CHAPTER IV

RESULTS AND DISCUSSION

1. Solid lipid nanoparticles (SLN)

1.1 Formulation of drug-free SLN

1.1.1 Preparation of drug-free SLN by solvent emulsification diffusion method

Physical appearance

In preliminary study on solvent diffusion method, two types of solid lipids were used; GC and GB. The surfactants used were P-188, T-80, PL-40 and PL-90. The physical appearance of both SLN dispersion, GC-SLN and GB-SLN with various surfactant ratios are shown in Table 9 and 10 respectively.

From the result, GC could form SLN dispersion when using T-80 as single surfactant at 5-15 %. The formulations showed good physical stability after storage at room temperature for 3 months. P-188, PL-40 and PL-90 as single surfactant could not stabilize GC-SLN that gel formation was displayed after storage. For GB-SLN using single surfactant, the preparations of GB-SLN containing P-188, T-80, PL-40 and PL-90 showed physical instability after storage at all formulations.

When using combined surfactants of T-80 with PL-40 or PL-90 and T-80 with P-188, the GC-SLN exhibited good physical stability after storage for 3 months. At 5 % of T-80 with PL-40 or PL-90 and T-80 with P-188, the combined surfactants of ratios 4:1 and 3:2 could stabilize GC-SLN for 3 months while 10 % combined surfactants using T-80:PL-40, T-80:PL-90 and T-80:P-188 at the ratio 8:2 provided white fluid dispersion over storage time. At 15 % of these combined surfactants the ratio of 12:3 displayed good physical stability for 3 months. For GB-SLN, the combined surfactants could not stabilize all preparations.

Effect of type of solid lipid

Various GB-SLN with surfactant and co-surfactants could not be prepared. The formulations using single and combined surfactants showed physical instability in all preparations. In contrast, GC-SLN could be obtained in all formulations. The longer chain of GB in which much difference between the melting point and room temperature could promote rapid lipid recrystallization in GB-SLN. Lipids exhibit a pronounced polymorphism. Depending on the conditions, glycerides may crystallize in three different polymorphic forms-alpha (α), beta prime (β') and beta (β). These polymorphic modifications characterized by the particular carbon chains packing may differ significantly in their properties such as solubility, melting point and thermal stability. The β form, a triclinic subcell structure, is the melting and thermodynamically stable polymorph. Whereas α is the least stable with a loosely packed hexagonal subcell structure. The α form therefore has a tendency to be quickly transformed to a form with a better chain packing β' and β (Eldem, Speiser, and Altorfer, 1991). This transformation is accompanies by a change of physiochemical properties. This was attributed to their chemical compositions; GB a more lipophilic mixture, contains C_{16} (<3%), C_{18} (<5%), C_{20} (<10%), C_{22} (>83%), $C_{22:1}$ (<3%) and C_{24} (<3%) while GC contains C_8 (<15%), C_{10} (<12%), C_{12} (30-50%), C_{14}(5-25%), C_{16} (4-25%) and C_{18} (5-35%). The GC melting point is in range of 42.5-47.5°C while GB melting point is in range of 69.0-74.0°C (Quintanar-Guerrero et al 2005).

Effect of single surfactant

T-80

T-80 of 5-15 % could stabilize all GC-SLN formulations after autoclaving and over storage time while GB-SLN with T-80 as single stabilizer showed white precipitates after storage for 1 month. This result agreed with previous reports Quintanar-Guerrero et al (2005). Summarizes the results obtained using Tween 80 for SLN prepared with Compritol® ATO 888. Tween 80 did not allow the preparation of submicronic particles, however, Tween 80 could be used as stabilizer. The metrotrexate-loaded nanoparticles was coated with polysorbate 80 displayed particle

size in rage of 70 nm (Gao and Jiang 2006) while Viriyaroj (2001) found that the preparations containing 5 % glycerol behenate and 1-5 % Tween 80, the mean particle sizes were in nanometer. The $D_{(v, 0.5)}$ values were below 1 μm both before and after autoclaving.

Tween is a nonionic surfactant which can stabilizes the suspensions through a steric mechanism from two forces, the osmotic force and the entropies effects. For osmotic force nonionic surfactants usually contained the polyethylene chain or hydrophilic polymer chain as the hydrophilic portions. When two droplets come in close contact, the polymer chain would over lab and the region became more concentrate. This led to the osmotic gradient resulting in the dilution of over lab area by water molecules and the solution force occurred which pushed the droplets apart. For the entropic effects when the polymer chain overlapped, the entropy of the system was lost. This resulted in thermodynamically unfavorable condition which forced the droplets to be separated (Duro et al., 1998).

Table 9. Physical appearance of SLN dispersion containing GC as solid lipid with different types and amount of surfactants and amount of co-surfactant prepared by solvent diffusion method

Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance		
	a	b	c		a	b	c		a	b	c		a	b	c
(T-80)				(P-188)				(PL40)				(PL90)			
(5)	+	+	+	(5)	+	+	G1M	(5)	+	+	G1M	(5)	+	+	G1M
(10)	+	+	+	(10)	+	+	G1M	(10)	+	+	G1M	(10)	+	+	G1M
(15)	+	+	+	(15)	+	+	G1M	(15)	+	+	G1M	(15)	+	+	G1M
(T80:PL40)				(P188:PL40)				(PL40:PL90)							
(4:1)	+	+	+	(4:1)	+	+	G3M	(4:1)	+	+	G1M				
(3:2)	+	+	+	(3:2)	+	+	G3M	(3:2)	+	+	G1M				
(1:4)	+	+	G3M	(1:4)	+	+	G3M	(1:4)	+	+	G1M				
(T-80:PL90)				(P188:PL90)				(PL40:PL90)							
(4:1)	+	+	+	(4:1)	+	+	G3M	(8:2)	+	+	G1M				
(3:2)	+	+	+	(3:2)	+	+	G3M	(5:5)	+	+	G1M				
(1:4)	+	+	G3M	(1:4)	+	+	G3M	(2:8)	+	+	G1M				
(T-80:P188)				(P188:PL40)				(PL40:PL90)							
(4:1)	+	+	+	(8:2)	+	+	G1M	(12:3)	+	+	G1M				
(3:2)	+	+	+	(5:5)	+	+	G1M	(8:7)	+	+	G1M				
(1:4)	+	+	G3M	(2:8)	+	+	G1M	(3:12)	+	+	G1M				
(T80:PL40)				(P188:PL90)											
(8:2)	+	+	+	(8:2)	+	+	G1M								
(5:5)	+	+	G3M	(5:5)	+	+	G1M								
(2:8)	+	+	G1M	(2:8)	+	+	G1M								

a, b, c: before and after autoclaving, after storage for 3 moths; +: white fluid dispersion; GAS, G1M, G3M: gel formed after solvent evaporated, gel formation in 1, 3 months

Table 9. Physical appearance of SLN dispersion containing GC as solid lipid with different types and amount of surfactants and amount of co-surfactant prepared by solvent diffusion method (cont.)

Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance		
	a	b	c		a	b	c		a	b	c		a	b	c
(T80:PL90)				(P188:PL40)											
(8:2)	+	+	+	(12:3)	GAS	-	-								
(5:5)	+	+	G3M	(8:7)	GAS	-	-								
(2:8)	+	+	G1M	(3:12)	GAS	-	-								
(T80:P188)				(P188:PL90)											
(8:2)	+	+	+	(12:3)	GAS	-	-								
(5:5)	+	+	G3M	(8:7)	GAS	-	-								
(2:8)	+	+	G1M	(3:12)	GAS	-	-								
(T80:PL40)															
(12:3)	+	+	+												
(8:7)	+	+	G1M												
(3:12)	+	+	G1M												
(T80:PL90)															
(12:3)	+	+	+												
(8:7)	+	+	G1M												
(3:12)	+	+	G1M												
(T80:P188)															
(12:3)	+	+	+												
(8:7)	+	+	G1M												
(7:8)	+	+	G1M												

a, b, c: before and after autoclaving, after storage for 3 moths; +: white fluid dispersion; GAS, G1M, G3M: gel formed after solvent evaporated, gel formation in 1, 3 months

Table 10. Physical appearance of SLN dispersion containing GB as solid lipid with different types and amount of surfactants and amount of co-surfactant prepared by solvent diffusion method

Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance		
	a	b	c		a	b	c		a	b	c		a	b	c
(T-80)				(P-188)				(PL40)				(PL90)			
(5)	+	+	P1M	(5)	+	+	P1M	(5)	+	+	G1M	(5)	+	+	G1M
(10)	+	+	P1M	(10)	+	+	G1M	(10)	+	+	G1M	(10)	+	+	G1M
(15)	+	+	P1M	(15)	+	+	G1M	(15)	+	+	G1M	(15)	+	+	G1M
(T80:PL40)				(P188:PL40)				(PL40:PL90)							
(4:1)	+	+	P1M	(4:1)	+	+	P1M	(4:1)	+	+	G1M				
(3:2)	+	+	P1M	(3:2)	+	+	P1M	(3:2)	+	+	G1M				
(1:4)	+	+	G3M	(1:4)	+	+	G3M	(1:4)	+	+	G1M				
(T-80:PL90)				(P188:PL90)				(PL40:PL90)							
(4:1)	+	+	P1M	(4:1)	+	+	P1M	(8:2)	GI	-	-				
(3:2)	+	+	P1M	(3:2)	+	+	P1M	(5:5)	GI	-	-				
(1:4)	+	+	G3M	(1:4)	+	+	G3M	(2:8)	GI	-	-				
(T-80:P188)				(P188:PL40)				(PL40:PL90)							
(4:1)	+	+	P1M	(8:2)	+	+	G1M	(12:3)	GI	-	-				
(3:2)	+	+	P1M	(5:5)	+	+	G1M	(8:7)	GI	-	-				
(1:4)	+	+	G3M	(2:8)	GI	-	-	(3:12)	GI	-	-				
(T80:PL40)				(P188:PL90)											
(8:2)	+	+	P1M	(8:2)	+	+	G1M								
(5:5)	+	+	G3M	(5:5)	+	+	G1M								
(2:8)	GI	-	-	(2:8)	GI	-	-								

a, b, c: before and after autoclaving, after storage for 3 moths; +: white fluid dispersion; GI, G1M, G3M: gel immediately, gel formation in 1, 3 months; P1M: precipitate after storage for 1 month.

Table 10. Physical appearance of SLN dispersion containing GB as solid lipid with different types and amount of surfactants and amount of co-surfactant prepared by solvent diffusion method (cont.)

Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance			Formulation	Physical appearance		
	a	b	c		a	b	c		a	b	c		a	b	c
(T80:PL90)				(P188:PL40)											
(8:2)	+	+	P1M	(12:3)	GI	-	-								
(5:5)	+	+	G3M	(8:7)	GI	-	-								
(2:8)	GI	-	-	(3:12)	GI	-	-								
(T80:P188)				(P188:PL90)											
(8:2)	+	+	+	(12:3)	GI	-	-								
(5:5)	+	+	G3M	(8:7)	GI	-	-								
(2:8)	+	+	G1M	(3:12)	GI	-	-								
(T80:PL40)															
(12:3)	+	+	P1M												
(8:7)	GI	-	-												
(3:12)	GI	-	-												
(T80:PL90)															
(12:3)	+	+	P1M												
(8:7)	GI	-	-												
(3:12)	GI	-	-												
(T80:P188)															
(12:3)	+	+	P1M												
(8:7)	+	+	G1M												
(7:8)	GI	-	-												

a, b, c: before and after autoclaving, after storage for 3 moths; +: white fluid dispersion; GI, G1M, G3M: gel immediately, gel formation in 1, 3 months; P1M: precipitate after storage for 1 month.

P-188

Visually, GC-SLN containing P-188 had tendency to form gelation. Their viscosity visibly increased. In most cases, gel formation was irreversible. This data showed that the gel formation occurred within 1 month after storage. This might be resulted from high temperature exposure during autoclaving. Introduction of energy to the GC-SLN systems accelerated particle growth and subsequently gelation. Similar result had been report by Freitas and Muller (1998). They found that steam-sterilization induced a significant increase in particle size for poloxamer 188. This destabilization can be attributed to the decreased steric stabilization by the emulsifier poloxamer. It is well known for emulsifier of this type, that increased temperatures lead to dehydration of the ethylene glycol chains which a decrease of the thickness of the protecting layer. Solid lipid floated on the top of dispersion and later brought about larger particles after being kept at room temperature. At concentrations of 5-15 % P-188, a large surface area of the solid lipid was available for adsorption of stabilizer. Bridging between particles occurred as a result of simultaneous adsorption of P-188 molecules onto the surfaces of different solid lipid particles. However, the number of particle-particle bridges was relatively low. Therefore, these systems had uncovered lipid surface particles which could contact other particles and resulted in gel formation. This result disagree with previous research by Quintanar-Guerrero et al (2005). They revealed that using poloxamer 188 as stabilizer could form submicron particles of both GB-SLN and GC-SLN while Scholer et. al. (2001) found that Dynasan 114 was dispersed in poloxamer 188 solution. The average diameter of SLN was in nanometer size range with narrow polydispersity index.

PL-40

GC-SLN containing PL-40 could be prepared. Yellowish fluid dispersion were observed due to the color of lecithin. Their physical appearance are shown in Table 9 . The experiment found that gel formation occurred in all preparations containing PL-40 after 1 month of storage. This could be assumed that semi-solid gel structures immobilized the whole aqueous phase of 90 %. This indicated that high energy during autoclaving could only retard gel formation but could not prevent the process. This might be explained that high energy upon autoclaving promoted disruption of the

bilayers, reducing the diffusional pathways and accelerating the diffusion mobility. Thus the stabilizer could cover the interface of droplets and retarded gel formation in comparison to that before autoclaving.

PL-90

GC-SLN containing PL-90 could be prepared by solvent diffusion method. The emulsion containing PL-90 at concentrations 5-15 % as an emulsifier became gel formation after storage at room temperature for 1 month. Due to the limited mobility of phospholipids molecules in excess which form predominantly vesicles in the aqueous phase these emulsifiers are not able to cover the newly created interface during platelet formation in an efficient way. Phospholipids molecules seem to be preferable associated with specific crystal interfaces during re-crystallization causing variations in polarity and atomic/molecular order of different nanocrystal faces. Crystal interfaces with low concentrations of adsorbed emulsifier molecules represent preferred sites of particle aggregation over which gel formation can proceed (Westesen and Siekmann, 1998). Gel formation can be prevented by the addition of co-emulsifying agent to the aqueous phase provide the concentration of co-surfactant is sufficiently high to constitute a reservoir of molecule immediately available for interfacial stabilization during re-crystallization. Moreover, the co-emulsifier should preferably adsorb on crystal interfaces not or only incompletely covered by phospholipids. From the results of PL-40 and PL-90, PL-90 was tendency to form gelation higher than PL-40. This might be the quantity of lecithin in each type (PL-40 containing phosphotidylcholine $\geq$ 40 % while PL-90 cointaining phosphotidylcholine $\geq$90 %). This results accordance with Westensen, Sekmann (1997). They found that hot tripalmitin emulsions containing exclusively the phosphotidylcholine rich soya lecithin product Lipoid® S100 as an emulsifier became semisolid already on cooling whereas dispersions stabilized by the egg lecithin mixture Lipoid® E 80 formed within 1 month after preparation.

Effect of combined surfactants

The type and amount of co-stabilizer were varied and had effect on the characteristic of SLN. In some rare cases a single emulsifier can yield the desired

SLN. More often, through, in the case of oil in water emulsion, mixed surfactants have been reported to have a synergistic effect on SLN stability in term of coalescence rate. The combination using of two or more emulsifying agents appears to produce mixed surfactant film at the interface having high surfactant coverage as well as sufficient to promote stability (Trotta et al 2003). For GC-SLN dispersions, T-80 with PL-40 or PL-90 at the concentration of 5 % could prepare and stabilize GC-SLN except T-80: PL-40 (1:4) and T-80:PL-90 (1:4) that showed gel formation after storage for 3 months while T-80 with P-188 as co-surfactant could prepare GC-SLN at (4:1) and (3:2). Using high composition of P-188 (1:4) could not stabilize over the storage time at room temperature and displayed gel formation. P-188with PL-40 or PL-90 as co-surfactant at all concentrations could prepare GC-SLN but could not stabilize GC-SLN. The systems exhibited gel formation similar to high composition of T-80 with P-188 as co-surfactant. This might be high temperature, exposure to light and mechanical stress promoted gel formation in SLN. Gel formation is an irreversible process which involves the loss of the colloidal particle size. It can be stimulated by increasing contact of the SLN dispersion with other surface and shear force.

At 10 % of combined surfactant, when using T-80 with either PL-40 or PL-90 as co-surfactant at the ratio of 5:5 and 2:8 exhibited gel formation after storage for 3 months and 1 months, respectively. This phospholipids molecules representing the excess emulsifier during the homogenization process form small particles. Phospholipids molecules bound to vesicles exhibited only a limited mobility. Therefore, they were not able to immediately cover the newly created interfaces during recrystalization (Westesen and Siekman 1997). While GC-SLN containing P-188 with PL-40 or PL-90 at the ratio of 5:5 and 8:2 showed gelation after standing at room temperature for 1 month in all formulations. Increasing of the P-188 concentration as combined surfactant with PL-40 or PL-90 would affect gel formation. Several mechanism might be involved in the gelation process. All promoters of gelation (high temperature, light, shear stress) increased the kinetic energy of the particle and favor collision of the particles. The surfactant film might

change its performance with temperature especially PEG surfactants (Mehnert, Mader 2001).

T-80:PL-40 (8:2) and T-80:PL-90 (8:2) at 10 % could stabilize GC-SLN and showed good physical stability after storage at room temperature for 3 months. This could be assumed that dissolved surfactant (T-80) with PL-40 or PL-90 in optimum ratio were able to diffuse to the particle surface in a much shorter time than vesicles diffuse. The results were similar to previous work of Westesen and Siekman (1997). Gel formation could be prevented by the addition of co-emulsifying agents to the aqueous phase provide the concentration of co-surfactant was sufficiently high to constitute a reservoir of molecule immediately available for interfacial stabilization during recrystalization. Moreover, the co-emulsifier should preferably adsorb on crystal interface not or only incompletely covered by phospholipids. Using T-80 with P-188 as co-stabilizer (8:2) showed good physical stability after kept at room temperature for 3 months. P-188 is polymeric molecules. A combination of T-80 with P-188 could stabilize by adsorption on the droplet surface act as a steric barrier, preventing close contact of the droplets and later particles. Combination of stabilizer was also preferred for long term stability (Muller and Keck 2004, Rabinow 2004).

T-80:P-188 (12:3), T-80:PL-40 (12:3) and T-80:PL-90 (12:3) at 15 % provided good physical stability of SLN while the preparations using T-80:P-188 (8:7), T-80:P188 (3:12) and T-80 with either PL-40 or PL-90 at (8:7), (3:12) formed gel after storage at room temperature for 1 month. This was due to the increasing lecithin concentrations. Lecithin mobility increased with regarding to a formation of liposomes within the aqueous phase and/or multiple bilayer structures around the particles. When those particles of mobile lecithin moved in close contact with each other by Brownian motion, lecithin bilayer fusion was likely to occur as well as particles agglomeration and gel formation (Schubert, Muller-Goymann 2005). Increasing of P-188 bridging effects was also possible. Free water could be incorporated intralamellar (swelling) which would explain the hardening of the gel with time (Freitas, Muller 1998).

pH measurement and Osmolality

The pH of drug free- SLN preparations are shown in Table 11. The pH of all preparations was moderately acidic in range of 3-4. The pH after autoclaving was lower than before autoclaving. This might be due to the high temperature accelerated the hydrolysis of GC leading to the formation of free fatty acid which gradually reduced the pH of the system.

The osmolality of drug free-SLN with various type and amount of single surfactant are shown in Table 11. The osmolality of GC-SLN examined both before, after and after storage for 3 months were rather constant. Increasing the percentage of single and combined surfactants could slightly increased the osmolality. All data showed very low osmolality of these preparations.

Particle size and zeta potential

The particle sizes and zeta potential of drug free GC-SLN with combined surfactants (T-80:PL40), (T-80:PL-90) both before, after and after storage for 3 months are shown in Table 12, 13, 14 respectively. The particle sizes of both formulations were also in the nanometer range with narrow size distribution. The particle sizes of T-80:PL-40 (8:2) was lower than T-80:PL-90. This might be due to the lower composition of phosphotidylcholine of PL-40 displayed high mobility to cover the newly creased interfaces during recrystalization faster than PL-90.

The determination of the zeta potential of drug free GC-SLN, the zeta potential of both formulations was rather constant at all before, after and after storage time. It was found that T-80:PL40 showed higher zeta potential thanT-80:PL-90. This might be that small particle could adsorb negative charge from the medium higher than large particle.

Table 11.The pH and osmolality of GC- SLN prepared by solvent diffusion method before, after autoclaving and after storage for 3 months

Formulation	Before autoclaving		After autoclaving		After storage for 3 months	
	pH	Osmolality	pH	Osmolality	pH	Osmolality
T-80 (5)	3.70±0.00	0.022±0.000	3.41±0.01	0.023±0.001	3.35±0.01	0.022±0.001
T-80 (10)	3.74±0.02	0.022±0.002	3.44±0.02	0.023±0.002	3.32±0.01	0.022±0.002
T-80 (15)	3.69±0.01	0.025±0.001	3.46±0.02	0.025±0.001	3.40±0.02	0.027±0.001
P-188 (5)	3.65±0.01	0.020±0.001	3.42±0.01	0.019±0.001	ND	ND
P-188 (10)	3.72±0.02	0.022±0.001	3.45±0.02	0.021±0.001	ND	ND
P-188 (15)	3.73±0.01	0.024±0.001	3.52±0.01	0.022±0.002	ND	ND
PL-40 (5)	3.75±0.01	0.022±0.002	3.55±0.01	0.022±0.001	ND	ND
PL-40 (10)	3.78±0.01	0.024±0.001	3.43±0.02	0.024±0.002	ND	ND
PL-40 (15)	3.75±0.01	0.025±0.001	3.55±0.01	0.024±0.001	ND	ND
PL-90 (5)	3.82±0.01	0.021±0.001	3.58±0.01	0.020±0.001	ND	ND
PL-90 (10)	3.76±0.02	0.019±0.002	3.52±0.01	0.022±0.001	ND	ND
PL-90 (15)	3.70±0.01	0.022±0.001	3.48±0.01	0.022±0.001	ND	ND
T-80:P-188(4:1)	3.67±0.02	0.023±0.001	3.37±0.03	0.023±0.001	3.32±0.01	0.022±0.001
T-80:P-188(3:2)	3.70±0.01	0.024±0.000	3.42±0.03	0.023±0.002	3.35±0.01	0.024±0.001
T-80:P-188(1:4)	3.59±0.02	0.023±0.001	3.33±0.02	0.025±0.001	ND	
T-80:PL-40(4:1)	3.71±0.00	0.023±0.000	3.42±0.01	0.022±0.002	3.35±0.02	0.023±0.001
T-80:PL-40(3:2)	3.74±0.02	0.024±0.001	3.38±0.02	0.022±0.001	3.32±0.01	0.022±0.002
T-80:PL-40(1:4)	3.71±0.01	0.022±0.002	3.41±0.01	0.021±0.001	ND	ND
T-80:PL-90(4:1)	3.71±0.02	0.021±0.001	3.44±0.01	0.021±0.001	3.32±0.02	0.022±0.001

ND: Not determined

Table 11.The pH and osmolality of GC- SLN prepared by solvent diffusion method before, after autoclaving and after storage for 3 months(cont)

Formulation	Before autoclaving		After autoclaving		After storage for 3 months	
	pH	osmolality	pH	osmolality	pH	osmolality
T-80:PL-90(3:2)	3.66±0.02	0.025±0.002	3.36±0.02	0.027±0.001	3.31±0.01	0.025±0.001
T-80:PL-90(1:4)	3.63±0.02	0.025±0.001	3.42±0.01	0.023±0.003	3.32±0.01	0.023±0.001
P-188:PL-40(4:1)	3.69±0.02	0.025±0.001	3.41±0.01	0.027±0.002	ND	ND
P-188:PL-40(3:2)	3.59±0.01	0.026±0.001	3.43±0.01	0.023±0.001	ND	ND
P188:PL-40(1:4)	3.62±0.01	0.022±0.002	3.42±0.02	0.024±0.002	ND	ND
P-188:PL-90(4:1)	3.67±0.02	0.020±0.001	3.42±0.01	0.021±0.001	ND	ND
P-188:PL-90(3:2)	3.64±0.02	0.021±0.001	3.32±0.02	0.020±0.002	ND	ND
P-188:PL-90(1:4)	3.63±0.01	0.021±0.002	3.38±0.02	0.020±0.001	ND	ND
T-80:PL-40(8:2)	3.69±0.02	0.024±0.002	3.40±0.01	0.026±0.001	3.32±0.01	0.025±0.002
T-80:PL-40(5:5)	3.65±0.01	0.026±0.001	3.45±0.02	0.025±0.002	ND	ND
T-80:PL-40(2:8)	3.61±0.02	0.024±0.001	3.42±0.01	0.022±0.002	ND	ND
T-80:PL90(8:2)	3.67±0.02	0.021±0.001	3.36±0.01	0.021±0.001	3.35±0.01	0.021±0.001
T-80:PL-90(5:5)	3.62±0.01	0.023±0.002	3.38±0.01	0.023±0.001	ND	ND
T-80:PL-90(2:8)	3.59±0.01	0.020±0.001	3.31±0.02	0.021±0.002	ND	ND
T-80:P-188(8:2)	3.72±0.01	0.019±0.002	3.44±0.02	0.020±0.001	3.32±0.01	0.021±0.002
T-80:P-188(5:5)	3.65±0.02	0.022±0.001	3.38±0.01	0.020±0.002	ND	ND
T-80:P-188(2:8)	3.68±0.01	0.021±0.002	3.45±0.02	0.022±0.001	ND	ND
T-80:PL-90(12:3)	3.67±0.02	0.021±0.001	3.44±0.02	0.021±0.002	3.35±0.01	0.022±0.002
T-80:PL-90(8:7)	3.62±0.01	0.022±0.002	3.42±0.01	0.020±0.002	ND	ND
T-80:PL-90(3:12)	3.71±0.02	0.020±0.001	3.39±0.01	0.022±0.001	ND	ND
T-80:PL-40(12:3)	3.67±0.02	0.023±0.002	3.39±0.02	0.023±0.003	3.35±0.01	0.025±0.001
T-80:PL-40(8:7)	3.58±0.01	0.021±0.002	3.41±0.02	0.0019±0.002	ND	ND
T-80:PL-40(3:12)	3.65±0.01	0.022±0.001	3.38±0.01	0.0021±0.001	ND	ND
T-80:P-188(12:3)	3.65±0.01	0.022±0.001	3.36±0.02	0.024±0.002	3.32±0.01	0.0021±0.002
T-80:P-188(8:7)	3.52±0.02	0.0021±0.002	3.35±0.01	0.022±0.001	ND	ND
T-80:P-188(3:12)	3.58±0.01	0.0021±0.002	3.38±0.01	0.023±0.002	ND	ND

ND: Not determined

Table 12. Particle sizes, zeta potential, pH and osmolality of GC-SLN prepared by solvent diffusion method before autoclaving

Formulation	Z-average±SD nm	Zeta potential±SD (mV)	pH average±SD	Osmolality±SD (Osmol/kg)
GC+T80: PL40(8:2)	103.6±0.5	-32.5±0.2	3.72±0.02	0.026±0.001
	103.4±0.3	-32.8±0.5	3.68±0.01	0.026±0.001
	103.8±0.3	-33.0±0.1	3.68±0.02	0.022±0.002
GC+T80: PL90(8:2)	386.6±1.8	-10.5±0.1	3.65±0.01	0.022±0.002
	390.4±2.3	-9.8±0.0	3.68±0.02	0.024±0.002
	382.6±2.8	-10.6±0.1	3.68±0.02	0.024±0.002

Table 13. Particle sizes, zeta potential, pH and osmolality of GC-SLN prepared by solvent diffusion method after autoclaving

Formulation	Z-average±SD nm	Zeta potential±SD (mV)	pH average±SD	Osmolality±SD (Osmol/kg)
GC+T80: PL40(8:2)	105.6±0.2	-31.7±0.2	3.42±0.02	0.026±0.001
	106.4±0.6	-32.8±0.3	3.39±0.01	0.026±0.001
	106.6±0.4	-30.0±0.2	3.39±0.02	0.028±0.002
GC+T80: PL90(8:2)	394.1±1.5	-10.5±0.1	3.35±0.01	0.022±0.002
	391.4±2.5	-10.2±0.0	3.38±0.02	0.022±0.002
	392.7±2.2	-10.4±0.1	3.38±0.02	0.021±0.002

Table 14. Particle sizes, zeta potential, pH and osmolality of GC-SLN prepared by solvent diffusion method after storage for 3 months

Formulation	Z-average±SD nm	Zeta potential±SD (mV)	pH average±SD	Osmolality±SD (Osmol/kg)
GC+T80: PL40(8:2)	105.6±0.4	-31.2±0.1	3.45±0.01	0.025±0.001
	103.4±0.5	-30.6±0.5	3.43±0.01	0.026±0.001
	103.0±0.1	-30.0±0.2	3.43±0.02	0.028±0.002
GC+T80: PL90(8:2)	393.0±1.2	-10.5±0.2	3.32±0.01	0.022±0.002
	395.5±2.2	-10.6±0.2	3.30±0.01	0.022±0.002
	394.1±2.8	-10.4±0.1	3.35±0.01	0.020±0.001

1.1.2 Preparation of drug-free SLN by HPH method

In the preliminary study, SLN prepared by HPH method with 1-5 % single surfactant and 1-5 % combined surfactants. Two type of lipid were used, GB and GC. From the results, when using single surfactant could not stabilize GC-SLN and GB-SLN at all preparations. When using combined surfactants, the results showed that 1000 mg of GB and GC containing T-80:PL-40(4:1) and T-80:PL-90(4:1) could display good physical appearance after storage for 3 months at room temperature. The physical appearance are shown in Tables 15, 16 respectively.

Effect of single surfactant

SLN containing P-188 of 1-5 % could not stabilize both GB and GC-SLN. Gelation and precipitations occurred after storage at room temperature. This might be resulted from high temperature exposure during homogenization and autoclaving. Introduction of energy to the SLN systems accelerated particle growth and subsequently gelation.

When using PL-40 and PL-90 3-5 % as single surfactant, the preparations of GB and GC SLN became semisolid immediately after the addition of the heated aqueous phase to the oil phase under shear force by high speed homogenizer whereas using PL-40, PL-90 of 1 and 2 % displayed gelation after storage for 1 month. This might be that lecithin was used as emulsifier alone. It was easy to form vesicles to slow down the molecule movements and could not cover the naked new surface immediately due to the surface of particles without the protection of emulsifier was easy to flocculate under the effect of intermolecular Van der Waals forces and form gel under the effect of gravity potential energy (Han et al 2008).

When using T-80 as surfactant in GC-SLN, solid lipid floated on the top of dispersion occurred after storage while GB-SLN precipitation appeared after storage at room temperature in all formulations. T-80 is a water soluble non-ionic surfactant. It could dissolve as free molecules in dispersion medium and form micelles at relatively high concentrations. After addition of oily phase and reduction by homogenization, the oily droplets could be broken to be nanoparticles, and the

surfactant molecules would diffuse to the surface of oily droplets and formed monolayer or multilayers in the case of high concentration of stabilizers. The diffusion velocity of the macromolecules in the low viscous aqueous phase was fast for T-80. Thus, it could rapidly adhere to the lipid surface and the particles were kept in the nanometer size range. After autoclaving, higher temperature produced high kinetic energy and might affect the layer of stabilizer. T-80, which was a small molecule and showed higher hydrophilic part in the molecule, might diffuse to the aqueous medium and then rearrange to form new layer on the surface of droplets, which might form weak intermolecular bonding between T-80 molecules that adhered to the oil droplets. This might cause aggregation and the larger particle size was obtained (Krog 1990).

Effect of combined surfactants

For GC and GB-SLN dispersions, 5 % of combined surfactant of T-80 with PL-40 or PL-90 at 4:1 provided good physical stability. The combination of two emulsifying agents to produce mixed surfactant films at the interface had high surfactant coverage as well as sufficient viscosity to promote stability. If lecithin was used as single emulsifier, phospholipid molecules bound to vesicle exhibited that, they were not able to immediately cover the newly created interface during recrystalization. Crystal interface with low concentration of adsorbed emulsifier molecule represented preferred sites of particle aggregation over which gel formation could proceed. Mobile surfactant was required to achieve optimum particle size stability in phospholipids stabilized lipid nanoparticles. Dissolved surfactant was able to diffuse to the particle surfaces in a much shorter time than vesicles diffused. SLN with T-80 and PL-40 or PL-90 of (3:2), (1:4) could not be prepared. Gel formation occurred during the preparation of coarse emulsion. This might be that increasing amount of lecithin, the slow mobility could not adhere to the new surface droplet after recrystalization. This caused in a sudden local lack of emulsifier on the particle surface and resulting in an instability of the dispersed state and gelation.

Five % of GB and GC-SLN using T-80 with P-188 of (4:1) displayed precipitation after storage for 3 months. When increasing the concentration of P-188

to the ratio of 3:2 and 1:4 gel formation occurred in 1 month. The instability of formulation was explained by two reasons. T-80 was of small molecular size. It could form films on the surface of the droplets, but exhibited lower steric effect. secondly While increasing of P-188, high temperature led to dehydration and decreased the mobility of ethylene glycol chains which mean a decrease of thickness of the protecting layer. When using PL-40 with PL-90 gel formation displayed immediately after mixing water phase to the oil phase. This indicated the lecithin did not have sufficient steric or electrostatic stabilization. The difference in SLN product using lecithin as stabilizer or co-stabilizer resulted from different experimental condition e.g. lecithin source, method of preparation, type of lipid matrix, quantity of lecithin and co-emulsifier in formulations. Whereas GB and GC-SLN containing P-188 with PL-40 or PL-90 as co-surfactant showed gel formation at room temperature. SLN using high concentration of PL-40 or PL-90 (1:4) could not be prepared in all formulations. This might be that high concentration of lecithin and P-188 increased the viscosity of preparation and could be observed under room temperature. This affected particle size. High viscosity retarded the movement of surfactant layers of lecithin around the droplet thus formed gel after storage for 1 month.

In the case of solvent diffusion method, GC-SLN could be prepared and showed good physical stability in many formulations than HPH method. This might be that the GC dissolved in a water-miscible organic solvent. The solution was emulsified in an aqueous phase using high speed homogenizer. This was caused by an increase of homogenization efficiency while high temperature in HPH method would affect the mobility and hydrophilicity of all emulsifiers. When using GB as a solid lipid, GB could not dissolve in a water miscible organic solvent thus GB-SLN displayed physical instability. This might be that the high speed homogenizer could not reduce the particle size into the nanoparticle size range and the longer chain of GB could promote rapid recrystalization. For HPH method, very high shear stress could disrupt the particles down to the submicron range when using optimum ratio of combined surfactants.

pH and osmolality

For HPH method, the pH and osmolality of SLN dispersion were not affected by type of lipid as shown in Tables 17, 18. The physical stability formulations showed no different of pH and osmolality at before, after and after storage time. This was because of lipids were not soluble in water.

1.2 Formulation of AA, AS load SLN

1.2.1 AA, AS Formulation prepared by solvent diffusion method

From the result of drug free GC-SLN and GB-SLN dispersion, formulations of good physical appearance contained 1000 mg GC with 5-15 % of T-80 as surfactant. The SLN containing 1000 mg GC with 5-15 % of various co-surfactants were selected to loaded AA, AS due to good physical stability after storage at room temperature for 3 months.

Physical appearance

The physical stability of dispersion of GC-SLN containing AA, AS are shown in Tables 19 and 20, respectively. Translucent colloidal dispersion of AA loaded GC-SLN were obtained at 10 % of both T-80 with PL-40 and PL-90 while 10 % of both T-80 with PL-40, PL-90 could not stabilize AS loaded GC-SLN that white precipitate appeared after storage for 3 months. The decrease of particle sizes of GC-SLN loaded AA were explained by two possible mechanisms: improvement of wetting characteristics and micellar solubilization of AA. AA solubilization may be influenced by the specific types of chemical interactions that can form depending on the structures of both AA and solvent molecules. Specific interactions in mono-, di-, and tri-glycerides, fatty alcohol, polyglycolized fatty acid esters were reported to increase in drug solubilities (Karatas and Farmaco 2005). On the other hand, the tertiary amine part of phospholipon could interact with the carboxylic part of AA and GC by ionic force (Figure 9). These could increase solubility and resulted in decreasing the particle size.

Using T-80 with PL-40 or PL-90 exhibited sufficient system stability. In accordance with result reported by Han et al (2008), the Monostearin formulation could not be covered by lecithin molecule thus flocculated rapidly to form gel. If lecithin was used as emulsifier alone, it was easy to form vesicles to slow down the molecular movements and could not cover the naked new surface immediately due to emulsifier molecules were fixed inside the vesicles. But there was no gelation phenomenon in the samples with addition of Tween 80 as co-emulsifier. It could be

explain that the emulsifier can increase the steric stabilization of its synergism. In accordance with Attama and Muller-Goymann (2007), phospholipon G® was anchored almost entirely on the nanoparticle surface, with polysorbate 80 providing further synergistic stabilization. Therefore, particle-particle interaction and subsequent agglomeration was greatly reduced. At 5 % (Table 18, 19) of all single surfactants and combined surfactants, SLN displayed white precipitate after autoclaving. This might be that the low concentration of stabilizer and co-stabilizer could not cover the interface of droplets after autoclaving.

T-80 with P-188 (8:2) at 10 % could not stabilize AA and AS formulation after autoclaving and exhibited white precipitates during standing at room temperature. This might be resulted from high temperature exposure during autoclaving. Introduction of energy to the SLN systems accelerated particles growth and subsequently gelation. At 15 % of surfactant and co-surfactant, using T-80 as single surfactant showed translucence in both AA and AS formulations. But the formulation containing 15 % of T-80 as surfactant showed white precipitates after storage at room temperature for 3 months. While T-80 with PL-40(12:3) and T-80 with PL-90 (12:3) displayed good physical stability after autoclaving. Translucence dispersions were obtained in AA and AS loaded GC-SLN whereas the formulation showed white precipitates after kept at room temperature for 3 months.

pH and osmolality

The pH and osmolality of AA loaded GC-SLN dispersions are shown in Table 21. The pH of all preparations were moderately acidic to neutral and similar to drug

free preparations. Considering to the osmolality of SLN dispersions, AA did not affect osmolality of GC SLN dispersion when compared with drug free SLN dispersions. These caused by the very low amount of AA that dissolved in dispersion medium at all formulations. Nevertheless, the osmolality of all preparations were obviously low which was inappropriate to the physiological fluid. Thus, selected preparation should add osmolality agents such as glycerol or be added to intravenous fluids for i.v. infusion before used.

Particle size and zeta potential

The particle sizes of SLN containing 750mg of AA with combined surfactants, showing good physical stability formulations are shown in Table 23. The particle of the preparations obtained by using T-80 with either PL-40 or PL-90 at the ratio 8:2 were lower than drug free SLN. These preparations of the particle size was in disagree with the result of drug free GC- SLN.

The particle sizes of formulations were also in the nanometer range with narrow size distribution and lower than 1 μm that was suitable for parenteral product. Among T-80 with either PL-40 or PL-90, the particle sizes of SLN were not different ($P<0.05$).

Zeta potential is an important and useful indicator of particle surface charge, which can be used to predict and control the stability of colloidal suspensions or emulsions. The zeta potential of selected formulations were measured. These formulation showed the negative charge in the range of -10.5, -4.5 millivolt which was lower than from drug free SLN. It was possible that some AA could dissolve in dispersion medium to be ionized form. This ionized proton could reduce the negative charge of particles. This led to decrease of electrostatic repulsion.

1.2.2 AA, AS Formulation prepared by HPH method

Physical appearance

The visual observation of the SLN dispersion containing 5 mg/ml of AS and AA are shown in Table 24. The AS loaded SLN with T-80 and either PL-40 or PL-90 as co-surfactant could not be prepared by hot homogenization. Hot emulsions containing AS became semisolid after standing at room temperature for both lipids. The preparation using T-80 with either PL-40 or PL-90 as co-stabilizer were physical unstable and displayed white precipitates after 1 month of storage at room temperature. In this study, the formulations of AA and AS loaded SLN could not be stabilized by HPH method.

This might be that glycoside part in AS. For HPH method, which used high, temperature the ester bond in the structure of AS was rapidly hydrolyzed by hydronium ion. The sugar moiety, glucose and rhamnose were obtained. This sugar part might diffuse into the medium and formed gel after standing at room temperature. In the case of AA, this might be that HPH method could not form micelles similar to solvent diffusion method and low efficiency to emulsify the preparations. This were increasing particle agglomeration and precipitation.

pH and osmolality

The pH and osmolality of AA and AS loaded SLN dispersions are shown in Table 25. The pH of all preparations was moderately acidic in the range 3-4 and lower than that of drug free preparations. This might be due to the acidic property of AA. AA could dissolve in dispersion medium affecting the pH of preparation directly. The osmolality of AA and AS SLN dispersions prepared was still too low to be used in intravenous administration. This caused by the very low amount of AA that dissolved in dispersion medium in all formulations. Thus, it was necessary to add some osmotic agents before used. However, the physical incompatibility and chemical stability of the intravenous mixtures should be further examined in order to assure safety in clinical use.

Table 15. Physical appearance of SLN dispersion containing GB as solid lipid with different types and amount of surfactant and co-surfactant prepared by HPH method

Formulation	Macroscopic			Formulation	Macroscopic			Formulation	Macroscopic			Formulation	Macroscopic		
	a	b	c		a	b	c		a	b	c		a	b	c
(P188)				(PL40)				(PL90)				(T80)			
(1)	+	+	P1M	(1)	+	+	P1M	(1)	+	+	P1M	(1)	+	+	P1M
(2)	+	+	P1M	(2)	+	+	P1M	(2)	+	+	P1M	(2)	+	+	P1M
(3)	+	+	G1M	(3)	GI	-	-	(3)	GI	-	-	(3)	+	+	P3M
(4)	+	+	G1M	(4)	GI	-	-	(4)	GI	-	-	(4)	+	+	P3M
(5)	+	+	G1M	(5)	GI	-	-	(5)	GI	-	-	(5)	+	+	P3M
(T80+P188)				(T80+PL40)				(T80+PL90)				(P188+PL40)			
(4:1)	+	+	P3M	(4:1)	+	+	+	(4:1)	+	+	+	(4:1)	+	+	G1M
(3:2)	+	+	G1M	(3:2)	GI	-	-	(3:2)	GI	-	-	(3:2)	+	+	G1M
(1:4)	+	+	G1M	(1:4)	GI	-	-	(1:4)	GI	-	-	(1:4)	GI	-	-
(P188+PL90)				(PL90+PL40)											
(4:1)	+	+	G1M	(4:1)	GI	-	-								
(3:2)	+	+	G1M	(3:2)	GI	-	-								
(1:4)	GI	-	-	(1:4)	GI	-	-								

a, b, c: before and after autoclaving, after storage for 3 months; +, white fluid dispersion; P1M, precipitation appeared after 1 month; F1M, F3M: solid lipids floated on the top of dispersion after1, 3 months; GI, G1M: gel immediately, gel formation within 1 month.

Table 16. Physical appearance of SLN dispersion containing GC as solid lipid with different types and amount of surfactant and co-surfactant prepared by HPH method.

Formulation	Macroscopic			Formulation	Macroscopic			Formulation	Macroscopic			Formulation	Macroscopic		
	a	b	c		a	b	c		a	b	c		a	b	c
(P188)				(PL40)				PL90)				(T80)			
(1)	+	+	P1M	(1)	+	+	P1M	(1)	+	+	P1M	(1)	+	+	F1M
(2)	+	+	P1M	(2)	GI	-	-	(2)	GI	-	-	(2)	+	+	F1M
(3)	+	+	G1M	(3)	GI	-	-	(3)	GI	-	-	(3)	+	+	F3M
(4)	+	+	G1M	(4)	GI	-	-	(4)	GI	-	-	(4)	+	+	F3M
(5)	+	+	G1M	(5)	GI	-	-	(5)	GI	-	-	(5)	+	+	F3M
(T80+PL40)				(T80+PL90)				T80+P188)				(PL90+PL40)			
(4:1)	+	+	+	(4:1)	+	+	+	(4:1)	+	+	F3M	(4:1)	GI	-	-
(3:2)	GI	-	-	(3:2)	GI	-	-	(3:2)	+	+	F1M	(3:2)	GI	-	-
(1:4)	GI	-	-	(1:4)	GI	-	-	(1:4)	+	+	F1M	(1:4)	GI	-	-
(P188+PL40)				P188+PL90)											
(4:1)	+	+	G1M	(4:1)	+	+	G1M								
(3:2)	GI	-	-	(3:2)	GI	-	-								
(1:4)	GI	-	-	(1:4)	GI	-	-								

a, b, c: before and after autoclaving, after storage for 3 months; +, white fluid dispersion; P1M, precipitation appeared after 1 month; F1M, F3M: solid lipids floated on the top of dispersion after1, 3 months; GI, G1M: gel immediately, gel formation within 1 month.

Table 17. The pH and osmolality of GB- SLN prepared by HPH method both before, after autoclaving and after storage for 3 months

Formulation	Before autoclaving		After autoclaving		After storage for 3 months	
	pH	osmolality	pH	osmolality	pH	osmolality
T-80 (1)	3.82±0.01	0.020±0.001	3.80±0.01	0.020±0.001	ND	ND
T-80 (2)	3.85±0.01	0.021±0.001	3.82±0.02	0.022±0.001	ND	ND
T-80 (3)	3.81±0.02	0.020±0.001	3.75±0.01	0.022±0.002	ND	ND
T-80 (4)	3.83±0.01	0.024±0.002	3.84±0.01	0.024±0.001	ND	ND
T-80 (5)	3.85±0.01	0.025±0.001	3.80±0.01	0.026±0.001	ND	ND
P-188 (1)	3.78±0.02	0.020±0.001	3.72±0.01	0.021±0.002	ND	ND
P-188 (2)	3.85±0.01	0.022±0.001	3.80±0.02	0.023±0.001	ND	ND
P-188 (3)	3.82±0.01	0.022±0.001	3.81±0.02	0.022±0.001	ND	ND
P-188 (4)	3.80±0.01	0.024±0.001	3.82±0.01	0.023±0.001	ND	ND
P-188 (5)	3.78±0.01	0.022±0.002	3.75±0.01	0.021±0.002	ND	ND
PL-40 (1)	3.78±0.01	0.021±0.001	3.79±0.01	0.022±0.001	ND	ND
PL-40 (2)	3.83±0.02	0.024±0.002	3.80±0.01	0.023±0.001	ND	ND
PL-90 (1)	3.75±0.01	0.023±0.001	3.78±0.02	0.023±0.001	ND	ND
PL-90 (2)	3.78±0.01	0.022±0.002	3.75±0.01	0.022±0.002	ND	ND
P-188:PL-40 (4:1)	3.85±0.01	0.023±0.001	3.83±0.01	0.024±0.001	ND	ND
P-188:PL-90 (4:1)	3.80±0.02	0.022±0.001	3.82±0.01	0.022±0.001	ND	ND
T-80:PL-40 (4:1)	3.88±0.01	0.026±0.002	3.85±0.01	0.026±0.001	3.82±0.01	0.026±0.001
T-80:PL-90 (4:1)	3.83±0.01	0.022±0.001	3.80±0.00	0.023±0.001	3.72±0.01	0.021±0.001
T-80:P-188 (4:1)	3.78±0.01	0.023±0.001	3.80±0.00	0.022±0.001	ND	ND
T-80:P-188 (3:2)	3.80±0.01	0.020±0.002	3.75±0.01	0.023±0.001	ND	ND
T-80:P-188 (1:4)	3.81±0.01	0.024±0.001	3.78±0.01	0.022±0.001	ND	ND

ND: Not determined

Table 18. The pH and osmolality of GC- SLN prepared by HPH method both before, after autoclaving and after storage for 3 months

Formulation	Before autoclaving		After autoclaving		After storage for 3 months	
	pH	osmolality	pH	osmolality	pH	osmolality
P-188 (1)	3.72±0.01	0.021±0.001	3.75±0.01	0.022±0.001	ND	ND
P-188 (2)	3.75±0.02	0.022±0.001	3.73±0.01	0.021±0.001	ND	ND
P-188 (3)	3.71±0.01	0.023±0.001	3.71±0.02	0.022±0.001	ND	ND
P-188 (4)	3.75±0.01	0.022±0.001	3.75±0.01	0.022±0.001	ND	ND
P-188 (5)	3.78±0.01	0.022±0.001	3.72±0.01	0.024±0.002	ND	ND
T-80 (1)	3.85±0.01	0.020±0.001	3.81±0.01	0.018±0.001	ND	ND
T-80 (2)	3.88±0.02	0.021±0.001	3.82±0.01	0.022±0.001	ND	ND
T-80 (3)	3.81±0.01	0.024±0.001	3.78±0.01	0.025±0.001	ND	ND
T-80 (4)	3.83±0.01	0.026±0.001	3.85±0.02	0.026±0.001	ND	ND
T-80 (5)	3.82±0.01	0.025±0.001	3.83±0.01	0.023±0.002	ND	ND
PL-40 (1)	3.75±0.02	0.022±0.001	3.78±0.01	0.021±0.001	ND	ND
PL-90 (1)	3.72±0.01	0.023±0.001	3.68±0.02	0.022±0.001	ND	ND
T-80:PL-40 (4:1)	3.76±0.02	0.023±0.001	3.75±0.01	0.024±0.001	3.72±0.01	0.026±0.001
T-80:PL-90 (4:1)	3.72±0.01	0.020±0.001	3.72±0.02	0.021±0.001	3.71±0.02	0.022±0.001
P-188:PL-40(4:1)	3.82±0.01	0.022±0.001	3.85±0.01	0.024±0.001	ND	ND
P-188:PL-90(4:1)	3.78±0.01	0.024±0.001	3.80±0.02	0.024±0.002	ND	ND
T-80:P-188(4:1)	3.82±0.01	0.021±0.001	3.80±0.01	0.022±0.001	ND	ND
T-80:P-188(3:2)	3.78±0.01	0.021±0.001	3.79±0.01	0.023±0.001	ND	ND
T-80:P-188(1:4)	3.73±0.01	0.022±0.001	3.75±0.01	0.022±0.001	ND	ND

ND: Not determined

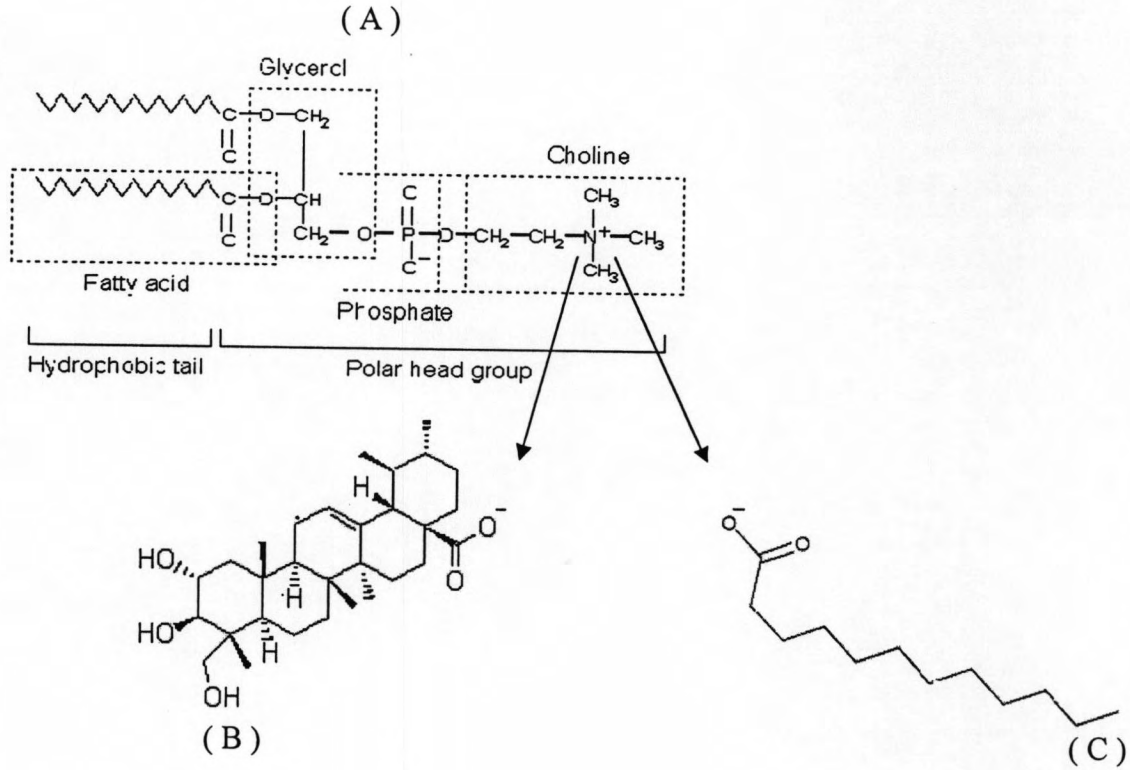


Figure 9. Ionic interact between (A) quaternary amine with (B) Asiatic acid and quaternary amine with (C) gelucire 44/14

Table 19. The physical appearance of AA loaded GC-SLN dispersion with various surfactant and combined surfactant prepared by solvent diffusion method

Formulation	Macroscopic			Formulation	Macroscopic			Formulation	Macroscopic		
	a	b	c		a	b	c		a	b	c
(T80)				(T80+PL40)				(T80+PL90)			
(5)	+	+	P1M	(4:1)	+	PA	-	(4:1)	+	PA	-
(10)	+	+	P3M	(3:2)	+	PA	-	(3:2)	+	PA	-
(15)	+	+	P3M								
(T80+P188)				(P188+PL40)				(P188+PL90)			
(4:1)	+	PA	-	(4:1)	+	PA	-	(4:1)	+	PA	-
(3:2)	+	PA	-	(3:2)	+	PA	-	(3:2)	+	PA	-
(1:4)	+	PA	-								
(T80+P188)				(T80+PL40)				(T80+PL40)			
(8:2)	+	+	P1M	(8:2)	+	+	+	(12:3)	+	+	P3M
(T80+P188)				(T80+PL90)				(T80+PL90)			
(12:3)	+	+	P1M	(8:2)	+	+	+	(12:3)	+	+	P3M

a, b, c: before and after autoclaving, after storage for 3 months; +, translucence fluid dispersion; PA, P1M, P3M: precipitation after autoclaving, precipitation after storage for 1, 3 months

Table 20. The physical appearance of AS loaded GC-SLN dispersion with various surfactant and combined surfactant prepared by solvent diffusion method

Formulation	Macroscopic			Formulation	Macroscopic			Formulation	Macroscopic		
	a	b	c		a	b	c		a	b	c
(T80)				(T80+PL40)				(T80+PL90)			
(5)	+	+	P1M	(4:1)	+	PA	-	(4:1)	+	PA	-
(10)	+	+	P3M	(3:2)	+	PA	-	(3:2)	+	PA	-
(15)	+	+	P3M								
(T80+P188)				(P188+PL40)				(P188+PL90)			
(4:1)	+	PA	-	(4:1)	+	PA	-	(4:1)	+	PA	-
(3:2)	+	PA	-	(3:2)	+	PA	-	(3:2)	+	PA	-
(1:4)	+	PA	-								
(T80+P188)				(T80+PL40)				(T80+PL40)			
(8:2)	+	+	P1M	(8:2)	+	+	P3M	(12:3)	+	+	P3M
(T80+P188)				(T80+PL90)				(T80+PL90)			
(12:3)	+	+	P1M	(8:2)	+	+	P3M	(12:3)	+	+	P3M

a, b, c: before and after autoclaving, after storage for 3 months; +: translucence fluid dispersion; PA, P1M, P3M: precipitation after autoclaving, precipitation after storage for 1, 3 months

Table 21. The pH, osmolality of GC-SLN loaded AA before, after autoclaving and after storage for 3 moths

Formulation	Before autoclaving		After autoclaving		After storage for 3months	
	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)
T80:P188(4:1)	3.37±0.01	0.021±0.001	ND	ND	ND	ND
T80:P188(3:2)	3.37±0.02	0.022±0.000	ND	ND	ND	ND
T80:P188(1:4)	3.34±0.01	0.025±0.001	ND	ND	ND	ND
T80:PL40(4:1)	3.46±0.02	0.024±0.001	ND	ND	ND	ND
T80:PL40(3:2)	3.40±0.00	0.025±0.001	ND	ND	ND	ND
T80:PL90(4:1)	3.35±0.04	0.022±0.000	ND	ND	ND	ND
T80:PL90(3:2)	3.34±0.02	0.023±0.000	ND	ND	ND	ND
P188:PL40(4:1)	3.67±0.02	0.026±0.001	ND	ND	ND	ND
P188:PL40(3:2)	3.56±0.01	0.024±0.001	ND	ND	ND	ND
P188:PL90(4:1)	3.33±0.02	0.022±0.001	ND	ND	ND	ND
P188:PL90(3:2)	3.33±0.04	0.022±0.001	ND	ND	ND	ND
T80:P188(8:2)	3.34±0.04	0.024±0.001	3.26±0.02	0.023±0.001	ND	ND
T80:PL40(8:2)	3.40±0.01	0.026±0.001	3.36±0.02	0.026±0.001	3.00±0.02	0.028±0.002
T80:PL90(8:2)	3.33±0.02	0.024±0.001	3.28±0.01	0.022±0.002	3.23±0.02	0.021±0.001
T80:P188(12:3)	3.35±0.02	0.025±0.001	3.30±0.02	0.026±0.001	ND	ND
T80:PL40(12:3)	3.36±0.02	0.025±0.001	3.26±0.01	0.028±0.002	ND	ND
T80:PL90(12:3)	3.38±0.01	0.023±0.001	3.25±0.02	0.025±0.001	ND	ND
T80(5)	3.52±0.02	0.022±0.001	3.38±0.01	0.020±0.001	ND	ND
T80(10)	3.69±0.02	0.023±0.001	3.42±0.01	0.021±0.001	ND	ND
T80(15)	3.64±0.01	0.024±0.001	3.35±0.02	0.026±0.001	ND	ND

ND: Not determined

Table 22. The pH, osmolality of GC-SLN loaded AS before, after autoclaving and after storage for 3 moths

Formulation	Before autoclaving		After autoclaving		After storage for 3months	
	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)
T80:P188(4:1)	3.57±0.01	0.020±0.001	ND	ND	ND	ND
T80:P188(3:2)	3.39±0.02	0.018±0.000	ND	ND	ND	ND
T80:P188(1:4)	3.44±0.01	0.019±0.001	ND	ND	ND	ND
T80:PL40(4:1)	3.56±0.02	0.022±0.001	ND	ND	ND	ND
T80:PL40(3:2)	3.50±0.01	0.024±0.001	ND	ND	ND	ND
T80:PL90(4:1)	3.55±0.02	0.021±0.000	ND	ND	ND	ND
T80:PL90(3:2)	3.58±0.02	0.022±0.001	ND	ND	ND	ND
P188:PL40(4:1)	3.47±0.01	0.022±0.001	ND	ND	ND	ND
P188:PL40(3:2)	3.46±0.02	0.020±0.001	ND	ND	ND	ND
P188:PL90(4:1)	3.55±0.01	0.022±0.001	ND	ND	ND	ND
P188:PL90(3:2)	3.53±0.02	0.024±0.001	ND	ND	ND	ND
T80:P188(8:2)	3.56±0.04	0.022±0.001	3.46±0.01	0.023±0.001	ND	ND
T80:PL40(8:2)	3.50±0.01	0.028±0.001	356±0.01	0.025±0.001	ND	ND
T80:PL90(8:2)	3.54±0.02	0.022±0.001	3.42±0.01	0.024±0.002	ND	ND
T80:P188(12:3)	3.55±0.02	0.024±0.001	3.46±0.02	0.024±0.001	ND	ND
T80:PL40(12:3)	3.56±0.02	0.022±0.001	3.46±0.01	0.022±0.002	ND	ND
T80:PL90(12:3)	3.50±0.01	0.022±0.001	3.42±0.01	0.023±0.001	ND	ND
T80(5)	3.52±0.01	0.022±0.001	3.39±0.01	0.022±0.001	ND	ND
T80(10)	3.59±0.02	0.026±0.001	3.48±0.01	0.021±0.001	ND	ND
T80(15)	3.55±0.01	0.028±0.001	3.45±0.02	0.028±0.001	ND	ND

ND: Not determined

Table 23. The particle sizes, zeta potential of AA loaded GC-SLN of both before and after storage at room temperature for 3 months

Formulation	After autoclaving		After storage for 3 months	
	Z-average±SD nm	Zeta potential ±SD(mV)	Z-average±SD nm	Zeta potential ±SD (mV)
T-80:PL-40 (8:2)	19.00±0.52	-13.6±1.25	22.50±0.32	-9.90±0.22
	20.50±0.25	-13.7±0.35	22.90±0.25	-10.50±0.10
	20.00±0.00	-13.0±0.20	22.20±0.10	-10.55±0.05
T-80:PL-90 (8:2)	26.50±0.32	-1.54±0.23	26.10±0.15	-4.50±0.25
	25.00±0.20	-1.92±0.15	26.25±0.10	-4.45±0.10
	25.00±0.50	-2.54±0.05	26.05±0.35	-4.25±0.20

Table 24. The physical appearance of AA, AS loaded GC-SLN and AA, AS loaded GB-SLN dispersion with various co-surfactant prepared by HPH method

Formulation	Macroscopic observation a	b	c	Formulation	Macroscopic observation a	b	c
(AS loaded GC) (T80+PL40,4:1)	+	PA	-	(AS loaded GB) (T80+PL40,4:1)	+	PA	-
(AS loaded GC) (T80+PL90,4:1)	+	PA	-	(AS loaded GB) (T80+PL90,4:1)	+	PA	-
(AA loaded GC) (T80+PL40,4:1)	+	+	P1M	(AA loaded GB) (T80+PL40,4:1)	+	+	P1M
(AA loaded GC) (T80+PL90,4:1)	+	+	P1M	(AA loaded GB) (T80+PL90,4:1)	+	+	P1M

a, b, c: before and after autoclaving, after storage for 3 months; +: translucence fluid dispersion; PA, P1M: precipitation after autoclaving, precipitation after storage for 1 month

Table 25. The pH, osmolality of GC-SLN loaded AA and GB-SLN loaded AA prepared by HPH method before, after autoclaving and after storage for 3 moths

Formulation	Before autoclaving		After autoclaving		After storage for 3 months	
	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)
(AA loaded GC) (T80+PL40)	3.85±0.02	0.028±0.001	3.78±0.01	0.026±0.001	ND	ND
(AA loaded GC) (T80+PL90)	3.78±0.01	0.025±0.003	3.72±0.02	0.026±0.002	ND	ND
(AA loaded GB) (T80+PL40)	3.92±0.01	0.026±0.001	3.85±0.01	0.024±0.001	ND	ND
(AA loaded GB) (T80+PL90)	3.88±0.02	0.023±0.001	3.80±0.01	0.022±0.001	ND	ND

ND: not determined

2. Stability testing

The preparations of 5 mg/ml AA using T-80 with PL-40 and T-80 with PL-90 as co-surfactant loaded GC-SLN were further examined for the physical stability under accelerated condition. The heating and cooling cycle was performed under storing the samples at 4°C for 48 hours and at 45°C for 48 hours for 6 cycles. On exposure to accelerated condition, the translucence fluid dispersion became yellowish after testing when using PL-40 as co-surfactant due to the color of material while using PL-90 as co-surfactant showed no difference between before and after testing. From the data obtained in Table 26, no significant difference in mean particle size was found after storage under stress condition of both formulations ($P<0.05$). The relative non difference in distribution of particle size was desirable for good stability. These results might be the admixture of T-80 with PL-40 or PL-90 resulted in a stabilizations of the colloidal dispersed stated of the AA formulations. The common property of both amphiphiles is their ability to form micelles and to exhibit a significant solubility in aqueous media. T-80 is able to diffuse to the particle surfaces in a much shorter time than PL-40 or PL-90 diffuse.

Moreover, micelles, in contrast to phospholipids vesicles, represent highly dynamic colloidal structures and may serve as reservoir for mobile phase active molecules required for the immediate coverage of unprotected particle surface created during the recrystallization process. The accelerated condition had no influence to pH, zeta potential and osmolality as shown in Table 27. The lower pH after accelerated condition might be due to AA lost in activity. Very low negative zeta potential of T-80 with PL-40 and T-80 with PL-90 of both before and after accelerated condition was shown in Table 26. However, it was found to be the stable formulations for stability testing. Thus high steric effect of co-stabilizers was enough to stabilize the particles. While the osmolality was relatively constant since the osmotic agent was not added in system.

Table 26. Particle sizes and zeta potential of AA loaded GC-SLN both before and after accelerated condition

Formulation	Before accelerated condition		After accelerated condition	
	Z-average±SD nm	Zeta potential±SD (mV)	Z-average±SD nm	Zeta potential±SD (mV)
T-80:PL-40 (8:2)	21.00±0.40	-8.34±1.55	20.87±0.021	-10.93±0.32
	20.00±0.52	-7.35±1.23	20.63±0.06	-10.08±1.31
	22.00±0.35	-8.07±1.09	21.93±0.06	-10.00±0.60
T-80:PL-90 (8:2)	25.00±0.25	-2.92±0.81	25.57±0.29	-3.87±1.37
	26.00±0.36	-2.00±0.50	26.03±0.32	-3.37±0.33
	25.00±0.32	-1.77±0.57	25.97±0.25	-3.70±0.28

Table 27. pH and osmolality of AA loaded GC-SLN both before and after accelerated condition

Formulation	Before accelerated condition		After accelerated condition	
	pH±SD	Osmolality±SD (Osmol/kg)	pH±SD	Osmolality±SD (Osmol/kg)
T-80:PL-40 (8:2)	3.32±0.01	0.024±0.001	3.21±0.02	0.025±0.001
	3.35±0.01	0.022±0.001	3.22±0.01	0.026±0.002
	3.35±0.01	0.023±0.001	3.20±0.01	0.024±0.001
T-80:PL-90 (8:2)	3.34±0.01	0.019±0.001	3.30±0.01	0.023±0.001
	3.35±0.01	0.022±0.001	3.28±0.02	0.024±0.001
	3.35±0.01	0.020±0.002	3.31±0.02	0.025±0.002

3. In vitro drug release

In vitro drug release is important to understand the in vivo performance of dosage form. Drug release studies help in evaluation of sustained and prolonged release dispersion systems. For the present work, 5 mg/ml of AA loaded GC-SLN prepared by solvent diffusion method was selected. HPLC method was to determine the amount of drug in receptor compartment of Modified Franz Diffusion apparatus. AA quantity was measured at the wavelength of 210 nm and the amount of AA release was then calculated from calibration curve.

The amount of drug release of these preparations for 2 hours are shown in Table 28. The release of AA from SLN was low. After 2 hours, the AA released was about 30 % of T-80 with PL-40 and 20 % of T-80 with PL-90 as co-surfactant. This might be the slightly solubility of AA into medium (water:isopropyl alcohol, 70:30) that could result in the low AA release. The amount of T-80 with PL-40 was higher than T-80 with PL-90. This result might be described in term of the particle sizes of T-80 with PL-40 was smaller than T-80 with PL-90. Therefore, the small particle could rapidly passed through the membrane faster than larger particles.

Table 28. Percent drug release after 2 hours in Modified Franz Diffusion Apparatus of AA loaded GC-SLN

AA loaded GC-SLN with T-80:PL-40	Conc. in donor compartment (mg/ml)	Conc. in receptor compartment (mg/ml)	% Release
1	15	4.90	32.67
2	15	4.30	28.67
3	15	4.55	30.33
Average±SD	15	4.58±0.30	30.56±2.00
AA loaded GC-SLN with T-80:PL-90	Conc. in donor compartment (mg/ml)	Conc. in receptor compartment (mg/ml)	% Release
1	15	3.08	20.53
2	15	3.12	20.80
3	15	3.32	22.13
Average±SD	15	3.17±0.13	21.15±0.85

4. Effect of storage time

The suitable preparation to be used in parenteral applications was the preparation of AA using T-80 with PL-40 and AA containing T-80 with PL-90 as co-surfactant. Its particles size was sufficiently small to be used in intravenous. Therefore, the preparation of AA containing T-80 with PL-40 and T-80 with PL-90 were kept at room temperature, 4°C in refrigerator and accelerated condition at 45°C in incubator. The particle size, pH, zeta potential and osmolality were evaluated both before and after storage for 3 months as shown in Table 29 and 30 respectively.

Effect of storage time on particle size

The SLN of AA using T-80 with PL-40 and T-80 with PL-90 as co-surfactant exhibited mean particle size in range of 20±0.52 nm and 22.5±0.32 after storage for 3 months, respectively which were relatively constant over the storage time at room temperature. The formulations kept at 4°C in refrigerator showed mean particle size in range of 22.3±0.29 nm and 25.7±0.33 nm after storage for 3 months. The particle sizes of both formulations at room temperature with 4°C in refrigerator were not different ($P<0.05$). This might be that co-stabilizers should exhibit sufficient affinity for the droplet surface to enable preparation of emulsion and for the particle surface in order to stabilize the nanosuspension and the combination of surfactant would not only better the system stability but also decrease the particle size because of its synergism.

Formulations of T-80 with PL-40 and T-80 with PL-90 displayed white precipitate in both formulations after 15 days of storage time at 45°C. This result might be attributed to the high temperature. During elevated temperature at 45°C, T-80 might diffuse from interface as a result of higher solubility in aqueous phase. The less of stabilizer adhering to the solid lipids led to particle aggregated about larger particle.

Effect of storage time on pH, zeta potential and osmolality

As shown in Table 29 and 30. The pH of AA containing T-80 with PL-40 and AA using T-80 with PL-90 as co-stabilizer slightly decreased with time. The decrease of pH were possibly resulted from the presence of free fatty acid liberated in system and the AA loss in activity. The lowest pH of 2.85 found in sample stored at room temperature was more acidic compared to stored at 4°C at each interval observation, which was in- significant difference ($P<0.05$). The result suggested that there were more fatty acids liberated in system stored at room temperature than kept in 4°C. The zeta potential was affected by alteration of pH. The zeta potential tended to increase over storage time. The zeta potential of such formulation storage at room temperature became more negative than that stored at 4°C. The osmolality values were rather constant. The ranges of osmolality were 0.028±0.001 Osmol/kg in T-80 with PL-40 and 0.024±0.002 Osmol/kg in T-80 with PL-90. This result indicated that the osmolality values seemed to be independent on storage time.

Table 29. The particle sizes, zeta potential, pH and osmolality of AA loaded GC-SLN of both before and after storage at room temperature for 3 months

Formulation	Before storage for 3 month				After storage for 3 months			
	Z-average±SD nm	Zeta potential±SD (mV)	pH±SD	Osmolality±SD (Osmol/kg)	Particle sizes±SD nm	Zeta potential±SD (mV)	pH±SD	Osmolality±SD (Osmol/kg)
T-80:PL-40 (8:2)	19.00±0.52	-13.6±1.25	3.31±0.01	0.025±0.002	22.50±0.32	-9.90±0.22	2.98±0.02	0.027±0.001
	20.50±0.25	-13.7±0.35	3.31±0.01	0.027±0.001	22.90±0.25	-10.50±0.10	2.85±0.01	0.027±0.002
	20.00±0.00	-13.0±0.20	3.32±0.01	0.027±0.001	22.20±0.10	-10.55±0.05	3.01±0.01	0.026±0.001
T-80:PL-90 (8:2)	26.50±0.32	-1.54±0.23	3.28±0.01	0.023±0.001	26.10±0.15	-4.50±0.25	3.05±0.01	0.0023±0.001
	25.00±0.20	-1.92±0.15	3.31±0.01	0.025±0.001	26.25±0.10	-4.45±0.10	2.98±0.01	0.024±0.001
	25.00±0.50	-2.54±0.05	3.30±0.01	0.023±0.001	26.05±0.35	-4.25±0.20	3.08±0.01	0.0024±0.002

Table 30. The particle sizes, zeta potential, pH and osmolality of AA loaded GC-SLN of both before and after storage in refrigerator for 3 months

Formulation	Before storage for 3 month				After storage for 3 months			
	Z-average±SD nm	Zeta potential±SD (mV)	pH±SD	Osmolality±SD (Osmol/kg)	Z-average±SD nm	Zeta potential±SD (mV)	pH±SD	Osmolality±SD (Osmol/Kg)
T-80:PL-40 (8:2)	21.20±0.10	-7.72±0.15	3.29±0.01	0.0028±0.001	22.10±0.05	-10.00±0.05	3.01±0.01	0.027±0.002
	21.00±0.22	-9.29±0.18	3.31±0.001	0.028±0.001	22.20±0.10	-9.50±0.10	2.98±0.01	0.028±0.001
	22.05±0.15	-7.20±0.05	3.30±0.01	0.028±0.002	22.70±0.10	-9.20±0.02	3.05±0.01	0.028±0.002
T-80:PL-90 (8:2)	27.00±0.25	-2.88±0.10	3.32±0.01	0.0025±0.001	25.30±0.12	-4.00±0.02	3.05±0.01	0.022±0.002
	26.00±0.10	-2.13±0.05	3.35±0.01	0.022±0.002	25.8±0.10	-3.00±0.15	3.12±0.00	0.025±0.001
	26.25±0.35	-3.75±0.10	3.33±0.00	0.021±0.001	25.9±0.22	-3.60±0.05	3.02±0.01	0.022±0.001

5. Measurement of cell viability

The formulation of AA loaded GC-SLN using T-80 with PL-40 and T-80 with PL-90 as co-surfactant were chosen. The in vitro study in cell cultures revealed that AA loaded GC-SLN had toxicity as shown in Figure 10, 11 respectively. The 50 % cytotoxicity dose was 176.57 μg/ml of T-80 with PL-40 and 166.55 μg/ml of T-80 with PL-90.

The cell toxicity might be explained in the term of AA concentration and surfactant composition. For AA concentration, this might be due to the high concentration in specific small area when AA loaded GC-SLN attached and adhered on cell membrane induced cell apoptosis. The much lower concentrations than these values were used to evaluate uptake studies for certain of a viable cell cultures during the experiment. In the term of surfactant, Schubert et al., (2005) revealed that increasing lecithin content indicating an increase in cytotoxicity with lecithin concentration.

This finding suggested that cell damaging group of the surfactant on the particle interface might be exposed in a more pronounced manner in comparison to the particle free emulsifier solution being present as a micellar solution. Moreover, at the interface of SLN probably both tween 80 and lecithin were present, and the combination of both emulsifiers at the interface might exhibit a synergism effect on the cytotoxicity of the preparation. However, the extent of Softisan® SLN cytotoxicity was not far from that in the aqueous phase.

This result disagreed with Scholer et al., (2001). They revealed that surfactant used as component of SLN, when tested alone at equivalent concentrations to these use in macrophage cell (Table 31). Therefore, cytotoxic effects, induced by SLN formulation containing specific surfactants, did not appear to accrue solely from the surfactants used.

Table 31. Viability of peritoneal macrophage after 20 hours of in vitro culture with surfactant solutions at a concentration of 0.01 % (equivalent to a SLN concentration of 0.1%)

Surfactant	Viability±SD
Medium	100.0±12.6
Poloxamine 908	90.2±3.0
Poloxamer 407	90.4±7.6
Poloxamer 188	87.3±0.9
Solutol HS15	93.3±5.3
Tween 80	90.9±5.5
Lipoid S75	86.0±3.3
Sodium cholate	92.8±4.9
Lipoid S75 Sodium cholate	85.3±3.6
Cetylpyridinium chloride	87.8±2.7

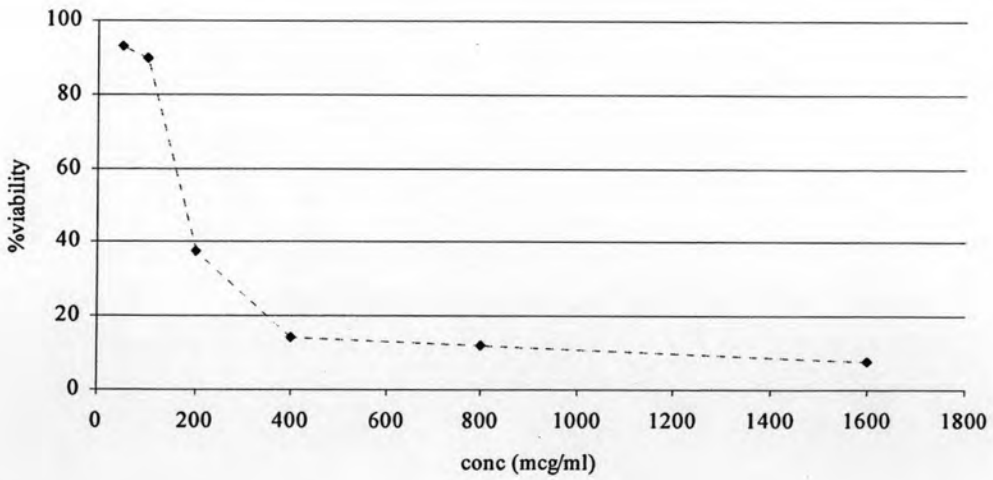


Figure10. Cell viability study of AA loaded GC-SLN using T-80 with PL-40 in ECV-304

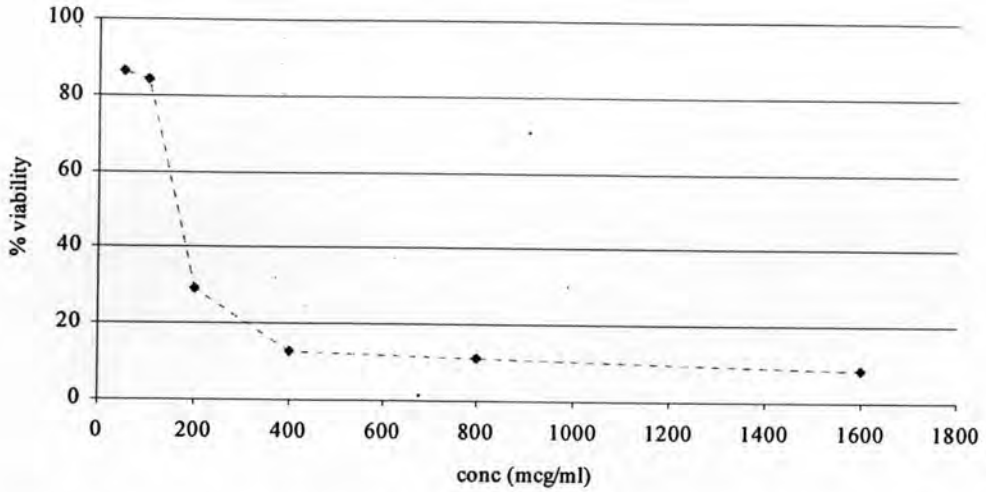


Figure 11. Cell viability study of AA loaded GC-SLN using T-80 with PL-90 in ECV-304

6. Transport and Cellular uptake of AA loaded SLN

The constant TEER value measured for 3 times was 125±5 ohm* cm^2 for ECV-304 cell monolayer. After adding AA loaded GC-SLN to upper compartment (200 µg/ml), the AA loaded GC-SLN was rapidly passed through the lower compartment within 10 minutes. The 40.45±0.75 and 35.86 µg/ml of AA loaded GC-SLN was measured from the lower compartment. This might be indicated the non integrity of ECV-304 cells monolayer.

However, the particles uptake was also studied to confirm the endocytosis of cells. The ECV-304 cells was evaluated by incubating with FITC solution, SLN without FITC solution and FITC loaded SLN for 2 hours.

Flow cytometer was used to count cells which had fluorescein intensity. The results are presented in Figure 15 for T-80 with PL-40 and Figure 16 for T-80 with PL-90, respectively. Both formulations showed high FITC intensity in term of % Gated.

The intensity of ECV-304 cells with AA loaded GC-SLN containing T-80:PL-40 and ECV-304 cells with AA loaded GC-SLN containing T-80:PL-90 exhibited low

intensity of both formulations. The FITC solutions with ECV-304 cells (Figure 14) showed intensity of FITC % Gated = 2.64±1.32 lower than AA loaded GC-SLN containing T-80:PL-90 % Gated = 4.28±0.28 (Figure 15) and AA loaded GC-SLN containing T-80:PL-40 % Gated = 3.74±0.48 (Figure 16).

From the results of AA loaded GC-SLN, T-80:PL-40 and T-80:PL-90 showed no different of FITC intensity ($P<0.05$) in both formulations. Increasing of FITC intensity might be that endocytosis mechanism could occur resulting in higher FITC uptake into cells.

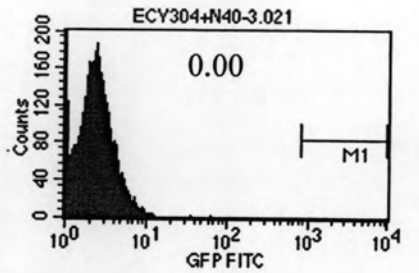
It might be that the added phospholipids synergism with polysorbate 80 at the interface would further improve the surface properties, since phospholipids have been shown to modify surface of SLN. Surface modifies SLN are potential delivery systems as biological macromolecules such as protein and peptides, and diagnostics could be tethered to the structures formed at the surface and their cellular trafficking improved. SLN could penetrate and could be used for intracellular delivery of some drugs (Attama et al 2007).

This result agreed with a previous research by Borchard et al., (1994). On in vitro experiments employing poly(methyl-2-C-methacrylate) nanoparticles. These particles were overcoated with several surfactants and their uptake by bovine brain microvessel endothelial cells was measured. The nanoparticle suspensions were incubated with the cell cultures and the radioactivity within cell cultures was determined after 30 min, 2 hours and 6 hours. The highest and fastest uptake (>300 % compared to uncoated controls) was observed after coating with polysorbate 80.

More recently, rat brain endothelial cells of the RBE4 cell line and poly (butyl cyanoacrylate) nanoparticles were used to study the nanoparticle uptake (Alyatdin et al., 2000). The poly (butyl cyanoacrylate) nanoparticles in the RBE4 cell in the experiment were produced by using FITC labeled nanoparticles over coated with polysorbate 80. The cells showed a punctuate appearance of fluorescene concentrated within the cell. In contrast, after treatment with nanoparticles at the same

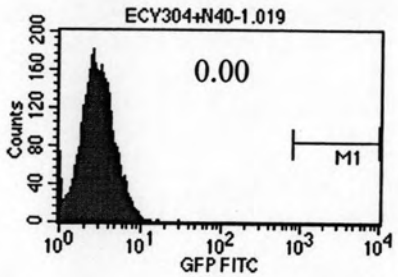
concentration without polysorbate 80, no fluorescene was observable within cells, even after the addition of a 10-fold higher concentrations of nanoparticles. After the addition of polysorbate 80 coated nanoparticles, fluorescene in the cells appeared rapidly after 10 min and reached a maximum after 48 min. The advantage of using nanoparticles for drug delivery resulted from their two basic properties. Firstly, due to their small size, nanoparticle uptake into even small capillaries and were taken up within cells, allowing an efficient drug accumulation at the targeted sites. Secondly, the use of biodegradable materials for nanoparticle preparation, allowed sustained drug release at the targeted site over period of day or even weeks after injection (Vinogradov et al., 2002).

Retarding the uptake of SLN the mechanism of uptake was highly dependant on several additional factors composition, particle size, charge ratio, time of incubation, activation of target cells, sterical stabilization and specific characteristics of homing devices. These aspects should also be kept in mind when optimizing drug carrier systems. Further investigation on the mechanism of nanoparticles uptake, and the kinetics of drug uptake and retention in the ECV-304 cells using nanoparticles as compared to a free drug in vivo would be useful to establish the efficacy of nanoparticles for various therapeutic applications (Labhasetwar 2002).



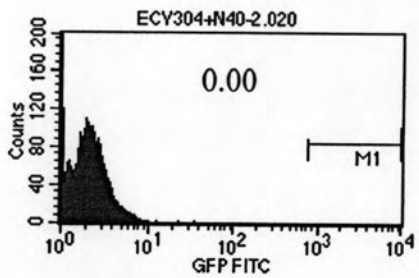
Sample ID: ECY304+N40-3
Gated Events: 17623
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	88.11	2.56
784, 9910	0.00	0.00	***



Sample ID: ECY304+N40-1
Gated Events: 17115
Total Events: 20000

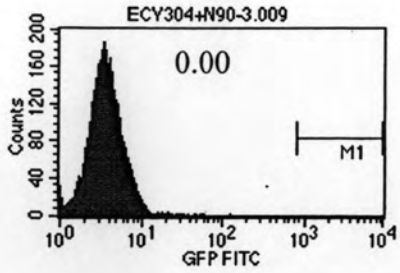
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	85.58	3.16
784, 9910	0.00	0.00	***



Sample ID: ECY304+N40-2
Gated Events: 9740
Total Events: 11655

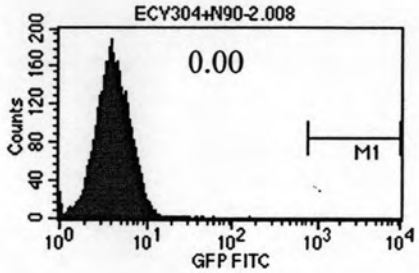
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	83.57	2.31
784, 9910	0.00	0.00	***

Figure 12. Cellular uptake of ECV-304 incubated with T-80:PL-40 particles.



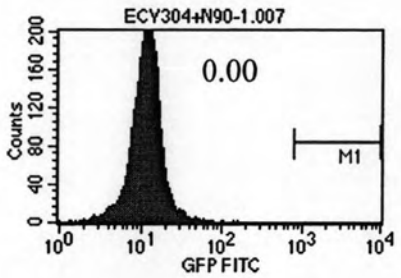
Sample ID: ECY304+N90-3
Gated Events: 17210
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	86.05	3.91
784, 9910	0.00	0.00	***



Sample ID: ECY304+N90-2
Gated Events: 17670
Total Events: 20000

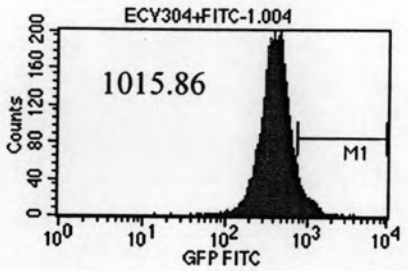
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	88.35	4.44
784, 9910	0.00	0.00	***



Sample ID: ECY304+N90-1
Gated Events: 18001
Total Events: 20000

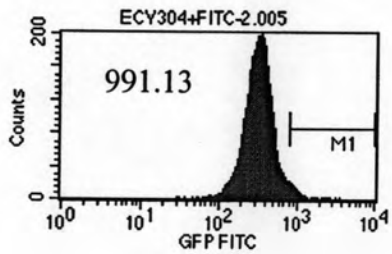
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	90.00	12.02
784, 9910	0.00	0.00	***

Figure13. Cellular uptake of ECV-304 incubated with T-80:PL-90 particles.



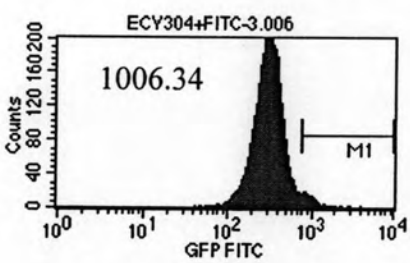
Sample ID: ECY304+FITC-1
Gated Events: 17370
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	86.85	430.51
784, 9910	4.74	4.12	1015.86



Sample ID: ECY304+FITC-2
Gated Events: 17428
Total Events: 20000

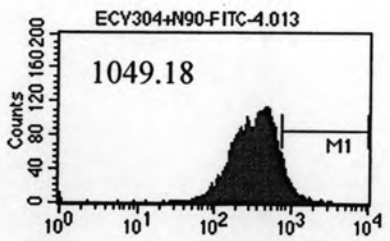
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	87.14	325.50
784, 9910	1.84	1.60	991.13



Sample ID: ECY304+FITC-3
Gated Events: 17706
Total Events: 20000

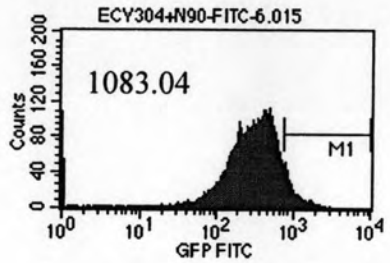
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	88.53	328.25
784, 9910	2.48	2.20	1006.34

Figure14. Cellular uptake of ECV-304 incubated with FITC.



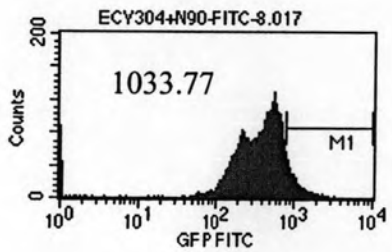
Sample ID: ECY304+N90-FITC-4
Gated Events: 16304
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	81.52	374.85
784, 9910	5.05	4.12	1049.18



Sample ID: ECY304+N90-FITC-6
Gated Events: 16768
Total Events: 20000

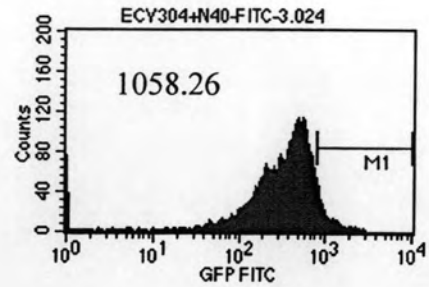
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	83.84	351.57
784, 9910	4.91	4.12	1083.04



Sample ID: ECY304+N90-FITC-8
Gated Events: 14305
Total Events: 20000

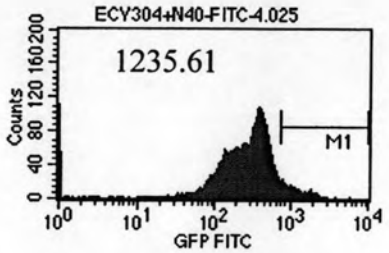
Left, Right	% Gated	% Total	Mean
1, 9910	100.00	71.53	410.89
784, 9910	6.44	4.61	1033.77

Figure15. Cellular uptake of ECV-304 incubated with T-80:PL-90 particles conjugated FITC.



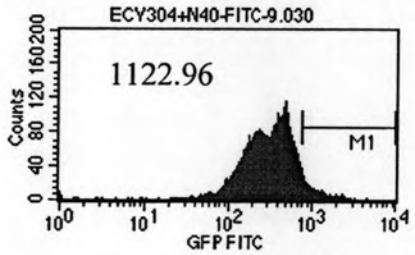
Sample ID: ECY304+N40-FITC-3
Gated Events: 14900
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	74.50	398.39
784, 9910	5.76	4.29	1058.26



Sample ID: ECY304+N40-FITC-4
Gated Events: 13665
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	68.33	336.25
784, 9910	4.97	3.40	1235.61



Sample ID: ECY304+N40-FITC-9
Gated Events: 14303
Total Events: 20000

Left, Right	% Gated	% Total	Mean
1, 9910	100.00	71.52	365.77
784, 9910	4.93	3.52	1122.96

Figure16. Cellular uptake of ECV-304 incubated with T-80:PL-40 particles conjugated FITC.