CHAPTER IV

RESULTS AND DISCUSSION

1. Evaluation of antimicrobials

1.1 Antimicrobial susceptibility test

The bacteria isolated on sheep blood agar are shown in Figure 10. Greyish, semi-transparent, slightly mucoid and exhibited α – hemolysis colonies were selected and used for the evaluation. *S. suis* was cultured on the sheep blood MHA presenting growth of the bacteria without and with antibacterial activity (Figure 11). Three reference strains, *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *Ps. aeruginosa* ATCC 27853, were used for quality control of antibacterial concentration. MIC ranges of the reference strains are shown in Table 3. The result was found that MIC values of reference strains were within the acceptable ranges (CLSI, 2011) except for CEF-3-MAX®, CEFOMAX®100 and EXCENEL® RUT, available products, were more than 1-3 logs.

The antimicrobial susceptibility testing of 16 local *S. suis* strains collected from pigs displaying clinical diseases in Thai swine herds, especially in the Western part, which 4 strains were *S. suis* serotype 2, was performed against fifteen antibacterial agents using agar dilution technique. The susceptibilities of the isolates, expressed as concentrations required to inhibition 50 and 90% of the isolates, were analyzed by WHONET 5.6 software and the data of MIC values are shown in the Table 4. The results from MIC value proved that *S. suis* isolates used in this study were more sensitive to β-lactam and fluoroquinolone groups than aminoglycoside group. Amoxicillin, cefazolin, ceftriaxone, cefotaxime, presented the MIC_{50} and MIC_{90} values not more than 2 μg/mL, suggesting that the *S. suis* isolates in the study were highly sensitive to such antibacterials. Whereas *S. suis* isolates were resistant to gentamicin and neomycin and the wide MIC ranges of

aminoglycosides were observed. *S. suis* was sensitive to aminoglycosides only at high concentration. On the other hand, β-lactams and fluoroquinolones provided narrow range of MIC values. It implied that both antibacterials can be used for the treatment of *S. suis* infected pigs better than aminoglycosides. This result was agreed with the study by Petcharat and Jasda (2008) which displayed that *S. suis* isolates from pigs in Western Thailand were sensitive to beta-lactams and fluoroquinolones such as amoxicillin, cephalothin, amoxicillin and clavulanic acid, ciprofloxacin and enrofloxacin. It also corresponded to studies by Han *et al.* (2001) and Marie *et al.* (2002) which reported that *S. suis* isolates were sensitive to amoxicillin. This is similar to studies done in China which found that 421 samples of *S. suis* extracted from tonsils of pigs in 9 provinces of China between 2005 and 2007 were sensitive to β-lactams (penicillin, ampicillin) and fluoroquinolones (enrofloxacin) (Zhang et al., 2008). *S. suis* isolates with very high resistance against gentamicin were found. It also corresponded to the report by Bilhmad *et al.* (2005) which reported that *S. suis* isolates in the South of Thailand resisted to neomycin and kanamycin. In addition, Marie *et al.* (2002) also found that *S. suis* isolates resisted to kanamycin. Drugs resistance may be due to the fact that drugs in aminoglycoside groups are widely used in pig farms without control. Most *S. suis* bacteria were collected from Western Thailand because there were high population of pig farms in the area. Hence, antibiotics were also widely used to prevent pig diseases in the area.

Regarding the sensitivity of the *S. suis* isolates, β-lactam and fluoroquinolone groups were selected for the synergistic activity assay. Within β-lactam group, *S. suis* isolates were highly sensitive to amoxicillin, cefazolin, cefotaxime and ceftriaxone since low MIC values were observed. The MIC_{50} and MIC_{90} values of ciprofloxacin and enrofloxacin were comparable. Thus, only enrofloxacin was selected to further study since it was widely used in animal more than ciprofloxacin and provided narrow MIC range. In addition, this result was agreed with the study of Groggel et al. (2007). Therefore, the

antibacterials used in the synergistic effect study were amoxicillin, cefazolin, cefotaxime and ceftriaxone combined with enrofloxacin.

Figure 10 The characteristic colonies of *S. suis* on sheep blood agar.

(A) Bacteria control plate

(B) Without the bacterial growth on the blood agar

(C) The bacterial growth on the blood agar plate

Figure 11 *S. suis* was cultured on the sheep blood Mueller Hinton agar presenting the growth of the bacteria without (A) and with antibacterial activity (B and C).

Table 3 MIC (µg/mL) ranges of the reference strains which have been obtained from the test, compared with CLSI accepted quality control ranges.

Antibiotics	*Staphylococcus aureus* ATCC 25923		*Escherichia coli* ATCC 25922		*Pseudomonas aeruginosa* ATCC 27853	
	R[a]	CLSI[b]	R[a]	CLSI[b]	R[a]	CLSI[b]
Amoxicillin*	<0.125	N/A	0.5	N/A	>512	N/A
Cefadroxil*	1	N/A	2	N/A	32	N/A
Cefalexin*	1	N/A	2	N/A	32	N/A
Cefazolin*	0.5	0.25 - 1	1	1 – 4	32	N/A
Cefotaxime*	1-2	1 – 4	<0.125	0.03 – 0.12	16	8 – 32
Ceftriaxone*	4	1 – 8	<0.125	0.03 – 0.12	16- 32	8 – 64
Ciprofloxacin**	0.25	0.12 – 0.5	<0.125	0.004 – 0.015	0.5	0.25 - 1
Dicloxacillin*	1	N/A	1	N/A	32	N/A
Enrofloxacin**	0.125-0.5	0.12 - 1	<0.125	0.008 – 0.03	2	1 - 4
Gentamicin*	1	0.12 - 1	0.5 - 1	0.25 – 1	2	0.5 - 2
Neomycin*	<0.125	N/A	0.25	N/A	<0.125	N/A
Cefotaxime*** (CEFOMAX® 100)	8 - 16	1 – 4	0.25 – 0.5	0.03 – 0.12	64 - 128	8 – 32
Ceftiofur*** (EXCENEL®RTU)	16	0.25 - 1	16	0.25 – 1	>512	16 - 64
Cefotaxime + Gentamicin*** (Cefralo® L.A.)	1	N/A	0.25 – 0.5	N/A	16	N/A
Ceftriaxone*** (CEF-3-MAX®)	8 -16	1 – 8	0.25 – 0.5	0.03 – 0.12	64 - 128	8 – 64

N/A : Have not been reported.

[a] R : The result obtained from this study.

[b]CLSI : Accepted quality control range taken from Clinical and Laboratory Standards Institute (CLSI, 2011).

* Reference standard, ** Raw material, *** Pharmaceutical product

Table 4 MICs of 15 antibacterial agents for 16 *S. suis* isolates.

Antibacterials	Field isolates (n=16)		
	Average MIC_{50} (µg/mL)	Average MIC_{90} (µg/mL)	Range of MIC (µg/mL)
Amoxicillin*	0.064	2	0.062 - 4
Cefadroxil*	2	64	0.062 - 128
Cefalexin*	8	128	0.062 - 128
Cefazolin*	0.5	2	0.062 - 4
Cefotaxime*	0.5	1	0.062 - 8
Ceftriaxone*	0.355	2	0.062 - 8
Ciprofloxacin**	0.5	16	0.062 - 32
Dicloxacillin*	2	8	0.5 - 32
Enrofloxacin**	0.5	16	0.5 - 16
Gentamicin*	256	256	8 - 512
Neomycin*	128	256	4 - 512
Cefotaxime*** (CEFOMAX® 100)	8	8	0.062 - 32
Ceftiofur*** (EXCENEL®RTU)	8	16	0.25 - 16
Cefotaxime + Gentamicin*** (Cefralo® L.A.)	16	32	0.062 - 32
Ceftriaxone*** (CEF-3-MAX®)	2	16	0.25 - 16

* Reference standard, ** Raw material, *** Pharmaceutical product

1.2 Testing of the antimicrobial combinations

The synergism of two antibacterials was tested using checkerboard method and agar dilution technique. Beta-lactam (amoxicillin, cefotaxime, cefazolin and ceftriaxone) and fluoroquinolone (enrofloxacin), drugs were selected regarding previous

study. Each β-lactam was combined to enrofloxacin and tested for the synergism. Gentamicin and cefotaxime combination were also tested synergistic effect since this combination was often employed for the treatment of *S. suis* infection in pigs by veterinarians and high rate of resistance associated with *S. suis* was reported (personal communication). Then, the following antimicrobial combinations were tested: amoxicillin + enrofloxacin, cefotaxime + enrofloxacin, cefazolin + enrofloxacin, ceftriaxone + enrofloxacin and cefotaxime + gentamicin. Checkerboard plot was constructed for each combination. Typical checkerboard plots presenting synergy, additivity and indifference in this study are shown in Figure 12. The synergy of the antibacterial combinations was interpreted as FIC index ≤ 0.5. Data of the synergistic effect of the antibacterial combinations against *S. suis* are shown in Table 5. Interpretation of the FIC index is shown in Table 6. The results demonstrated that the synergy of enrofloxacin and amoxicillin presented the highest frequency (68.75%). Indifference was found in the combination of ceftriaxone + enrofloxacin and cefotaxime + gentamicin. No antagonism was observed in any combinations.

Furthermore, no synergistic effect against *S. suis* no.11, 37, 51, 55, and 60 was observed in all β-lactam and enrofloxacin combinations including cefotaxime and gentamicin combination. It could be assumed that those *S. suis* isolates were collected from pig farms highly consuming antibacterials and consequently antimicrobial resistance was found.

Therefore, the combination of enrofloxacin and amoxicillin provided higher antibacterial activities against *S. suis* than combination of enrofloxacin combined ceftriaxone, cefotaxime or cefazolin and gentamicin combined to cefotaxime, respectively. Thereby, the combination of enrofloxacin combined to amoxicillin was selected to formulate as antibacterial suspensions in oil medium for parenteral administration.

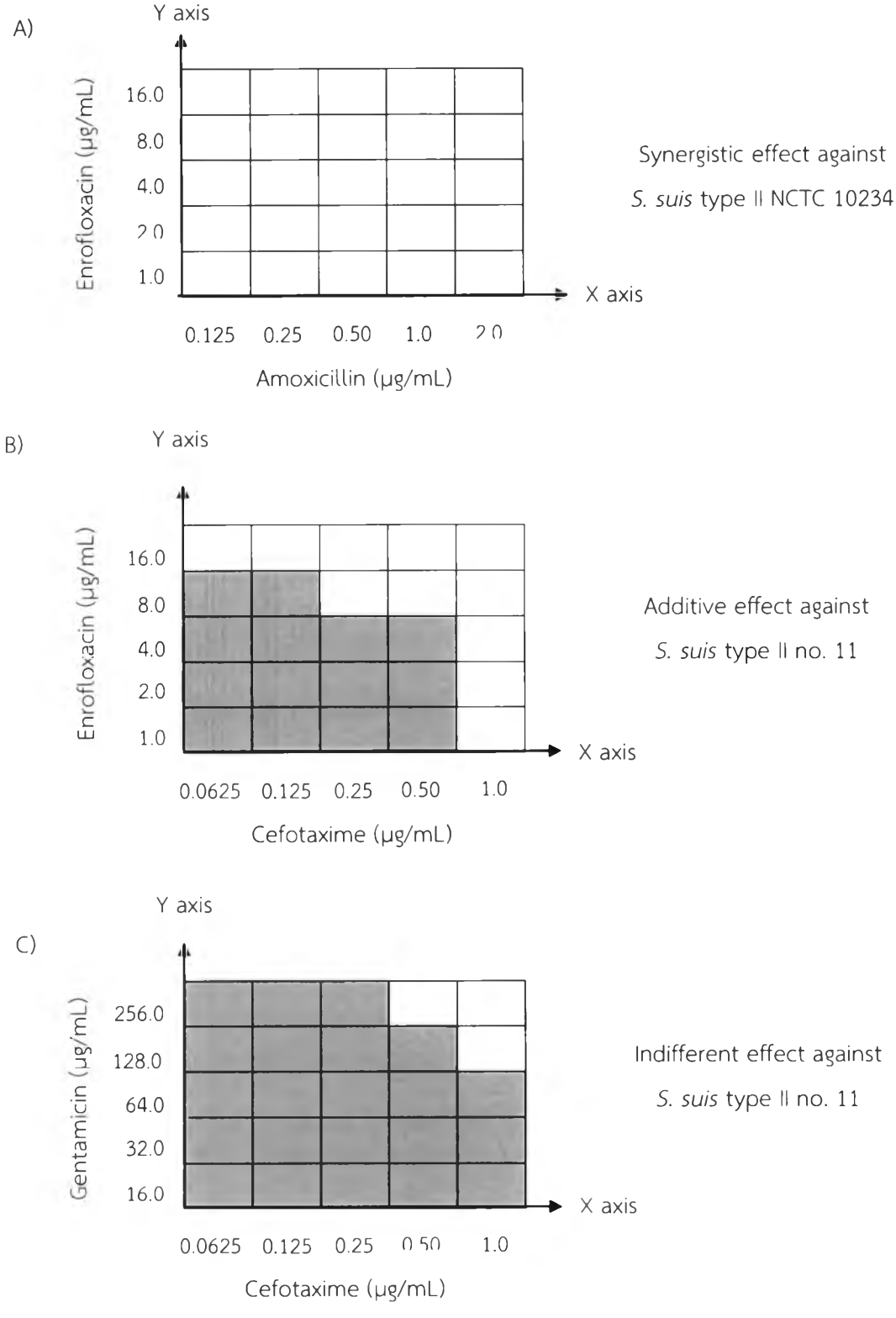

Figure 12 Typical checkerboard plots obtained from this study bacterial growth observed in the shaded area; A) synergy B) additivity C) indifferent.

Table 5 FIC index for the combinations with enrofloxacin and combination of gentamicin and cefotaxime.

Strains	FIC index (combination with Enrofloxacin)				FIC index (combination with Gentamicin)
	Amoxycillin	Cefazolin	Ceftiaxone	Cefotaxime	Cefotaxime
1. *S.suis* no. 2	0.125	1	0.375	0.625	0.125
2. *S.*suis no. 3	0.125	1	0.375	0.625	0.125
3. *S.suis* no. 4	0.125	0.125	0.125	0.125	1
4. *S.suis* type II no.5	0.5	0.5	0.5	0.5	0.5
5. *S.suis* type II no.11	0.625	0.625	0.75	0.625	1.5
6. *S.suis* no. 12	0.187	0.187	0.187	0.187	0.75
7. *S.suis* type II no.13	0.125	0.125	0.125	0.125	0.75
8. *S.suis* type II no.14	0.125	0.125	0.125	0.125	0.75
9. *S.suis* no. 37	0.625	0.625	0.625	0.625	1.5
10. *S.suis* no. 44	0.125	0.125	0.125	0.125	0.125
11. *S.suis* no. 48	0.125	0.125	0.125	0.125	0.125
12. *S.suis* no. 50	0.125	0.125	0.125	0.125	0.187
13. *S.suis* no. 51	0.625	0.625	0.625	0.625	0.125
14. *S.suis* no. 55	0.625	0.75	1.5	1	0.625
15. *S.suis* no. 60	0.625	1	1.5	1	0.625
16. *S.suis* no. 61	0.125	0.125	0.125	0.125	0.5
S.suis type II[a] NCTC 10234	0.125	0.125	0.125	0.125	0.125

[a] Reference standard of *S. suis* capsular serotype 2.

The FIC index was interpreted as follows; synergism: FIC index ≤ 0.5, additivity: 0.5 < FIC index ≤ 1, indifferent: 1 < FIC index ≤ 2 and antagonism: FIC index < 2.

Table 6 Interpretation of susceptibility testing results.

Antibacterial combinations	FIC index interpretation			
	% synergy	% additivity	% indifferent	% antagonism
Amoxycillin + Enrofloxacin	68.75	31.25	0	0
Cefazolin + Enrofloxacin	56.25	43.75	0	0
Ceftiaxone + Enrofloxacin	68.75	18.75	12.50	0
Cefotaxime + Enrofloxacin	56.25	43.75	0	0
Cefotaxime + Gentamicin	50.00	37.50	12.50	0

2. Characterization

2.1 DSC analysis

Thermal analysis is suitable to ascertain whether a drug mixture of ingredients in a particular product is compatible. The DSC thermogram of amoxicillin trihydrate, enrofloxacin base and physical mixture of both drugs were shown in Figure 13. The DSC of the enrofloxacin provided two peaks. The first peak was probably the melting point of enrofloxacin corresponding to Golovnev et al. (2012). They revealed that the melting point onset and melting enthalpy were 222.3 $^{\circ}$C and 90.75 J/g, respectively. From, the DSC result of enrofloxacin in this study, the melting point (peak) was at 224.57 $^{\circ}$C, the onset was at 223.77 $^{\circ}$C and melting enthalpy was about 98.28 J/g. The DSC thermogram of the amoxicillin trihydrate presented two peaks. The first peak was evaporation of water approximately at 100 $^{\circ}$C, which it was boiling point of water. The second peak was due to the melting point of amoxicillin at 189.39 $^{\circ}$C which was similar to the finding of Fernando

et al. (2012). This publication stated that the melting point of amoxicillin was about 194 °C. The endothermic peak of physical mixtures of amoxicillin trihydrate and enrofloxacin base should be due to dehydration (100 °C) and melting (189 °C) processes. Moreover, the second peak became broader than that of pure amoxicillin trihydrate. Endothermal peak of enrofloxacin base disappeared in physical mixture. It might be suspected that the drugs possibly interacted with each other. Firstly, the incompatibility between melted amoxicillin trihydrate and enrofloxacin base may occur. Another possibility might attribute to the melted amoxicillin trihydrate caused enrofloxacin to be dissolved at high temperature. However, this interaction should not be an incompatibility issue at room temperature.

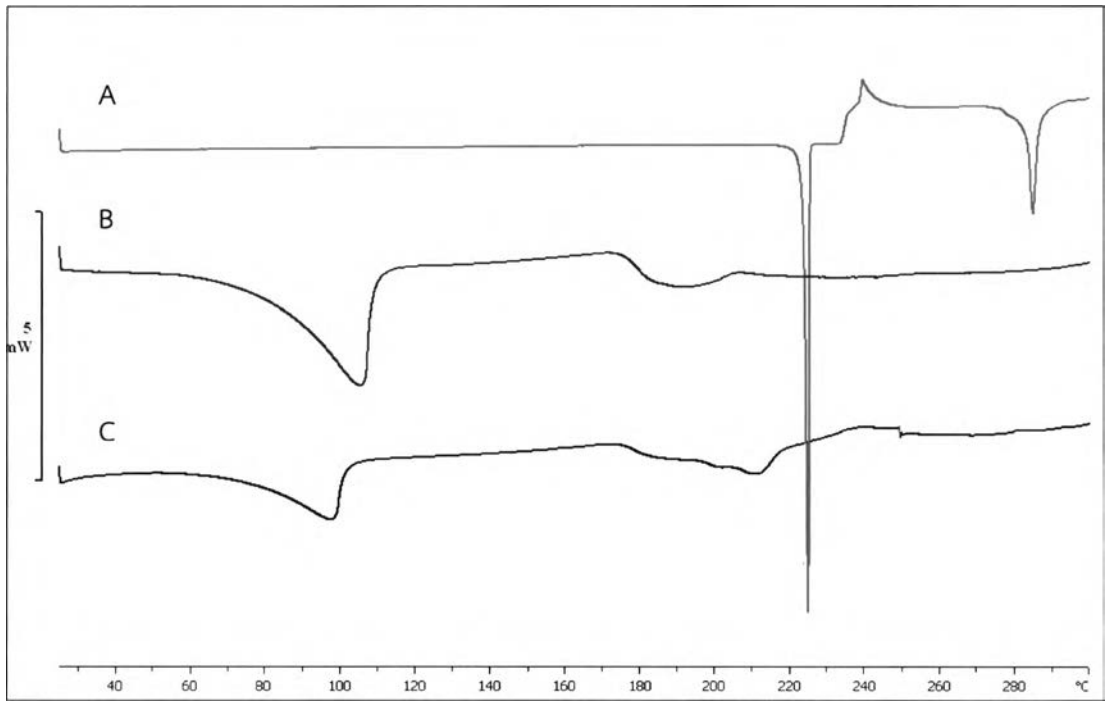

Figure 13 DSC thermograms of enrofloxacin base (A), amoxicillin trihydrate (B), and physical mixture of amoxicillin trihydrate and enrofloxacin base (C).

2.2 Quantitative analysis of amoxicillin and enrofloxacin by HPLC method

The HPLC method was developed and validated for determination of amoxicillin and enrofloxacin in oil suspensions since HPLC method for simultaneous determination of amoxicillin and enrofloxacin has not been reported. Wavelengths of amoxicillin and enrofloxacin were detected with UV spectrum, which photo diode array detector was operated in a range of wavelengths from 190 to 800 nm. The result as shown in Figure 14 and 15 revealed that the maximum absorption of amoxicillin trihydrate was found at 229 – 230 nm, which is in agreement with Numan et al. (2009). A wavelength of 277- 278 nm was an optimal enrofloxacin detection, which is in approval with Manceau et al. (1999) and Pierini et al. (2004). Therefore, the photo diode array detector of the HPLC system was set at 229 nm and 277 nm.

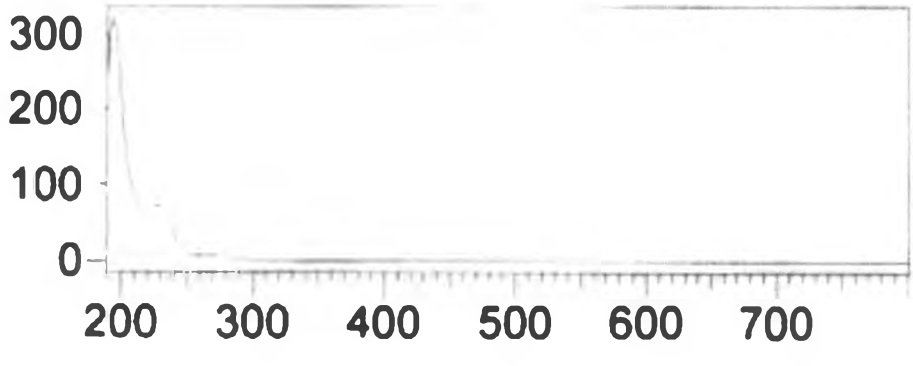

Figure 14 UV spectrum of amoxicillin trihydrate.

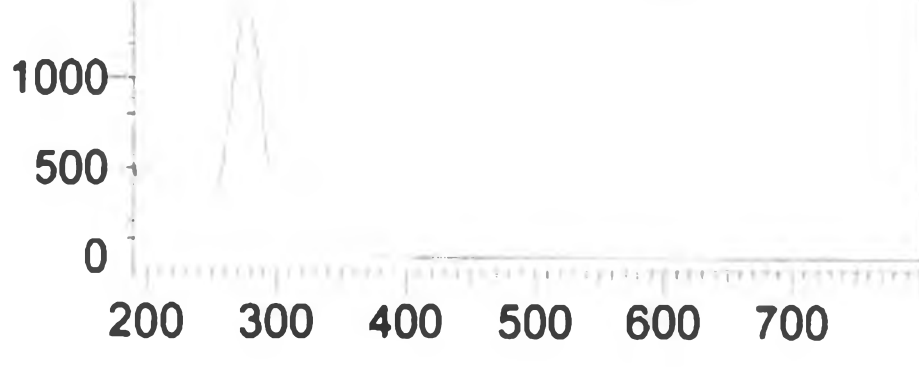

Figure 15 UV spectrum of enrofloxacin.

The standard mixture of amoxicillin and enrofloxacin was prepared and assayed by reversed-phase HPLC (RP-HPLC) method as shown in pages 41-42. The result displayed that both peaks were clearly separated and the retention times of amoxicillin trihydrate and enrofloxacin were around 5.0 and 12.7 minutes, respectively, which was shown in Figure 16 (λ = 229 nm) and Figure 17 (λ = 277 nm).

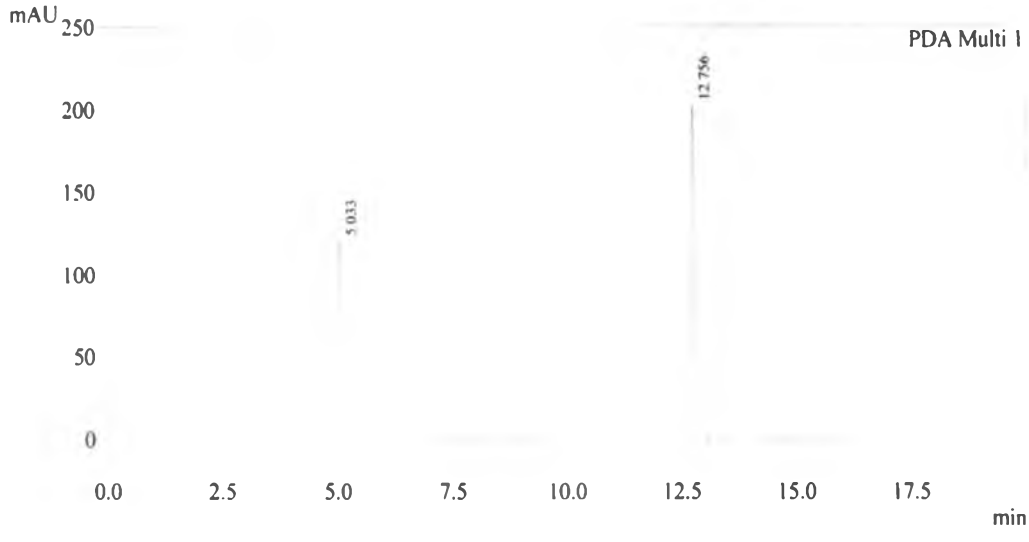

Figure 16 Chromatograms of simultaneously analyzed two drugs, amoxicillin trihydrate (peak at 5 min) and enrofloxacin base (peak at 12.7 min) at 229 nm.

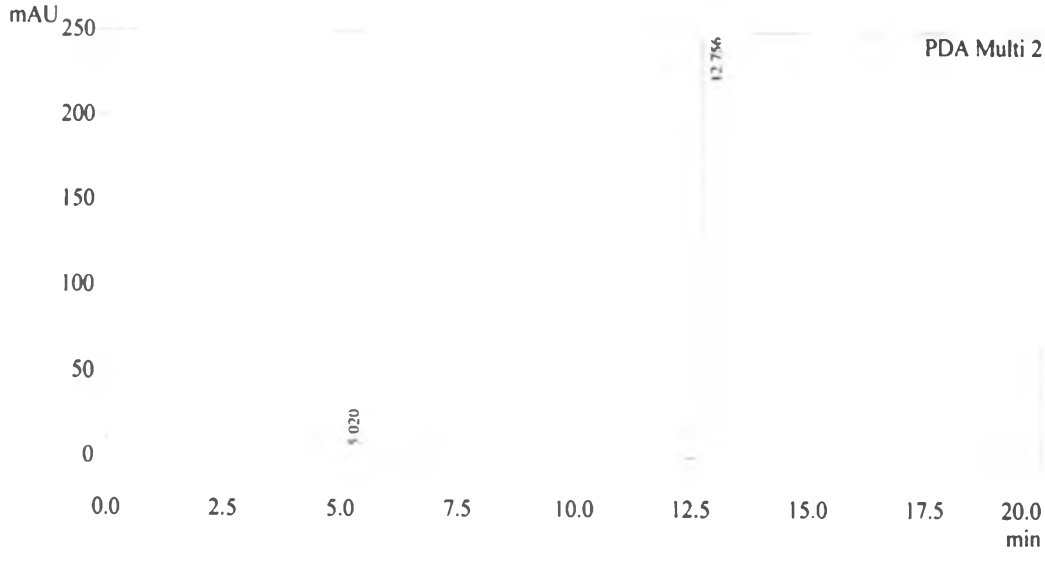

Figure 17 Chromatograms of simultaneously analyzed two drugs, amoxicillin trihydrate (peak at 5 min) and enrofloxacin base (peak at 12.7 min) at 277 nm.

2.3 Organic solvent for drug extraction

The antibacterials in oil suspension were extracted using various organic solvents. The percent extraction of amoxicillin and enrofloxacin is reported in Table 7. Our result implied that the percent extraction of amoxicillin and enrofloxacin was the highest when ethyl ether was used (n=1). Hexane and ethyl ether were selected for further experiment.

Table 7 The percentages of extraction of amoxicillin and enrofloxacin after the combination antibacterial suspensions in corn oil using organic solvents (n=1).

NO	Organic solvent	Amoxicillin trihydrate			Enrofloxacin base		
		Peak Area	Concentration (ug/mL)	Extraction (%)	Peak Area	Concentration (ug/mL)	Extraction (%)
1	Toluene	565092	21.08	70.28	9367594	67.41	28.09
2	Benzene	546930	20.39	67.97	5111619	36.59	15.25
3	Chloroform	171319	6.11	20.38	133288	0.54	0.22
4	Hexane	604489	22.58	75.27	28859675	208.58	86.91
5	Ethyl ether	634800	23.73	79.11	29402338	212.51	88.55

Extraction process was repeated using these two organic solvents. Percent extraction and variation of the result are reported in Table 8. Since hexane provided better percent recovery and less variation, this solvent was chosen for amoxicillin and enrofloxacin extraction from the corn oil suspension.

Table 8 The percentages of extraction of amoxicillin and enrofloxacin after the combination antibacterial suspensions in corn oil using ethyl ether and hexane (n=3).

Organic solvent	Amoxicillin trihydrate			Enrofloxacin base		
	Peak Area	Concentration (ug/mL)	%Extraction	Peak Area	Concentration (ug/mL)	%Extraction
Ethyl ether	578650 ± 25895	21.58 ± 0.97	71.93 ± 3.23	26786424 ± 797863	187.61 ± 5.18	78.17 ± 2.16
Hexane	623978 ± 23233	23.27 ± 0.87	77.57 ± 2.90	29271599 ± 559753	204.75 ± 3.91	85.31 ± 1.63

2.4 Solubility of amoxicillin trihydrate and enrofloxacin base

Solubilities of amoxicillin trihydrate and enrofloxacin base in the mixture of phosphate buffer saline (PBS) pH 7.4 and ethanol (9:1 by volume) at 37 °C were 7.0840 mg/mL and 1.1172 mg/mL, respectively (Figure 18). Amoxicillin trihydrate and enrofloxacin base are slightly soluble in water and reported solubilities are 3.4 mg/mL (Fernando et al., 2012) and 0.164 mg/mL (Seedher and Agarwal, 2009), respectively, which may lead to difficulty of HPLC analysis during *in vitro* release study. Therefore, the mixture of phosphate buffer saline pH 7.4 and ethanol (9:1 by volume) was used as receiver fluid in release study.

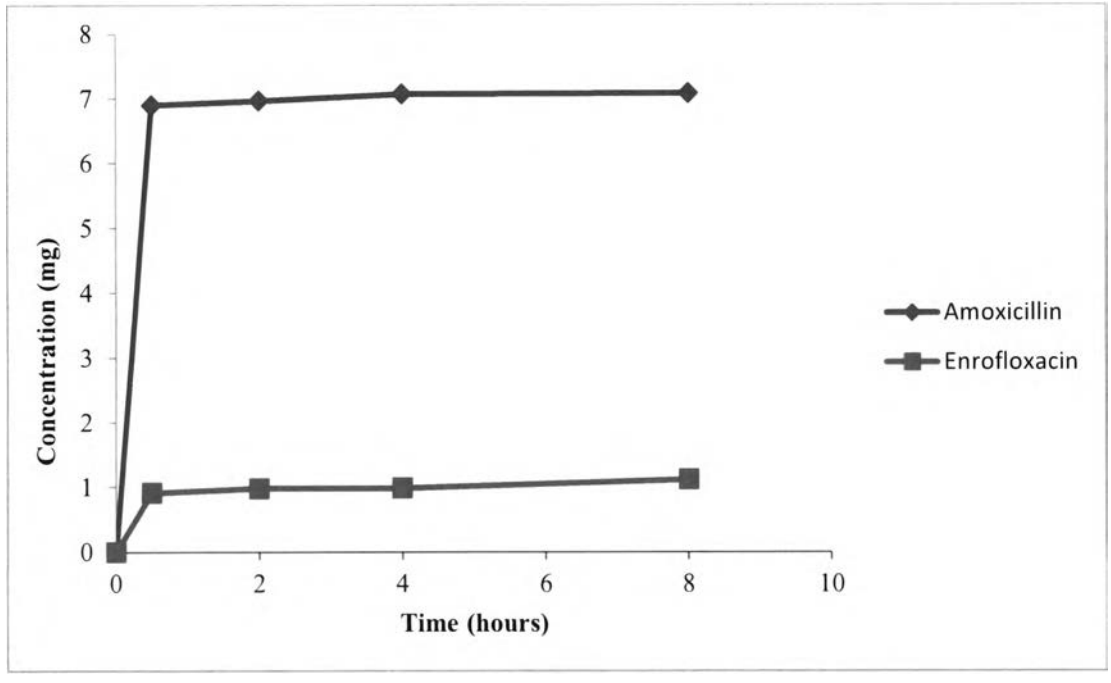

Figure 18 Solubility determination of amoxicillin trihydrate and enrofloxacin base in the mixture of phosphate buffer pH 3.0 and ethanol (9:1 by volume).

3. Preparation of antimicrobial suspensions in oil for IM injection

The combination antibacterial suspensions of amoxicillin trihydrate and enrofloxacin base in oil were prepared. The compositions of suspension were shown in Table 9.

Table 9 The compositions used in formulations for the preparation of antimicrobial suspensions in oil.

Ingredients	Formulations											
	CS20	CS80	CT20	CT80	COS20	COS80	COT20	COT80	IS20	IS80	IT20	IT80
Amoxicillin trihydrate (mg)	580	580	580	580	580	580	580	580	580	580	580	580
Enrofloxacin base (mg)	500	500	500	500	500	500	500	500	500	500	500	500
Span 20 (S20) (g)	0.5	-	-	-	0.5	-	-	-	0.5	-	-	-
Span 80 (S80) (g)	-	0.5	-	-	-	0.5	-	-	-	0.5	-	-
Tween 20 (T20) (g)	-	-	0.5	-	-	-	0.5	-	-	-	0.5	-
Tween 80 (T80) (g)	-	-	-	0.5	-	-	-	0.5	-	-	-	0.5
Corn oil (C) (mL)	5	5	5	5	-	-	-	-	-	-	-	-
Cottonseed oil (CO) (mL)	-	-	-	-	5	5	5	5	-	-	-	-
Isopropyl myristate (I) (mL)	-	-	-	-	-	-	-	-	5	5	5	5

Remark: Formulations were coded as Figure 19 where the first abbreviations are: C as corn oil and CO: cottonseed oil and other abbreviations are the same as shown in Table 10.

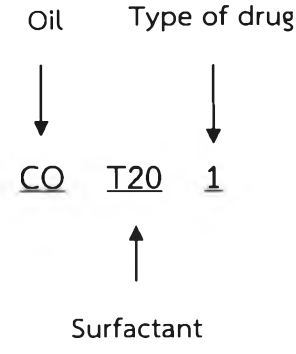

Figure 19 Formulation code.

Table 10 Composition abbreviation.

Oil	Surfactant	Type of drug
Corn oil (C)	Span 20 (S20)	drug as slightly soluble in
Cottonseed oil (CO)	Span 80 (S80)	water (1)
Isopropyl myristate (I)	Tween 20 (T20)	drug as very soluble in
	Tween 80 (T80)	water (2)

3.1 Oil and surfactant screening

According to Table 9, twelve antibacterial suspensions were prepared. They were evaluated as following.

3.1.1 Physical appearances and sedimentation volume

The physical appearances such as color and phase separation were visually observed. Photos of freshly prepared antibacterial suspensions in oil are shown in Figure 20. The suspensions in corn oil and cottonseed oil became yellowish while isopropyl myristate provided less color intensity. Corn oil and cottonseed oil as external phases could suspend the internal phase better than isopropyl myristate presenting precipitation of the internal phase. The same amount of each surfactant was added to

disperse the internal phase in all formulations. Less floating particles and homogeneously dispersed particles were observed when Tween 20 or Tween 80 was used.

Sedimentation volume of the suspensions was calculated after they were prepared and after they were stored for 24 hours and 1 week. The result is shown in Table 11. The formulations containing corn oil or cottonseed oil as external phase showed higher sedimentation volume than the formulations containing isopropyl myristate. The reason is that viscosity of isopropyl myristate (5-7 mPa.s) is less than viscosity of corn oil (37-39 mPa.s) and cottonseed oil (70.4 mPa.s). The internal phase settles at the bottom readily in low viscosity medium. Trend of sedimentation volume was in the following order; COS20 = COS80 > CS20 > CS80 > CT80 > COT80 > IS20 > IT80 > CT20 > COT20 > IT20 > IS80. Although the formulations containing Span 80 or Span 20 as dispersing agent presented high sedimentation volume, big lump and not homogeneous dispersion of the internal phase were observed.

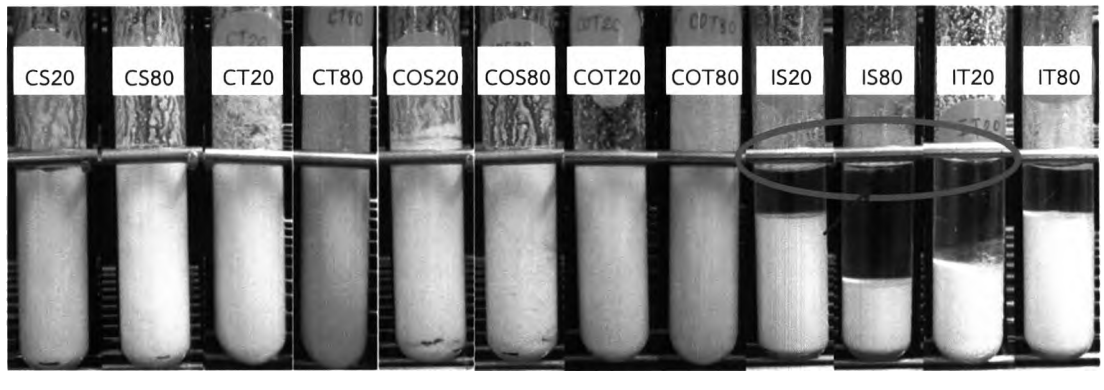

Figure 20 The appearances of suspension formulations freshly prepared and floating particle shown in the circle.

3.1.2 Redispersibility

Redispersibility represents ability of suspension to be uniformly redispersed with minimal shaking after it has stood for some time. Time duration to resuspend the suspensions is shown in Table 11. Longer period of time was needed to resuspend the centrifuged sample than the one-week stored samples. Time duration to

resuspend the stored suspensions is in the following order; COT20 < CT20 < COS20 < CS20 < COT80 < CT80 < IT20 and the rests needed time duration longer than 15 hours. The order of time duration to resuspend the centrifuged suspensions is following; IS20 < CT20 < IS80 < COT20 < CT80 < COT80 < CS80 < IT20 < IT80 < COS80 < COS20 < CS20. A longer time duration to resuspend the suspensions represents a higher risk for the suspensions to be caking and also causes difficulty to administer the suspensions to patients. The suspensions containing isopropyl myristate or Span 20 did not show good appearance and homogenous dispersion as mentioned earlier even though short time duration to resuspend was observed in some samples. From the results, good candidates in oil suspension formulation were corn oil, cottonseed oil Tween 20 and Tween 80.

Table 11 Sedimentation volume and redispersibility of all antimicrobial suspension formulations after 24 hours and 1 week at room temperature.

Code	Oil	Surfactant	Sedimentation volume, F		Time of redispersion	
			24 hr.	1 week	centrifuge (minutes)	1 week (minutes)
CS20	Corn oil	Span 20	1.00	0.91	76.20	4.22
CS80	Corn oil	Span 80	0.93	0.77	27.47	>24 h
CT20	Corn oil	Tween 20	0.68	0.68	12.58	1.25
CT80	Corn oil	Tween 80	0.78	0.78	24.13	9.75
COS20	Cottonseed oil	Span 20	1.00	1.00	70.08	1.45
COS80	Cottonseed oil	Span 80	1.00	1.00	42.23	>24 h
COT20	Cottonseed oil	Tween 20	0.64	0.64	15.12	0.58
COT80	Cottonseed oil	Tween 80	0.77	0.77	26.55	7.38
IS20	IPM	Span 20	0.73	0.73	9.38	>15 h
IS80	IPM	Span 80	0.34	0.34	12.75	>24 h
IT20	IPM	Tween 20	0.45	0.45	28.00	47.08
IT80	IPM	Tween 80	0.70	0.70	30.00	>24 h

3.1.3 Determination of particle size and particle size distribution

Particle size and particle size distribution were measured using a light microscope. Particle size and particle size distribution of all formulations were not different. Size range of the suspensions was 30 - 200 μm which was not greater than 250 μm in diameter. The average diameter of the particles was 66.08 ± 27.70 μm in all formulations.

3.1.4 Viscosity

Viscosities of the suspensions were measured using two viscometers, AND model SV-10 at room temperature and Brookfield: model-DV-II spindle no.0. At 30 $^{\circ}$C, and reported in Table 12 and Table 13. The viscosity values from both viscometers were different due to their principles AND viscometer measured probe vibration in the samples but Brookfield viscometer measured probe rotation in the samples. Moreover, suspensions commonly present plastic flow, a non-newtonian flow, in which their viscosity values can be changed depending on applied shear strength or shear rate. However, both methods showed that the viscosity of suspensions using IPM as dispersion medium was the lowest. The low viscosity of the suspensions caused fast sedimentation of the internal phase as shown in the previous result.

Table 12 Viscosity values of the suspensions using AND model SV-10 at room temperature.

Code	Viscosity ± SD (mPa.s)	Temperature ± SD (°C)
CS20	208.0 ± 2.1	30.0 ± 0.1
CS80	197.0 ± 3.0	28.9 ± 0.0
CT20	149.0 ± 1.0	29.8 ± 0.3
CT80	167.0 ± 2.6	29.4 ± 0.4
COS20	202.0 ± 2.3	30.2 ± 0.4
COS80	191.0 ± 1.0	29.3 ± 0.3
COT20	171.0 ± 1.0	30.5 ± 0.1
COT80	218.0 ± 1.5	30.1 ± 0.2
IS20	12.4 ± 0.5	29.8 ± 0.6
IS80	11.2 ± 0.1	29.8 ± 0.6
IT20	10.1 ± 0.5	29.0 ± 0.0
IT80	13.7 ± 0.1	30.0 ± 0.2

Table 13 Viscosity values of the suspensions using Brookfield: model-DV-II spindle no.0 at 30 °C.

Code	RPM	Shear rate	Shear stress	%T	Viscosity (mPa.s)	Temperature (°C)
CS20	1	1.22	0.62	84.8	508.7	30.2
CS80	1	1.22	0.74	100.0	647.5	30.0
CT20	1	1.22	0.72	97.3	583.7	29.8
CT80	1	1.22	0.67	91.2	541.7	29.7
COS20	1	1.22	0.63	85.4	512.3	30.0
COS80	1	1.22	0.73	98.7	626.4	30.0
COT20	1	1.22	0.74	100.0	602.9	29.9
COT80	0.7	0.856	0.74	100.0	858.7	29.8
IS20	1	1.22	0.12	16.6	99.6	30.0
IS80	1	1.22	0.03	0.1	30.6	30.0
IT20	1	1.22	0.18	24.8	150.0	30.0
IT80	1	1.22	0.06	7.6	45.6	30.2

Regarding physical appearances, sedimentation volume, redispersibility and viscosity, corn oil and cottonseed oil were selected as external phases whereas Tween 20 and Tween 80 were selected as dispersing agents for further study.

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

4.1 Physical stability of formulations in heating cooling cycle

Suitable combinations in suspensions were selected according to the previous study. Four suspension formulations were prepared, which were COT20, COT80, CT20 and CT80 and were kept in heating cooling cycle to evaluate their physical stabilities.

4.1.1 Physical appearances and sedimentation volume

After they were stored in the heating cooling cycle, physical appearance of the four suspensions did not change except for color of the suspensions becoming slightly yellowish. This could be instability of the oils since no color change was observed on the internal phase. The sedimentation volume was determined and displayed in Table 14. The results showed that the sedimentation volume of all samples became less as the samples were longer kept in the heating cooling cycle. Low sedimentation volume represented dense settlement of the internal phase.

Table 14 Sedimentation volume of antibacterial suspensions in oil after heating cooling cycle.

Formulations	Sedimentation volume, F		
	Cycle 2	Cycle 4	Cycle 6
CT20	0.62 ± 0.04	0.64 ± 0.02	0.59 ± 0.02
CT80	0.70 ± 0.03	0.67 ± 0.01	0.67 ± 0.02
COT20	0.70 ± 0.01	0.68 ± 0.04	0.67 ± 0.04
COT80	0.70 ± 0.03	0.68 ± 0.01	0.65 ± 0.01

4.1.2 Redispersibility

Redispersibility of antimicrobial suspensions in oil after they were kept in heating cooling cycle was reported in Table 15. The results showed that CT20 and COT20 were dispersed well when shaken. Even though, low sedimentation volume of CT20 was found in the previous study, this formulation was resuspended easily. Therefore, sedimentation volume was not the only parameter to indicate the ease of redispersibility.

Table 15 Redispersibility of antibacterial suspensions in oil after heating cooling cycle.

Formulations	Time of redispersion (Mean ± SD)		
	Cycle 2 (minutes)	Cycle 4 (minutes)	Cycle 6 (minutes)
CT20	1.54 ± 0.58	8.91 ± 5.95	6.94 ± 2.71
CT80	116.27 ± 54.87	190.68 ± 17.89	154.90 ± 42.46
COT20	4.85 ± 6.79	4.21 ± 1.03	7.31 ± 0.70
COT80	32.02 ± 12.32	127.04 ± 105.24	43.2 ± 6.06

4.1.3 Determination of particle size and particle size distribution

Particle size and particle size distribution were detected under light microscopic to evaluate aggregation or crystal growth of the dispersed particles. The result in term of particle size was shown in Figure 21 and the Table A-1 in Appendix A. Particle size distributions were delineated in Figures 22 – 25. Particle size of all suspensions did not show significant change throughout the heating cooling cycle ($p>0.05$). Average of the particle size was approximately 34 μm. However, the dispersed particles in CT20, CT80 and COT80 were prone to conglomerate and crystal growth since frequency of small particle size became lower and that of big particle size increased.

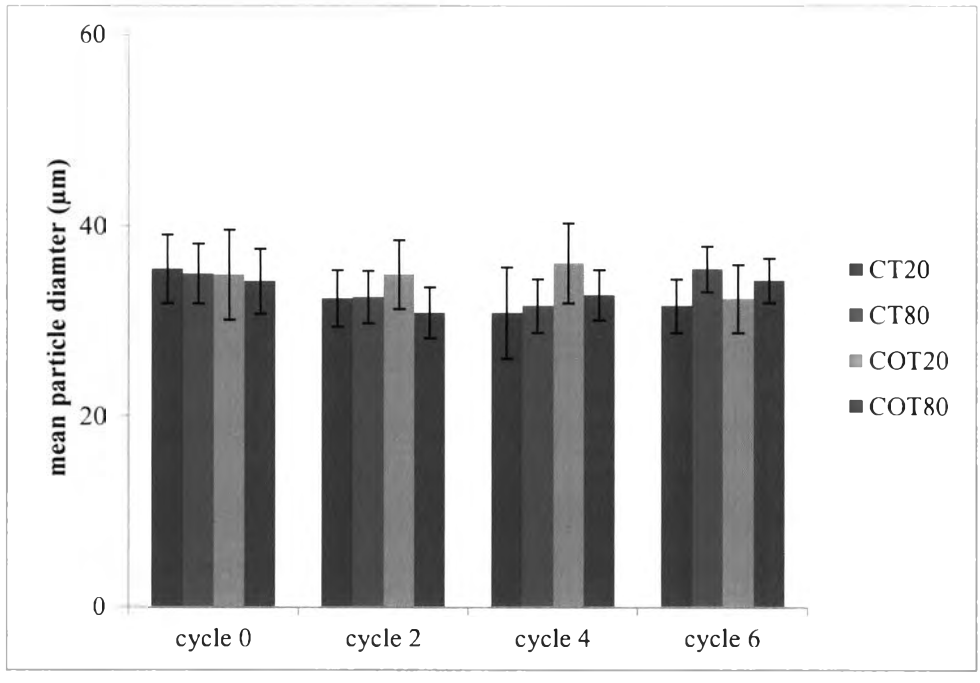

Figure 21 Particle size of all formulations after heating cooling cycle.

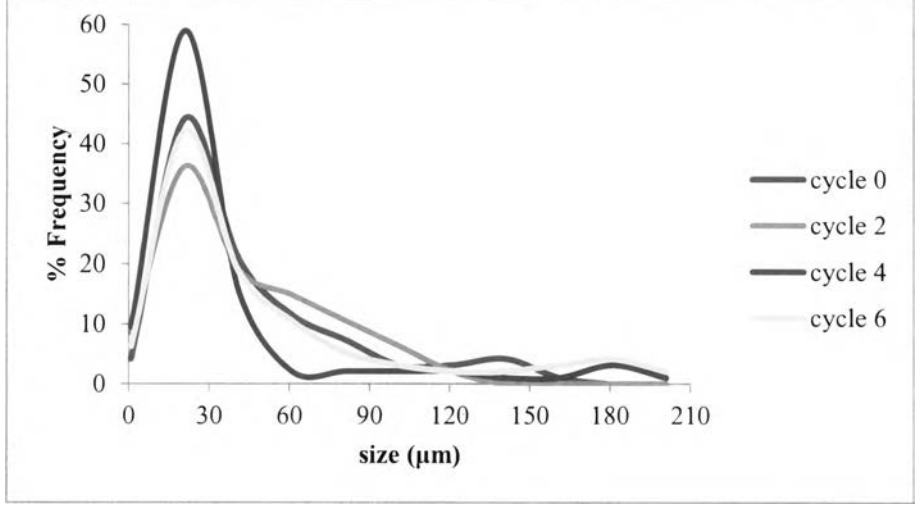

Figure 22 Particle size distribution of CT20 formulation after heating cooling cycle.

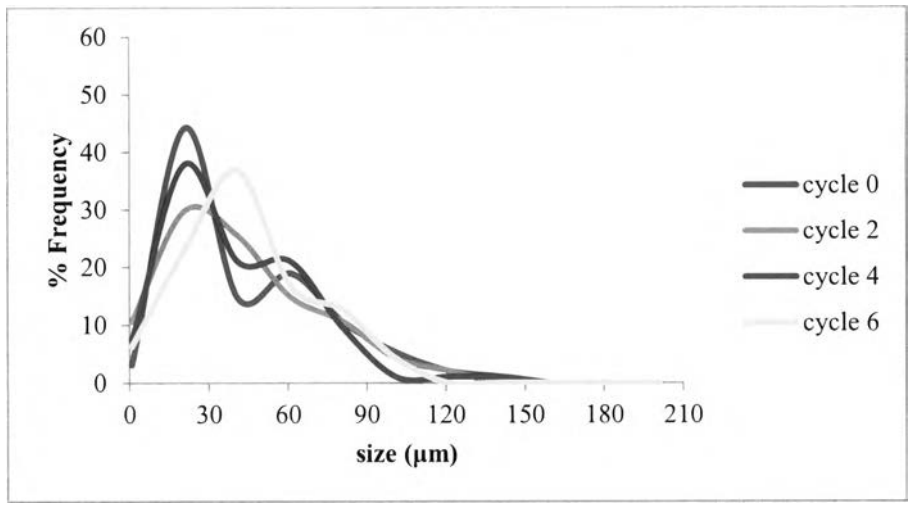

Figure 23 Particle size distribution of CT80 formulation after heating cooling cycle.

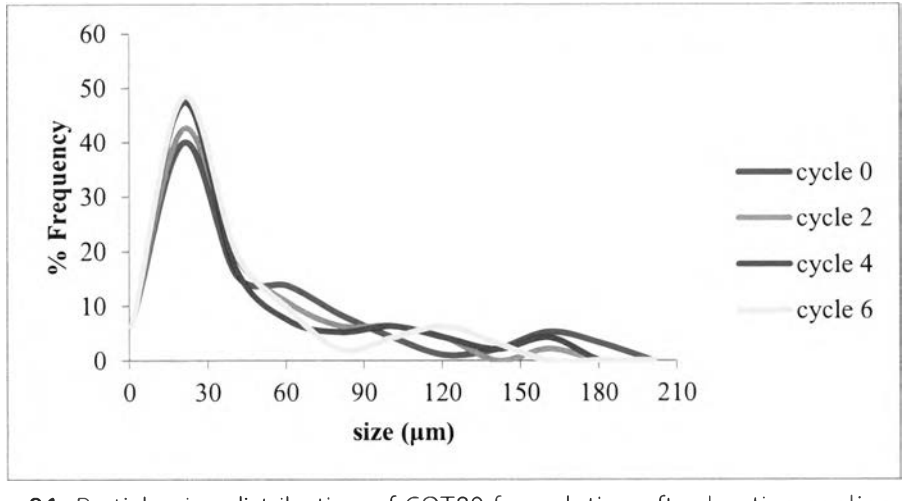

Figure 24 Particle size distribution of COT20 formulation after heating cooling cycle.

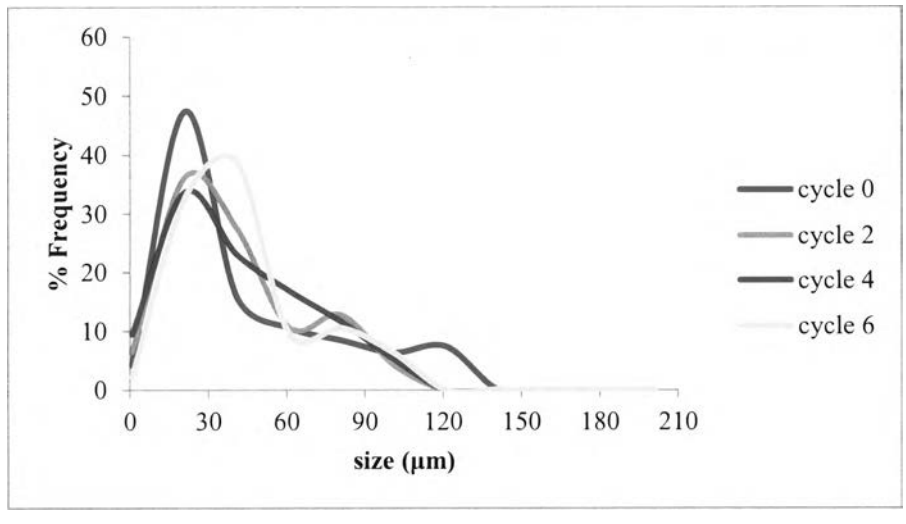

Figure 25 Particle size distribution of COT80 formulation after heating cooling cycle.

Therefore, COT20 was selected for further study based on its high sedimentation volume, short time period for resuspension, unchanged particle size and particle size distribution.

4.2 Physical and chemical stability of formulations

From the study of physical stabilities of formulations in heating cooling cycle, COT20 was selected for further study. Amoxicillin and enrofloxacin suspensions were prepared in cottonseed oil and Tween 20 was used as dispersing agent. COT201 contained amoxicillin trihydrate and enrofloxacin base as very slightly water soluble internal phase. COT202 contained amoxicillin sodium and enrofloxacin hydrochloride as water soluble internal phase. Both suspensions were stored at 30 °C and 40 °C for three months.

4.2.1 Physical appearances and sedimentation volume

Physical appearances of the suspensions were presented in Figure 26. All suspensions became yellowish upon storage at 30 °C and 40 °C. At 40 °C, the change of color was faster. Other physical appearances were still unchanged.

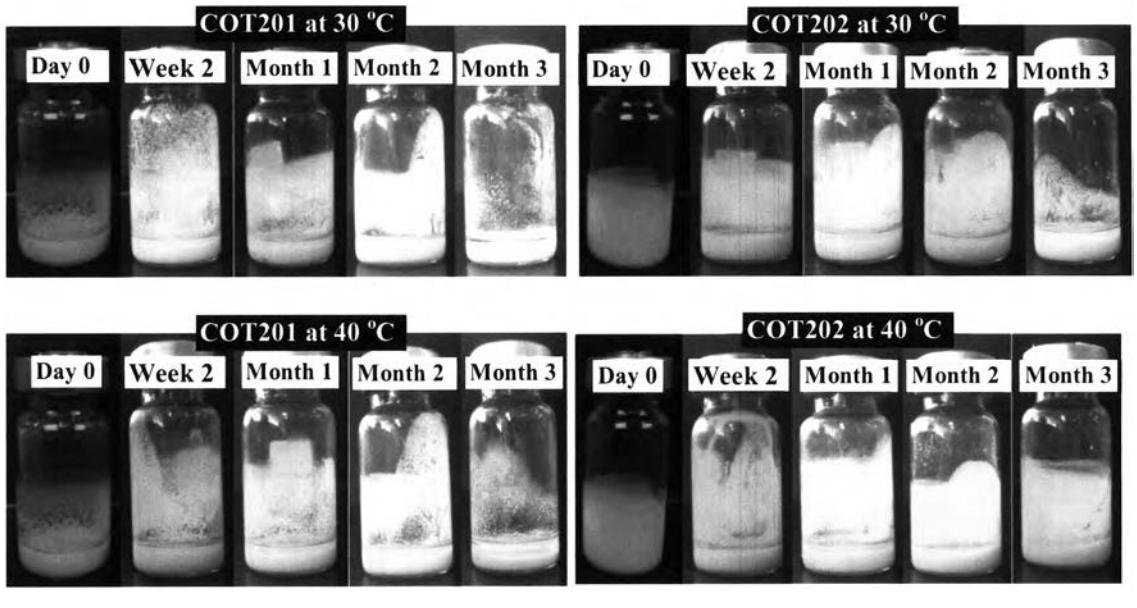

Figure 26 The appearances of COT201 and COT202 during stability test.

4.2.2 Redispersiibility

Redispersibility of all formulations after they were stored for three months was reported in Table 16. The results showed that COT201 at 30 °C was dispersed well when shaken even though its sedimentation volume was less than others. Temperature might induce change of internal phase properties causing difficulty to redisperse. Suspensions are prone to form cake when they are difficult to redisperse. The difficulty to redisperse of COT202 could be explained by different polarity of the internal phase and external phase. COT202 was poorly redispersed in oil medium because amoxicillin sodium and enrofloxacin hydrochloride, the internal phase, were much more polar than cottonseed oil, the external phase. The polar particles preferred to conglomerate or aggregate to each other rather than freely dispersed in non polar medium.

Table 16 Sedimentation volume and time of redispersion of antibacterial suspensions after 3 months.

Code	Conditions	Sedimentation volume, F				Time of redispersion (minutes)
		initial	1 month	2 months	3 months	
COT201	30 °C	0.73±0.01	0.73±0.01	0.72±0.01	0.70±0.00	19.03 ± 13.44
COT201	40 °C	0.73±0.01	0.73±0.02	0.74±0.02	0.75±0.01	183.43 ± 61.74
COT202	30 °C	0.85±0.01	0.84±0.01	0.83±0.01	0.82±0.01	>10 hours
COT202	40 °C	0.85±0.01	0 84±0.00	0.82±0.02	0.80±0.03	>12 hours

4.2.3 Determination of particle size and particle size distribution

Mean particle size of the suspensions upon 3 months storage at 30 °C and 40 °C is shown in Figure 27. The particle size of antibacterial particles became bigger when the suspensions were kept for 3 months at 30 °C and 40 °C. Mean particle diameter of all formulations showed significant increase in size (p<0.05). Particle size distributions were displayed in Figures 28-31. All formulations at 30 °C and 40 °C were likely to

conglomerate or crystal growth since frequency of the small particles became less and that of the bigger particles became more upon storage.

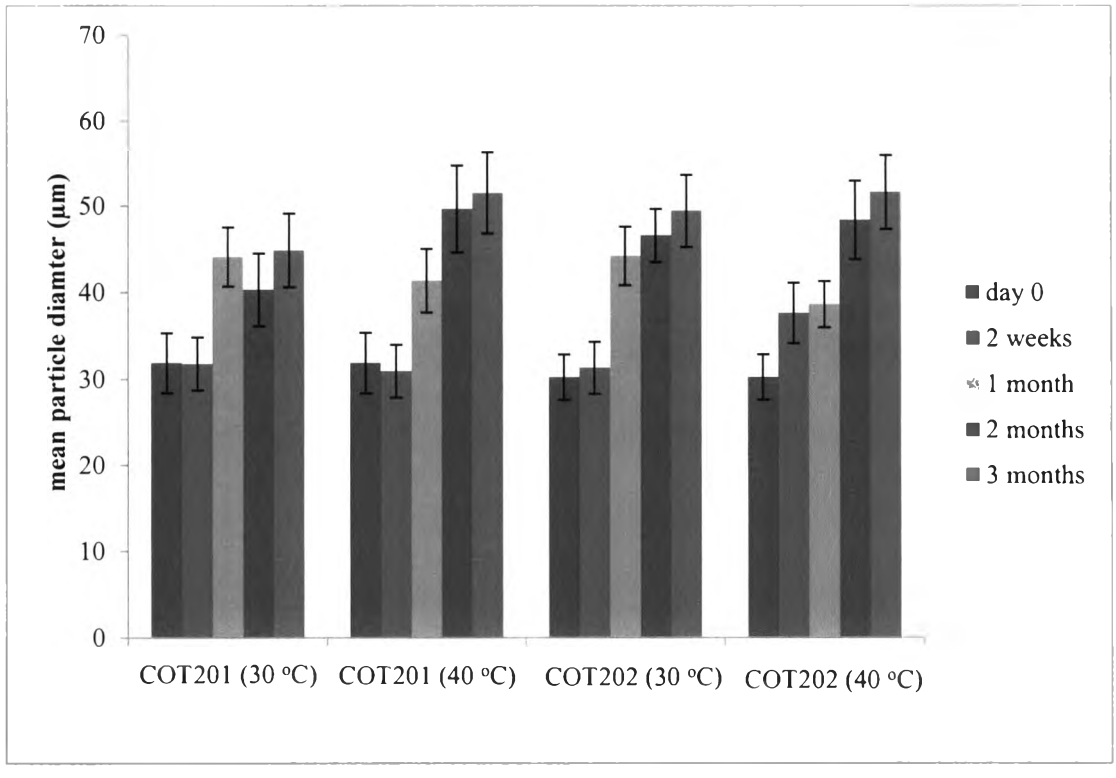

Figure 27 The particle size of antibacterial suspensions after they were stored for 3 months.

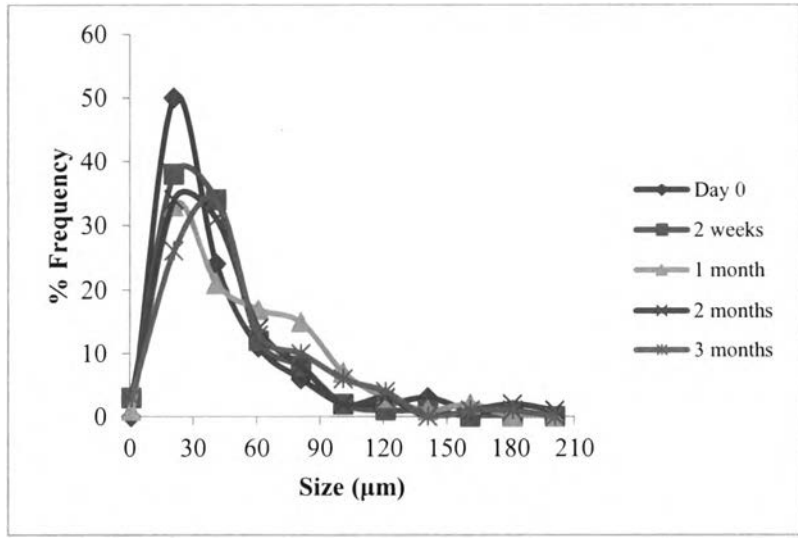

Figure 28 Particle size distribution of COT201 after 3 months at 30 °C.

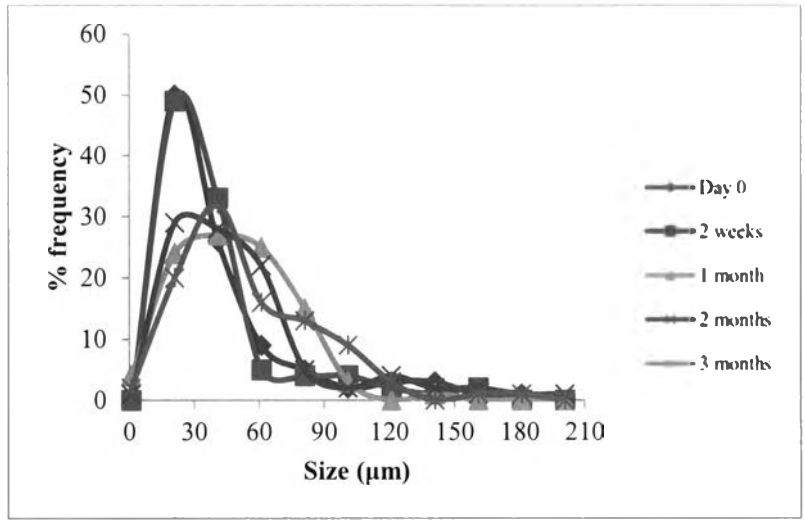

Figure 29 Particle size distribution of COT201 after 3 months at 40 °C.

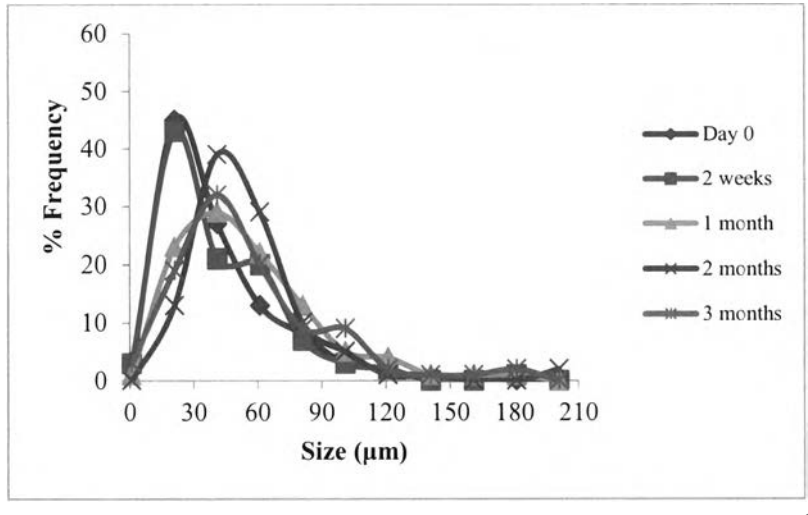

Figure 30 Particle size distribution of COT202 after 3 months at 30 °C.

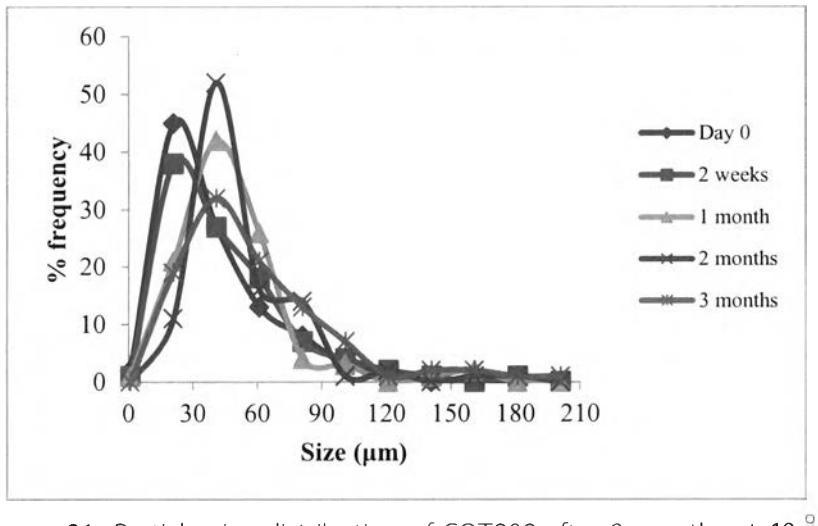

Figure 31 Particle size distribution of COT202 after 3 months at 40 °C.

4.2.4 Viscosity

As shown in Table 17, the increase of viscosity of the suspensions kept at 30 °C was unexpected. In suspensions, the change of viscosity could be due to the changes of particle size and particle size distribution of internal phase. This viscosity change of the suspensions kept at 40 °C was not observed although the changes of particle size and particle size distribution were also observed in previous result. However, this viscosity increase was not much and it would not affect stability and injectability of the suspensions.

Table 17 The viscosities of suspensions at day 0 and 3 months.

Formulations	conditions	Viscosity (mPa.s)	
		Day 0	3 months
COT201	30 °C	152.0 ± 2.0	174.3 ± 3.8
COT201	40 °C	153.0 ± 1.0	146.0 ± 4.0
COT202	30 °C	209.0 ± 1.0	238.0 ± 3.5
COT202	40 °C	212.0 ± 1.5	210.0 ± 3.5

4.2.5 Chemical stability of formulations

Percent remainings of the antibacterials in suspensions after they were kept at 30 °C and 40 °C for 3 months were shown in Tables A-3, A-4 and Figure 32. At 30 °C, the amoxicillin and enrofloxacin remainings after they were kept for 3 months were more than 95% in both suspensions. Degradation of amoxicillin and enrofloxacin were increased at high temperature. However, only amoxicillin in COT202 showed the degradation greater than 10 percent after 3 months storage at 40 °C. Amoxicillin sodium, a water soluble form, was degraded faster than amoxicillin trihydrate, a very slightly water soluble form at both temperatures (Kaur, Rao and Nanda, 2011; Alburyhi, Siaf and Noman, 2013). There was reported that amoxicillin is not quite stable. It can undergo hydrolysis

and becomes inactive. Surrounding moisture can induce its degradation. Amoxicillin water soluble form is prone to adsorb moisture form environment and undergoes hydrolysis (Kaur et al., 2011). Enrofloxacin is a fluoroquinolone which is quite stable in both forms (Altreuther, 1987). Generally, pharmacopoeia accepts injection preparation containing not less than 90.0% and not more than 105.0% of labeled amount of amoxicillin but enrofloxacin is not defined in the British pharmacopoeia (BP, 2013). Thus, the same criteria as amoxicillin are applied for enrofloxacin in this study. The content of amoxicillin and enrofloxacin remained in COT201 kept at 30 °C within the range of pharmacopoeia acceptance.

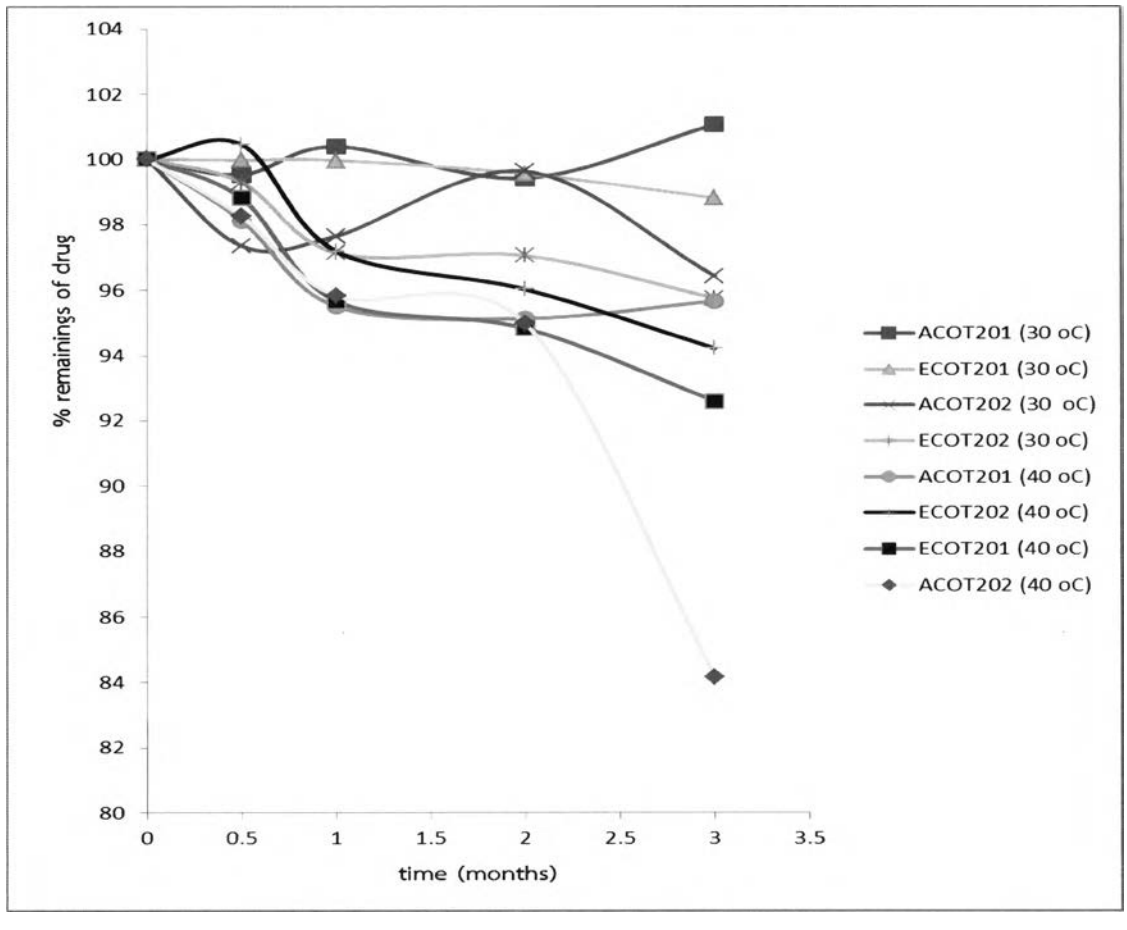

Figure 32 Percent remainings of amoxicillin and enrofloxacin in the suspensions after they were stored at 30 °C and 40 °C; amoxicillin trihydrate (ACOT201), amoxicillin sodium (ACOT202), enrofloxacin base (ECOT201) and enrofloxacin hydrochloride (ECOT202).

5. *In vitro* release study

In vitro release of COT201 and COT202 were studied. The release profiles were displayed in Figure 33. Release of enrofloxacin base was slowest and great different from that of enrofloxacin hydrochloride. Release profiles of amoxicillin trihydrate and amoxicillin sodium were not much different. Amoxicillin sodium and amoxicillin trihydrate releases reached a plateau in 14 and 10 hours, respectively. Enrofloxacin hydrochloride reached a plateau in 6 hours while enrofloxacin base took longer time than 24 hours. Less than 50 percent of loaded enrofloxacin base was released in 24 hours. Solubility of enrofloxacin base could be a factor affecting the release rate. However, release rate also depended on other factors such as particle size and particle size distribution which should be a reason in the case of amoxicillin trihydrate and amoxicillin sodium. In this experiment, the antibacterials were used as received. The particle size of enrofloxacin base was 140 μm while those of amoxicillin trihydrate, amoxicillin sodium and enrofloxacin hydrochloride were in the range of 30 - 70 μm. One hundred percent cumulative amounts at plateau were not observed in all samples. It could be explained that the internal phase of the suspensions were not evenly dispersed before being filled in the dialysis bag and some drug particles were trapped in the closure area.

Therefore, COT201 was selected based on unchanged physical appearance, short time period for resuspension and unchanged particle size distribution. Particle size of the antibacterials affected their release which was required to do further study.

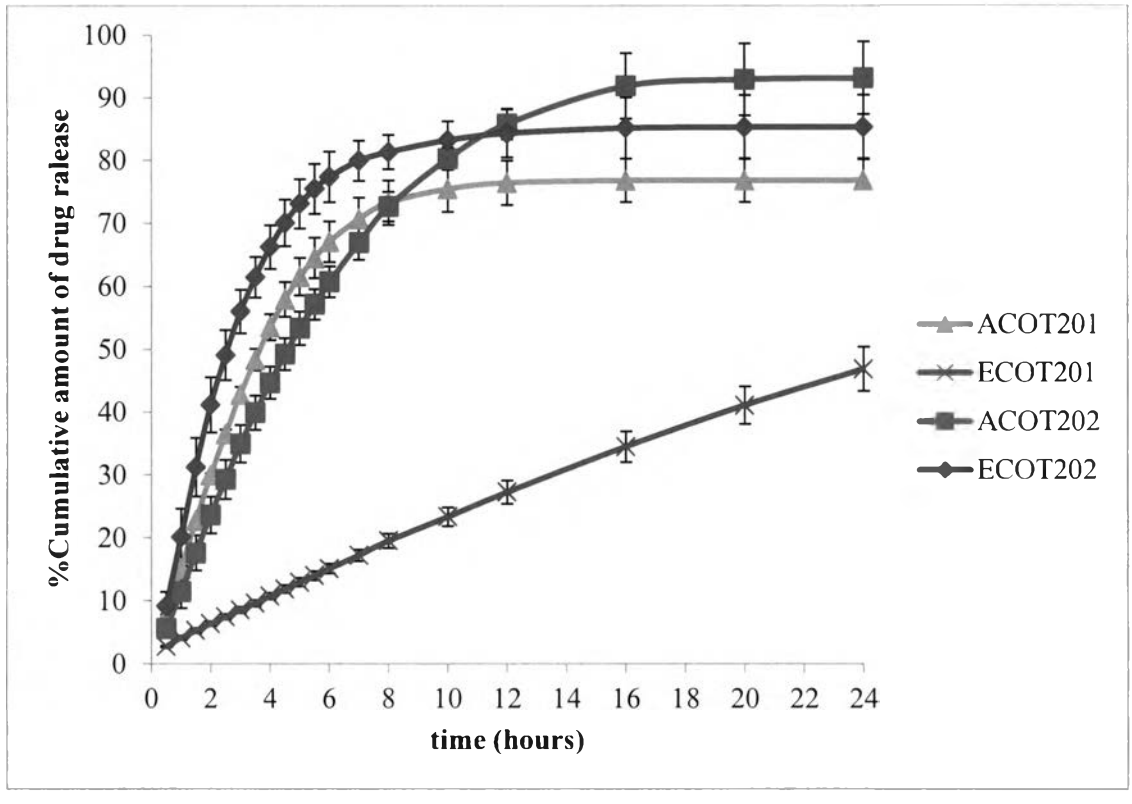

Figure 33 The release profiles of amoxicillin trihydrate (ACOT201), amoxicillin sodium (ACOT202), enrofloxacin base (ECOT201) and enrofloxacin hydrochloride (ECOT202) from the suspensions in oil (mean±SD, n=3).