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.

Solvents

1. Acetonitrile, HPLC grade, Labscan Analytical Science, Ireland
2. Methanol, HPLC grade, Labscan Analytical Science, Ireland
3. Propan- 2- ol, HPLC grade, Labscan Analytical Science, Ireland

Ingredients

1. Retinyl palmitate, Fluka Biochemika, $\geq$ 85% (HPLC), Switzerland, Lot No. 409730/1.
2. All-*trans* retinol acetate 2.8 m.I.U./g, Sigma, USA, Lot No. 70K0939
3. Vitamin D_2, Fluka Biochemika, ~ 99% (HPLC), Switzerland, Lot No. 415657/1.
4. Vitamin D_3, Fluka Biochemika, $\geq$ 99.0% (HPLC), Switzerland, Lot No. 415401/1.
5. Tocopherol acetate, Fluka Biochemika, $\geq$ 98% (HPLC), Switzerland, Lot No. 380607/1.
6. VitaminK_1, Fluka Biochemika, $\geq$ 99.0% (HPLC), Switzerland, Lot No. 419960/1.

7. Egg phospholipid, Lipoid E PC, Lipoid GMBH, Ludwigshafen, Germany, Lot No. 306-544-8

8. Soy phospholipid, Epikuron 200®, Lucas Meyer GMBH, Hamburg, Germany

9. Egg Phosphatidylglycerol, Lipoid E PG®, Lipoid GMBH, Ludwigshafen, Germany, Lot No. 899144-1

10. Octadecylamine, 98% (GC), Sigma Chemical Co., St. Louis, USA, Lot No. 20K 3674

11. Polyoxyethylene sorbitanmonooleate, Fluka Biochemika, Switzerland, Lot No. 93780

12. Miglyol 812, Sasol, Werk Witten, Germany, Lot No. 010-319

13. Purified soybean oil, Lipoid, Germany, Lot No. 700042

14. Butylated hydroxytoluene (2, [6]-Di-tert-Butyl-p-cresol), Sigma Chemical Co., St. Louis, USA, Lot No. 70K 0071

15. Vitalipid® N Adult, Vitalipid® Fresenius Kabi, Sweden, Batch No. 27070-02

16. 10% Intralipid®, Fresenius Kabi AB, Sweden, Batch No. 25914-5

17. 20% Intralipid®, Fresenius Kabi AB, Sweden, Batch No. 24956-51

18. Glycerol, BHD Laboratory Supplies, England, Lot No. K23624360708

19. Sodium chloride, Merck, Germany, Lot No. C034

20. HEPES (N- [2- hydroxyethyl] piperazine-N′- [2- ethanesulfonic acid], Sigma Chemical Co., St. Louis, USA, Lot No. 113H57311

21. Osmolality standard 100 mmol/kg, Opti-mole®, Wescor, Inc., USA, Lot No. 0101011

22. Osmolality standard 290 mmol/kg, Opti-mole®, Wescor, Inc., USA, Lot No. 0291018

23. Osmolality standard 1000 mmol/kg, Opti-mole®, Wescor, Inc., USA, Lot No. 1000354

24. Dimethyl sulphoxide, BDH Laboratory Supplies Poole, England, Lot No. K 28748588 107

25. Mannitol salt agar dehydreated, DIFCO, USA, Lot No. 86121 JJ.

26. Cetrimide agar base dehydrated, DIFCO, USA, Lot No. 96854 JC

27. Brilliant green agar dehydrated, DIFCO, USA, Lot No. 97139 JE

28. MacConkey agar dehydrated, DIFCO, USA, Lot No. 91063 JB

29. Sabouraud 4% dextrose agar, Merck, Germany, Lot No. VK 642638 106

30. Tryptic soy agar (Casein-peptone soymeal-peptone agar USP), Merck, Germany, Lot No. VL 606258 045

31. Tryptic soy broth (Casein-peptone soymeal-peptone broth USP), Merck, Germany, Lot No. VK 500959 014

32. Lactose broth, Merck, Germany, Lot No. VK 573261 034

33. Potassium dihydrogen phosphate, Merck, Germany, Lot No. A 645673

34. Sodium chloride, Merck, Germany, Lot No. K 21825904

35. Disodium hydrogen phosphate dodecahydrate, Merck, Germany, Lot No. A852279

36. Peptone (peptone from meat peptic digested), Merck, Germany, Lot No. V 255324 843.

Equipment

1. Autoclave (model ES-315, Tomy Seiko Co., LTD, Japan)

2. Analytical Balance (Mettler PJ 6000®, Mettler-Toledo AG, Switzerland)

3. Centrifuge (model CR 20B3, Hitachi Koki Co., Japan)

4. High speed homogenizer (Vstrol® model D- 79282, Ten-Dottingen , Germany)

5. High pressure homogenizer (Emulsiflex®C50, Avestin, Inc., Canada)

6. HPLC column, (Phenomenex Prodigy® 5u ODS –2, 150 * 4.6 mm, USA)

7. HPLC: equipped with degasser DGU - 14A, pump LC – 10 ADVP, UV- detector SPD – 10Avp/ 10Avvp, and system controller SCL – 10 AVP, (model Class- VP®, shimadzu Inc., Japan)

8. Spectrophotometer (Spectronic 3000 Array®, Milton Roy Company, USA)

9. Microbalance (Mettler AT 200®, Muttler-Toledo AG, Switzerland)

10. Microcentrifuge (Model Z 230 MA, Maschinenfabrik Berthod Hermel AG., Germany)

11. Osmometer (Vapropressure osmometer® model 5520, Wescor, Inc., USA)

12. Particle size analyzer (Mastersizer 2000 with Hydro 2000S, Malvern instruments, UK)

13. pH meter (pH 3000, Wissenschaftl-Techn Werkst™tten, Germany)

14. Shaker bath (Model TBVS HETOMIX, Heto, Denmark)

15. Sonicator (Tru-Sweep® model 275D, Cresp Ultrasonics Corp., USA)

16. Water bath (DT Hetotherm, Heto, Denmark)

17. Zeta potential analyzer (ZetaPlus®, Brookhaven Instruments Co., USA)

Methods

1. Formulation of oil-in-water lipid emulsion

1.1 Emulsion compositions

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 oil, and type of emulsifier and co-emulsifier were varied.

1.1.1 Oil

10% w/w or 20% w/w of oil were used in the formulation to study the effect of increasing oil content on emulsion stability. Soybean oil and a mixture of soybean oil and Miglyol 812 (50: 50) were the two types of oil used in the formulation.

1.1.2 Emulsifier

Egg or soy phospholipids was used as main emulsifier in emulsion formulation. The different source of phospholipids contains difference in phosphatides composition (See Appendix B). Egg phospholipids compose of 92% phosphatidylcholine or more, lysophosphatidylcholine 3%, and other lipids 2% while soy phospholipids compose of 98% phosphatidylcholine and a small amount of accompanying phospholipids.

1.1.3 Type of co-surfactant

Both, ionic and non-ionic surfactants were added in the formulation to study the effect of surfactant on the emulsion formulation. Tween 80 is a non-ionic surfactant that was added in the oil phase. The weight ratio of phospholipid to tween 80 used was 1.3: 1, achieved from the previous study by Rungthurakit (2000). Ionic surfactants were added to modify the surface properties or the zeta potential of emulsion droplets. Phosphatidylglycerol (PG) is an anionic surfactant that yields negatively charged oil droplets. Stearylamine, a cationic surfactant, provides positively charged oil droplets. The amount of phosphatidylglycerol and stearylamine used were 0.66 mM (0.06 g) and 11.1 mM (0.3 g), respectively, which had shown promising properties for emulsion formulation in the study of Chansiri, 1999.

1.1.4 Other additives

Glycerol was used for adjusting the isotonicity of the emulsion with an amount of 2.5%. Butylated hydroxytoluene (BHT) was an antioxidant in the formulation and was used at 0.2%. The amount of additives used was obtained from the study of Chansiri et al (1999).

The ingredients of emulsion studied are shown in Table 7.

Table 7. Ingredients of 10% and 20% lipid emulsions

Ingredients (g)	Rx1	Rx2	Rx3	Rx4	Rx5	Rx6	Rx7	Rx8	Rx9	Rx10	Rx11	Rx12	Rx13	Rx14	Rx15	Rx16
Miglyol 812	5	10	5	-	-	5	-	-	5	-	5	-	5	5	-	-
Soybean oil	5	10	5	10	20	5	10	20	5	10	5	10	5	5	10	10
Butylated hydroxytoluene (BHT)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Stearylamine (SA)	-	-	-	-	-	0.3	0.3	0.3	-	-	-	-	0.3	0.3	0.3	0.3
Phosphatidylglycerol (PG)	-	-	-	-	-	-	-	-	-	-	0.06	0.06	-	-	-	-
Tween 80 (T80)	-	-	-	-	-	-	-	-	0.9	0.9	-	-	0.9	0.9	0.9	0.9
Soy phospholipid	1.2	1.2	-	-	-	-	-	-	-	-	-	-	1.2	-	1.2	-
Egg phospholipid	-	-	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	-	1.2	-	1.2
Glycerol	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
Distilled water to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

1.2 Preparation of oil-in-water lipid emulsion

To prepare o/w emulsion, the aqueous phase was prepared by dispersing phospholipids in a mixture of glycerol and distilled water and PG (if needed) using a magnetic stirrer. Nitrogen was bubbled into the aqueous phase for 15 minutes in order to minimize the oxidation of lipid components from oxygen. The oil phase was prepared by mixing Miglyol 812 and/or soybean oil, BHT, SA and/or T80. The oil phase was heated to 60-65°C so as to dissolve the BHT and then bubbled with nitrogen for 15 minutes. The oil phase which cooled to approximately 45°C was added into water phase to make a primary emulsion under stirring. The 200 ml mixture was then homogenized by a homogenizer (Vstrol®) for 15 minutes and passed through a high-pressure homogenizer (Emulsiflex C50®) at 20,000 psi, for 10 recycle times. The final pH of the emulsion was adjusted to 8.0 by the addition of 0.2 N NaOH. The emulsion was then purged with nitrogen for 15 minutes before being kept in the vial which was sealed with a pulp and aluminum screw cap and refrigerated. The emulsions were divided into two groups. One was refrigerated without sterilization and another group was sterilized by autoclaving at 121°C, 15 psi for 15 minutes before storage in refrigerator. The scheme of emulsion preparation is shown in Figure 5.

Preparation Rx1 and 2 were formulated using blended oil emulsified by soy phospholipid and passed through Emulsiflex C50® for 5 recycle times. Preparation in Rx 3 and 4 were emulsified by egg phospholipids. Number of cycle of high-pressure homogenization (Emulsiflex C50®) was varied form 3, 5, 7, to 10 recycle times, at 20000 psi. The number of homogenization cycle,

which provided stable emulsion with optimal droplet size, was selected for further preparations (Rx 5-16).

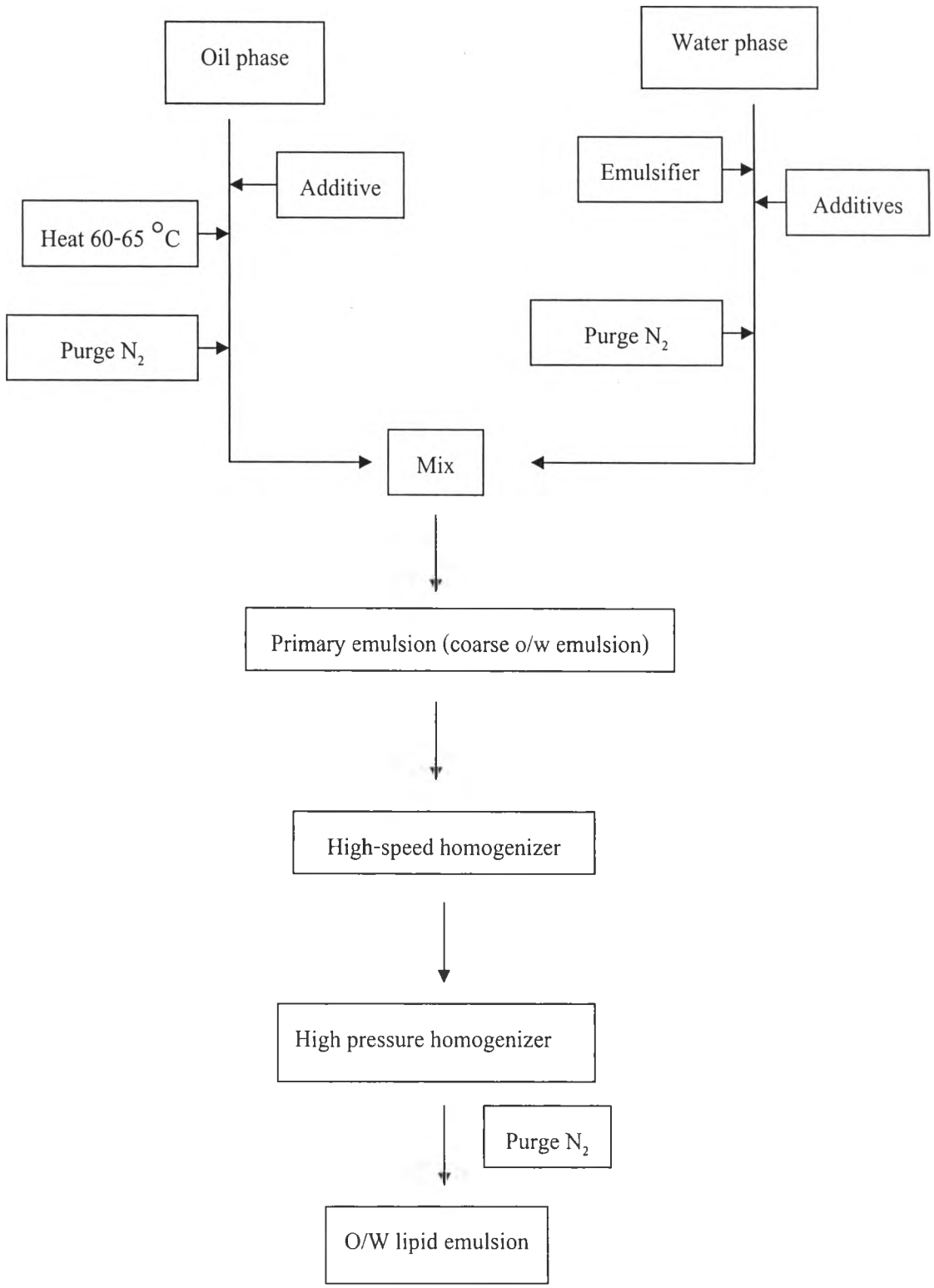

Figure 5. A schematic illustration of o/w lipid emulsion preparation

2. Formulation of lipid emulsion containing oil-soluble vitamins

Lipid emulsion containing vitamins were formulated using soybean oil, egg phospholipid, tween 80, PG, and SA. The oil-soluble vitamins added were vitamin A palmitate 3300 IU, vitamin D_3 200 IU, vitamin E acetate 10 IU, and vitamin K_1 0.15 mg. The amount of vitamins used was based on the US Food and Nutrition Board's recommended Dietary Allowance (RDA) for adults (Table 8). Vitamins used were protected from light and the yellow light was used in order to minimize the risk in degradation of vitamins.

The preparation method was similar to the preparation of lipid emulsion without oil-soluble vitamins. However, soybean oil was separated into 2 equal portions. BHT and SA were added in to a half of oil and heated to 55-60 $^{\circ}$C until BHT and SA completely dissolved. The mixture was cooled down to 45 $^{\circ}$C before being poured into the oil left, which composed of vitamins and T80. The water phase composed of EPC and PG that were dispersed in a mixture of glycerol and distilled water. Oil phase and water phase were bubbled separately with nitrogen for 15 minutes before mixing. Primary lipid emulsion was produced by constant stirring oil phase into a water phase for 15 minutes and then homogenized by a high-speed homogenizer for 15 minutes. The reduction of size was fulfilled by passing the emulsion through a high-pressure homogenizer at 20,000 psi for 10 recycle times. The pH of emulsion was adjusted to 8.0 by adding 0.2 N NaOH. Emulsion was purged with nitrogen for 15 minutes before being packed in the tightly sealed vial. The prepared emulsions were divided into 3 groups so as to study the effect of sterilization and method of sterilization on the properties of emulsions. The non-sterilized emulsion was kept in the refrigerator. In addition, emulsions were

sterilized by either steam sterilization at 121°C, 15 psi, for 15 minutes or filtration using 0.22 μm filter paper before storage in the refrigerator.

Table 8. The emulsion compositions (%w/w) of lipid emulsions containing oil-soluble vitamins

Ingredients	**Rx A**	**Rx B**	**Rx C**
Vitamin A palmitate	3,300 IU (0.33%)	3,300 IU (0.33%)	3,300 IU (0.33%)
Vitamin D_3	200 IU (0.002%)	200 IU (0.002%)	200 IU (0.002%)
Vitamin E acetate	10 IU (0.1%)	10 IU (0.1%)	10 IU (0.1%)
Vitamin K_1	0.15 mg	0.15 mg	0.15 mg
Soybean oil	10 %	10 %	10 %
BHT	0.2 %	0.2 %	0.2 %
SA	-	-	0.3 %
T80	0.9%	0.9%	0.9%
PG	-	0.06%	-
EPC	1.2%	1.2%	1.2%
Glycerol	2.5%	2.5%	2.5%
Distilled water to	100%	100%	100%

3 Study of physical stability

The physical stability of emulsions was visually observed. Any changes in physical appearance such as flocculation creaming, coalescence, and phase separation were recorded. The physical appearance of sterilized emulsion kept in refrigerator was observed at 24 hours, 1 week and 1 month after preparation. Some of emulsions were examined for long term stability up to 3-4 months.

4. Physicochemical characteristics of lipid emulsion.

The physicochemical properties of sterilized emulsion, i.e., particle size, zeta potential and pH, kept in refrigerator were examined at 24 hours, 1 week and 1 month after preparation.

4.1 Particle size analysis

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

Mastersizer® works by using the optical unit to capture the actual scattering pattern from a field of particles, and then calculates the size of particles that create that pattern. There are three distinct procedures involved in measuring a sample on the Mastersizer®. Firstly, the sample is prepared and dispersed in water (dispersant) to the correct concentration which provided the optimal laser obscuration and then delivered to the optical unit, which is used to collect the raw data that is used to

measure the size of the particles within a sample. 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] which is an internationally agreed method of defining the mean and other moments of particle size (British standard, 1993).

D (v, 0.5), D (v, 0.1) and D (v, 0.9) are standard percentile reading from the analysis.

- D (v, 0.5) is the size in microns at which 50% of the sample is smaller and 50% is larger. 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 diameter. D [3, 2] is the surface area mean diameter or the Sauter mean.

The measurement of the width of the distribution is called as Span. The narrower the distribution, the smaller the span becomes. The span is calculated as:

$$\text{Span} = \frac{d(0.9) - (0.1)}{d(0.5)}$$

4.2 Zeta potential measurement

The ZetaPlus® 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 field strength. Finally, the zata potential is calculated from the solution conditions and the mobility. A high zeta potential (> 30mV), either positive or negative, signifies a stable dispersion while a low zeta potential, either positive or negative, signifies an unstable dispersion. The solution conditions under which the zeta potential is zero are called isoelectric conditions.

For the measurement, lipid emulsion was diluted to 1: 1000 using a (N- [2-hydroxyethyl] piperazine-N′- [2- ethanesulfonic acid] (HEPES) buffer solution. One liter of HEPES solution consisted of 0.001 M of HEPES and 5 mM of sodium chloride (NaCl). The pH of final solution was adjusted to 7.4 using 0.2 N sodium hydroxide (NaOH).

4.3 pH measurement

pH of emulsion was determined by use of pH meter, pH 3000®.

4.4 Osmolality measurement

Osmolality of emulsion was determined by 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 for osmolality.

To measure osmolality, A 10 microliter of lipid emulsion was inoculated into a solute-free paper disc in 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 seeked 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 an explicit function of solution vapor pressure.

5. Assay of oil-soluble vitamins in lipid emulsion

High Performance Liquid Chromatography (HPLC) technique was used to identify and determine the amount of vitamins contained in the lipid emulsion.

To quantify the amount of vitamins contained in the lipid emulsion by HPLC method, the calibration curve of oil-soluble vitamins were established using a standard vitamin (from Fluka

Biochemica) in various concentrations, 10-50 μg/ml of vitamin A palmitate, 0.5-3.0 μg/ml of vitamin D_3 and vitamin K_1, and 60-140 μg/ml of vitamin E acetate, using vitamin A acetate as internal standard. The instrument was automatically calculated the peak area of each vitamin presented in the formulation. Peak area ratio between vitamins and vitamin A acetate was used to plot the curve. The amount of vitamins presented in the lipid emulsion was determined by extrapolation to the line of calibration curve.

HPLC parameters are as follows:

HPLC column	:	Prodigy^{TM} 5 μ ODS (2) 150 * 4.60 mm
Mobile phase	:	Acetonitrile: Methanol (75: 25)
Flow rate	:	2.0 ml/min
Detector	:	UV-Vis detector at λ 280 nm

5.1 Preparation of internal standard

A good internal standard should have similar structure and comparable physicochemical properties such as polarity, extractability and detectability to the analytes. Thus, vitamin A acetate was chosen to use as internal standard for analyzed oil-soluble vitamins.

To prepare the internal standard solution, vitamin A acetate 1 mg/ml stock solution was prepared by weighing and dissolving 100 mg of standard vitamin A acetate in propan-2-ol and then adjusted volume to 100 ml. Vitamin A acetate at the concentration of 2 μg/ml was used in an analyzed sample by adding 20 μl of stock solution of vitamin A acetate into 10 ml of sample dilution.

5.2 Sample preparation

Propan-2-ol was used to break the emulsion in order to analyze the amount of vitamin entrapped in the oil droplets of the emulsion. 10 ml of analyzed sample was prepared by mixing 1 ml of lipid emulsion containing vitamins with 20 µl of vitamin A acetate stock solution, and then adjusted volume to 10 ml using propan-2-ol. The analyzed was then degassed and filtered before analysis.

6. Sterility test

The emulsions sterilized by filtration were examined for microbial contamination using microbial limit tests for estimating the number of viable aerobic microorganisms.

The test procedure was separated into 3 parts, total aerobic microbial count, total aerobic mould count, and test for specific microorganism (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella species*, and *Escherichia coli*)

6.1 Media preparation

The method of preparation was followed microbiological tests in USP 23. 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 *Salmonella species*; Manitol-Salt Agar Medium (MSA) for *Staphylococcus aureus*; and Cetrimide agar Medium (CA) for *Pseudomonas aeruginosa*.

The media were purposed as follows (USP 23, 1995).

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.

6.2 Phosphate buffer solution preparation

Phosphate buffer solution was used as diluent for sterility test of emulsion. It was composed of the following ingredients (USP 23, 1995):

Potassium dihydrogen phosphate (KH_2PO_4)	1.78 g
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 adjusted to pH 7.0 by adding of a sodium hydroxide solution. The phosphate buffer was sterilized and stored under refrigeration.

6.3 Specimen preparation and test procedure (USP 23, 1995)

6.3.1 Total aerobic bacterial count

The plate method was used for specimen preparation. 1:10, 1:100 and 1:1000 dilution of lipid emulsion was prepared by accurately pipette 10 ml of lipid emulsion into 90 ml of phosphate buffer. 10 ml from previous dilution was used to prepare the next dilution (Figure 5). Each dilution, two plates were prepared by pipette 1 ml of each dilution into each of two sterile petri dishes and then 20 ml of TSA medium cooled to approximately 45 $^{\circ}$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 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.

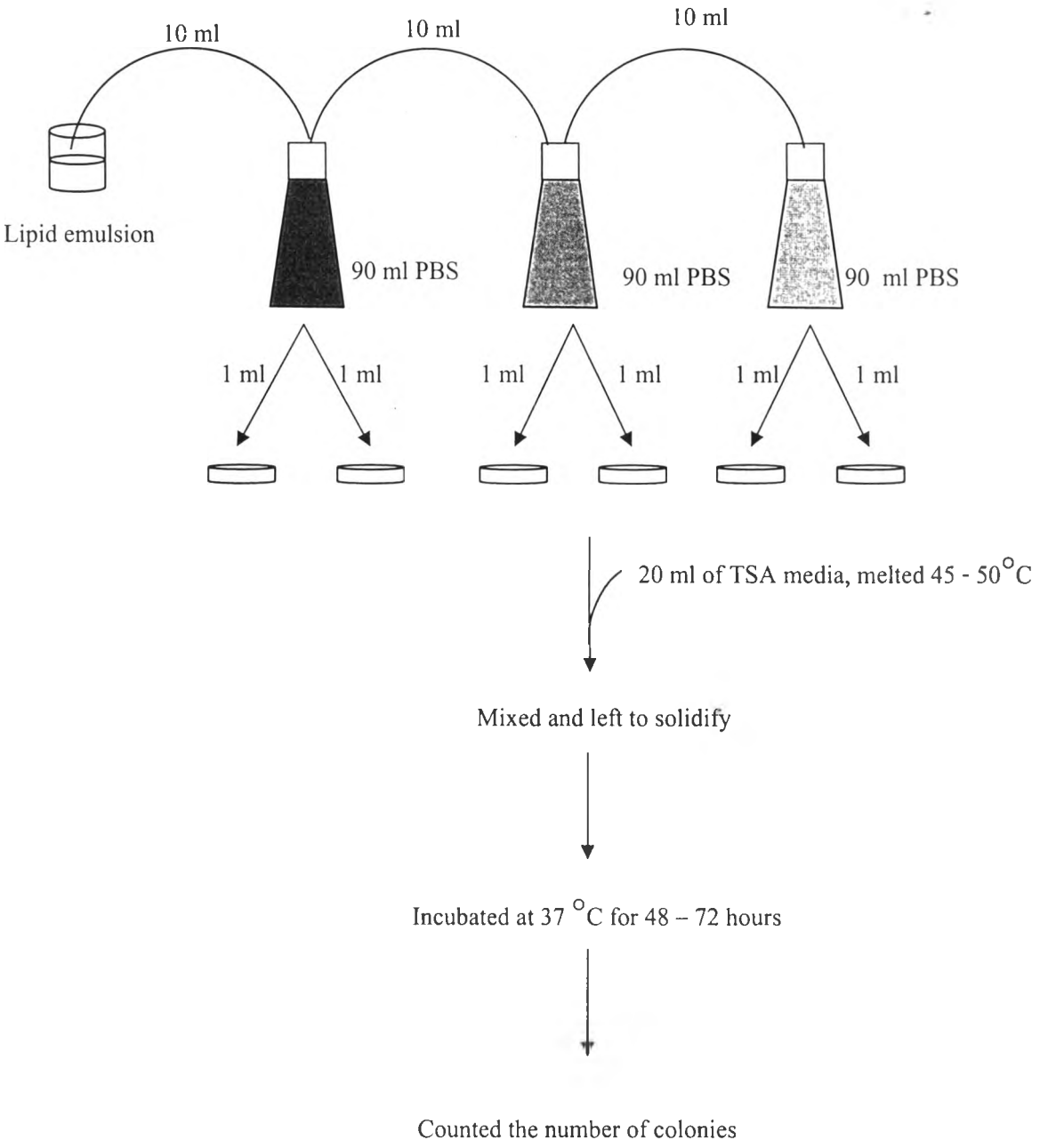

Figure 6. Schematic diagram of total aerobic microbial count

6.3.2 Total aerobic mold count

The test procedure was similar to 6.3.1. 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 6.

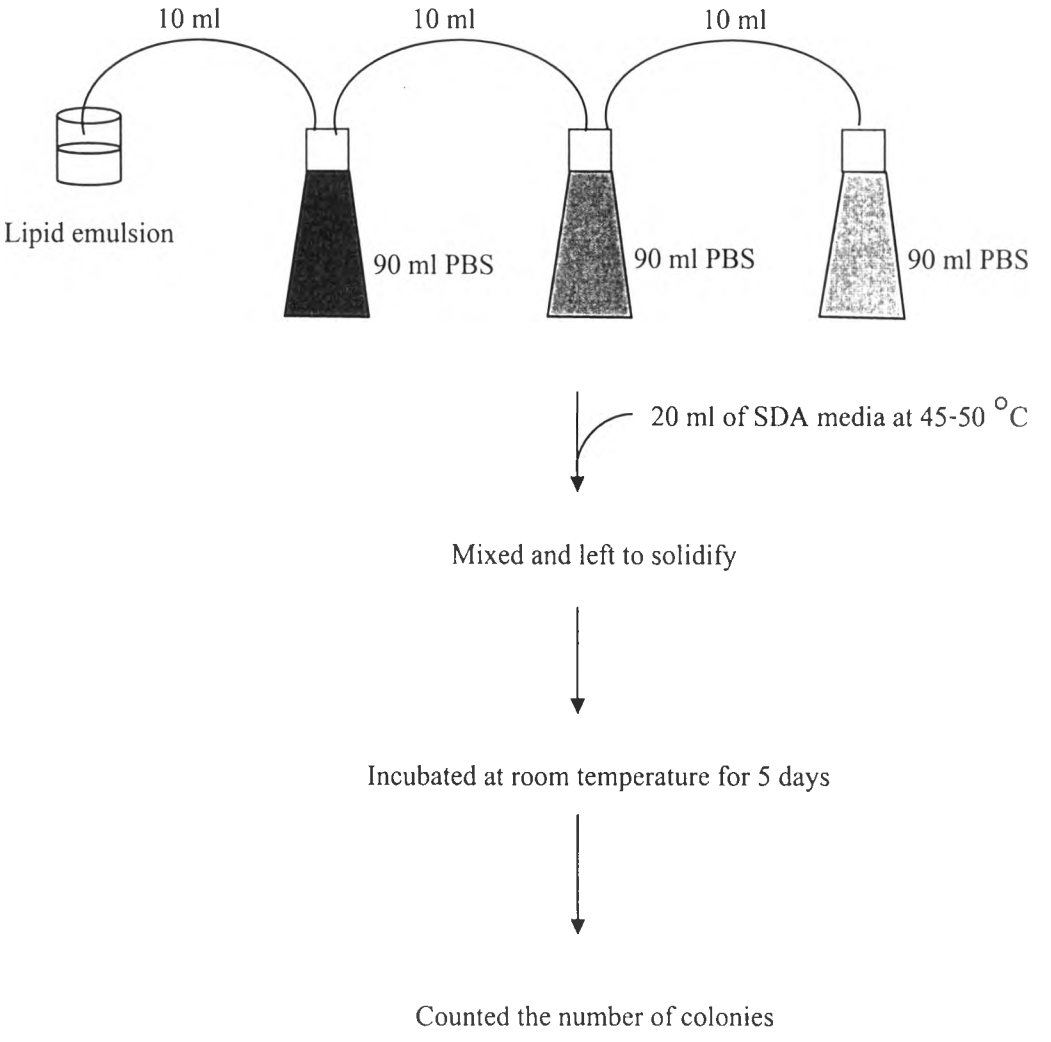

Figure 7. Schematic diagram of total aerobic mold count

6.3.3 Test for specific microorganism

1) *Staphylococcus aureus* and *Pseudomonas aeruginosa*

10 ml of lipid emulsion was added into TBS medium to make 100 ml. The mixture was mixed, and incubated at 37 °C for 24 hours. The medium was examined for organism growth. If organism growth was present, inoculating 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 for 24-48 hours. If, upon examination, none of the plates contained colonies having the morphologic characteristics as shown in Table 9, the test lipid emulsion met the requirements for the absence of these organisms.

2) Test for *Salmonella species* and *Escherichia coli*

10 ml of lipid emulsion was added into LB medium to make 100 ml. 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 MCA medium. The mixture was incubated at 37 °C for 24-48 hours. If, upon examination, none of the plates contained colonies having the morphologic characteristics as shown in Table 9, the test lipid emulsion met the requirements for the absence of these organisms.

Table 9. Morphologic characteristics of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* species, and *Escherichia coli* on selective agar Media

Selective 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)	*Salmonella species*	No growth	Small, transparent, colorless or pink to white opaque, frequently surrounded by pink to red zone

7. Haemolysis study

To assess the safety of lipid emulsion for parenteral administration, the haemolysis caused by emulsion was studied. The lipid emulsions in the presence and absence of vitamins were investigated. The method of haemolysis study was followed the method of Gould et al. (2000).

7.1 Drabkin's solution preparation

1.25 g of Drabkin's reagent and 0.5 ml of Brij 35 solution (30%w/v) were dissolved in 1 liter of distilled water.

7.2 Phosphate buffer solution preparation for haemolysis

The buffer solution pH 7.4 was prepared by dissolving 2.38 g of di-sodium hydrogen orthophosphate dihydrate, 0.19 g of potassium dihydrogen orthophosphate and 8 g of sodium chloride in distilled water. The solution was adjusted to the final volume of 1 liter.

7.3 Blood preparation

5 ml of the human blood collected from a healthy volunteer with normal blood chemistry was washed to remove plasma and buffy coated from the red blood cells (erythrocytes) by

centrifuging at 2,200 g, 10 minutes for 3 times using buffer solution at the volume of five times of erythrocytes volume. Then, the erythrocytes were adjusted to contain approximately 12% haematocrit by re-suspending erythrocytes in buffer to gain weight about 3.33 times of their initial weight.

7.4 Haemolysis test of emulsion

Dimethyl sulphoxide (DMSO) was used to dissolve the emulsion with a dilution of 1: 1000 to obtain a clear test solution. A 0.2 ml of red blood cell suspension was added to 0.2 ml of test solution. Then, the mixture was incubated at 37 ^{o}C for 15 minutes. The mixture was spun in a microcentrifuge for 15 seconds then a 0.2 ml of supernatant was drawn and mixed to 3 ml of Drabkin's solution to assay for the amount of haemoglobin released. The absorbance of sample was determined using UV detector at wavelength of 540 nm.

Positive control represented 100% haemolysis was prepared by adding 0.2 ml buffer and 0.2 ml uncentrifuged blood suspension in 3 ml of Drabkin's solution.

Negative control was prepared in order to subtract the value of spontaneous haemolysis. The mixture of 0.2 ml buffer and 0.2 ml blood suspension was microcentrifuged for 15 seconds. 0.2 ml of supernatant was added to 3 ml of Drabkin's solution and the absorbance was measured.

Three replications of each test sample were prepared and tested. The absorbance achieved from test sample, positive control and negative control were used in calculation the degree of

haemolysis. The percent haemolysis induced by the test sample was calculated using the following relationship.

$$\% \text{ Haemolysis} = \frac{(A_S - A_O)}{A_{100}} * 100$$

Where

A_S = Absorbance of test sample

A_O = Absorbance of negative control

A_{100} = Absorbance of positive control

The haemolytic activity is measured of the amount of haemoglobin release. In fact, the erythrocyte lyses with adding Brij 35 in erythrocyte solution. The Drabkin's reagent contains potassium ferricyanide, potassium cyanide and sodium bicarbonate. In nature, the ferrous ions of the haemoglobins are oxidized to the ferric state by potassium ferric cyanide to form methemoglobin. The methemoglobin then reacts with cyanide to form cyanomethemoglobin, which can detect the absorbance at 540 nm.