CHAPTER III
MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources and used as received. Distilled water was used throughout the experiments.

Chemicals

1. Purified soybean oil (Ph. Eur., USP) (Lipoid GMBH, Germany) Lot no. 700087/906
2. Egg phospholipids (Lipoid E80) (Lipoid GMBH, Germany) Lot no.1031233-12
3. Sodium oleate (Lipoid GMBH, Germany) Lot no. 204070-1
4. Tween® 80 polyoxyethylene sorbitanmonooleate (Acros Organics, USA) Lot no. A0206384001
5. *d-α*-tocopheryl polyethyleneglycol-1000-succinate (Vitamin E-TPGS) (Eastman, UK) Lot no. 00007000
6. Glycerine (USP) (UNIQEMA, Belgium) Lot no. 21324M
7. *dl-α*-Tocopherol (DSM, Switzerland) Lot no. UT04100049
8. Sodium hydroxide (Merck, Germany) Lot no. B582798
9. sodium dihydrogen orthophosphate (Ajax Finechem, Australia) Lot no. AF502342
10. 10% Intralipid® (Fresenius Kabi AB, Sweden) Batch no. 0411073
11. 20% Intralipid® (Fresenius Kabi AB, Sweden) Batch no. SL1595

12. Vitalipid® N Infant (Fresenius Kabi AB, Sweden) Batch no. 1024825
13. Osmolality standard 100 mmol/kg, Opti-mole® (Wescor, Inc., USA)Lot No. 0104033
14. Osmolality standard 290 mmol/kg, Opti-mole® (Wescor, Inc., USA) Lot No. 0294063
15. Osmolality standard 1000 mmol/kg, Opti-mole® (Wescor, Inc., USA) Lot No. 1004093
16. Zeta potential transfer standard -50 mV ± 5 mV (Malvern Instruments, UK) Lot no. 160401/S

Equipments

1. Analytical Balance (AT200, Mettler, Germany)
2. High speed homogenizer (D-500, Wiggen Hauser, Germany)
3. High pressure homogenizer (Emulsiflex® C50, Avestin, Inc., Canada)
4. High pressure homogenizer (Emulsiflex® C5, Avestin, Inc., Canada)
5. Water bath (GFL, Germany)
6. pH meter (Model 3000, WTW, Germany)
7. Autoclave (Model SS320, Tomy, Japan)
8. Osmometer (Vapropressure osmometer® Model 5520, Wescor, Inc., USA)
9. Particle size analyzer (Mastersizer 2000 with Hydro 2000S, Malvern instruments, UK)
10. Zeta potential analyzer (Zetasizer 4, Malvern Instruments, UK)
11. Centrifuge (Model K3 system, Centurion, UK)
12. UV-visible Spectrophotometer (UV-160A, Shimadzu, Japan)

Methods

1. Preparation of lipid emulsions

The effects of emulsion compositions in formulation were investigated for the differences in physicochemical properties of the emulsion, including physical stability. The amount and type of emulsifiers and cosurfactants were varied.

In this experiment, egg phospholipid was used as a main emulsifier in the emulsion formulation. It composes mainly of 80% phosphatidylcholine, 3% lysophosphatidylcholine, 9.5% phosphatidylethanolamine and others (see Appendix B). Three types of cosurfactant were used, i.e. sodium oleate, polyoxyethylene-20-sorbitan monooleate (Tween® 80) and *d*-α-tocopheryl polyethyleneglycol-1000-succinate (Vitamin E-TPGS). Glycerine at a concentration of 2.5% was used for isotonicity adjustment of the emulsion. *dl*-α-tocopherol was added as an concentrations of antioxidant at 0.001 and 0.002% for 10 and 20% oil respectively. The formulations of emulsion studied are shown in Table 3.

Table 3. Compositions of 10% and 20% lipid emulsions.

Ingredients (%w/w)	Rx 1	Rx 2	Rx 3	Rx 4	Rx 5	Rx 6	Rx 7	Rx 8	Rx 9	Rx 10	Rx 11	Rx 12	Rx 13	Rx 14	Rx 15	Rx 16	Rx 17	Rx 18	Rx 19	Rx 20	Rx 21	Rx 22
Soybean oil	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	20	20	20	20	20	20	20
dl-α- tocopherol	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
Egg phospholipids	1.2	1.5	2.0	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Tween® 80	-	-	-	1.2	0.9	0.6	0.4	-	-	-	-	-	-	-	-	1.2	0.9	0.6	-	-	-	-
Vitamin E-TPGS	-	-	-	-	-	-	-	1.2	0.9	0.6	0.4	-	-	-	-	-	-	-	1.2	0.9	0.6	0.4
Sodium oleate	-	-	-	-	-	-	-	-	-	-	-	0.06	0.03	0.02	0.015	-	-	-	-	-	-	-
Glycerine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
D.I.water to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

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1.1 Preparation of coarse emulsion

The emulsions were prepared by using the method shown in Figure 4. The four formulations, Rx1, Rx5, Rx8 and Rx13 were selected to study the method of preparation of a coarse emulsion. To prepare the aqueous phase, 1.2% w/w of egg phospholipid and 2.5% w/w of glycerol were dispersed in the water phase at 55-60°C. The oil phase was a mixture of 10% w/w of purified soybean oil and 0.001% w/w of *dl-α*-tocopherol. Then, the oil phase was heated to 55-60°C before adding into the water phase under stirring at 55-60°C. The mixture was then homogenized by a high speed homogenizer at homogenization speed in the range of 10,000 to 18,000 rpm for 5 or 10 minutes. The pH of emulsion was adjusted to 8 by adding the solution of 0.2 N sodium hydroxide. The sample was then visually observed for the emulsion formation and the stability after storage for 24 hours at room temperature.

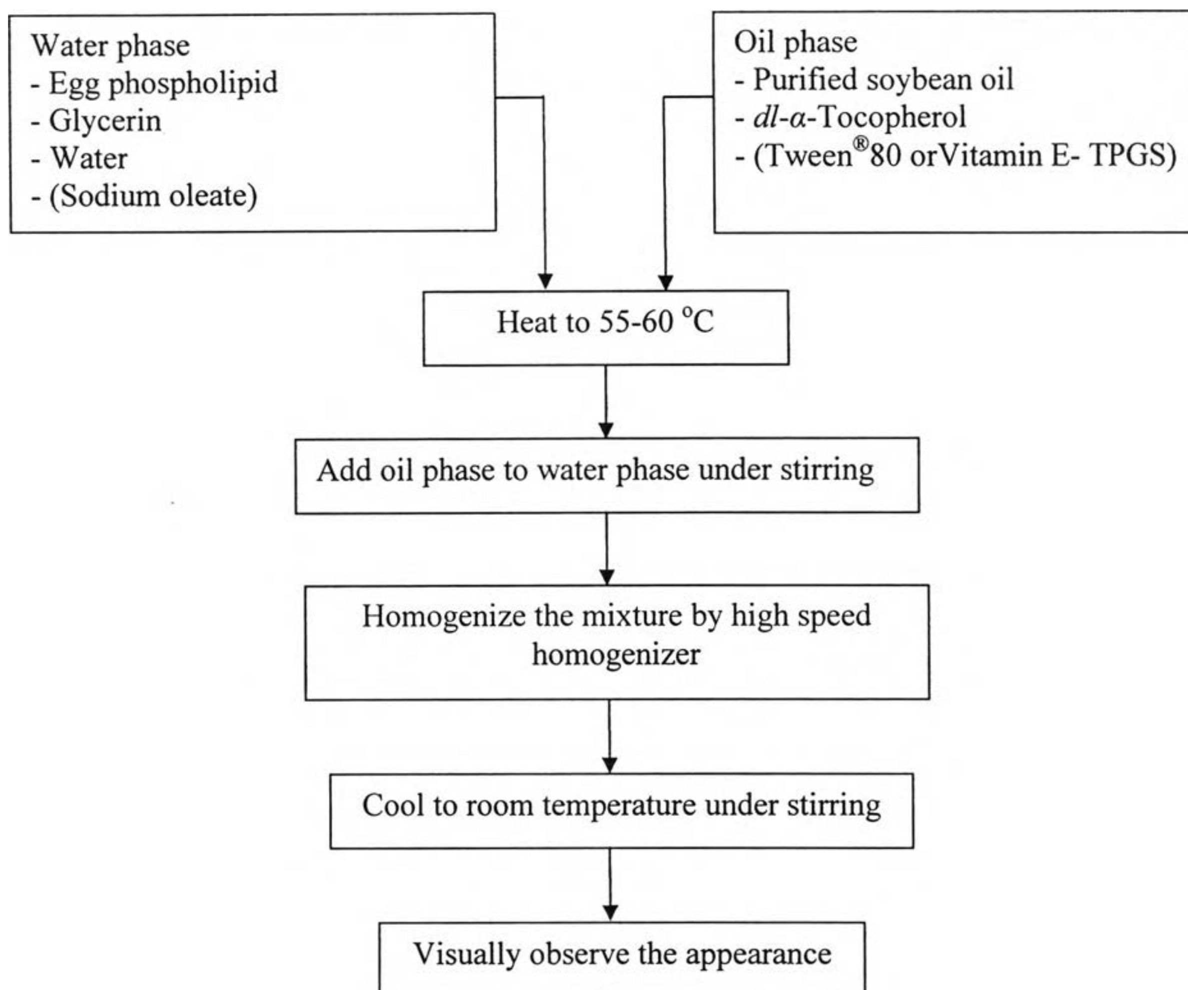

Figure 6. Schematic diagram of the preparation process of a coarse emulsion.

1.2 Preparation of lipid emulsions by high pressure homogenization

The size reduction of a coarse emulsion was done and the effects of homogenization process were studied.

1.2.1 Number of homogenization cycle

The coarse emulsion was passed through a high pressure homogenizer (Emulsiflex C-50 and Emulsiflex C-5) at 15,000 psi. (Ruangthurakit, 2000) for 3, 5, 7 and 10 cycles respectively. The particle size of emulsions was determined by Mastersizer 2000. The number of cycles that provided optimal stability was selected to study the effect of homogenization pressure.

1.2.2 Pressure of homogenization

The coarse emulsion was passed through a high pressure homogenizer at pressures of 10,000, 15,000 and 20,000 psi for 5 cycles. The pressure of homogenization that provided optimal stability and small particle size is selected for further investigation.

1.3 Preparation of autoclaved emulsions

The emulsion was purged with nitrogen gas for a few minutes before being kept in the vial that was sealed with a rubber and aluminum cap. The emulsion was sterilized by autoclaving at 121° C for 15 minutes. The physicochemical properties of the emulsion were determined as stated in 3.

2. Effect of cosurfactants on emulsion formation

2.1 Weight ratio of EPC to cosurfactants

The effect of type and amount of cosurfactant on the emulsion formation were studied by using the preparation process obtained from the previous experiment. The formulations ranging from Rx1 to Rx22 were prepared and their physicochemical properties were determined as stated in 3. The ionic surfactant (sodim oleate) and nonionic surfactants (Tween® 80 and Vitamin E-TPGS) were added in the formulation to study the effect of their emulsifying properties on the emulsion formation. Sodium oleate is often used in commercial parenteral emulsions, for example Lipofundin® MCT, Abbolipid®, Schiwalipid® and others, to obtain stable formulations during the autoclaving process (Joseph, 1990). Due to a hemolytic effect, the low concentrations of sodium oleate less than 0.1% are generally used in commercial parenteral emulsions (Collins-Gold, Lyons and Bartholow, 1990). In the present study, the concentrations of sodium oleate were ranging from 0.015% to 0.06% w/w. Tween® 80 is a nonionic surfactant that could improve the stability of the emulsions by utilizing the steric stability of the surfactant layer (Benitaharacteri and Levy, 1989; Yamaguchi *et al.*, 1995). Vitamin E-TPGS is a water soluble derivative of natural source vitamin E. Structurally, it comprises a dual nature of lipophilicity and hydrophilicity, similar to a surface-active agent, moreover, it is stable under heat sterilization conditions (Mu and Feng, 2002). The weight ratio of Tween® 80 to egg phospholipids was varied from 1:1 to 1:3. Likewise, the weight ratio of Vitamin E-TPGS to egg phospholipids was varied from 1:1 to 1:3. Consequently, the concentration of Tween® 80 and Vitamin E-TPGS used were 0.4% to 1.2% w/w while the concentration of EPC was fixed at the concentration of 1.2% w/w.

2.2 Effect of total emulsifier concentration

The total emulsifier concentration of EPC and cosurfactant was optimized by preparing emulsions with varying amounts of total emulsifier (1.5% to 3.0%) at a weight ratio of EPC to cosurfactant obtained from 2.1.

3. Investigation of the physicochemical properties of emulsions

The sterile and non-sterile emulsions were characterized for the physicochemical properties. The properties of commercial parenteral emulsions were also studied.

3.1 Particle size analysis (Candido, 2001)

The mean droplet size and size distribution were examined by use of a computerized laser light scattering apparatus (Mastersizer 2000®).

The laser light scatterer contains the optical unit to capture the actual scattering pattern from a field of particles, and then calculates the size of particles that create the pattern. There are three distinct procedures involved in measuring a sample. Firstly, the sample is prepared and dispersed in water (dispersant) to the correct concentration which provides the optimal laser obscuration and then delivers to the optical unit, which is used to collect the raw data for measuring the size of the particles. Secondly, the scattering pattern of the particles from the prepared sample is captured. The detector array within the optical unit is made up of many individual detectors. Each detector will collect the light scattering from a particular range of angles. Finally, when the measurement is complete, the raw data contained in the measurement can be analyzed by Malvern software, which is set to make a

measurement and then analyses the data automatically. Interpretation of results, the instrument used the volume of the particle to measure the size. The statistics of the distribution are calculated from the results using the derived diameters D[m,n] define the mean and other moments of particle size. The derived diameters include d (v, 0.5), d (v, 0.1), d (v, 0.9), D[4,3] and D[3,2] where

- d (v, 0.5) is the size in microns at which 50% of the sample is smaller and 50% is larger than the given size. This value is also known as Mass Median Diameter (MMD).
- d (v, 0.1) is the size of particle below which 10% of the sample lies.
- d (v, 0.9) gives a size of particle below which 90% of the sample lies.
- D[4,3] is the volume weighted mean diameter.
- D[3,2] is the surface area mean diameter or the Sauter mean

3.2 Determination of zeta potential

The Zetasizer® 4 is an instrument for measuring the velocity of charged, colloidal particles in liquid. The electrophoretic mobility is determined by dividing the measured velocity by the electric filed strength. Finally, the zeta potential is calculated from the solution conditions and the mobility. A high zeta potential (>30 mV), either positive or negative, signifies a stable dispersion while a low zeta potential(< 30 mV), either positive or negative, signifies an unstable dispersion (Heurtault *et al.*, 2003).

For all the measurements, the lipid emulsions were diluted in ultra pure water and measure at the count rate of 100 – 500 KCps.

3.3 Determination of pH

pH of emulsion was determined by use of pH microprocessor 3000 (WTW).

3.4 Determination of osmolality

Osmolality of the emulsion was determined by using Osmometer (Vapro® 5520). It is an electronic adaptation of the hygrometric method of vapor pressure determination. Vapro osmometer was calibrated using 100 mmol/kg, 290 mmol/kg and 1000 mmol/kg standard solutions.

To measure osmolality, a 10 microliter of lipid emulsion was transferred into a solute-free paper disc placed on the sample holder, whereupon the sample holder was pushed into the instrument and the sample chamber was locked. A fine-wire thermocouple hygrometer was the sensing element that was suspended in a metal mount. As vapor pressure equilibrated in the chamber airspace, the thermocouple sensed the ambient temperature of the air, thus establishing the reference point for the measurement. Under electronic control, the thermocouple then seeks the dew point temperature within the enclosed space, giving an output proportional to the differential temperature. The difference between the ambient temperature and the dew point temperature was the dew point temperature depression that was and explicit function of solution vapor pressure.

4. Determination of emulsion stability

After being stored for 24 hours, 1 week and 4 week at room temperature, 4°C and 40°C, emulsions were visually observed for any changes in physical appearance

such as flocculation, creaming, coalescence and phase separation. The emulsions were also determined for any changes in physicochemical properties as described in 3. The samples were also kept at 4°C and 40°C at the same time interval for accelerated stability test.

5. Sterility Test

The below sterile emulsions were examined for microbial contamination using a microbial limit test for sterility of parenteral product (USP 27). The test procedure includes total aerobic microbial count, total aerobic mould count and test for specific microorganism, *Stapphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella spp.* and *Escherichia coli*.

Rx	Emulsifiers	Total emulsifier concentration (%)
LE1	EPC:Vitamin E-TPGS at 3:1	2.0
LE2	EPC:Vitamin E-TPGS at 2:1	1.5
LE3	EPC:Vitamin E-TPGS at 2:1	2.0
LE4	EPC:Tween® 80 at 2:1	1.5
LE5	EPC:Tween® 80 at 2:1	2.0

5.1 Media preparation

The method of preparation was followed microbiological tests in USP 27. The dehydrated media used were Tryptic Soy Agar (TSA) and Sobouraud Dextrose Agar (SDA). TSA was used to estimate the number of total aerobic bacterial while SDA was used to estimate the number of total aerobic mould. Lactose

Broth (LB), and Triptic Soy Broth (TSB) were used for specific organisms; MacConkey Agar Medium (MAC) for *Escherichia coli*; Brilliant Green Agar Medium (BGA) for *Samonella species*; Mannitol-Salt Agar Medium (MSA) for *Staphylococcus aureus*; and Cetrimide Agar Medium (CA) for *Pseudomonas aeruginosa*.

The media were purposed as follows (USP 27, 2004)

A. Tryptic Soy Agar (TSA) Medium (Casein-peptone soymeal-peptone agar USP)

To prepare 200 ml of Tryptic Soy Agar media, 8 g of tryptic soy agar was suspended in distilled water and then adjusted volume to 200 ml.

B. Sabouraud Dextrose Agar (SDA) Medium

Sabouraud Dextrose Agar media was prepared by suspending 13 g of SDA in 200 ml of distilled water. The mixture was then boiled to effect solution.

C. Cetrimide Agar (CA) Medium

Cetrimide Agar media was prepared by suspending 4.53 g of CA in 100 ml of distilled water and then boiled for 1 minute to effect solution.

D. MacConkey Agar (MAC) Medium

To prepare MacConkey Agar media, 5 g of MAC was suspended and boiled with 100 ml of distilled water for 1 minute to effect solution.

E. Brilliant Green Agar (BGA) Medium

To prepare Brilliant Green Agar media, the solution of 5 g BGA in 100 ml of distilled water was boiled for 1 minute to effect solution.

F. Mannitol-Salt Agar (MSA) Medium

To prepared Mannitol-Salt Agar media, 11.1 g of MSA was suspended in 100

ml of distilled water. The mixture was then heated with frequent agitation and boiled for 1 minute to effect solution.

G. Fluid Lactose Medium

Fluid Lactose media was prepared by suspending 1.17 g of LB in 90 ml of distilled water.

H. Fluid Soybean-Casein digest medium

Fluid Soybean-Casein Digest media was prepared by suspending 2.7 g of TSB in 90 ml of distilled water.

5.2 Phosphate buffer solution preparation

Phosphate buffer solution was used as diluent for sterility test of emulsion. It was composed of the following ingredients (USP 27, 2004):

Ingredient	Amount
Potassium dihydrogen phosphate (KH_2PO_4)	1.78g
Di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.10H_2O$)	2.88 g
Sodium chloride (NaCl)	2.15 g
Peptone	0.5 g
Tween® 80	2.5 ml
Purified water to make	500 ml

These components were dissolved together and the pH adjusted to 7.0 by adding of a sodium hydroxide solution. The phosphate buffer was sterilized and stored at 4°C before use.

5.3 Specimen preparation and test procedure (USP 27, 2004)

5.3.1 Total aerobic bacterial count

The plate method was used for specimen preparation. The different dilution, 1:10, 1:100 and 1:1000 of lipid emulsion was prepared by accurately pipette 10 ml of lipid emulsion into 90 ml of phosphate buffer. The 10 ml of a previous dilution was used to prepare the next dilution (Figure 7). After the dilution were made, two plates were prepared by pipetting 1 ml of each dilution into each of two sterile petri dishes and then 20 ml of TSA medium at approximately 45-50°C was added to each dish. The sample was mixed with the medium by rotating the dishes and then allowed the mixture to solidify at room temperature. The plates were incubated at 37°C, 50 ± 15% RH for 48 to 72 hours in an incubator and examined for bacterial growth. The number of colonies was counted, and the average of colonies of the two plates was expressed in terms of the number of microorganisms per ml of sample. If no microbial colonies were found, it could be concluded that the initial 1:10 dilution of the emulsion contained less than 10 microorganisms per ml.

10 ml 10 ml 10 ml

90 ml PBS 90 ml PBS 90 ml PBS

Lipid emulsion

1 ml 1 ml 1 ml 1 ml 1 ml 1 ml

20 ml of TSA media, melted 45-50°C

Mixed and left to solidify

Incubated at 37°C, 50 ± 15 %RH for 48-72 hours

Counted the number of colonies

Figure 7. Schematic diagram of total aerobic microbial count.

5.3.2 Total aerobic mold count

The test procedure was similar to 5.3.1. However, the medium used was SDA medium and the incubation time was increased to 5 days. The scheme for total aerobic mold count test is shown in Figure 8.

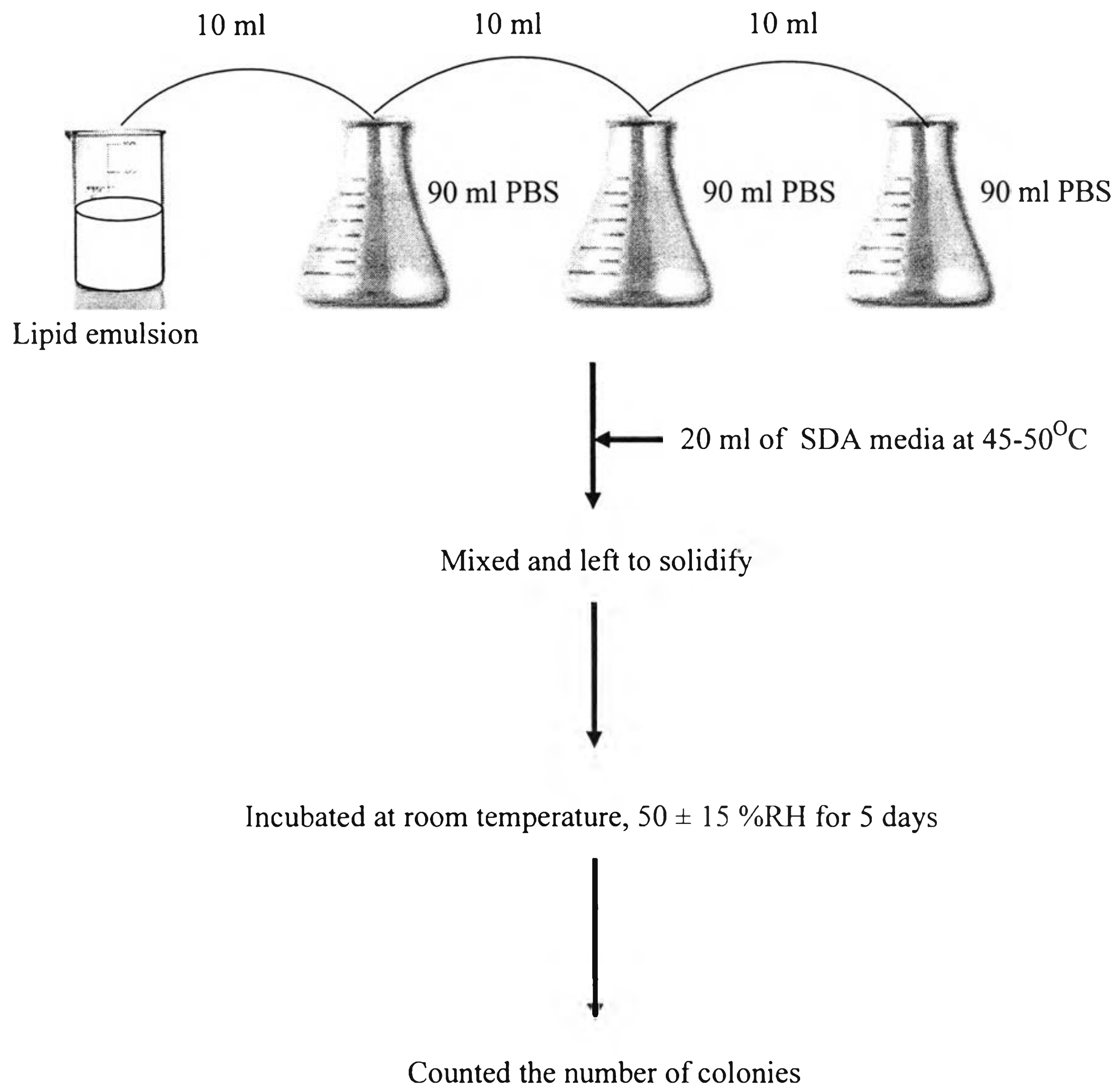

Figure 8. Schematic diagram of total aerobic mold count.

5.3.3 Test for specific microorganism

1) *Staphylococcus aureus* and *Pseudomonas aeruginosa*

A 10 ml sample of lipid emulsion was added into TSB medium to make a 100 ml mixture. The mixture was mixed, and incubated at 37°C for 24 hours. The medium was examined for organism growth. If organism growth was present, inoculation loop was used to streak a portion of the medium onto the surface of MSA medium and of CA medium. The mixture was incubated at 37°C, 50 ± 15 %RH for 24-48 hours. If, upon examination, none of the plates contained colonies having the morphologic characteristics as shown in Table 4, the test lipid emulsion met the requirements for the absence of these organisms.

2) *Samonella species* and *Escherichia coli*

A 10 ml sample of lipid emulsion was added into LB medium to make a 100 ml mixture. The mixture was mixed, and incubated at 37°C for 24 hours. The medium was examined for growth. If organism growth was present, inoculating loop was used to streak a portion of the medium onto the surface of BGA medium and of MCA medium. The mixture was incubated at 37°C, 50 ± 15 %RH for 24-48 hours. If, upon examination, none of the plates contained colonies having he morphologic characteristics as shown in Table 4, the test lipid emulsion met the requirements of the absence of these organisms.

Table 4. Morphologic characteristics of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Samonella species* and *Escherichia coli* on selective agar media.

Selective agar medium	Type of organism	Morphologic characteristic of selective organism	
		Negative result	Positive result
Mannitol-Salt Agar (MSA)	*Staphylococcus aureus*	No growth	Yellow colonies with yellow zones
Cetrimide Agar (CA)	*Pseudomonas aeruginosa*	No growth	Generally greenish colonies
MacConkey Agar (MCA)	*Escherichia coli*	No growth	Brick-red colonies, may have surrounding zone of precipitated bile
Brilliant Green Agar (BGA)	*Samonella species*	No growth	Small, transparent, colorless or pink to white opaque, frequently surrounded by pink to red zone

6. Red blood cell hemolysis

To assess the safety of lipid emulsion for parenteral administration, the hemolysis caused by emulsion was studied. The method of hemolysis study was followed the method of Bock and Müller (1994)

6.1 Phosphate buffer solution preparation for hemolysis test

The isotonic phosphate buffer solution pH 7.4 was prepared by dissolving 1 tablet of phosphate buffer saline tablet in 200 ml of distilled water. Then adjust pH to 7.4 by using disodium hydrogen orthophosphate dehydrate or sodium dihydrogen orthophosphate

6.2 Blood preparation

Red Blood Cells (RBC) were isolated by centrifugation (1000g at 20°C for 5 minutes) of heparinized human blood and redispersed in isotonic phosphate buffer saline (PBS) pH 7.4. The RBC were washed three times with buffer (redispersion followed by centrifugation) and, redispersed to give a final concentration of 2% w/v in PBS. The RBC were stored at 4°C and used in the test within 48 hours.

6.3 Hemolytic test of emulsion

1 ml of each of the isotonic samples containing equidistantly increasing concentrations of the test agent was pipetted into an Eppendorf vial (1.5 ml). 100 μl of the RBC stock dispersion was added to each sample. After incubation at 37°C for 30 minutes (Nagasaka and Ishii, 2001), the samples were remove intact

RBC and cell debris by centrifugation at 3000g for 5 min. 100 μl of the supernatant was added to 2.0 ml of an ethanol/HCl mixture [1part HCl (37% w/w) + 39 parts ethanol (99% v/v)]. The absorption of hemoglobin was determined at 398 nm by photometric monitoring against blank samples. Control samples of 0% lysis (RBC dispersed in buffer) and 100% lysis (RBC dispersed in distilled water) were employed in all experiments. The mean value of three measurements using different samples was recorded.

7. Transmission Electron Microscopy (TEM)

The shape and the surface morphology of stable lipid emulsions containing EPC with either Vitamin E-TPGS or Tween® 80 at the weight ratio of 2:1 at the total emulsifier concentration of 1.5%, namely LE2 and LE4, respectively were investigated compared to commercial product, 10% Intralipid® by means of the transmission electron microscope (TEM).

The procedure for negative staining of a lipid emulsion preparation sample was as follows. A drop of lipid emulsion was applied onto carbon coated grids. After leaving for 1-3 min to allow adsorption of lipid emulsion to a grid, the excess was removed by filter paper. A drop of 2% phosphotungstic acid was applied onto the grid, leaving for 1 min, drawn off by filter paper. Then the grid was air-dried and examined under a transmission electron microscope.