CHAPTER IV

RESULTS AND DISCUSSION

1. Formulation and preparation of lipid emulsion

In emulsion preparation, not only compositions of emulsion affected the physicochemical properties of emulsion, but the methods of preparation also influenced on the stability of emulsion. To produce stable emulsion, these factors should be considered.

1.1 Phospholipid as main emulsifier

Generally, phospholipid is used in the commercial lipid emulsion to stabilize the emulsion. Phospholipids obtain from animal (egg) or vegetable (soybean) sources. Soy phospholipids were purposed in the study because it is another source of natural phospholipids and also less expensive than egg phospholipid. However, the investigation of Schuberth and Wretlind (1961) revealed that egg phospholipids is supposed to have better tolerability than soy phospholipids. Emulsion stabilized by egg phopholipids was well tolerated without any clinical abnormalities, whereas severe falls in blood pressure and apnea were observed in emulsion stabilized by soy phospholipids. Yeadon et al. (1958) purposed that these effects were dependent on the degree of purity of the phospholipids that were used. Moreover, the differences in the degree of unsaturation between egg and soy phospholipids influence

on the stability of emulsion. Soy phospholipids contains a greater proportion of unsaturated bonds resulting in more susceptible to peroxidation. However, soy phospholipids can also form a stable emulsion, especially in the presence of further negatively charged phospholipids. However, the most commonly used emulsifier in parenteral emulsions is egg phospholipids because it provides a stable emulsion and is less toxic upon administration compared with the synthetic emulsifiers.

The comparison between the sources of phospholipid was done in emulsions Rx 1 to Rx 4 after autoclaving. Emulsions formulated using soy phospholipids as emulsifier with 5 cycles of homogenization (Rx 1 and Rx 2) showed creaming within 1 week. The emulsion separated into two parts, but it became reversible by simply shaking. Rx 3 and Rx 4, which used egg phospholipids as emulsifier with 10 cycles of homogenization, exhibited stable emulsion within 1-month storage. Soy phospholipids contained higher percent of PC (92%) than the amount found in egg phospholipids (98%). PC is zwitterionic surfactant exhibiting no charge at physiological pH. However, the emulsions containing SPC were prepared using less recycle times of homogenization. The instability found might possibly due to the effect of insufficient input energy. Egg phospholipids was used as main emulsifier for further preparation in stead of soy phospholipids and the homogenization cycle of 10 times was also a device of method of preparation.

1.2 Effect of recycle times of homogenization

The volume weight mean droplet size [D 4, 3] was mainly used for interpretation of particle size measurement in this study because it provides the mean value in volume which is the proportion of the oil droplets with respect to the whole internal phase volume.

The effect of recycle times on droplet size of emulsion was studied in emulsion Rx 3 and Rx 6 before autoclaving. The droplet sizes of formulation with 10% blended oil (1: 1 Miglyol 812 to soybean oil) emulsified by EPC (Rx 3) prepared upon increasing a number of homogenization cycles of Emulsiflex C50® from 3, 5, 7 to 10 cycles was investigated immediately after preparation. The results showed that the oil droplet sizes decreased when the cycles of homogenization increased. The same trend was found in the formulation using 10% blended oil, emulsified by EPC and SA (Rx 6). The results were shown in Table 10. It was noted that the pressure of the Emulsiflex C50® was fixed at 20,000 psi.

Although the emulsions were passed through the homogenizer with different cycles, the physical appearances of emulsion were similar to that emulsion which passed homogenizer for 10 times. The white homogeneous emulsion was observed in all formulations after preparation.

Table 10. The volume weighted mean size [D 4, 3] of the oil droplets when using different cycle times of homogenization of non-autoclaved emulsion containing 10% blended oil

Rx	Emulsifiers	Mean droplet size (μm)*			
		3 cycles	5 cycles	7 cycles	10 cycles
3	EPC	0.665±0.048	0.477±0.000	0.283±0.000	0.232±0.001
6	EPC+SA	11.486±3.431	0.465±0.002	0.312±0.002	0.263±0.000

* = Mean ±SD; n=3

The results showed that the cycle times of 10 provided the smallest droplet size and was used for further preparation.

The size reduction from recycling the emulsion through the homogenizer was similar to that obtained from the study of Davis et al. (1987) which indicated that a small particle size could be achieved using an optimal number of recycling times of homogenization that provided the size reduction. Many workers studied the effect of homogenization used in emulsion preparation. Washington and Davis (1987) revealed that the droplet size achieved from using Microfluidizer at pressure greater than 8000 psi in emulsion preparation were similar to the droplet size of commercial products, 10% and 20% Intralipid®. The study of Chansiri et al. (1999) showed that the average particle size of 0.28-0.4 μm were obtained using Microfluidizer at pressure of 20000 psi for 5 recycle times in emulsion preparation. The investigation of Bock et al. (1998) showed the effect of recycle time (1 to 10 recycle times) and pressure of homogenizer (4,800 to 13,200 psi) on droplet size of the

emulsion. The lower in average size of oil droplets was achieved using higher pressure in emulsion preparation and increasing the recycle time provided the more narrow size distribution.

1.3 Effect of steam sterilization

The effect of steam sterilization was studied by many workers (Chansiri et al., 1999; Groves et al., 1985; Herman and Groves, 1993; Washington and Davis, 1987). Elevated temperature accelerated the hydrolysis of the oil and phospholipid, corresponding to free fatty acids formed which led to a decrease in pH of emulsions (Chansiri et al., 1999). The zeta potential would be expected to become more negative following autoclaving. This might be the result of an increase in free fatty acids concentration and/ or the redistribution of the excess phospholipid, which presented as mutilamellar liposomes in the aqueous phase, to the interface during autoclaving (Groves and Herman, 1992). It was also found that the existence of these phospholipid reservoirs makes fat emulsions exhibit good stability when expose to high temperature as autoclaving (Lucks et al., 2000).

Idson (1988) and Klang and Benita (1994) revealed that the pH of the emulsion normally decreased after autoclaving or upon storage and the toxicity of emulsions could be correlated to free fatty acids levels in the product. The rate of free fatty acids production was minimal if the pH of the emulsion was between 6 and 7, after sterilization.

A series of emulsions containing different types and amounts of oil as well as emulsifiers (Rx 3 to Rx 16), was prepared and the effect of steam sterilization on the physicochemical

properties of emulsions was investigated. The emulsions were examined immediately before and after autoclaving.

Physicochemical properties

From the experiments, it was found that a slight change in the white color of emulsion to soymilk-like emulsion after autoclaving was observed in emulsion containing SA (Rx 6-8 and Rx13-16). The physical appearance of other formulations (Rx 3-Rx 5 and Rx 9-Rx 12) did not change after autoclaving. There was no sign of instability observed in all systems both before and after autoclaving except for Rx 11 and Rx 12, which a few oil droplets were visibly observed on the surface.

From the results, it was found that 10 % blended oil emulsion which stabilized by EPC, EPC with T80, and EPC with PG exhibited an increase in oil droplet size after autoclaving while others showed no obvious change in oil droplet size. For 10 % soybean oil system, except for emulsion stabilized by EPC alone, no change in mean droplet size was found after autoclaving. The results are shown in Table 11. In the presence of other surfactant, SPC was able to be used instead of EPC.

The steam sterilization method caused an increase in the mean droplet size (Chaturvedi et al., 1992). The emulsifying ability was found to be altered by a reduction in an aqueous solubility resulting in final phase separation (Benita and Levy, 1993). High temperature has a great effect on the properties of interfacial film, especially if nonionic emulsifiers are used. Nonionic

emulsifiers are very sensitive to temperature. As temperature increases, the hydrophilic chain of emulsifier loses waters and becomes more lipophilic (Weiner, 2000a). The HLB values are completely changed and tend to be decreased. The lower in HLB value results in instability of the emulsion. It was found that the use of co-emulsifier seemed to improve the emulsifier efficiency. It could imply that in some cases, the main emulsifier did not provide enough stable formulation as the weak, thin film around the droplet was easily broken offer autoclaving. The co-emulsifier was needed to enhance the physical stability of the emulsion (Sworbrick and Boylan, 1992). The system contained a mixture of emulsifiers could form stronger interfacial film and were superior to those formed using a single emulsifier (Lund, 1994). Jumaa and Müller (1998) suggested that nonionic surfactants, i.e., Cremophor EL, poloxamer 188, Solutol HS15, and Tween 80 were usually combined with phospholipids to improve the stability of the surfactant layer. A close-packed mixed film was obtained by combination of emulsifiers which conferred stearic stability to the dispersed droplets.

The negativity of surface charge of oil droplets increased after sterilization in all emulsions containing EPC, EPC with T80 and EPC with PG. The systems containing EPC with SA, EPC with T80 with SA and SPC with T80 with SA, the positively zeta potential became less positive (Table 12).

Table 11. Particle size of unautoclaved or autoclaved emulsions formulated with 10% blended oil (Rx 3, 6, 9, 11, 13 and 14) and 10% soybean oil (Rx 4, 7, 10, 12, 15 and 16) using various emulsifiers

Rx	Emulsifiers	Particle size of emulsion (μm)*	
		Before autoclaving	After autoclaving
3	EPC	0.232±0.001	0.775±0.024
6	EPC+SA	0.263±0.000	0.256±0.000
9	EPC+T80	0.192±0.000	1.582±0.004
11	EPC+PG	0.673±0.149	0.857±0.003
13	SPC+T80+SA	0.195±0.000	0.195±0.000
14	EPC+T80+SA	0.197±0.000	0.198±0.000
4	EPC	0.383±0.001	0.577±0.071
7	EPC+SA	ND	0.497±0.026
10	EPC+T80	0.200±0.000	0.209±0.000
12	EPC+PG	0.791±0.020	0.787±0.011
15	SPC+T80+SA	0.198±0.000	0.197±0.001
16	EPC+T80+SA	0.202±0.000	0.201±0.001

* = Mean±SD; n = 3

ND = not determined

Table 12. Zeta potential of unautoclaved or autoclaved emulsions formulated with 10% blended oil (Rx 3, 6, 9, 11, 13 and 14) and 10% soybean oil (Rx 4, 7, 10, 12, 15 and 16) using various emulsifiers

Rx	Emulsifiers	Zeta potential (mV) *of emulsion	
		Before autoclaving	After autoclaving
3	EPC	-8.03±1.17	-25.89±0.72
6	EPC+SA	50.01±2.76	39.49±1.31
9	EPC+T80	-13.42±2.44	-10.79±3.31
11	EPC+PG	-18.85±2.04	-20.51±2.03
13	SPC+T80+SA	22.96±0.54	20.75±3.66
14	EPC+T80+SA	25.15±0.75	22.85±8.77
4	EPC	-26.39±1.34	-15.51±4.13
7	EPC+SA	31.67±1.66	6.20±5.60
10	EPC+T80	-14.79±0.76	-14.16±1.49
12	EPC+PG	-17.81±2.39	-30.39±2.11
15	SPC+T80+SA	20.31±1.25	11.37±1.28
16	EPC+T80+SA	23.17±2.90	10.42±1.98

* = Mean±SD; n = 4

Although the zeta potential of emulsion in some systems did not exhibit high zeta potential (>30 mV), there was no any instability observed in emulsions. No oil droplet was observed on the surface of the emulsion after autoclaving, except for Rx 11 and Rx 12. The lowering of zeta potential was also observed in the study of Chansiri et al. (1999) who formulated the emulsion using a mixture of

oil that consisted of Miglyol 812 and olive oil at the amount of 50: 50, egg phospholipid as emulsifier. The emulsions were added with anionic fractions of egg phospholipid (PA, PG and PI) and, also, cationic emulsifier (SA) to modify the surface charge of the oil droplets. They found that the zeta potential of emulsion stabilized by egg phospholipid was changed from -10.50 ± 2.04 to -26.60 ± 0.80 mV after autoclaving. The system stabilized by addition of PG with egg phospholipids was found to exhibited higher negatively zeta value than that obtained from this study. They showed that zeta potential of emulsion incorporating PG was changed from -39.40 ± 1.20 to -40.30 ± 0.80 mV after autoclaving. The emulsion system using 11.1 mM of SA also showed a decrease in zeta potential from 64.90mV to 31.50 mV after autoclaving. Similarly, Chansiri et al. (1999) revealed the effect of autoclaving on zeta potential that the zeta potential became more negative following autoclaving.

The pH of all formulations decreased after autoclaving due to the hydrolysis of PC leading to the formation of free fatty and the chemical changes in phospholipid constituents (Idson, 1988; Herman and Groves, 1993; Washington and Davis, 1987). The results are shown in Table 13.

It could be concluded that the physicochemical properties of emulsion were changed after autoclaving. The use of optimal emulsifier could minimize the physicochemical changes leading to instability. Many investigations revealed that using a combination of emulsifiers, ionic lipids or nonionic emulsifiers provided a synergistic effect on stability, which will be discussed in the following section.

Table 13. pH of unautoclaved or autoclaved emulsions formulated with 10% blended oil (Rx 3, 6, 9, 11, 13 and 14) and 10% soybean oil (Rx 4, 7, 10, 12, 15 and 16) using various emulsifiers

Rx	Emulsifiers	pH of emulsion	
		Before autoclaving	After autoclaving
3	EPC	8.06	6.21
6	EPC+SA	8.01	6.25
9	EPC+T80	8.04	6.66
11	EPC+PG	8.02	7.44
13	SPC+T80+SA	8.04	6.34
14	EPC+T80+SA	8.04	6.22
4	EPC	8.01	6.68
7	EPC+SA	8.01	5.54
10	EPC+T80	8.01	6.68
12	EPC+PG	8.05	7.26
15	SPC+T80+SA	8.07	6.62
16	EPC+T80+SA	8.03	6.47

1.4 Composition of emulsion

The effect of the amount of oil and surfactant on physicochemical properties and stability of emulsion at different storage time was investigated.

Physicochemical properties

The zeta potential in emulsion containing 20% oil was not markedly different from that in 10% emulsion. Nonetheless, the particle size found in 20 % emulsion was almost twice larger than of 10 % emulsions. It is possible that the large particle size found in 20% emulsion might lead to less stable formulations compared to the 10% emulsions (Table 14).

Table 14. The physicochemical characteristics of 10% and 20% soybean oil emulsions stabilized by EPC and EPC with SA after stored at 4°C for 4 weeks

Rx	% Oil	Emulsifiers	ζ (mv)*	D(4,3) (μm)♦	pH
4	10	EPC	-19.86±2.79	0.409±0.001	6.77
7	10	EPC+SA	12.22±3.35	0.388±0.000	5.75
5	20	EPC	-16.53±2.35	0.764±0.00	6.84
8	20	EPC+SA	18.33±1.52	0.580±0.001	5.85

* = Mean ±SD; n=3

♦ = Mean ±SD; n=4

Physicochemical properties

The physical stability of autoclaved emulsions formulated using 10% oil and EPC as main emulsifier was observed. It was found that emulsions emulsified by EPC alone (Rx 3 and

Rx 4) had a few oil droplets on the surface while creaming occurred in blended oil emulsion (Rx 3) after 4 week storage. Blended oil/soybean oil emulsions (Rx6 and Rx 7) stabilized by EPC+SA exhibited physically stable emulsion. For emulsion containing blended oil/soybean oil stabilized with EPC+T80 (Rx9 and Rx 10), creaming and oil film was finally occurred, respectively. Similarly, Rx11 and Rx 12 using EPC+PG as emulsifiers showed a few oil droplets and or the oil film showing instability of systems. Additionally, stable emulsions were observed in emulsions stabilized by a mixture of phospholipids, nonionic surfactant and cationic lipid (EPC+T80+SA or SPC+T80+SA). The results are shown in Table 15.

Table 15. The physical stability of emulsions using different oils and emulsifiers

Rx	Oil system	Emulsifiers	Physical appearance of emulsion			
			24 hours	1 week	4 weeks	16 weeks
3	10% Blended oil	EPC	White emulsion	White emulsion	Creaming	Creaming
6		EPC+SA	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion
9		EPC+T80	White emulsion	Oil droplets on the surface	Creaming	Creaming
11		EPC+PG	White emulsion	White emulsion	Oil droplets on the surface	Oil droplets on the surface
13		SPC+T80+SA	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion
14		EPC+T80+SA	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion
4	10% Soybean oil	EPC	White emulsion	White emulsion	Few oil droplets on the surface	Creaming
7		EPC+SA	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion	Creaming
10		EPC+T80	White emulsion	Oil film on the surface	Oil film on the surface	Oil film on the surface
12		EPC+PG	White emulsion	Oil droplets on the surface	Oil film on the surface	Oil film on the surface
15		SPC+T80+SA	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion
16		EPC+T80+SA	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion	Soy milk-like emulsion

When only pure phospholipids (PC) were used, emulsions showed poor physical stability. It can be explained that PC which exhibits no charge at neutral pH, hence oil droplets are prone to flocculate or coalesce from attractive force (Chansiri et al, 1999), while other emulsifying agents are likely to produce repulsive forces between oil droplets and reduce the coalescence.

Changing in physicochemical characteristics of the emulsion resulting in emulsion instability. The stability of the emulsion can be easily monitored by measuring the changes in the droplet size. Table 16 shows the changes of particle size during storage for up to 1 month in refrigerator and Table 17 shows the percent of particle size greater than 1 μm found in the emulsion.

It was noted that all emulsions studied had a various size distribution dependent upon the emulsion compositions. The size distribution of soybean oil emulsion stabilized by EPC with SA (Rx 7) exhibited wider distribution than that found in a blended oil emulsion (Rx 6). Emulsion stabilized by EPC with PG (Rx 11and Rx 12) had the widest distribution. Emulsions using EPC, T80 and SA and SPC, T80 and SA (Rx13-16) exhibited a similar size distribution to that obtained from EPC with T80 (Rx 9 and Rx 10) emulsifier system (See Appendix D).

Table 16. Effect of emulsifier and co-emulsifiers on droplet size [D (4,3)] of emulsions prepared using 10 % blended oil or soybean oil after storage in refrigerator for 24 hours, 1 and 4 weeks

Rx	Oil	Emulsifiers	Mean droplet size (μm)*		
			24 hours	1 week	4 weeks
3	10% Blended oil	EPC	0.775±0.024	ND	ND
6		EPC+SA	0.256±0.000	ND	ND
9		EPC+T80	1.582±0.004	1.251±0.094	1.740±0.004
11		EPC+PG	0.857±0.003	0.698±0.013	0.671±0.008
13		SPC+T80+SA	0.195±0.000	0.194±0.000	0.194±0.000
14		EPC+T80+SA	0.198±0.000	0.196±0.000	0.196±0.001
4	10% Soybean oil	EPC	0.577±0.071	ND	0.409±0.001
7		EPC+SA	0.497±0.026	ND	0.388±0.000
10		EPC+T80	0.209±0.000	0.207±0.001	0.212±0.001
12		EPC+PG	0.787±0.011	0.786±0.049	0.674±0.013
15		SPC+T80+SA	0.197±0.001	0.197±0.000	0.197±0.000
16		EPC+T80+SA	0.201±0.001	0.200±0.000	0.202±0.001

* = Mean ±SD; n=3

ND = not determined

Table 17. The percent of emulsion having the particle size bigger than 1 μm

Rx	Oil	Emulsifiers	Amount of droplet size of emulsion greater than 1 μm*		
			24 hours	1 week	4 weeks
3	10% Blended oil	EPC	13.35%	ND	ND
6		EPC+SA	0.00%	ND	ND
9		EPC+T80	64.87%	57.02%	71.35%
11		EPC+PG	14.73%	13.61%	12.61%
13		SPC+T80+SA	0.00%	0.00%	0.00%
14		EPC+T80+SA	0.00%	0.00%	0.00%
4	10% Soybean oil	EPC	10.98%	ND	6.57%
7		EPC+SA	8.93%	ND	5.40%
10		EPC+T80	0.00%	0.00%	0.00%
12		EPC+PG	20.04%	19.26%	17.58%
15		SPC+T80+SA	0.00%	0.00%	0.00%
16		EPC+T80+SA	0.00%	0.00%	0.00%

* = Mean ±SD; n=3

ND = not determined

For longer storage time (almost 4 months), the emulsions using EPC, EPC withT80, and EPC with PG had almost 3 times wider size distribution compared to those stored for 1 month (See Appendix D).

The emulsions formulated using blend or soy oil and emulsified with EPC+T80+SA (Rx 14 and Rx 16) or SPC+T80+SA (Rx 13 and Rx 15) exhibited stable emulsion with the mean droplet size of emulsion approximately 0.2 μm with no particle size greater than 1 μm was observed. Using EPC+T80 as emulsifiers, the smaller size (0.209 μm) of emulsion was found when 10% soybean oil was used as oil phase. In contrast, blended oil emulsion had almost 10 times larger in droplet size with approximately 70 % particles greater than 1 μm. It might be explained as the difference in viscosity between soybean oil and blended oil which affect the physical instability of emulsion. Surprisingly, using EPC+SA as emulsifiers, emulsions containing 10% blended oil provided smaller droplet size than that containing soybean oil. However, the results were determined only after 24 hours storage for 10% blended oil emulsion. Emulsion prepared using EPC alone exhibited large oil droplets in all emulsions.

The changes in particle sizes of emulsion as a period of time are shown in Figure 8. The emulsions stabilized by EPC alone were unstable and became creaming within 6 weeks. The instability was observed in emulsions containing soybean oil and blended oil, although the droplet size of emulsion exhibited no significant changes. Therefore, the use of EPC alone was insufficient to stabilize emulsion investigated.

Neither creaming nor coalescence was occurred in emulsions emulsified by EPC with SA and the sizes were rather stable. The 10% blended oil emulsion emulsified with EPC with T80 (Rx 9) was not stable while emulsion containing soybean oil with the same emulsifiers (Rx 10) was stable. The particle sizes above 1 μm were found in blended oil emulsion (Rx 9) immediately after steam sterilization was 64.87%, while absence of the particle size greater than 1 μm was observed in

system using soybean oil (Rx 10). Undoubtedly, blended oil emulsion had high tendency to flocculate resulting in creaming. There were only a few studies using the nonionic surfactant plus phospholipids system to stabilize the emulsion. One of studies included the study of Kan et al. (1999) which investigated a synergistic effect of EPC and Tween80 on emulsion stability. The result indicated that emulsion made from the optimum weight ratio of 1: 1 EPC to T80 was found to pose small droplet size and good stability. Also, the studies of Jumaa and Müller (1998), Lund (1994) and Yamagachi et al. (1995) found that the emulsion containing lecithin as primary emulsifier and nonionic surfactant as secondary emulsifier had smaller particle size and more stability than emulsion containing only lecithin. A synergistic effect of Tween 80 and egg phospholipids was observed which resembled the behavior of polymer such as poloxamer (Kan et al., 1999). The stability of emulsions in the presence of nonionic polymer has been interpreted in term of stearicstabilization arising from the presence of the adsorbed chains of emulsifier. For nonionic surfactant, osmotic (solvation) forces and entropic effects involved in the stability of emulsions. The osmotic effect occurs as each droplet comes closer and the concentration is increased resulting in osmotic repulsion (Davis et al., 1985). Entropic effects occurred as the result from the overlapping of hydrophilic chain appear on the droplets. For emulsion containing EPC+T80+SA and SPC+T80+SA as a mixture of emulsifiers, emulsions were stable regardless of the oil used. No particles greater than 1 μm were found through the storage time. Similarly, Korner et al (1994) revealed the molecular interactions among phospholipids, poloxamer (nonionic surfactant) and stearylamine in emulsion. It was found that the existence of molecular interactions among these agents prolonged stability of the o/w emulsion. These interactions result in molecular arrangements in the

interfacial regions leading to an effective combined steric-electrical energy barrier capable of preventing droplet coalescence upon random collision.

The study on the effect of time on particle size distribution is perhaps the single most important test to evaluate emulsion stability. Typically, emulsions showed the change in their size distributions with time in that the distribution was polydispersed (See Appendix D for details).

The charges of the zeta potential on emulsion droplets are mainly due to the charge of surfactant coating droplets. If an anionic surfactant is used i.e., PG, zeta potential shows negative value. Positive zeta potential can be achieved by addition of cationic surfactant i.e., SA. The emulsion can be stabilized by electrostatic repulsive force by these charged molecules. When only nonionic surfactant stabilizes an emulsion, no electrostatic charges present to stabilize droplet. Thus, nonionic surfactants stabilize emulsion by osmotic (solvation) forces and entropic effects, as called stearic stabilization.

The results shown in Table 18 indicated that zeta potential of emulsion was changed with the storage time. In emulsion emulsified with EPC alone with 10% soybean oil, it was found that the negative surface charge of oil droplet increased. The surface charge of oil droplets was reversed to positive value for the systems using SA as co-emulsifier (Rx 6-8 and Rx 13-16). An increase in more positive value of zeta potential was observed in 10% soybean oil formulating using SA as co-emulsifier, while the corresponding 10% blended oil emulsion exhibited a decrease in positive value of zeta potential. Emulsions using PG as co-emulsifier (Rx 11 and Rx 12), the zeta

potential of 10% emulsions were approximately the same which were in the range of –20.20 to –21.32 mV for emulsions containing blended oil and –28.45 to –30.44 mV for emulsions containing soybean oil.

Table 18. Zeta potential of emulsions prepared using 10% blended oil or 10% soybean oil after storage in refrigerator for 24 hours, 1 and 4 weeks

Rx	Oil	Emulsifiers	Zeta potential (mV) of emulsion		
			24 hours	1 week	4 weeks
3	10% Blended oil	EPC	-25.89±0.72	ND	ND
6		EPC+SA	39.49±1.31	ND	ND
9		EPC+T80	-10.79±3.31	-9.72±3.13	-14.59±0.31
11		EPC+PG	-20.51±2.03	-20.20±1.75	-21.32±1.31
13		SPC+T80+SA	20.75±3.66	15.13±2.53	8.49±2.69
14		EPC+T80+SA	22.85±8.77	12.68±2.01	16.39±0.76
4	10% Soybean oil	EPC	-15.51±4.13	ND	-19.86±2.79
7		EPC+SA	6.20±5.60	ND	12.22±3.35
10		EPC+T80	-14.16±1.49	-7.98±1.10	-12.31±1.69
12		EPC+PG	-30.39±2.11	-28.45±1.83	-30.44±2.63
15		SPC+T80+SA	11.37±1.28	15.98±2.04	15.23±0.96
16		EPC+T80+SA	10.42±1.98	16.10±5.61	20.46±3.74

* = Mean ±SD; n=4

ND = not determined

Normally, the absolute value of high zeta potential or more than 30 mV, positive or negative, should be achieved in order to ensure a high energy barrier, which causes the repulsion of adjacent droplets and the formation of a stable emulsion (Benita and Levy, 1993).

The study of Chansiri et al. (1999) demonstrated the relation between emulsion stability and the zeta potential. The emulsions which the zeta potential were in the range of -32.7 to -39.4 mV by incorporating of anionic emulsifier (PA, PG and PI), or approximately 31.5 mV by the use of SA showed the stable emulsions in spite of exposure to high temperature as autoclaving. While the system using egg phospholipids and that incorporated PC had the zeta potential of –10.5 and –12.2 mV respectively, instability of emulsion occurred. Oil droplets were visibly observed after autoclaving.

The changes in zeta potential of emulsions after storage for 4 months were determined and drawn in Figure 8. The positive value of zeta potential of emulsion using EPC+SA, EPC+T80+SA, and SPC+T80+SA remained nearly constant with slight decrease in the 16-week storage, while that of emulsion containing EPC, EPC+T80, and EPC+PG as emulsifiers seemed to increase to more negative at the end of the storage.

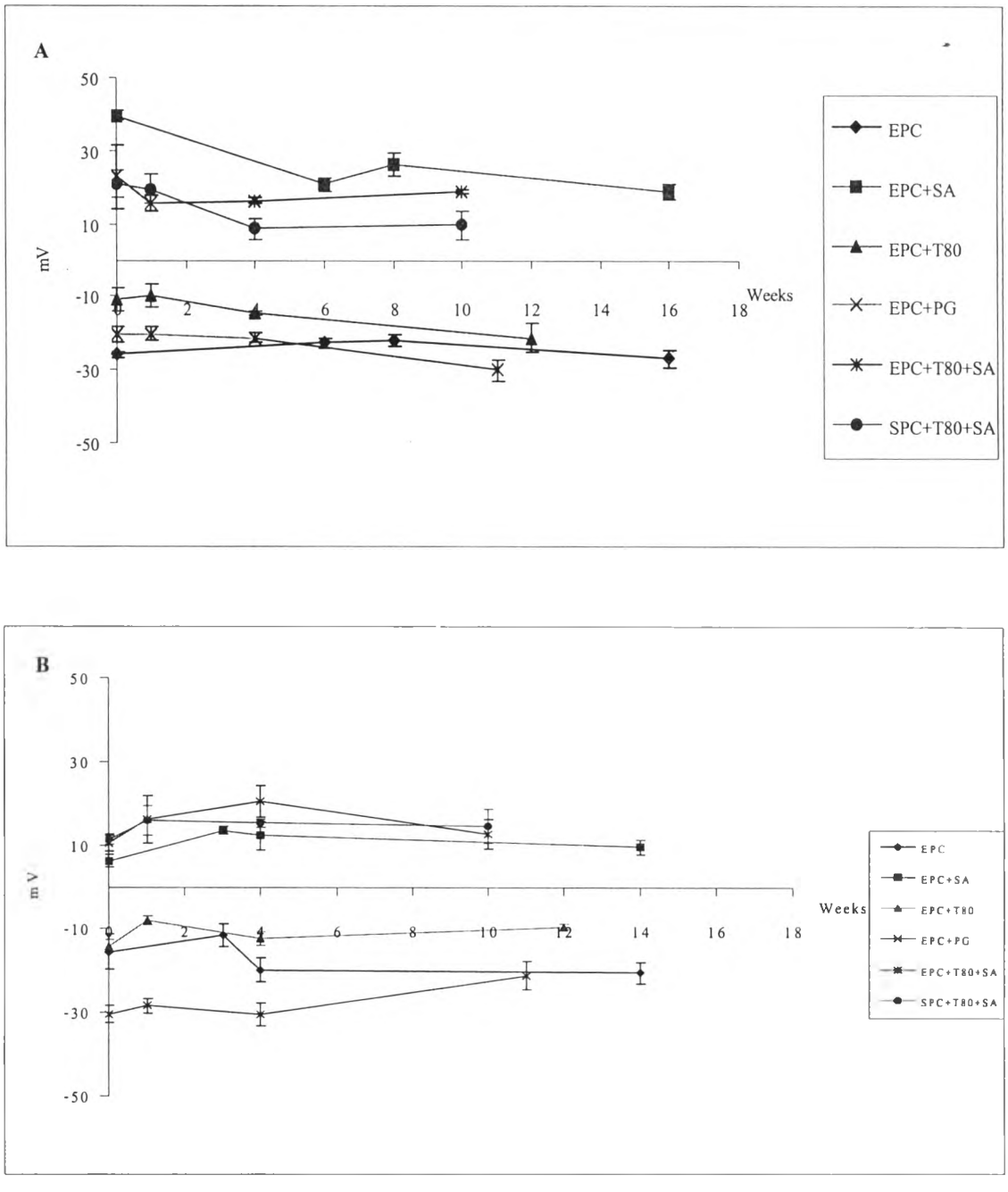

Figure 8. Zeta potential of (A) 10 % blended oil emulsions and (B) 10% soybean oil emulsions using various emulsifiers at different storage times

The results showed that pH of all formulations remained stable and were in the range of 5.54 to 7.84 and no further reduction was noted following 1 month of storage (Table 19). The decrease in pH is known to affect significantly the physical stability of the emulsion since it reduces the ionization of some phospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid, resulting in a diminution of the negative zeta potential value (Bangham, 1968; Davis, 1982; Rydhag and Wilton, 1981). Moreover, changes in pH can change the properties of an ionic emulsifier.

The pH values of all emulsions stored for 4 weeks were also rather stable, however, the lower pH compared to initial pH was clearly found in systems containing EPC+T80+SA and SPC+T80+SA as emulsifiers.

From the physical stability and physicochemical characteristics investigated, it was indicated that formulations with 10% soybean oil stabilized by egg or soy phospholipid, stearylamine and Tween 80 (EPC+T80+SA and SPC+T80+SA) provided stable emulsions for at least 4 months with suitable physicochemical properties. The emulsion was stabilized by a combination of electrostatic repulsive force produced by ionic surfactant (SA) and stearic repulsive force produced by non-ionic surfactant (T80). Stearylamine changed the surface charge of the oil droplets to positive value while Tween 80 stabilized the emulsion by stericeffect achieved from polyoxyethylene chains of its molecule. Combination of these forces enhanced emulsion stability. Phospholipid stabilized systems by long range repulsive electrostatic forces and short range repulsive hydration forces. An emulsion was

Table 19. pH of emulsions using 10 % blended oil or 10% soybean oil after storage in refrigerator for 24 hours, 1 and 4 weeks

Rx	Oil	Emulsifiers	pH of emulsion		
			24 hours	1 week	4 weeks
3	10% blended oil	EPC	6.21	ND	ND
6		EPC+SA	6.25	ND	ND
9		EPC+T80	6.66	6.84	7.12
11		EPC+PG	7.44	7.51	7.84
13		SPC+T80+SA	6.34	6.62	6.03
14		EPC+T80+SA	6.22	5.87	5.91
4	10% soybean oil	EPC	6.68	ND	6.77
7		EPC+SA	5.54	ND	5.75
10		EPC+T80	6.68	6.79	6.95
12		EPC+PG	7.26	7.23	7.17
15		SPC+T80+SA	6.62	6.81	6.40
16		EPC+T80+SA	6.47	6.69	6.19

ND = not determined

stabilized by the addition of emulsifying agents which lowered the interfacial tension and formed a film at the oil-water interface which acted as a mechanical barrier to droplet coalescence.

In this investigation, blended oil emulsions seemed to be unstable compared to soybean oil emulsions. Medium chain triglycerides (MCT) are known to be oxidized more quickly and

more completely (Lucks et al, 2000) and probably more sensitive to hydrolysis than long chain triglyceride (LCT), soybean oil. Correspondingly, the degradation products as free fatty acid are more soluble in water, and should contribute to a further decrease in pH of emulsion (Bangham, 1968; Davis, 1982; Rydhag and Wilton, 1981). As can be seen in the present study, emulsion prepared using soybean oil exhibited better physical stability than that using a mixture of soybean oil and Miglyol 812. In contrast, the study of Lucks et al (2000) found the better stability of MCT/LCT emulsion than LCT emulsions in TPN preparation.

The physicochemical properties (droplet size and zeta potential) of formulated emulsions containing 10% and 20% soybean oil were compared with the commercial product, Intralipid® 10% and 20 %, which the ingredients labeled consists of 10% soybean oil, 1.2% egg phospholipids and 2.5% glycerol. The physicochemical characteristics of commercial products were measured at least 6 months before the expiry date. The investigation revealed that the droplet size of commercial product containing 10% or 20% oil is approximately equally, 0.303 ± 0.008 and 0.344 ± 0.001 μm, respectively. When the amount of oil increased, the zeta potential exhibited more negatively value, -24.22 ± 5.07 and -31.84 ± 4.30 mV for 10% and 20% Intralipid, respectively. While the formulated emulsions with the same compositions showed almost twice bigger in droplet size when the amount of oil increased from 10% to 20% (0.409 ± 0.001 and 0.764 ± 0.000 μm, respectively). Moreover, the lower in zeta potential was also observed. The 10% and 20% prepared emulsions showed a slightly different in zeta potential value (-19.86 ± 2.79 and -16.53 ± 2.35 mV, respectively)

It was assumed that stabilizer as oleic acid might be added in the commercial product to protect emulsion from oxidation and increase the electrostatic repulsive force (Benita and Levy, 1993; Chansiri et al., 1999). Oleic acid was found to increase the surface potential of the mixed monolayer among phospholipid-poloxamer monolayers which prolonged the stability of o/w emulsion formulation (Benita and Levy, 1993). However, oleic acid is not used in the present study because the excess use of oleic acid might induce haemolysis.

The method of addition of oil and water phases, rate of mixing, temperature of each phase, and the rate of cooling after mixing of the phases are considerable to have some effects on the droplet size distribution, and stability of the final emulsion (Hashida et al, 1977). In addition, storage temperature should also be concerned. The work of Klang et al. (1994) revealed that the emulsion stored at $4^{\circ}C$ appeared to be more stable than emulsion stored at room temperature or higher. High temperature accelerated the chemical reaction occurring in oil and phospholipid (oxidation of oil and hydrolysis of emulsifier) and also resulted in destroy the physical stability of the emulsion (Benita and Levy, 1993; Rosoff, 1988). The physicochemical changes and physical instability were most pronounced for the emulsion stored at $40^{\circ}C$ (Davis et al., 1985).

2. Formulation of lipid emulsion containing oil-soluble vitamins.

2.1 Effect of oil-soluble vitamins on emulsion stability

The results from previous studies demonstrated that soybean oil emulsions using SPC+T80+SA (Rx 15) and EPC+T80+SA (Rx 16) as emulsifiers exhibited emulsion stability through the storage time observed. Lipid emulsions containing oil-soluble vitamins were prepared using soybean oil as oil phase and EPC+T80, EPC+T80+SA and EPC+T80+PG as emulsifiers to study the effect of surface charge on vitamin stability. All emulsions were prepared by the method described in Chapter III, part 2.

The effect of the methods of sterilization on the physical stability and physicochemical properties of emulsions was investigated by measuring the droplet size, zeta potential and pH after storage in the refrigerator for 24 hours, 1 and 4 weeks. The results are reported in Table 20.

Sterility test

The sterility test was assured to the sterility of emulsion after sterilization by filtration through 0.22 μm membrane. The filtrated systems were tested to evaluate the number of viable aerobic microorganisms present and for freedom from designated microbial species in pharmaceutical articles of all kinds. The results indicated that all formulations were free from restrict

viable microorganisms and molds. Thus, the filtration was the alternative sterilization method for parenteral lipid emulsion.

It was noted that filtration might exclude the droplet sizes greater than 0.22 μm from the emulsion. However, the mean droplet size of the formulated emulsions was smaller than 0.22 μm (discussed later).

Physicochemical properties

The physical appearance of emulsions containing vitamins formulated is reported in Table 20. It was found that all systems provided stable emulsion for at least 4 weeks. The method of sterilization, either autoclaving or filtration showed no difference in the physical stability of emulsions containing vitamins. However, little difference in color between emulsions was observed. Only, lipid emulsion containing stearylamine as co-emulsifier exhibited the color of slightly yellowish and was referred to as a soymilk-liked emulsion.

Table 20. Physical appearance of 10% emulsions containing oil-soluble vitamins after storage for 24 hours, 1 and 4 weeks

Rx	Emulsifiers	Sterilization	Physical appearance of emulsion		
			24 hours	1 week	4 weeks
A	EPC+T80	None	White emulsion	White emulsion	White emulsion
		Filtration	White emulsion	White emulsion	White emulsion
		Autoclaving	White emulsion	White emulsion	White emulsion
B	EPC+T80+PG	None	Soymilk-like emulsion	Soymilk-like emulsion	Soymilk-like emulsion
		Filtration	Soymilk-like emulsion	Soymilk-like emulsion	Soymilk-like emulsion
		Autoclaving	Soymilk-like emulsion	Soymilk-like emulsion	Soymilk-like emulsion
C	EPC+T80+SA	None	White emulsion	White emulsion	White emulsion
		Filtration	White emulsion	White emulsion	White emulsion
		Autoclaving	White emulsion	White emulsion	White emulsion

The droplet sizes achieved from non-sterilized emulsions stabilized by different emulsifiers were similar which were in the range of 0.192 to 0.202 μm (Table 21). The method of sterilization had no effect on the particle size of emulsion and the droplet sizes of sterilized emulsions were similar to those achieved from non-sterilized emulsions prepared using the same co-emulsifiers.

The particle sizes of emulsions remained approximately the same throughout the period of storage. There was no particle size greater than 1 μm presented in all emulsions studied.

In addition, the oil droplets of emulsions containing oil-soluble vitamins had approximately the same sizes as those in emulsions formulated using the same co-emulsifier but without the addition of vitamins (Rx 10, 15 and 16) as previously reported in Table 16.

The negative zeta potential of emulsion was observed in formulations stabilized by EPC+T80 and EPC+T80+PG emulsifier system due to the amine charge provided by EPC and PG (Table 22).

Table 21. Effect of emulsifier and co-emulsifiers on droplet size [D (4,3)] of emulsions containing oil-soluble vitamins prepared using 10% soybean oil after storage in refrigerator for 24 hours, 1 and 4 weeks

Rx	Emulsifiers	Sterilization	Mean droplet size (μm)*		
			24 hours	1 week	4 weeks
A	EPC+T80	None	0.199±0.000	0.199±0.000	0.197±0.001
		Filtration	0.199±0.001	0.199±0.000	0.196±0.000
		Autoclaving	0.199±0.000	0.197±0.000	0.199±0.000
B	EPC+T80+PG	None	0.202±0.001	0.198±0.000	0.199±0.001
		Filtration	0.198±0.000	0.199±0.000	0.198±0.000
		Autoclaving	0.197±0.001	0.199±0.000	0.200±0.001
C	EPC +T80+SA	None	0.202±0.001	0.200±0.000	0.200±0.001
		Filtration	0.199±0.000	0.198±0.001	0.198±0.000
		Autoclaving	0.191±0.001	0.201±0.001	0.200±0.001

* = Mean ± SD; n=3

Table 22. Zeta potential of emulsions containing oil-soluble vitamins prepared using 10% soybean oil after storage in refrigerator for 24 hours, 1 and 4 weeks

Rx	Emulsifiers	Sterilization	Zeta potential (mV)* of emulsions		
			24 hours	1 weeks	4 weeks
A	EPC +T80	None	-11.36±4.14	-6.68±2.85	-13.07±2.54
		Filtration	-14.06±2.36	-12.26±4.11	-13.00±1.23
		Autoclaving	-11.95±2.39	-8.11±1.85	-15.17±4.06
B	EPC +T80+PG	None	-15.23±3.74	-14.85±2.93	-15.86±1.22
		Filtration	-12.68±1.82	-14.24±1.89	-17.59±1.13
		Autoclaving	-14.69±3.81	-16.55±2.44	-22.23±4.74
C	EPC +T80+SA	None	31.82±2.24	24.24±1.63	15.32±1.30
		Filtration	24.91±2.96	22.75±1.60	21.16±1.85
		Autoclaving	20.73±2.16	20.34±2.74	14.21±2.90

* = Mean ± SD; n=4

On the other hand, emulsion emulsified by EPC+T80+SA showed positive zeta potential.

The zeta potential seemed to be more negative after storage for 4 weeks compared to the freshly prepared emulsions. Autoclaving caused an increase in negative zeta potential compared to filtration as high temperature during autoclaving would lead to the hydrolysis of phospholipids resulting in free fatty acid released.

Addition of vitamins did not affect on the surface charge of negatively charged emulsion. Conversely, the surface charge of positively charged emulsion decreased with addition of vitamin.

pH of all formulations were in the range of 6.55 to 8.01 (Table 23) which were higher compared to the formulations without vitamins and using the same emulsifiers (Rx 10 and Rx 16 in Table 19). However, the lowering of pH was observed after storage for 4 weeks especially in emulsion using EPC+T80+SA as emulsifiers. It could be a result from the presence of hydrogen ions from phospholipid hydrolysis.

Emulsions containing ionic co-emulsifier, autoclaving had a greater effect on a decrease in pH compared with system using only T80 as co-emulsifier.

Table 23. pH of emulsions containing oil-soluble vitamins prepared using 10% soybean oil after storage in refrigerator for 24 hours, 1 and 4 weeks

Rx	Emulsifiers	Sterilization	pH of emulsion		
			24 hours	1 week	4 weeks
A	EPC +T80	None	7.99	7.43	7.95
		Filtration	7.02	6.72	6.59
		Autoclaving	7.19	7.18	7.48
B	EPC +T80+PG	None	8.01	7.60	7.46
		Filtration	6.81	6.61	6.55
		Autoclaving	6.99	7.19	7.14
C	EPC +T80+SA	None	8.01	7.55	6.84
		Filtration	7.96	8.03	6.78
		Autoclaving	7.09	7.26	6.77

Osmolality

In order to use emulsion for intravenous route, it is necessary to investigate osmolality of the emulsions. In the study, the osmolality of emulsion containing oil-soluble vitamins was measured after storage for 4 weeks. The results are shown in Table 24.

Table 24. Osmolality of emulsions containing oil-soluble vitamins prepared using 10% soybean oil after storage in refrigerator for 1 month

Rx	Emulsifiers	Sterilization	Osmolality (mOsmol/kg)
A	EPC+T80	None	306
		Filtration	309
		Autoclaving	309
B	EPC+T80+PG	None	328
		Filtration	324
		Autoclaving	324
C	EPC+T80+SA	None	309
		Filtration	363
		Autoclaving	307

The value of blood serum osmolality is ranging from 275 to 306 mOsmol/L (Reich et al, 2000). The results showed that the lipid emulsions formulated had the maximum osmolality about 363 mOsmol/kg which was considered to be acceptable for use as intravenous administration.

In this study, the method of sterilization (autoclaving or filtration) exhibited slight differences in zeta potential and pH of the emulsions. However, autoclaving was more convenient and universally accepted in sterilization of large and small volume parenteral nutrition.

In fact, the incorporation of oil-soluble vitamins might promote stability of emulsion. Vitamin E was an antioxidant of oil and phospholipids and might collaborate with BHT in

antioxidation. The results revealed that vitamins provided the positive effect on the physicochemical properties of the emulsions formulated compared to the emulsion formulated without the addition of vitamins.

The physicochemical properties of commercial lipid emulsion containing vitamins, Vitalipid®, which was composed of vitamin A, D_2, E and K_1 was also examined at 6 months before the expiry date. The results were as follows: size, 0.524±0.122 μm; zeta potential, -28.00±4.28 mV; pH, 8.3 and osmolality, 292 mmol/kg.

3. Analysis of oil-soluble vitamins in lipid emulsions.

In this study, a commercial oil-soluble vitamins emulsion (Vitalipid) was used to test if the developed HPLC technique and conditions could be applied to determine the amount of vitamins added in the studied formulations, e.g., vitamin A palmitate and vitamin K_1. It was found that the % remaining of vitamin A palmitate in Vitalipid was markedly high (8 3%) and 100% for vitamin K_1. Thus, such HPLC conditions and methods were used throughout to determine the amount of oil-soluble vitamins in prepared formulations. The sample chromatogram is as shown in Figure 9.

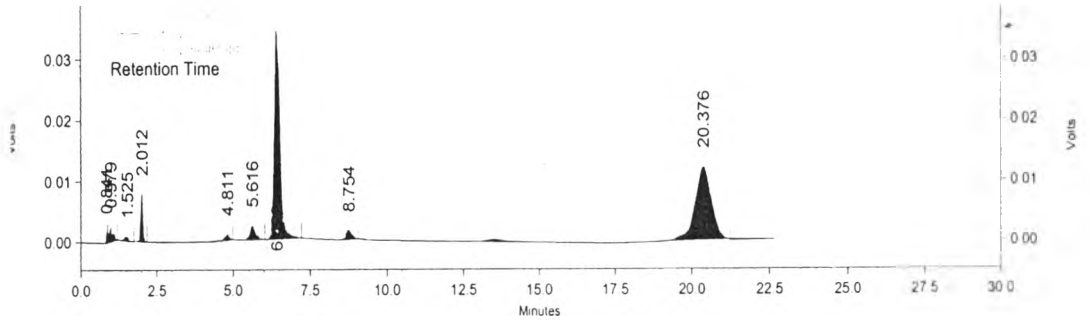

Figure 9. Chromatogram of commercial product, Vitalipid® N Adult, using HPLC technique

The amount of vitamins remained in the autoclaved formulations containing EPC+T80+PG and EPC+T80+SA as emulsifiers were assayed for vitamin content on day 2 and after 2-month storage by HPLC technique. Filtrated emulsions were also examined for the vitamin content using the same technique. Yellow light was used throughout in order to minimize the loss of vitamins owing to light exposure.

To quantify the amount of vitamins in the emulsions, calibration curves of each vitamin were performed as shown in Figure 10-13. Peak area ratio of vitamin to internal standard was plotted as y-axis against the vitamin concentration. The regression analysis was done and the correlation of determination (R^2) as well as equations is reported as shown in Table 25.

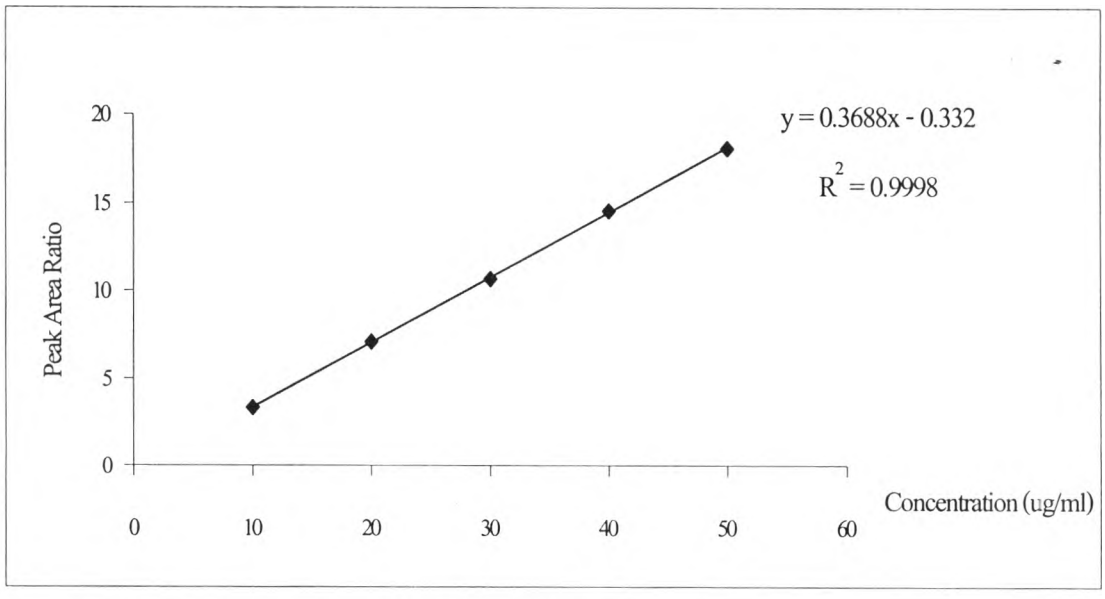

Figure 10. Calibration curve of standard vitamin A palmitate

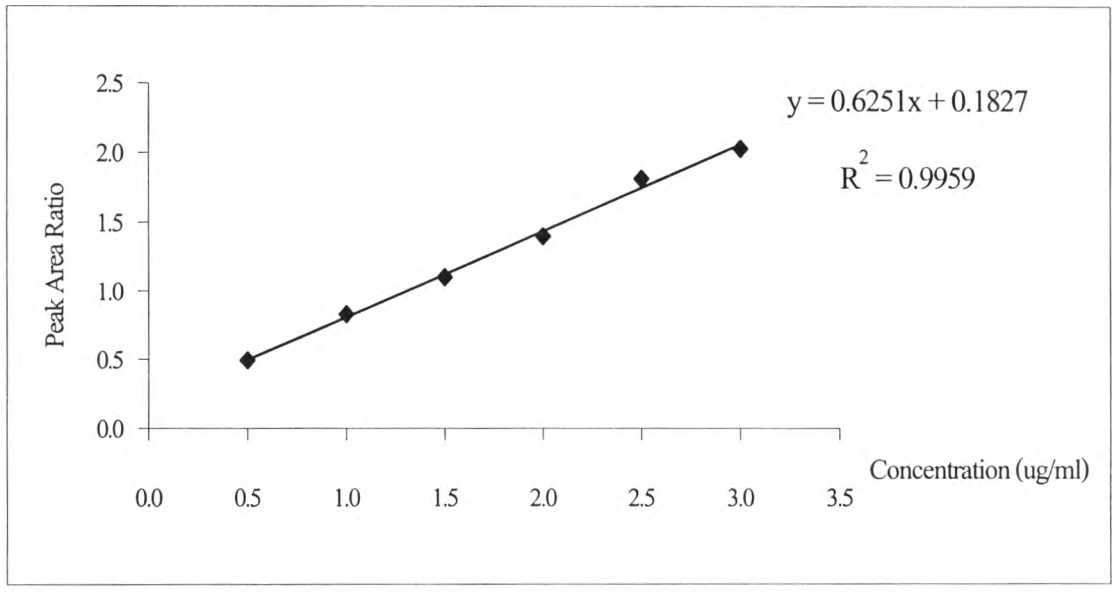

Figure 11. Calibration curve of standard vitamin D_3

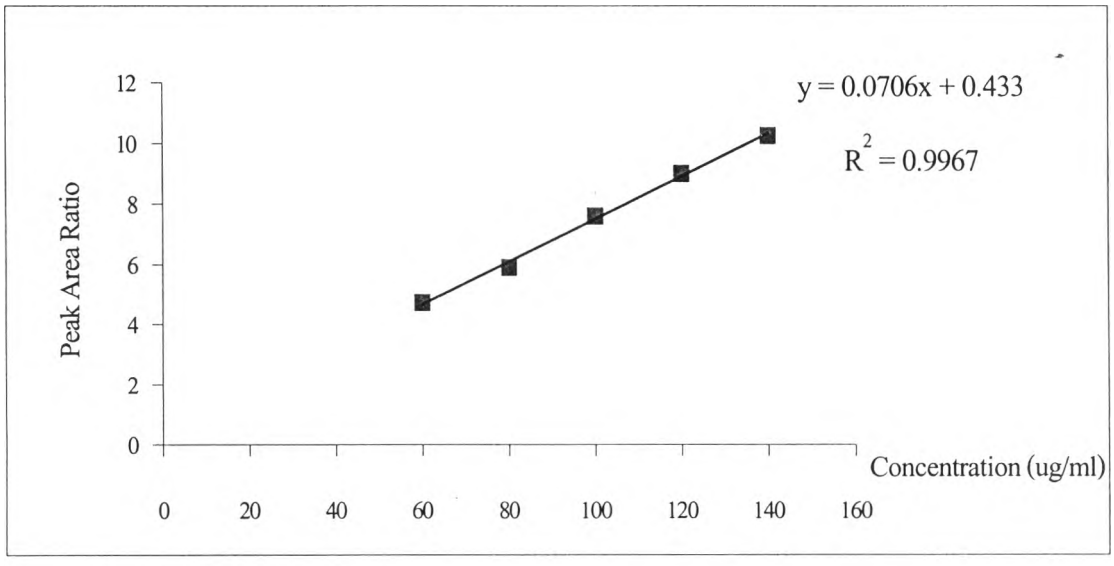

Figure 12. Calibration curve of standard vitamin E acetate

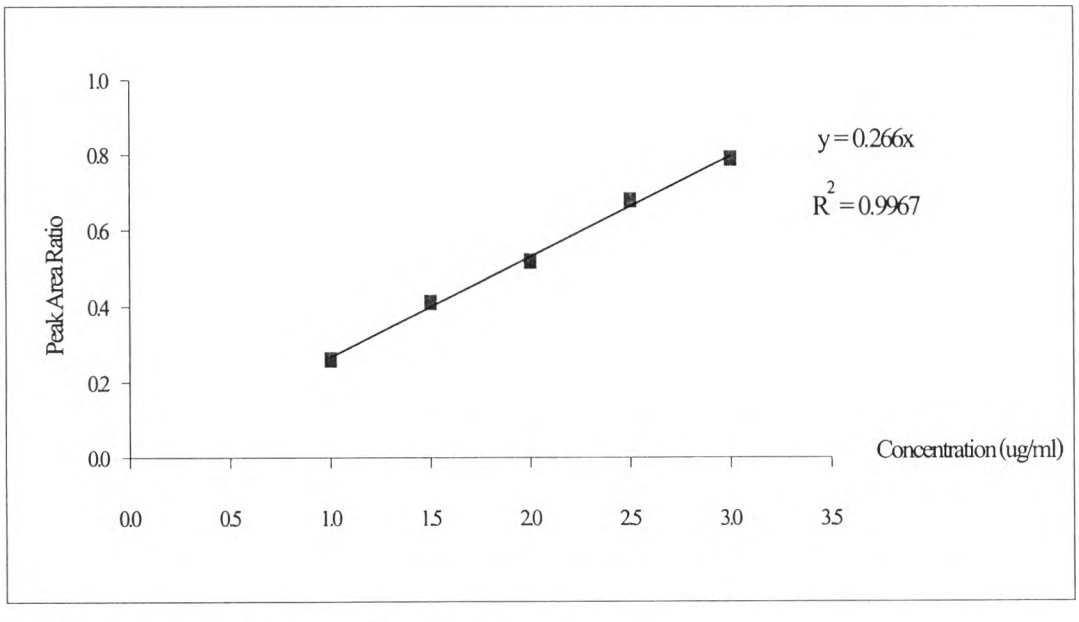

Figure 13. Calibration curve of standard vitamin K_1

Table 25. The regression equation and correlation of determination (R^2) of standard curve of oil-soluble vitamins analyzed by HPLC method

Oil-soluble vitamin	Equation*	Correlation of determination (R^2)
Vitamin A palmitate	$^{a}Y = 0.3688^{b}x - 0.332$	0.9998
Vitamin D_3	$^{a}Y = 0.6251^{b}x + 0.1827$	0.9959
Vitamin E acetate	$^{a}Y = 0.0706^{b}x + 0.433$	0.9967
Vitamin K_1	$^{a}Y = 0.266^{b}x$	0.9969

^{a}Y represents the concentration of oil-soluble vitamin in μg/ml; ^{b}x represents the peak area ratio achieved from injection of sample

The amount of vitamins was calculated by substitution the peak area ratio obtained from the HPLC analysis to the "y"in the equation. Table 26 shows the peak area ratio and the calculated amount and percent remaining of each vitamin in emulsion.

The retention times of vitamin A palmitate, vitamin D_3, vitamin E acetate, and vitamin K_1 were approximately 21, 6, 7 and 9 minutes, respectively, as illustrate in Figure 14 and 15.

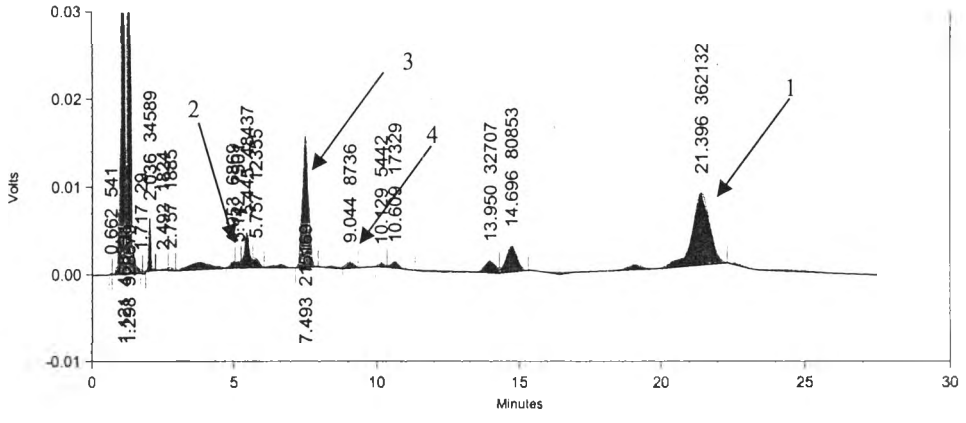

Figure 14. Chromatogram of lipid emulsion containing oil-soluble vitamins using EPC+T80+PG as emulsifiers (1= vitamin A palmitate; 2= vitaminD_3; 3= vitamin E acetate; 4= vitamin K_1)

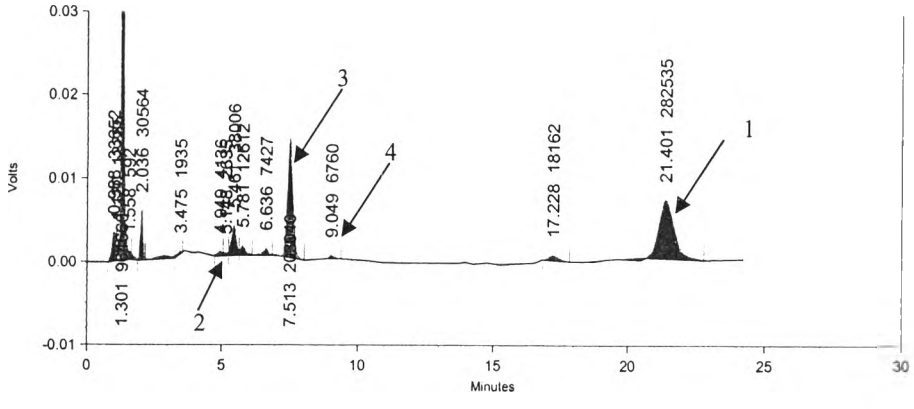

Figure 15. Chromatogram of lipid emulsion containing oil-soluble vitamins using EPC+T80+SA as emulsifiers (1= vitamin A palmitate; 2= vitaminD_3; 3= vitamin E acetate; 4= vitamin K_1)

It was noted that the amount of vitamin K_1 could not be accurately determined as the amount of vitamin K_1 incorporated in this study was out of the calibration concentration range.

The results revealed that % remaining of oil-soluble vitamins after autoclaving was approximately 80%. The loss of vitamins might be resulted from the degradation of vitamins during autoclaving although the physicochemical properties of emulsion remained unchange.

Sforzini et al. (2001) investigated the stability of vitamin A palmitate and tocopherol in all-in-one parenteral admixtures after compounding with lipid emulsion within 24-72 hours at 4 ^{o}C. They found that the amount of vitamin A palmitate and tocopherol were lost approximately 11% compared to the initial formulation. In this study, vitamin A palmitate was lost approximately 18% in Rx B and 26% in Rx C, respectively, while vitamin E acetate was lost about 18% in Rx B and 12.5% in Rx C, respectively. It is possible that vitamin C contained in such admixture prevented degradation of vitamin A palmitate. The preparation method of adding the vitamins in the formulation seemed to have an affect on the stability of lipid emulsion. Brown et al. (1986) recommended that the addition of vitamins was possible to be added during or after administrating amino acid and dextrose solutions. However, Ruangthurakit (2000) suggested that vitamins should be added immediately prior to administration.

In the study, the error might occur in the emulsion preparation because the amount of emulsions formulated was in experimental scale (100 ml to 200 ml). The oil-soluble vitamins were added in the oil phase, thus, any loss of oil phase exhibits great effect on vitamin content. Therefore, in practice, the small excess amount of vitamins should be added in the formulation to achieve the amount of vitamins required on the label and through the shelf life of emulsion.

Table 26. The peak area ratio and calculated amount of oil-soluble vitamins in the emulsions

Rx	Emulsifiers	Vitamins	Peak area ratio*	Amount of vitamins (μg/ml)*	% Remaining*
B	EPC+T80+PG	Vitamin A palmitate	9.60±0.76	26.94±2.70	81.64±6.26
		VitaminD_3	1.17±0.21	1.57±0.34	78.67±16.99
		Vitamin E acetate	6.25±0.06	82.40±0.86	82.40±0.86
		Vitamin K_1	0.26±0.01	0.97±0.02	64.44±1.54
C	EPC+T80+SA	Vitamin A palmitate	8.68±0.94	24.43±2.55	74.02±7.73
		VitaminD_3	1.17±0.08	1.59±0.13	79.33±6.53
		Vitamin E acetate	6.61±0.09	87.44±1.34	87.44±1.34
		Vitamin K_1	0.22±0.01	0.83±0.04	55.11±2.34

* = Mean±SD

The long-term stability of oil-soluble vitamins contained in the lipid emulsions was determined using the same technique. Lipid emulsions containing oil-soluble vitamins studied using EPC+T80, EPC+T80+PG and EPC+T80+SA as emulsifiers. The method of sterilization used was studied for any differences in the vitamins solubilized in prepared emulsions kept in the refrigerator for 2 months. The results are shown in Tables 27 and 28. The retention times of all vitamins were approximately the same compared to emulsion previously studied.

Table 27. Peak area ratio of oil-soluble vitamins found in the emulsions after storage for 2 months analyzed by HPLC method

Rx	Emulsifiers	Method of sterilization	Peak area ratio *			
			Vitamin A palmitate	Vitamin D_3	Vitamin E acetate	Vitamin K_1
A	EPC +T80	Filtration	6.86±0.26	0.56±0.02	8.87±0.09	0.29±0.01
		Autoclaving	6.16±0.57	0.47±0.00	8.17±0.04	0.34±0.01
B	EPC +T80+PG	Filtration	7.11±0.28	0.48±0.02	8.91±0.06	0.47±0.18
		Autoclaving	6.40±0.41	0.44±0.01	7.72±0.06	0.38±0.02
C	EPC +T80+SA	Filtration	5.64±0.08	0.47±0.01	7.25±0.07	0.27±0.06
		Autoclaving	5.71±0.62	0.48±0.08	7.26±0.11	0.26±0.02

* = Mean±SD; n=3

Table 28. The amount of oil-soluble vitamins in emulsions after storage for 2 months analyzed by HPLC method

Rx	Emulsifiers	Sterilization	Concentration, μg/ml (% Remaining)			
			Vitamin A palmitate	Vitamin D_3	Vitamin E acetate	Vitamin K_1
A	EPC +T80	Filtration	19.50±0.71 (59.09±2.16%)	[S]0.61±0.04 (30.45±1.85%)	[S] 119.46±1.27 (119.46±1.27%)	[S] 1.08±0.04 (71.85± 2.89%)
		Autoclaving	17.06±1.54 (53.34±4.68%)	[S] 0.46±0.00 (22.98±0.00%)	[S] 109.64±0.50 (109.64±0.50%)	[S] 1.29±0.02 (86.05±1.45%)
B	EPC +T80+PG	Filtration	20.62±0.83 (62.49±2.52%)	0.49±0.03 (24.58±1.39%)	[S] 120.68±0.43 (120.68±0.43%)	2.16±0.66 (143.69±44.15%)
		Autoclaving	18.25±1.12 (55.31±3.40%)	0.41±0.02 (20.58±0.80%)	[S] 103.22±0.88 (103.22±0.88%)	1.42±0.08 (94.40±5.22%)
C	EPC +T80+SA	Filtration	16.19±0.20 (49.07±0.62%)	0.46±0.02 (22.98±0.80%)	96.56±1.02 (96.56±1.02%)	1.00±0.21 (66.83±13.80%)
		Autoclaving	16.39±1.68 (49.67±5.08%)	0.47±0.12 (23.51±6.00%)	96.65±1.53 (96.65±1.53%)	0.98±0.07 (65.16±4.34%)

*= Mean ±SD; n=3

[S]= significantly different

The result indicated that the amount of vitamins were lost after storage. Vitamin A palmitate was lost approximately 40%-50 %, almost 80% for vitamin D_3 and 35% for vitamin K_1. Conversely, there was no any much loss of vitamin E acetate and vitamin K_1 found in some formulations. However, there were no significant differences in the physicochemical properties (size, pH, osmolality) of emulsions found as emulsion formulated using EPC+T80+PG and EPC+T80+SA as emulsifiers.

The amounts of vitamin contained in the formulations prepared by different methods of sterilization were compared using 1-way ANOVA analysis at 95 % confidence level. The vitamin contents in Rx A was found to be significant different in that emulsion sterilizes by filtration method provided the lower level of vitamin K_1 than that in autoclaving method. Conversely, the higher level of vitamin A, vitamin D_3 and vitamin E were found when filtration was used. However, there were no significant differences found regarding to the method of sterilization in the amount of vitamins assayed in the Rx B and Rx C, except in Rx B which autoclaving significantly decreased the amount of vitamin E acetate from 120.68 to the value of 103.22 μg/ml in filtrated emulsion. The use of ionic co-emulsifier might increase the entrapment and stability of vitamins. In the point of views of preparation, autoclaving was easier and more convenient than filtration method.

It was noted that the results obtained in Tables 26 and 28 from the present study were, however, unable to compare as they were prepared at the different batches and emulsions were formulated in different experimental scale. Thus, it is expected that the amount of vitamins would be lost more in the preparation of 100 ml emulsion than that of 200 ml emulsion.

4. Haemolysis study

The lipid emulsions containing oil-soluble vitamins were tested for the effect on human red blood cells in order to assure the suitability of co-emulsifiers used in the formulation. Lipid emulsion studied were the emulsions emulsified by EPC+T80+SA with and without the addition of oil-soluble vitamins and emulsion containing oil-soluble vitamins emulsified by EPC+T80+PG. All emulsions were autoclaved and tested after preparation for 2 months. The result was showed in Table 29.

Table 29. The percent of haemolysis induced by the different types of emulsion systems

Rx	Emulsifiers	Vitamins	% Haemolysis*
16	EPC+T80+SA	-	29.82±1.57
B	EPC+T80+PG	added	42.92±14.98
C	EPC+T80+SA	added	23.38±7.79

* = Mean±SD; n=3

The results indicated that the haemolysis were found in every systems investigated. Rx B exhibited significantly higher degree of haemolysis ($P \leq 0.05$) than Rx 16 and Rx C. The haemolytic effect was also found in Vitalipid® with the haemolysis of 25.76±7.03%. It might be that lysophosphatidylcoline and oleic acid, which were the degradation products resulting from the degradation of phosphatidylcholine, caused haemolytic activity (Gould et al., 2000) and only 0.2 mM of lyso-phosphatidylcoline was reported to induce 100% haemolysis. All formulated lipid emulsions

were sterilized using autoclaving, therefore, might accelerate in the degradation of egg phosphatidylcholine resulting in high concentration of degradation products. Moreover, the surfactant and co-surfactant might cause haemolysis. Jumaa and Müller (2000) reported that haemolytic effect was dependent on the type and concentration of nonionic surfactant used in the emulsion.

However, such high percent of haemolysis observed might be due to the method of emulsion preparation used for haemolysis study. A high amount of DMSO was used to solubilize emulsion before the absorbance was able to measure. Such a large amount of DMSO could be toxic to erythrocyte. The haemolysis of commercial emulsion (Intralipid®) was also reported recently (Pongcharoenkiat, 2001). Unfortunately, toxicity of DMSO at the concentration used in the study was not tested on erythrocytes. Hence, there was an uncertainty to make any conclusion of the effect of compositions in emulsion used on haemolysis.

However, there were some differences among prepared emulsions studied. The addition of PG in formulation B presented significantly higher haemolytic activity. The toxicity observed might be due to the degradation of inherent phosphatidylcholine in PG to form lyso-PC. In contrast, an addition of SA caused less haemolysis even the higher amount of SA (11.1 mM) was used compared to PG (0.66 mM). Similarly, the study of Klang et al. (1994) demonstrated their results about the toxicity occurring by injected positively-charged emulsion intravenously to the mice. They found that no marked acute toxicity was observed after the injection and also no difference was noted between the use of positively-charged emulsion and commercial lipid emulsion. Also, no any evidence revealed the haemolytic toxicity induced by oil-soluble vitamins.