CHAPTER III

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources and used as received.

1. Metronidazole (Lot No. R1-44/00835, The Government Pharmaceutical Organization, Thailand)
2. Soybean oil (Lot No. 99H0081, Sigma Chemical, USA)
3. Isopropyl myristate (Lot No. 405657/1, Fluka Chemical, USA)
4. Castor oil (Lot & Control No. OAF 60/1194, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
5. Cremophore EL (Lot No. 423975, BASF, Germany)
6. Cremophore RH_{40} (Lot No. 584451, BASF, Germany)
7. Brij 35 (Lot No. 44/0120, EAC, Thailand)
8. Brij 72 (Lot No. 44/0835, EAC, Thailand)
9. Brij 721s (Lot No. 44/0628, EAC, Thailand)
10. Lutrol F-68 (Lot No. 46863824UO, BASF, Germany)
11. Lutrol F-127 (Lot No. 180026, BASF, Germany)
12. Tween 80 (Lot No. 392141/1, Fluka Chemical, USA)
13. Glycerin (Lot & Control No. LDLG192U, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
14. Propylene glycol (Lot & Control No. RPO835S, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
15. Polyethylene glycol 400 (Lot & Control No. PEO401AACC, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
16. Potassium dihydrogen phosphate (Batch No. F2H145, Fisher Scientific, UK)
17. Butanol (Lot No. K23035184632, Fluka Chemie AG, Buchs, Switzerland)
18. Ethanol (Lot No. 65282, Sappasamitr, Thailand)
19. Cetyl alcohol (Lot No. 00918, EAC, Thailand)
20. Sodium hydroxide pellets (Lot No. 7708, Mallinckrodt, Mexico)
21. Ultrapure water® equipped with filter system (Balson, Balson Inc.,USA)
22. *Porphyromonas gingivalis* (strain 381)

Equipment

1. Analytical balance (Model PB 8001, Mettler Toledo, Switzerland)
2. Magnetic stirrer (GEM HS-100, Thailand)
3. Hot air oven (Model 110, Mammert, USA)
4. Water bath (Model TBVS01, Hetomix and DT Hetoterm, Heto, Denmark)
5. Conductivity meter (portable Consort C535 version1.1, UK)
6. pH meter (Model 420A, Orion, USA)
7. UV visible spectrophotometer (Model UV-1601, Shimadzu, Japan)

8. Viscometer (Model LVDVI^{+}, Brookfield Engineering Laboratories, Inc. Massachusetts, USA)
9. Polarized light microscope (The KHC Olympus, Japan)
10. Transmission Electron Microscopy (Model JEM-200CX, Jeol®, Japan)
11. Vortex mixer (Vortex-genie, model G 560E, USA)
12. Modified Franz Diffusion Cell
13. Anaerobic glove box (Forma Scientific, 1029 S/N 13457-201, Ohio, USA)
14. Incubator (Model TS 5410, Termaks, Germany)
15. Hot plate
16. Micropipette

Glassware and Miscellaneous

1. Dialysis membrane (Lot No. 10B040530, molecular weight cut off 12,000 Dalton, Sigma Chemical, USA)
2. Beaker (Pyrex, USA)
3. Cylinder (Pyrex, USA)
4. Volumetric flask (Pyrex, USA)
5. Stirring rod (Pyrex, USA)
6. Test tube (Pyrex, USA)
7. Transferring pipette (HBG, Western Germany)
8. Disposable syringes and needle (Terumo, Thailand)
9. Hypodermic glass syringes (2 ml, S-T Company, Thailand)
10. Spinal needle (0.8×40mm, no $21\times1^{1/2}$, Lot No. 01G31, Nissho Nipro Corp.,Ltd. Thailand)
11. Plastic petri dish (10 cm diameter)
12. Anaeropack® Anaero (Lot No. 20A03-13, Mitsubishi gas chemical Co.,Inc.)

Methods

1. Formulation study design of microemulsion gel (MEG)

Partial pseudo-ternary phase diagrams were constructed to examine the formation of microemulsion gel (MEG) composing of four components: oil [isopropyl myristrate (IPM), soybean oil (SBO), castor oil (CO)]; surfactant [tween 80 (T_{80}), Cremophor EL (C_{EL}), Cremophor RH40 (C_{RH})]; co-surfactant [Lutrol F-68 (L_{68}), C_{EL}, butanol (B), Brij 35 (B_{35}), Brij 72 (B_{72}), Brij 721S (B_{721S}), cetyl alcohol (C), glycerin (G)] and water (W) or mixture of propylene glycol (PG) and water. Required amount of oil phase was added to the mixture of surfactant and co-surfactant to obtain oil:surfactant weight ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. Different ratio of each surfactant and co-surfactant were prepared by accurately weighing each composition into the flask. Oil was added to make the total weight of each preparation of 10 grams. The type and amount of oil, surfactant and co-surfactant systems are listed in Table 4-6. The mixture was mixed thoroughly until homogeneous dispersion was obtained. Then each mixture system was titrated with ultrapure water. The end point was detected whether the transparent gel became turbid gel.

Table 4 Study design of microemulsion gel systems.

System	Oil	Surfactant (E1)	Co-surfactant (E2)	Water phase	% E1:E2
1	IPM	T_{80}	L_{68}	W: PG (4:1)	2:1
2	IPM	T_{80}	L_{68}	W: PG (4:1)	1:1
3	IPM	T_{80}	L_{68}	W	2:1
4	IPM	T_{80}	L_{68}	W	1:1
5	IPM	T_{80}	-	W	-
6	IPM	T_{80}	C_{EL}	W	1:1
7	IPM	T_{80}	B_{72}	W	1:1
8	IPM	T_{80}	B_{721S}	W	1:1
9	IPM	T_{80}	B_{35}	W	1:1
10	IPM	T_{80}	C	W	1:1
11	IPM	C_{EL}	-	W: PG (4:1)	-
12	IPM	C_{RH}	-	W: PG (4:1)	-
13	IPM	C_{RH}	B	W	1:1
14	CO	T_{80}	G	W	2:1
15	CO	T_{80}	G	W	1:1
16	CO	T_{80}	-	W: PG (4:1)	-
17	CO	C_{EL}	G	W	1:1
18	CO	C_{EL}	-	W: PG (4:1)	-
19	SBO	T_{80}	-	W	-
20	SBO	T_{80}	G	W	1:1

(E1: E2 = surfactant: co-surfactant)

Table 5 Amount of ingredients in MEG system using 1:1 ratio of E1:E2.

Weight ratio of oil:surfactant mixture	Amount (g)		
	Oil	Surfactant (E1)	Co-surfactant(E2)
1:9	1.00	4.50	4.50
2:8	2.00	4.00	4.00
3:7	3.00	3.50	3.50
4:6	4.00	3.00	3.00
5:5	5.00	2.50	2.50
6:4	6.00	2.00	2.00
7:3	7.00	1.50	1.50
8:2	8.00	1.00	1.00
9:1	9.00	0.50	0.50

Table 6 Amount of ingredients in MEG system using 2:1 ratio of E1:E2.

Weight ratio of oil:surfactant mixture	Amount (g)		
	Oil	Surfactant (E1)	Co-surfactant(E2)
1:9	1.00	6.00	3.00
2:8	2.00	5.33	2.67
3:7	3.00	4.67	2.33
4:6	4.00	4.00	2.00
5:5	5.00	3.33	1.67
6:4	6.00	2.67	1.33
7:3	7.00	2.00	1.00
8:2	8.00	1.33	0.67
9:1	9.00	0.67	0.33

*** The amount of oil, surfactant (E1) and co-surfactant (E2) of each system was varied by following the composition in Table 4.

2. Preparation of microemulsion gel (MEG)

2.1 Construction of partial pseudo-ternary phase diagram

The existence of MEG was monitored by the corresponding pseudo-ternary phase diagram with the mixture of water phase on the top of the phase diagram, the oil phase and the mixture of surfactant and co-surfactant on the bottom left and right corners, respectively. The MEG and liquid crystal phase (LC) were identified as the shaded area in the phase diagram where clear, transparent gel with birefringent property were obtained based on visual inspection and polarized light microscopy. The boundary of MEG and LC region was determined using the end point from the water titration and confirmed by polarized light microscopy. The MEG systems that possessed desired property for periodontal drug delivery (such as changing structure from liquid to semi-solid, rigid, stiffing or jelly-like transparent gel when contact certain amount of water) were selected to further investigation. This system in intra-pocket of periodontal disease could increase the retention time and control drug release at the site of action. Consideration of MEG and LC as an appropriate system for periodontal drug delivery also focused on the toxicity and irritation of surfactant at high concentration. The MEG systems using suitable and allowable amount of surfactant that had the desired properties were selected for further studies.

2.2 Preparation of microemulsion gel base

Representative formulations of each system from partial pseudo-ternary phase diagrams were selected and prepared for characterization and stability studies. The criteria for selected formulation was the point when most series were superimposed in which stable, clear and transparent gel had suitable viscosity for use in intra-pocket of periodontal disease and the four compositions could be compared.

The composition and ratio of the selected MEG are shown in Table 7. Each composition was precisely weighed and heated before being allowed to melt in water bath at 60°C for 15 minutes and then mixed at room temperature with a vortex mixer at a maximum speed for 10 minutes. The systems were examined under a polarized light microscope after 7 days of storage at room temperature to allow the system to reach equilibrium. Systems which exhibited MEG and LC structure were selected for further investigation on physicochemical properties and physical stability.

2.3 Preparation of microemulsion gel containing metronidazole

Several systems were prepared to obtain the appropriate system for periodontal use. The selected formula that possessed viscous-stiffing, transparent and stable gel base were selected to incorporate metronidazole. The saturation solubility of drug in MEG and LC was performed by varying concentrations of metronidazole incorporate into MEG base of each system from 0.50, 0.75, 1.00, 1.50, 2.00, 2.50 to 5.00 %w/w following system in Table 7. The saturation solubility of metronidazole in each MEG base and LC systems was determined by visual observation and polarized light microscopy. Then the formulas were observed for 1 week. The highest drug concentration at which drug crystals were not found was considered to represent the saturation solubility of metronidazole in that system.

Table 7 Representative formulation of each system for preparing MEG base and MEG containing metronidazole.

Composition of formula	System	Ratio of oil:surfactant:(co-surfactant)
IPM : T_{80} : W	1/1	5:5 (water 10%)
	1/2	4:6 (water 8%)
	1/3	4:6 (water 10%)
	1/4	3:7 (water 8%)
	1/5	3:7 (water 10%)
	1/6	1:9 (water 7%)
CO : C_{EL} : W: PG (4:1)	2/1	2:8 (water 23%)
	2/2	2:8 (water 20%)
	2/3	3:7 (water 10%)
	2/4	3:7 (water 25%)
	2/5	5:5 (water 13%)
	2/6	5:5 (water 20%)
IPM : T_{80} : L_{68} : W (T_{80}:L_{68} = 2:1)	3/1	3 : 4.67 : 2.33 (water 15%)
	3/2	3 : 4.67 : 2.33 (water 20%)
	3/3	3 : 4.67 : 2.33 (water 25%)
	3/4	2 : 5.33 : 2.67 (water 20%)
	3/5	2 : 5.33 : 2.67 (water 25%)
	3/6	2 : 5.33 : 2.67 (water 17%)
IPM : C_{EL} : W: PG (4:1)	4/1	1:9 (water 10%)
	4/2	2:8 (water 25%)
	4/3	3:7 (water 25%)
	4/4	3:7 (water 20%)
IPM : C_{RH}: W: PG (4:1)	5/1	3:7 (water 14.52%)
	5/2	4:6 (water 20%)
	5/3	4:6 (water 15%)
	5/4	5:5 (water 15%)
	5/5	5:5 (water 15%)
IPM : T_{80} : C_{EL} : W	6/1	3 : 3.5 : 3.5 (water 15%)
	6/2	2 : 4 : 4 (water 9%)
	6/3	1 : 4.5 : 4.5 (water 15%)
	6/4	1 : 4.5 : 4.5 (water 20%)
IPM : T_{80} : B_{35} : W	7/1	3 : 3.5 : 3.5 (water 15%)
SBO : T_{80} : W	8/1	1:9 (water 7%)

The non-syneresis, non-separated, non-precipitated and stable formulas were selected. The MEG containing metronidazole systems were studied for their loading capacity and physical stability. For each system, the drug was incorporated into MEG and LC system by mixing with other components in the systems and melting or warming in water bath using the protocol as described in 2.2.

3. Physicochemical characterization of microemulsion gel (MEG)

3.1 Physical appearance

The visual observation of each MEG and drug-containing MEG were recorded and described general properties of each system such as color (transparent, clear or turbid gel), viscosity (stiffness, viscous, homogeneous or syneresis), appearance (lamellar gel, liquid, semisolid, jelly-like, rigid gel or ringing gel). After preparation for one week, these selected formulations were reevaluated.

3.2 Polarized light microscopy

A microscope with polarized lens and analyzer was employed to examine the birefringent property of formulation and the boundary of MEG in the phase diagram at room temperature. Microscopic pattern of selected MEG was verified under cross-polarized light. A small amount of each sample was placed between a cover slip and glass slide and then examined under polarized light by turning polarized lens at 90^{o} or cross polarizing, under magnifications of 100x and 400x. The sample that appeared dark and exhibited non-birefringent property would be classified as microemulsion (ME). The sample showing birefringent property would be classified as liquid crystal (LC). The texture of liquid crystal phase was identified as described by Radler et al (1989). Photographs were taken during cross polarizing with a camera attached to the microscope in order to identify microscopic pattern that would be ME, MEG, LC phase of the formulation.

3.3 Determination of microemulsion type

Two different tests were used to determine the type of MEG ;

- Dilution test

The dilution test was performed by adding water or oil (SBO or IPM) to the microemulsion. Each component was weighed at the equal amount and mixed together. If water was easily dispersed in the external phase, the microemulsion was o/w type. On the other hand, if oil (SBO or IPM) was dispersible in the external phase, the microemulsion was w/o type.

- Dye solubility test

Dye solubility test was performed by adding a drop of water-soluble or oil-soluble dye to microemulsion. The intense staining of the external phase after addition of a water-soluble dye (Tartrazine) or an oil-soluble dye (D&C red NO.17) indicated an o/w microemulsion or w/o microemulsion respectively.

3.4 pH

The pH of MEG base and microemulsion containing metronidazole were detected both before and after stability testing. The selected formula was measured at room temperature by using a pH meter that calibrated at pH 4 and pH 7 with standard buffer solution before measured.

3.5 Conductivity measurement

The conductance of MEG was determined at room temperature using a portable conductivity meter (Consort C535 V 1.1, UK). Standard solution of 12.88 mS and 1413 μS from Mettler-Toledo were used to calibrate the instrument.

3.6 Viscosity measurement

The viscosity of selected systems were monitored by a Brookfield LVDV-I$^+$ viscometer using at least three different spindle number (NO. 61, 62, 63, 64) depending on sample viscosity. All systems were measured at room temperature. The instrument was calibrated with viscosity standard calibration Lot No. 101002 before used. Each sample was measured by varying spindle number and speed depended on the viscosity of each sample. Viscosity had been measured at low shear rate or speed, in order to avoid slipping effect at the wall surface of sample. Each sample used the same container and sample volume (100 ml). Measurement was performed by interval of one minute until five minutes. Then the relationship between time and viscosity were plotted to show rheological property of sample.

3.7 Syringeability

Syringeability of each MEG and LC system both before and after stability was performed by withdrawing one ml of sample from container into two ml glass syringes that standed forward and locked with clamp. The accurately forces (1 kg.) was put toward the plunger of two ml glass syringes. The time that the entire MEG passed through a 21-gauge needle of internal diameter 0.3 mm was recorded and diverted value into ml per second. Tripicate measurement of each sample were performed.

3.8 Particle size determination

The mean particle diameter, size and shape of MEG before and after stability were measured at room temperature by a transmission electron microscope (TEM) following negative staining techniques. A drop of sample was placed on the coated copper grid (size 400 mesh) for 15 minutes. Excess sample was absorbed with filter paper. The sample on grid was then stained with 1% phosphotungstic acid solution for 30 seconds. Excess solution was absorbed. When the sample was dried, pictures were taken at various magnifications. The particle diameter of each sample both before and after stability testing was measured from pictures of 300 particles/sample by a computer program *SemAfore version 4.01*. Then, the average particle size of each sample was calculated.

SemAfore is an economical, very easy to use digital image recording system. The system is based on a personal computer with a program that runs under a windows operating system. *SemAfore* acquires slow scan images from the TEM into the computer. A digitizer converts the signal into a digital stream of data. The images have the same high resolution as the photographs. The images can be zoomed and the gray scale can be adjusted in order to correct exposure errors and to enhance the image. Prior to use, the scale of picture from TEM was calibrated following each magnification.

The measurement of particle diameter was based on Martin's diameter which was the length of a line that bisected the particle image and the line may be drawn in any direction but should be in the same direction for all particles measured (Martin, 1993). This program measured the distance between two horizontals on the opposite sides of the particle by clicking mouse of computer on the left side and then drag to the right of the particle, after that the diameter was shown. The bar scale from TEM image must be recalibrated with computer scale, the recalibrated value of each picture was filled in the computer program before measuring the diameter of particle with *SemAfore*. The results that were obtained from *SemAfore* program were tested statistically significant difference both before and after stability test.

4. *In vitro* drug diffusion studies

All selected 1.5% w/w metronidazole MEG systems were freshly prepared and precisely weighed. The *in vitro* drug diffusion studies of MEG containing metronidazole 1.5% w/w were carried out using modified Franz diffusion cell (Norling et al, 1992) as shown in Figure 4 The apparatus consisted of two glass compartments, donor and receptor compartments. The inner diameter of each cell was 1.8 cm, corresponding to an effective permeable surface area of 2.55 cm^2. The receptor compartment contained 13-14 ml of phosphate buffer solution pH 6.8 as release medium that was calibrated prior to use. Two compartments were separated by dialysis membrane that had molecular weight cut-off of 12,000 dalton. Before placing up dialysis membrane onto a diffusion cell, the dialysis membrane was cut into a circular shape and soaked in deionized water for 12 hours and then rinsed with boiling water to wash off any water soluble contaminants. The membrane was then soaked for 30 minutes in the release medium and clamped between the donor and the receptor compartment of the cell.

The diffusion cell with circulating jacket and diffusion medium in the receptor compartment that covered with membrane were allowed to equilibrate and maintained the temperature at 37±0.5°C about 30 minutes before studying and throughout the experiments.

After equilibration, 1.0 gm of sample was carefully weighed into the donor compartment. The two compartments were clamped with the treated membrane in between. The release medium was carefully filled into the receptor part without air bubble. The study was operated continuously for 48 hours by a magnetic stirring bar rotating at 750 rpm. A 10-ml aliquot of receptor medium was withdrawn at appropriate time intervals (0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 18 and 24 hours) and replaced immediately with an equal volume of fresh release medium. A portion of withdrawn solution was diluted and determined for the amount of drug diffused using a UV-visible spectrophotometer. The amount of drug release was calculated from calibration curve and corrected for the amount previously withdrawn for assay. The tripicate determination of each sample was measured.

The general models of diffusion kinetics: Zero order, Higuchi, Cube root, Power expression, and Weibull models were use to elucidate the drug diffusion model. (Siepmann and Peppas, 2001). Model with the highest coefficient of determination (R^2) was accepted as a model of drug diffusion.

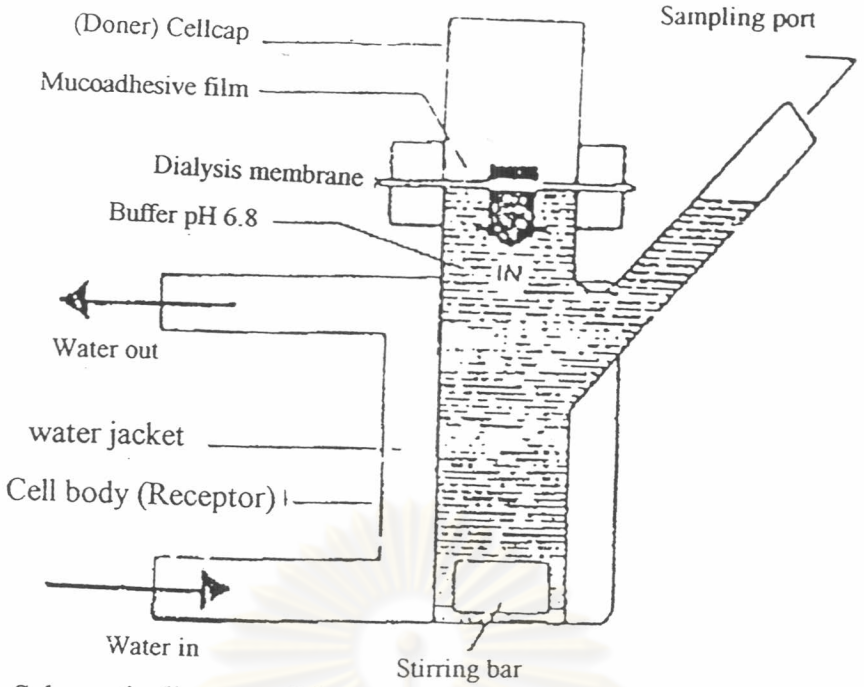

Figure 4 Schematic diagram of the apparatus for *in-vitro* diffusion studies. (from Siepmann and Peppas, 2001).

4.1 UV-visible assay for amount of metronidazole diffused from MEG

There are several methods available for determination of metronidazoleincluding non-aqueous titration, spectrophotometry, colorimetry, high performance liquid chromatography (HPLC), gas-liquid chromatography and thin layer chromatography (Reynolds, 1996). In diffusion studies, spectrophotometry was used because it was convenient and rapid.

The standard curve of metronidazole in phosphate buffer solution of pH 6.8 as release medium was performed to calculate the amount of drug dissolved in drug release studies. 100 mg of metronidazole was accurately weight into a 100-ml volumetric flask. Phosphate buffer solution of pH 6.8 was used to dissolve and adjust to volume. Ten milliliters of this solution were pipetted into a 100-ml volumetric flask and adjusted to volume with phosphate buffer solution of pH 6.8. This solution was stock solution of metronidazole.

Standard solutions of metronidazole were prepared by pipetting 2, 3, 4, 5, 6, 7 and 8 ml of metronidazole stock solution into 50-ml volumetric flasks. These solutions were accurately diluted with phosphate buffer solution pH 6.8 to volume so that the concentrations of standard solutions were 4, 6, 8, 10, 12, 14 and 16 μg/ml, respectively. The absorbance of standard solutions were performed by using UV visible spectrophotometer at 320 nm. Phosphate buffer solution of pH 6.8 was used as blank. The relationship of metronidazole concentration and absorbance was fitted using linear regression.

4.2 Validation characteristic for determination of metronidazole release from the MEG and liquid crystal system by UV spectroscopy

The parameters evaluated to ensure the acceptability of performance of the selected analytical method were accuracy, precision, specificity and linearity

4.2.1 Accuracy

Metronidazole solution were prepared at 4-16 μg/ml. Three sets of each concentration were prepared. Each individual sample was analyzed by UV spectrophotometry and percentage of analytical recovery of each sample was calculated.

4.2.2 Precision

a) Within run precision

The within run precision was determined by analyzing of three sets of the calibration curve in the same day. Inverse concentrations of metronidazole were compared. Then, the percent coefficient of variation (% CV) for each concentration was calculated.

b) Between run precision

The between run precision was determined by comparing each concentration of three sets of the calibration curve prepared on different day for six days. Inverse concentration for the three standard curves on different days were determined and the percent coefficient of variation (% CV) for each concentration was calculated.

4.2.3 Specificity

Under the condition selected for *in-vitro* metronidazole release studies, the peaks of other components in the MEG and liquid crystal systems must not interfere with the peak of metronidazole. This validation was made by comparing the peak scan from UV spectrophotometer between the receptor fluid taken from the MEG and liquid crystal system without incorporated metronidazole with the one taken from the drug-containing system of the similar composition.

4.2.4 Linearity

Linear regression analysis of the absorbances versus the corresponding concentrations was performed. The coefficient of determination was calculated.

5. Physical stability testing

Two different methods were performed to study stability of MEG system. First method, the MEG formulations were observed under accelerated conditions (heating and cooling cycle) by storing the sample at 4°C for 48 hours and 45°C for 48 hours for 6 cycles (Prince, 1977). The physicochemical properties of all formulations were studied and compared with those before stability testing as in 3.1-3.8 (physical appearance, microscopic pattern, MEG type, pH, conductivity, viscosity, syringeability, particle size and inhibition zone diameter). The sign of unstable formulation was also visually investigated.

Second method, shelf-life stability of MEG both as functional of time for 4 months and storage temperature at 45°C, 75% RH was evaluated by visual inspection and as in 3.1-3.8, the same as the first method. Stable systems were identified as those free of any physical change such as phase separation, syneresis, precipitation, discoloration, viscosity change and appearance. Both MEG base and MEG containing 1.5%w/w metronidazole were investigated by using two different method as aforementioned.

6. Anti-microbial activity of selected MEG system and 1.5% w/w metronidazole MEG.

Porphyromonas gingivalis (strain 381) or *P. gingivalis (Pg)* was used to be a representative of anaerobic bacteria in adult periodontal pocket. *P. gingivalis (Pg)* is a short rod-shape gram-negative anaerobe, general cells form black brown to black colonies on blood agar.

The effectiveness of MEG base and 1.5 %w/w metronidazole MEG against *P. gingivalis (Pg)* were evaluated by using the agar diffusion method, according to the procedure described by Midolo et al (1995). This method is a modified susceptibility test where the sample was in direct contacted with an inocculum of a specified organism and then the sample was evaluated for inhibition zone diameter. *Porphyromonas gingivalis* (strain 381) was cultured with plaque agar in an anaerobic glove box containing 85% nitrogen, 10% hydrogen and 5% carbondioxide.

6.1 Collection of *Porphyromonas gingivalis* and subculture

Gingival fluid of periodontal disease patient was collected from periodontalpocket by filter paper strip (Harco periopaper), then *Porphyromonas gingivalis* was isolated to pure prior collected in plaque that kept at –80°C (deep freeze) recommended by National Committee for Clinical Laboratories (NCCLS) standard procedure. Deep frozen *Porphyromonas gingivalis* in plaque was recovered from frozen condition and melted then inoculated and sub-cultured onto Tryptic soy agar supplemented with 5% defribrinated sheep blood by streaking. This process was done in an anaerobic glove box to generate anaerobiosis and control anaerobic conditions then incubated at 37°C for 5-7 days.

6.2 Plaque agar plates preparation

Media culture of plaque agars using in this study was tryptic soy agar (BBL),synnonym ; trypticase soy agar, tryptone soya agar, soybean-casein digest USP. The tryptic soy agar was composed of trypticase soy agar (BBL) 40 gm, yeast extract 5 gm, hemin 5 gm, sodium chloride 5 gm, L-cystein 400 mg, agar 15 gm,5% sheep blood. The total volume was adjusted by distilled water to 1000 ml that obtained pH 7.3. Each composition was suspended in distilled water, then was dissolved and heated with agitation until boiled. After the media were sterilized by autoclaving at 121°C, 15psi for 15 minutes, 5% defribrinated sheep blood was added after cooling media to 45°C and mix well. Tryptic soy agar media was then transferred into plastic petri-dish. The plates were prepared by adding 25 ml of plaque agar to each 10 cm petri-dish.

6.3 Culture media

The colony of *Porphyromonas gingivalis* was incubated until black pigment was seen, then it was collected by loop into nutrient broth tube and gently mixed by vortex mixer to distribute throughout nutrient broth tube. The culture was diluted with nutrient fluid to give density equivalent to 10^7 microbials/ml by comparing with a standard Mc farland No.1. Inoculated a properly obtained clinical specimen onto the medium and then streak to obtain isolated colonies.

6.4 Antimicrobial activity test of MEG and LC system usingmodified agar diffusion method

Each composition, MEG base and MEG containing 1.5% w/w metronidazole were subjected to test microbial activity by modified agar diffusion method. The agar plates were perforated a hole per plate, then a sterile cotton-tipped swab was dipped into the adjusted culture and twisted damply beside the tube before streaking throughout the surface of plaque agar. The inoculum plates were dried for 10 to 15 minutes. The sample was devided into three group; first: the composition of each system (castor oil, soybean oil, isopropyl myristate, tween 80, Cremophor RH40, Cremophor EL and propylene glycol), second: the microemulsion gel base and third group: MEG containing 1.5% w/w metronidazole. The amount of 0.10 gm of each sample was filled into the perforated hole of plaque agar with diameter 6 mm one by one following three group of sample. The plates were incubated in anaerobic glove box at 37°C for 3-5 days. At the end of incubation period, the diameter of inhibition zone was measured. The sizes of zones of inhibition were recorded and diverted to mm scale.

6.5 Microbial contamination test for non-sterile microemulsion gel

The MEG base and MEG containing metronidazole were prepared by aseptic technics by the following procedure; the equipment that would not be damaged at high temperature was heated in hot air oven at 220°C for 2 hours, otherwise the equipment would be rinsed with 70% ethanol and dried at low temperature. Prior to prepare, the hand had to be cleaned and a hat, mask and gloves had to be worn, including the surrounding that was cleaned with 70% ethanol. Then the MEG and MEG containing 1.5% metronidazole were prepared as condition above.

The microbial contamination tests were performed according to directions in the individual monographs of the Thai Pharmacopoeia; non-sterile preparations were used for broken skin, lesion and mucous membrane (nose, throat, ear, vagina). One gram of the MEG gel base or MEG containing 1.5% metronidazole was weighed. Each gel was diluted with distilled water in ratio of 1:10 (MEG:water) and using vortex mixer to distribute throughout the tube. One milliliter of sample was drawn using pipette and carefully dispensed onto petri-dish with ready made medium. Then each plate was incubated at 37°C, 48 hours for aerobic bacterial and 20-25°C, 3 days for yeast and 5 days for mould. The colonies became red for aerobic bacterial, blue-green for yeast and black or blue larger than yeast for mould.