CHAPTER III

MATERIALS AND METHODS

Materials

1. Absolute ethanol, AR grade (RCI Labscan Ltd., Thailand) Batch no. 12080104
2. Acetonitrile, HPLC grade (RCI Labscan Ltd., Thailand) Batch no. 11060267
3. Amoxicillin trihydrate, ASEAN Reference Substance (ARS, Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. V 309014
4. Amoxicillin trihydrate, DMSc Reference standard (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. 551013
5. Amoxicillin trihydrate, micronized form (Aurobindo pharma Ltd., India) Batch no. KAX01110173
6. Amoxicillin trihydrate, Reference standard (Sigma, USA) Lot no. SZB7346XV
7. Benzene (Merck, Germany)
8. Cefadroxil, ASEAN Reference Substance (ARS, Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. V 106135
9. Cefazolin sodium, ASEAN Reference Substance (ARS) (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. T 308043
10. Cefotaxime sodium salt, Reference standard (Sigma, USA) Lot no. 050M0748
11. Cefotaxime sodium 10 g (CEFOMAX 100®)
12. Cefotaxime 100 mg and gentamycin 75 mg (CEFRALO® L.A. suspension, A.H.A Pharmaceuticals, A.H.A international. Co. Ltd., China)
13. Ceftiofur hydrochloride 50 mg/mL (EXCENEL® RTU, Pfizer Inc., USA)
14. Ceftriaxone sodium, ASEAN Reference Substance (ARS) (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. T 108139

15. Ceftriaxone 10 g (CEF-3-MAX®)
16. Cephalexin, ASEAN Reference Substance (ARS) (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. T 206042
17. Chloroform, AR grade (RCI Labscan Ltd., Thailand) Batch no. 11080300
18. Cottonseed oil, (Sigma, USA) Lot no. MFCD00130872
19. Corn oil (Sigma, USA) Lot no. MKBG9426V
20. Dimethysulfoxide (DMSO) (Merck, Germany)
21. Dicloxacillin sodium, DMSc Reference standard (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. 08A53039
22. Disodium hydrogen orthophosphate, AR grade (Ajax Finechem Pty Ltd., Australia) Batch no. 1101369

23. Dipotassium hydrogen phosphate (Merck, Germany) Lot no. 321 A712801
24. Enrofloxacin base, Raw material (Zhejiang guobang Pharmaceutical Co., Ltd., China) Batch no. 101130-2
25. Enrofloxacin HCl, Raw material (Zhejiang guobang pharmaceutical Co., Ltd., China) Batch no. 1001082
26. Enrofloxacin, Reference standard (Sigma, USA) Lot no. S2BA336XV
27. *Escherichia coli* (*E. coli*) ATCC 25922 (ATCC®, American Type Culture Collection, USA)
28. Ethyl ether (Merck, Germany)
29. Gentamicin sulfate, DMSc Reference standard (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. 01A53154
30. Hexane, AR grade (RCI Labscan Ltd., Thailand) Batch no. 12080015
31. Isopropyl myristate (Sigma, USA) Lot no. STBB3338V
32. Isopropanol, HPLC grade (RCI Labscan Ltd., Thailand) Batch no. 11010375
33. Methanol, HPLC grade (RCI Labscan Ltd., Thailand) Batch no. 11060197

34. Mueller Hinton Agar (Difco™, Becton, Dickinson and Company, USA) Lot no. 0078729
35. Neomycin sulfate, DMSc Reference standard (Reference standard center Bureau of Drug and Narcotic, Thailand) Control no. 01A53063
36. Phosphoric acid (Merck, Germany) Lot no. K42661473 133
37. Polysorbate 80 or Tween™ 80 (CRODA Co., Ltd., Thailand) Batch no. 0000367734
38. Polysorbate 20 or Tween™ 20 (CRODA Co., Ltd., Thailand) Batch no. OP10-219
39. Potassium dihydrogen phosphate (Ajax Finechem Pty Ltd., Australia) Batch no. 1102146
40. *Pseudomonas aeruginosa* (*Ps. Aeruginosa*) ATCC 27853 (ATCC®, American Type Culture Collection, USA)
41. Sodium chloride (Merck, Germany) Lot no. K41653304 049
42. Sorbitan laurate or Span™ 20 (CRODA Co., Ltd., Thailand) Batch no. 0000571380
43. Sorbitan oleate or Span™ 80 (CRODA Co., Ltd., Thailand) Batch no. 0000424289
44. *Staphylococcus aureus* (*S. aureus*) ATCC 25923 (ATCC®, American Type Culture Collection, USA)
45. *Streptococcus suis (S. suis)* serotype II NCTC 10234 (NCTC, National Collection of Type Cultures, Central Public Health Laboratory, England)
46. Toluene (RCI Labscan Ltd., Thailand)
47. Tryptic Soy Agar (Difco™, Becton, Dickinson and Company, USA) Lot no. 0356973

Apparatus

1. Analytical balance (Model AG285, Mettler Toledo, Switzerland)
2. Analytical balance (Model PL 3002, Mettler Toledo, Switzerland)
3. Analytical balance (Model AB104-S, Mettler Toledo, Switzerland)
4. Centrifuge (Model MX-305, Tomy, USA)
5. Differential scanning calorimeter (Model 822^{e}, Mettler Toledo, Switzerland)
6. High performance liquid chromatography
    a. Liquid chromatography pump (LC-20AD, Shimadzu, Japan)
    b. Automatic sample injector (SIL-20AC, Shimadzu, Japan)
    c. Column (Luna C18, 5 µm, 250 mm x 4.6 mm, Thermo Electron Corporation, England)
    d. Column oven (CTO-20A, Shimadzu, Japan)
    e. UV-VIS detector or PDA detector (SPD-20A, Shimadzu, Japan)
    f. Communication bus model (SPD-20A, Shimadzu, Japan)
7. Incubator (Model UFE 500, Memmert, Germany)
8. Light microscope (KHC, Olympus, Japan)
9. Magnetic stirrer (CAT, Germany)
10. Micropipettes (Biohit proline®, Biohit , Inc., USA)
11. Milli-Q reverse osmosis (Millipore, Milford, MA, USA)
12. Modified Franz diffusion apparatus (Science Service, Thailand)
13. pH meter (Model 420A, Orion Research, Inc., USA)
14. Refrigerator (Model SR-F518 MS, SANYO Electric Co., Ltd, Japan)
15. Refrigerated incubator (FOC 2251,VELP Scientifica, Italy)
16. Sonicator (Model TP680DH, Elma, Germany)
17. Stability cabinet (Eurotherm Axyos, Germany)

18. Suspension mixture (Glas-Col Culture Rotator Orbital Mixer Shaker, Rotator 099A RD4512, Ratex Instrument Pty Ltd., Australia)
19. Thermostat water bath (Model W6, Grant, England)
20. Vacuum Oven (WTC binder, Germany)
21. Vortex mixer (Vortex Ginies-2, Scientific industries, USA)
22. Water bath (Model WB22, Becthai Co., Ltd., Thailand)

Accessories

1. Aluminum pan
2. Injection vial (Tan Soon Huat Products Co., Ltd., Thailand)
3. Injection vial 10 ml (Chai Saeng Huat Ltd., Part., Thailand)
4. Nylon membrane filter 47 mm., pore size 0.45 μm (Lubitech Technologies Ltd., China)
5. Nylon syringe filters 13 mm., pore size 0.45 μm (Lubitech Technologies Ltd., China)
6. Parafilm® M (Pechiney Plastic Packaging, Inc., USA)
7. Regenerate cellulose dialysis membrane, MWCO 3,500 Da (Spectra/Por® 3 Dialysis Membrane, spectrum Laboratories Inc., USA) Lot no. 3255104
8. Syringe, size 3, 5, 10 mL (Nipro Crop., Ltd., Thailand)
9. Plate sterile 90 mm. (Hycon plastics®, Biomed Co., Ltd., Thailand)

Methods

1. Evaluation of antimicrobials

1.1 Microorganism preparation

1.1.1 Microorganisms

Sixteen *S. suis* strains were isolated from pigs in Western Thailand at 6 – 8 weeks of age displaying clinical signs associated with *S. suis* infection including arthritis and convulsion. All strains were identified by the clinical microbiology laboratory using standard methods (สมบูรณ์, 2010; CLSI, 2011) and stored frozen at -40 °C at Department of Microbiology laboratory, Faculty of Veterinary Medicine, Chulalongkorn University until used.

1.1.2 Media

Media used in this study was Tryptic soy agar (TSA) (Difco™, USA) mixed with 5% sheep blood, also called as sheep blood agar to ensure that the bacteria grow well. This media was used in *S. suis* subculture process to determine MICs using agar dilution method. Pure *S. suis* from the subculture process was diluted in 0.85% saline solution to obtain a proper density of the bacteria for further studies. Mueller Hinton agar (MHA) (Difco™, USA) with 5% mixture of sheep blood (sheep blood MHA) was used as a medium for inoculums preparation and growing bacteria for checkerboard method analysis (CLSI, 2011).

1.1.3 Bacterial isolate on Sheep blood agar

To isolate pure culture of individual bacterial cells, *S. suis* samples were stored in the stock agar. *S. suis* was inoculated onto sheep blood agar using streak plate technique as following; pipetted 20 µl of stock agar, dropped and streaked on the sheep blood agar. Then, the bacteria were incubated at 37°C in an atmosphere with 5-7% of CO_2

for 24 hours. After that, 1-2 colonies which were greyish, semi-transparent, slightly mucoid and exhibited α – hemolysis, were picked from each plate to subculture on sheep blood agar plates and incubated in the same manner to obtain pure bacteria (CLSI, 2011).

1.1.4 Quality control strains of susceptibility test

Three standard strains (*Escherichia coli* (*E. coli*) ATCC 25922, *Staphylococcus aureus* (*S. aureus*) ATCC 25923 and *Pseudomonas aeruginosa* (*Ps. aeruginosa*) ATCC 27853) were used for quality control to determine the validity of the drug concentration. *E. coli* and *Ps. aeruginosa* were inoculated onto TSA agar plates whereas *S. aureus* was inoculated onto sheep blood agar plates. The MICs values obtained from the experiments were compared with the MICs values in standard tables, which the MICs of each drug should not be less than or greater than 1 log of MICs value of the reference standard to ensure reproducibility of the test and to confirm the performance of the drugs and the person conducting the test (CLSI, 2011).

1.1.5 Preparation of stock solutions

Stock solutions of antibacterials were prepared at concentration of 10240 μg/mL. The twelve antimicrobials used in this experiment were dicloxacillin sodium, gentamicin sulfate, neomycin sulfate, cefadroxil, cefazolin sodium, ceftriaxone sodium, cefotaxime sodium, ciprofloxacin lactate, CEF-3-MAX®, CEFOMAX®100, CEFRALO® (L.A.) and EXCENEL® RTU. The drugs were dissolved in distilled water. Moreover, 50% DMSO, acetic acid, and 1 N hydrochloric acid were solvents for amoxicillin trihydrate, enrofloxacin base and cephalexin, respectively. Then, distilled water was added to obtain the desired concentration and volume. Serial dilutions of antibacterial stock solutions were prepared by two-fold dilution (Table 1). Intermediate concentrations were 5120, 2560, 1280, 640, 320, 160, 80, 40, 20, 10, 5, 2.5 and 1.25 μg/ml and desired final concentrations were 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg/ml (CLSI, 2011).

Table 1 The dilution process of antibacterial drugs to be used in agar dilution susceptibility test.

Antimicrobial solutions			Volume (mL)	Solvent (mL)	Intermediate concentration (μg/mL)	Final concentration (μg/mL)	Log_2
Step	Concentration (μg/mL)	Source					
	10240	Stock	-	-	10240	-	-
1	10240	Stock	6	6	5120	512	9
2	10240	Stock	2	6	2560	256	8
3	10240	Stock	2	14	1280	128	7
4	1280	Step 3	6	6	640	64	6
5	1280	Step 3	2	6	320	32	5
6	1280	Step 3	2	14	160	16	4
7	160	Step 6	6	6	80	8	3
8	160	Step 6	2	6	40	4	2
9	160	Step 6	2	14	20	2	1
10	20	Step 9	6	6	10	1	0
11	20	Step 9	2	6	5	0.5	-1
12	20	Step 9	2	14	2.5	0.25	-2
13	2.5	Step 12	6	6	1.25	0.125	-3

(CLSI, 2011)

1.2 Antimicrobial susceptibility test

The antimicrobial susceptibility testing was performed using agar dilution technique according to the Clinical and Laboratory Standards Institute (CLSI, 2011). All fifteen antibacterial drugs were used in this test. The two-fold serial dilution of each drug

was prepared as following; mixing Mueller Hinton agar with 5% sheep blood, pipetting the to-be-tested drug, mixing along and then placing in an agar plate, consecutively.

To-be-tested bacteria were incubated at 37 ^{o}C in an atmosphere under 5-7 % CO_2 for 24 hours. Colony was diluted using 0.85 % saline solution. Turbidity of suspension was compared with 0.5 McFarland representing 1 x 10^{8} CFU/mL. Then, the bacteria were diluted to provide density of 1 x 10^{7} CFU/mL. Replicator inoculum block was used to spot the bacteria on agar plates which had different drugs and the plates were incubated at 37 ^{o}C in an atmosphere under 5-7 % CO_2 for 24 hours. The experiment was run in triplicate.

Interpretation of susceptibility testing results

The bacterial growth in each agar plate should be read with naked eye and optimum lighting. The MIC was taken as the first lowest concentration at which no growth occurred. When the drug inhibits the growth of bacteria, the agar surface must be smooth and no colonies are seen on surface of the agar. The MIC_{50} and MIC_{90} values (the minimum inhibitory concentration at 50% and 90% of bacterial growth inhibition, respectively) were calculated using WHONET 5.6 (WHONET 5.6, 2011) and compared the MIC_{50} and MIC_{90} values of antibacterials in each group, β-lactams, fluoroquinolones and aminoglycosides. After that, the antibacterial in each group providing the lowest of MIC value was selected for the synergistic activity assay in section 1.3.

1.3 **Testing of the antimicrobial combinations** (Pillai et al., 2005)

Synergy of two antibacterials was evaluated using checkerboard method. MHA with 5% mixture of sheep blood was prepared and steriled under pressure steamer at 121 ^{o}C and 15 pounds per square inch for 15 minutes. When the agar was cooled down, the antibacterials were mixed to the agar at 55 ^{o}C. The mixture of drugs and agar were poured onto plates and left until solidified. The antibacterials used in this study were selected from section 1.2 and the concentration of the drugs was decided from the MIC_{90} obtained

from section 1.2. Serial dilutions of the drugs in agar were prepared at concentrations of MIC_{90}, $MIC_{90}/2$, $MIC_{90}/4$, $MIC_{90}/8$, $MIC_{90}/16$. Two antibacterials were combined in each plate. Figure 8 shows concentrations of the antibacterial combination on a checkerboard plot. Then, purified *S. suis* was prepared in 0.85% saline solution at turbidity of 0.5 McFarland Standard (amount of bacteria approximately 1×10^8 CFU/mL) and diluted to 1×10^7 CFU/mL. The diluted *S. suis* was spotted on the agar surface using replicator inoculum block and the final concentration of *S. suis* was 1×10^5 CFU/point. The inoculated agar plates were incubated at 37 °C for 24 hours.

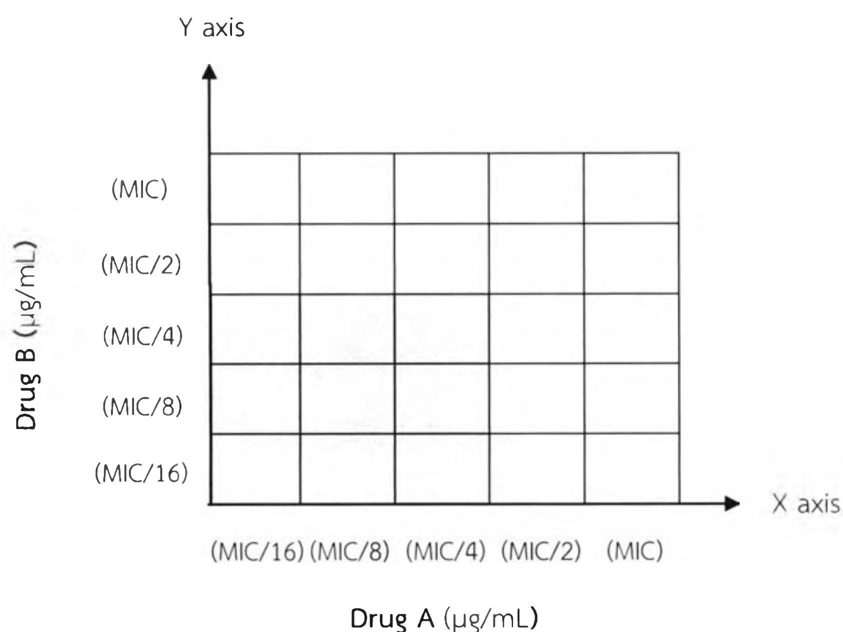

Figure 8 A trypical checkerboard plot and no bacterial growth observed in this gray area representing synergistic effect.

Interpretation of synergistic effect testing results

The bacterial growth in agar plate at different levels of drug concentrations was recorded. At the lowest concentration of antimicrobials without visible bacterial growth on the agar, Fractional Inhibitory Concentration (FIC) index of antibacterial drugs was calculated according to Equation 3. The FIC index was interpreted as follows; if both

drugs are synergistic with one another, the FIC index should be less than or equal 0.5 (FIC index $\leq$ 0.5), additivity was defined as a FIC index of more than 0.5 and not more than 1 (0.5 < FIC index $\leq$ 1), indifference was determined as a FIC index of more than 1 but less than 2 (1 < FIC index > 2). FIC index of 2 was interpreted as antagonism (Pillai et al., 2005; An et al., 2011).

$$\text{FIC index} = FIC_A + FIC_B = \frac{(A)}{(MIC_A)} + \frac{(B)}{(MIC_B)} \quad \text{.....Equation 3}$$

Whereas (A), (B) = the concentration of drug A or B, respectively, which was the lowest inhibitory concentration in this study.

(MIC_A), (MIC_B) = the MIC_{90} of drug A or B alone.

FIC_A, FIC_B = the fractional inhibitory concentration of drug A or B, respectively.

The percent synergism was calculated as following;

$$\%\text{Synergism} = \frac{\text{Numbers of isolates expressing synergistic effect} \times 100}{\text{Total numbers of } S.Suis \text{ isolated}} \quad \text{.....Equation 4}$$

Moreover, the percent additivity, indifference and antagonism were calculated using numbers of isolates expressing additive, indifferent and antagonistic effects, respectively, in place of the numbers of isolates expressing synergistic effect.

A pair of antimicrobial combination, which provided the least FIC index, was selected for further experiments.

2. Characterization

2.1 Thermal analysis by differential scanning calorimetry (DSC)

Thermal analysis was determined by using differential scanning calorimetry (DSC) (Model 882^{e}, Mettler Toledo, Switzerland). Standard aluminium pan (40 µl) was used. The lid pan was reamed. The powder of each drug and physical mixture between two drugs were tested. The sample approximately 2-3 mg were weighted into an aluminum

pan, sealed with a lid for analysis, and placed in DSC unit. An empty aluminum pan was used as reference. The sample was heated from 25 °C to 300 °C at rate of 2 °C/min. High pure nitrogen was flushed at a flow rate of 200 mL/min (Ghassempour et al., 2007; Calsavara, Zanin and Moraes, 2012; Golovnev, Vasiliev and Kirik, 2012). Then, melting point, melting enthalpy (ΔH) and onset temperature were evaluated by STARe software (Mettler Toledo, Switzland). The melting point was compared between each drug and physical mixture of the drugs.

2.2 Quantitative analysis of amoxicillin and enrofloxacin by HPLC method

HPLC technique was used to analyze the selected antibacterials, amoxicillin and enrofloxacin. Solutions of the antibacterials were prepared in phosphate buffer: acetonitrile (90:10) and UV absorption spectra of the solutions were scanned. HPLC method was validated using the guideline in the United States Pharmacopeia 34/National Formulary 29 (USP34/NF29) (USP, 2011). The analytical parameters used in the assay validation for HPLC method were specificity, linearity, accuracy and precision as described in Appendix B. Each concentration of amoxicillin and enrofloxacin was determined in triplicate.

2.2.1 *HPLC condition*

The chromatographic condition was modified from Numan et al. (2009) and Manceau et al. (1999) as follows:

Column : Phenomenex® Luna 5μ C_{18}(2) 100A (250 x 4.6 mm, 5 micron)

Guard column : Phenomenex®

Mobile phase : 0.02M dibasic potassium phosphate buffer pH 3.0 and acetonitrile using linear gradient as shown in Table 2.

Table 2 HPLC linear gradient method for amoxicillin and enrofloxacin analysis.

Time (min)	% phosphate buffer	% acetonitrile	Elution
0 - 9	90 → 10	69 → 31	Linear gradient
9 - 11	69	31	Isocratic
11 - 11.30	69 → 31	90 → 10	Linear gradient
11.30 - 20	90	10	Isocratic

Injection volume : 20 µL

Flow rate : gradient elution, 1.0 mL/min

Detector : PDA detector at 229 nm for amoxicillin and at 277 nm for enrofloxacin

Run time program : 20 minutes

2.2.2 Preparation of potassium phosphate buffer

Potassium phosphate buffer solution pH 3, the mobile phase, was prepared from 6.9672 mg of potassium hydrogen phosphate dibasic in 1000 ml of water. Then, the solution was adjusted to pH 3 using phosphoric acid or sodium hydroxide (NaOH). After that, the buffer solution was filtered under vacuum through a 0.45 µm membrane filter and then degassed by sonication for 30 minutes before used.

2.2.3 Stock and working standard solution preparation

A stock standard solution of amoxicillin and enrofloxacin was prepared by correctly weighing 5 mg of each drug into a 25 ml volumetric flask. The mobile phase, 90% dibasic potassium phosphate buffer solution pH 3.0 and 10% acetonitrile, was added to dissolve the drugs and adjust to the final volume. This stock solution had a concentration of 200 µg/mL of each drug. Working standard solutions of amoxicillin and enrofloxacin were prepared by diluting and adjusting the stock solution using the mobile phase so that the concentrations of amoxicillin and enrofloxacin were 60, 50, 40, 20, 10 and 5 µg/ml. Three replications were injected to HPLC for each concentration. Peak areas were reported

for all solutions. Standard curves were prepared from the relationship between peak area responses of amoxicillin and enrofloxacin and their concentrations.

2.2.4 Samples preparation

Sample solutions were further diluted to obtain appropriate concentration using the mobile phase. The diluted sample concentration was within the concentration range of the standard solutions. The amount of amoxicillin and enrofloxacin were analyzed using HPLC and calculated according to the standard curve.

2.3 Organic solvents for drug extraction

The selected antibacterials, amoxicillin and enrofloxacin, from the previous study were prepared to be suspensions in oil. Five hundred milligrams of each antibacterial was mixed together and 5 milliliters of corn oil was added. The suspension was mixed and poured in a 10 mL screw cap test tube. Then, 5 mL of an organic solvent was added into the suspension and a vortex mixer was used to shake the sample. The preliminary was centrifuged 6,000 rpm for 15 minutes and the sample was centrifuged at 8000 rpm for 20 minutes. The organic solvent and the oil were removed from the sample. The sediment was washed using three 5 mL portions of the fresh organic solvent (USP, 2011). Finally, the residue was dried and stored in vacuum with silica gel. The organic solvents were evaluated in this experiment including benzene, chloroform, ethyl ether, hexane and toluene. To determine percent extraction of the antibacterials, the dried residue was dissolved in 55 mL of methanol, 7 mL of water and 20 mL of isopropyl alcohol and then methanol was added to adjust the volume to 100 mL. The residue was further diluted to a suitable concentration within the concentration range of the standard curve. The content of antibacterials was determined using HPLC method and the percentages of extraction were calculated using Equation 5.

$$\%Extraction = \frac{\text{Concentration of extracted} \times 100}{\text{Concentration of added}} \quad \text{.....Equation 5}$$

The organic solvent providing the highest percent of extraction was selected to use for the extraction.

2.4 Solubilities of amoxicillin trihydrate and enrofloxacin base

The solubilities of amoxicillin trihydrate and enrofloxacin base were experimentally determined. Solvent was 90:10% (v/v) of phosphate buffer saline (PBS) pH 7.4: ethanol (BP, 2007). Excess amount of amoxicillin trihydrate or enrofloxacin base was added in 5 g of the solvent in a 20 ml test tube. The test tube was kept in a shaking water bath controlled at 37 °C. The sample solution was taken at 0.5, 2, 4 and 8 hours and filtered through a syringe membrane filter. The filtrate was collected and diluted with phosphate buffer pH 3.0. The drug content in the diluted solution was analyzed using HPLC method in section 2.2 and the solubility amoxicillin trihydrate and enrofloxacin base in the solvent were calculated.

3. Preparation of suspensions in oil for intramuscular injection

The antibacterial combination suspension of amoxicillin trihydrate and enrofloxacin base in an oil was prepared. The two antibacterial dry powders were mixed using a vortex mixture until homogenized and then the appropriate amount of a surfactant was dispersed into the antibacterial agents. After that, the oil was added into the mixture. The suspension was homogenized using a vortex mixture. The suspension was characterized for physical appearance, sedimentation volume, redispersibility, particle size, particle size distribution and viscosity. Chemical stability of the formulation was also determined.

3.1 Surfactant and oil screening

Various surfactants and oils were used to evaluate wettability. Various surfactants (Tween 20, Tween 80, Span 20 and Span 80) were tested. Corn oil, cottonseed oil or isopropyl myristate were used as dispersion media in this study. The powder of 580 mg of amoxicillin trihydrate and 500 mg of enrofloxacin was mixed with 0.5 g of a surfactant. Then, 5 ml of each oil was added into the mixture of antibacterial combination and surfactant using the process mentioned previously. Antibacterial suspensions were prepared from these various surfactants and various oils. The suspensions were evaluated physical appearance. The surfactants in the suspensions providing homogeneously dispersed internal phase, the antibacterial particles, without floating particles on the surface were selected for further study. Physical properties of the suspensions were determined as following.

3.1.1 Physical appearance and sedimentation volume

Appearance of the antibacterial suspensions including color and homogeneity was visually observed immediately, 24 hours and/or 1 week. Sedimentation volume of the suspensions was also measured after 5 ml of the suspensions were placed in 10 ml test tube and undisturbedly kept for 24 hours and/or 1 week. Then, ratio of sedimentation volume (F) was calculated using Equation 6. Ratio of sedimentation volume should be close to or equal to 1 (กรีพล, 2008; Patel, 2010).

$$F = {}^{V_u}/_{V_o} \qquad \text{...... Equation 6}$$

Whereas F = ratio of sedimentation volume

V_u = the final volume of sediment

V_o = the original volume of suspension before settling

3.1.2 Redispersibility

Redispersibility represents ability of a suspension to uniformly disperse with minimal shaking after it has stood for some time. Two tubes of 5 ml antibacterial suspensions were prepared. The first tube was undisturbedly kept at room temperature for a week. The other tube was centrifuged at 3,500 rpm for 30 minutes. Then, both tubes were rotated top to bottom at speed of 20 rpm to uniformly disperse the suspension. Suspension taking longer time to redisperse is unsatisfied and likely to form cake in the future (กรีพล, 2008).

3.1.3 Determination of particle size and particle size distribution

Particle size and particle size distribution were measured using light microscope (Olympus, Japan) after preparing the formulation. Approximate 30 μL of each sample was diluted with 1 mL of mineral oil. The prepared sample was placed under the light microscope with 40x optical lens and 40x object lens. The particle size was measured using the light microscope and the particle size distribution was also determined. The experiment was done in triplicate. The formulations providing less aggregation of the particles and narrow particle size distribution were selected (กรีพล, 2008; Patel, 2010).

3.1.4 Viscosity

The viscosity of antibacterial suspension providing the best characteristics in section 3.1.1 and 3.1.2 was monitored using viscometers, Brookfield: model-DV-II spindle no.0 and AND model SV-10, at room temperature. Briefly, the antibacterial suspensions of amoxicillin trihydrate and enrofloxacin base in oil were prepared by weighing 1.740 g and 1.518 g of amoxicillin trihydrate and enrofloxacin base, respectively into a 50-mL test tube. It was mixed using a vortex mixture. Then, 1.500 g of surfactant was added into the antibacterials and 15 mL an oil was added into the mixture. After that, the sample was measured using Brookfield viscometer and AND model SV-10. For Brookfield viscometer (model-DV-II spindle no.0, speed at 50, 40, 30, 20, 10, 5, 2.5, 2,

1.5, 1.2, 1, 0.8, 0.7 and 0.5 rpm), approximately 13 mL of each suspension was put in a small sample cup. The temperature of sample was controlled at 30 ± 0.5 °C using water bath. Then, the sample was determined. For AND model SV-10, 11 mL sample was poured into a plastic small sample cup. The sample was measured at room temperature. Finally, the obtained viscosity values were compared. The instruments were calibrated with purified water before used. Each sample was measured in triplicate.

4. Physical and chemical stability study of suspensions in oil for IM injection

4.1 Physical stability of formulations in heating cooling cycle

The suspensions in oil selected from section 3 were tested. The suspensions in oil were loaded in tightly closed amber glass bottles. The bottles were kept in accelerated condition, heating cooling cycle. The samples were stored at 4 °C for 48 hours followed by 45 °C for 48 hours as complete one cycle. This test was repeated for 6 cycles (Shafiq et al., 2007). Physical appearance, redispersibility, particle size and particle size distribution of the suspensions were monitored as described in section 3.1.1, 3.1.2, 3.1.3 and compared to these of the suspensions at the beginning. The stable formulation was then selected for further study.

4.2 Physical and chemical stability of formulations

The suspension providing the best physical stability in section 4.1 was selected to study in this section. The suspension was prepared and kept in tightly closed 10-mL amber glass bottles. The filled bottles were separated into 2 groups and placed in 2 different conditions modified from ASEAN guidelines (ASEAN guidelines, 2005; Henal et al., 2011) as following; 1) 30 ± 2 °C /75 ± 5 %RH and 2) 40 ± 2 °C /75 ± 5 %RH. Samples were taken from both conditions at 0 day, 2 weeks, 1 month, 2 months and 3 months. Physical appearance, particle size, particle size distribution and viscosity of the samples were evaluated in the same manner as section 3.1.3 and 3.1.4. Then, the samples were

analyzed using HPLC method in section 2.2 to determine amoxicillin and enrofloxacin contents. The experimental was performed in triplicate. Then, concentration vs time profiles of the antibacterials were delineated.

5. *In vitro* release study

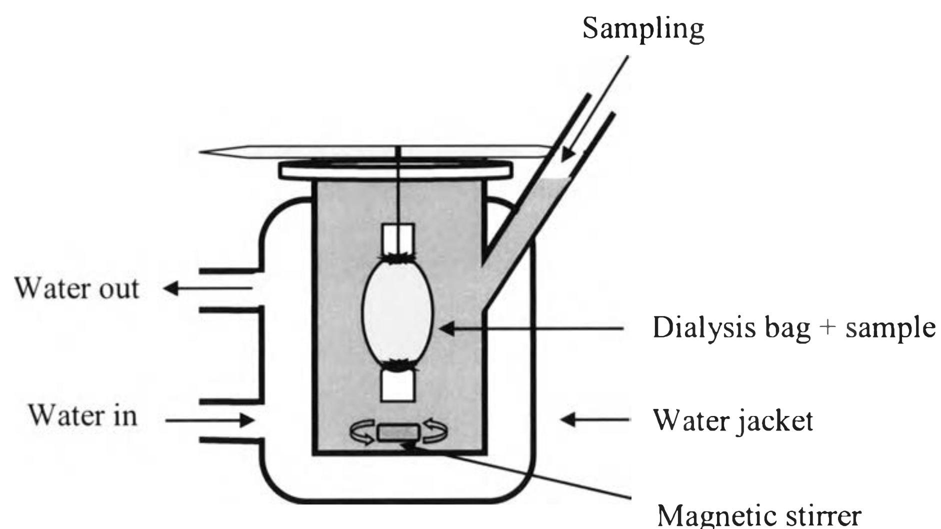

Figure 9 Modified Franz diffusion cell.

The release of amoxicillin trihydrate and enrofloxacin base suspension was compared with that of amoxicillin sodium and enrofloxacin hydrochloride suspension. Amoxicillin trihydrate and enrofloxacin base are slightly soluble in water with solubility of 3.4 mg/ml (Fernando, Joe and Lynn, 2012) and 0.164 mg/ml (Seedher and Agarwal, 2009), respectively while amoxicillin sodium and enrofloxacin hydrochloride are soluble in water with solubility of 50 mg/ml and 10 mg/ml, respectively (Toku-e, 2013). The suspensions were prepared in the surfactant and the oil selected from the previous study. Dialysis bags (1.15 cm diameter and 3.0 cm length) with molecular weight cut off of 3,500 daltons were soaked in purified water for 24 hours and rinsed many times with boiling water. Then, the bags were soaked in a mixture of 90 %v/v PBS pH 7.4 and 10 %v/v ethanol for 1 hour. Three hundred µl of the suspensions was loaded into the dialysis bags and the bags were

tightly closed. Each filled bag was hung in a receiver chamber of modified Franz diffusion cell as shown in Figure 9. The chamber was filled with 11 ml of the mixture of 90 %v/v PBS pH 7.4 and 10 %v/v ethanol. Temperature in the chamber was equilibrated at 37 ± 0.5 ^{o}C prior to hanging the dialysis bag and throughout the experiment. The receiver chamber was equipped with a magnetic stirring bar rotating rate at 600 rpm. All fluid in the receiver chamber was taken and replaced with fresh 90 %v/v PBS pH 7.4 and 10 %v/v ethanol mixture at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 10, 12, 16, 20, and 24 hours to maintain sink condition in the receiver chamber throughout the experiment. The taken receiver fluid was diluted at suitable concentration and then analyzed using HPLC method to determine amoxicillin and enrofloxacin contents. The experiment was run in triplicate. Percentage of drugs released was calculated using Equation 7.

$$\%\text{Released} = \frac{\left(V_s \cdot \sum_{n=1}^{n} C_{n-1} + V_m \cdot C_n\right)}{M} \times 100 \qquad \text{.....Equation 7}$$

Whereas V_s = the volumes of sample

V_m = the volumes of release medium

C_n = the drug concentration in sample n

M = the total amount of drug initially added to dialysis bag

6. Statistical analytical

The data in this study were statistically analyzed by descriptive statistics (mean ± standard deviation) and one-way analysis of variance (ANOVA). When the significant difference ($p < 0.05$) was indicated, the data were subjected to multiple comparisons by Tukey or Dunnett test to compare the difference. The statistical package for social sciences (SPSS) software version 17.0 was used in this study.