desirable dry product was not achieved. So, the cryoprotective agents, dextrose and mannitol in final concentration of 1, 2 and 4%, were used and then the appearance (puffy dry) and size (in nanometric range) were eveluated. Moreover, the short term stability of freeze-dried Coenzyme Q_{10}-loaded nanoparticles was performed after storage in test tube and wrapped with aluminum foil for 4 weeks at room temperature (25°C) and in refrigerator (4°C). The stability of Coenzyme Q_{10} in freeze-dried product was expressed as the percentage of Coenzyme Q_{10} remaining at 4 weeks in relation to the initial amount.

6.3 Stability of mixed-cream containing Coenzyme Q_{10}-loaded nanoparticles

The freeze-dried Coenzyme Q_{10}–loaded nanoparticles were mixed with commercial cream base (o/w) for investigation of the physical appearance. Coenzyme Q_{10}-loaded nanoparticles mixed-cream was observed in physical change under accelerated condition by storing the product at 4°C in refrigerator for 48 hours and then at 45°C in an hot air oven for 48 hours. The heating-cooling cycling was continued for 6 cycles. Absence of phase separation and color change were expected

in stable formulations. Coenzyme Q_{10} crystal powder was also mixed with cream to use for comparison. The compositions of o/w cream base were shown as follow:

Rx	Glyceryl monostearate	10	grams
	Cetyl alcohol	5	grams
	Stearyl alcohol	2.5	grams
	Liquid paraffin	10	grams
	Preservative qs		
	Purified water qs	1000	grams

Moreover, the short term stability of Coenzyme Q_{10}-loaded nanoparticles mixed-cream was performed after being kept in light-resistant container for 4 week at room temperature (25°C). The remaining amount of Coenzyme Q_{10} in relation to initial amounts was determined by HPLC method.

7. Statistical analysis

The experiment for size analysis were carried out in triplicate (three different determination of the same sample, n=3) and the data were calculated as mean ± SD. Statistical comparisons of the experiment results were performed by one-way analysis of variance (ANOVA) at an α level of 0.05. The difference in results between the two groups was compared by Turkey's test at the significant levels of $P < .05$.