Formulation Development of a Filter-Sterilizable Lipid Emulsion for Lipophilic KW-3902, a Newly Synthesized Adenosine A_1-Receptor Antagonist

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KW-3902 (a newly synthesized adenosine A_1-receptor antagonist) has potent diuretic and renal protective activities. The objective of the present study was to develop an injectable formulation of KW-3902, that was water-insoluble and less than 1 μg/ml, and so lipid emulsion was selected as a favorable formulation. Changing the mixing ratio of oil to lecithin, the particle size of the lipid emulsion was controlled, and by adjusting the mixing ratio of oil/lecithin=1 : 1, the weight ratio, a lipid emulsion with a mean particle size of 130 nm was prepared. This small particle size makes this emulsion filter-sterilizable, which is a favorable feature for heat labile products. The stability of the KW-3902 lipid emulsion was assessed from the viewpoint of the electrostatic repulsion, and by including the oleic acid a stable lipid emulsion was developed, which was stable for at least 12 months at 10 and 25 °C and for 3 months at 40 °C. The feature of this small particle size emulsion was also characterized by comparing it with a conventional emulsion (oil/lecithin=1 : 0.12, the weight ratio, particle size is 220 nm). The release of KW-3902 from the oil particles was measured and the apparent permeability of KW-3902 was calculated from the equation according to Fick's theory. The apparent permeability, *P*, of KW-3902 was not affected by the particle size of the emulsion (1.78×10^{-11} cm/s for the small emulsion and 1.76×10^{-11} cm/s for the conventional emulsion). The distribution mode of KW-3902 in the lipid emulsion was also discussed by considering the findings of the permeability and solubility of KW-3902.

Key words lipid emulsion; injection; permeability; KW-3902; stability

Pharmaceutical scientists have been confronted with two difficulties of parenteral drug delivery technologies. One is the study of many injectable drugs on the drug delivery system (DDS), which has been investigated for the effective targeting of drugs and the prolongation of the drug concentration in the blood. The other is the problem regarding the formulation technologies of many injectable drugs with poor solubility or insolubility in water. The latter is a serious problem for the pharmaceutical industry because drug insolubility can delay or completely block new drug development and can prevent the much-needed reformulation of some currently marketed drugs.

Traditional approaches to formulating water-insoluble drugs have been addressed by solubilizing the drugs using detergents, organic solvents, and solutions with a pH outside the physiological range or by utilizing molecular complexes with a vehicle.[1—6] However, these approaches may cause local pain or phlebitis because of the detergents, organic solvents or precipitation of drugs upon dilution after injection, which would be major problems in clinical practice.[7,8] Moreover, some surfactants used as solubilizing agents in parenteral formulations of insoluble drugs have been implicated in undesirable biological reactions after injection.[9—11] An alternative approach for parenteral formulations of water-insoluble or poorly soluble drugs will be provided by dispersed systems, such as emulsions,[12—19] liposomes[20,21] and nanospheres,[22] which are making good progress toward more ideal DDSs. For instance, diazepam in a lipid emulsion recently became commercially available,[23,24] which was developed to reduce the incidence of local side effects after intravenous injection of conventional preparations of diazepam containing organic solvents. However, intravenously injected liposomes and emulsions are rapidly captured by the reticuloendothelial system (RES) rich organs such as the liver and spleen.[25—29] This is a major problem for researchers of liposomes and emulsions, because this is a significant disadvantage for delivery of drugs to non-RES tissues.

KW-3902 (1,3-dipropyl-8-(3-tricycle[3.3.1.$0^{3,7}$]nonyl)-3,7-dihydro-1*H*-purine-2,6-dione, see Fig. 1) is a newly synthesized adenosine A_1-receptor antagonist.[30,31] Recently, it was reported that KW-3902 has potent diuretic and renal protective activities in rats and dogs.[32—34] For these reasons, an injectable preparation is desired to evaluate the potential of KW-3902 under clinical trial. However, KW-3902 is a water-insoluble compound and has no dissociation group, which is ionized over the range of a physiologically acceptable pH. Therefore, we formulated a favorable and filter-sterilizable lipid emulsion of KW-3902 and studied the physicochemical characteristics and stability of this emulsion.

Experimental

Materials KW-3902 was synthesized at Kyowa Hakko Kogyo Co., Ltd. Egg yolk lecithin (containing 99% of phosphatidylcholine (PC)), oleic acid and concentrated glycerin were purchased from NOF Corporation (Tokyo, Japan). Soybean oil was purchased from The Nisshin Oil Mills, Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and obtained from Kanto Chemical Co. (Tokyo, Japan). Cellulose tubing (MW cut-off of 6—8 kDa) used for dialysis was purchased from Spectrum Med-

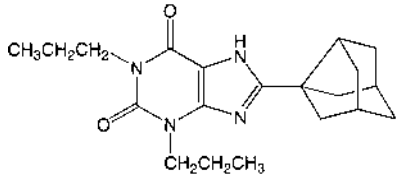

Fig. 1. Chemical Structure of KW-3902

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ical Industries, Inc. (Los Angeles, CA, U.S.A.).

Lipid Emulsion Preparation A primary emulsion was first prepared by mixing the soybean oil with or without KW-3902, and egg yolk lecithin, oleic acid, concentrated glycerin and water for injection at 50 °C for 15 min using a high-shear homogenizer (8000 rpm, Model PVQS-1(3), Mizuho Industrial Co., Ltd., Osaka, Japan). Then the pH of the emulsion was adjusted to 7 using 1 N NaOH. A primary emulsion was passed through a microfluidizer (Model M-110EH, Microfluidics Corp., Newton, MA, U.S.A.) 5 times at 120 MPa. The standard emulsion used in this study was as follows: soybean oil (5% w/v), egg yolk lecithin (0.6—10% w/v), oleic acid (0—0.48% w/v), concentrated glycerin (2.21% w/v), and KW-3902 (0.05% w/v), when expressed as a percentage of the total volume of the emulsion. The emulsion was sterilized by filtration through a 0.22 μm Ultipor filter (Pall Corp., East Hills, NY, U.S.A.), then packaged in 10 ml ampules (Type 1 glass) and sealed after nitrogen purging.

The mean particle size of the emulsion was determined by dynamic light scattering (DLS-700, Otsuka Electronics, Osaka, Japan). The ζ potential was determined by electrophoretic light scattering analysis (ELS-800, Otsuka Electronics, Osaka, Japan). The emulsion was diluted two hundred times with 0.01 M phosphate buffer at pH 7.4 before measuring the ζ potential.

Drug Release The emulsion was diluted twenty times with water and *in vitro* release of KW-3902 from oil droplets was monitored by membrane dialysis at 37 °C. The sample volume in the dialysis tube was 2 ml and the sink volume was 40 ml, which contained 2% BSA. The concentration of KW-3902 in the original emulsion was 0.5 mg/ml. Assuming 100% drug release, the concentration of KW-3902 in the sink solution will be 1.25 μg/ml, which is within the solubility of KW-3902 when existing with BSA at 17 μg/ml. A cellulose tube with a MW cut-off of 6—8 kDa, and thus freely permeable to KW-3902 (MW=356.47), was used. Concentrations of the drug in pre-/post-dialysis samples and aliquots at various time intervals were determined and drug release profiles (% drug released *vs.* time) were generated.

Physical and Chemical Stability The KW-3902 content in the emulsions was analyzed by high performance liquid chromatography on a Shimpack CLC-ODS column (5 μm, 150×6 mm). The mobile phase consisted of an acetonitrile/potassium dihydrogenphosphate (0.02 mol/l) with 13 : 7 (v/v) at a flow rate of 1.0 ml/min. A UV detector at 280 nm was used to detect and quantitate KW-3902. Emulsions were stored for an appropriate period at 10, 25 and 40 °C and assayed for physical and chemical stability. The mean particle size and pH were used as indicators of physical stability. KW-3902 potency was monitored during the stability program.

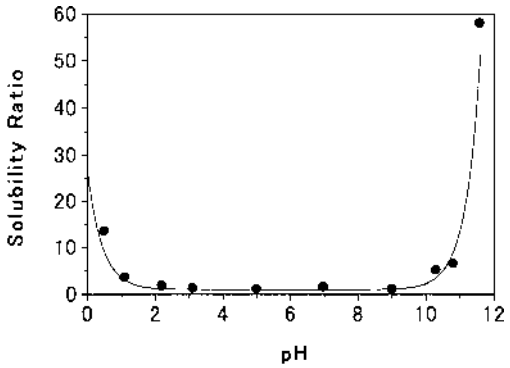

Fig. 2. The pH-Solubility Profile for KW-3902 at 25 °C

The solid line indicates a theoretical curve of the Henderson–Hasselbalch equation,
SR for a weak base and its salt=$S_{tot}/S_u=1+10^{(pKa-pH)}$
SR for a weak acid and its salt=$S_{tot}/S_u=1+10^{(pH-pKa)}$
where S_{tot} is the observed total solubility, S_u is the solubility of the unionized species, $pK_{a1'}=1.4$ (N of imidazole) and $pK_{a2'}=9.9$ (OH of enol form).

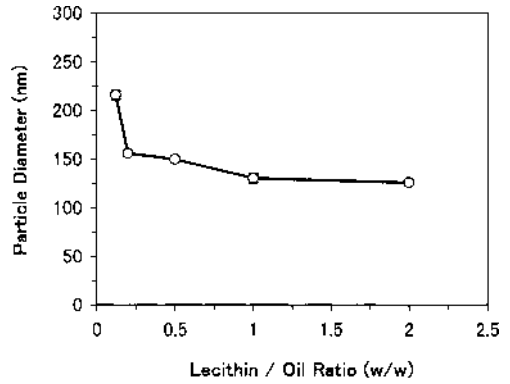

Fig. 3. Effect of the Lecithin/Oil Ratio on the Particle Size of Lipid Emulsions

The concentration of the soybean oil is 50 mg/ml. Each point represents the mean±S.D. of three to six experiments.

Results and Discussion

Physical and Chemical Characteristics of KW-3902 Table 1 shows briefly the physical and chemical characteristics of KW-3902, and Fig. 2 shows the solubility of KW-3902 as a function of pH. The apparent solubility of KW-3902 in water follows the Henderson-Hasselbalch equation,[35] which describes the effect of the pH and pK_a on the solubility ratio of a weak base and a weak acid, *i.e.*

$$SR \text{ (for a week base and its salt)} = \frac{S_{tot}}{S_u} = 1 + 10^{(pK_a - pH)} \quad (1)$$

$$SR \text{ (for a week acid and its salt)} = \frac{S_{tot}}{S_u} = 1 + 10^{(pH - pK_a)} \quad (2)$$

where *SR*, the solubility ratio, is defined as the observed total solubility S_{tot} divided by the solubility of the unionized species S_u. The solubility of KW-3902 in water is quite low, less than 1 μg/ml, over the physiological pH range.

One of the traditional approaches to prepare an i.v. formulation for a water-insoluble drug is cosolvent mixtures.[3,4] However, the numerous disadvantages of organic solvents such as local pain and the presence of phlebitis have been reported.[7,8] Moreover, some surfactants formulated in injection have been implicated in undesirable biological reactions.[9—11]

To develop a less toxic i.v. formulation of KW-3902, these traditional approaches appear to be risky. Therefore, we tried to develop an injectable lipid emulsion formulation since KW-3902 shows a somewhat high apparent solubility in soybean oil, 7 mg/ml. Lipid emulsions consisting of soybean oil and lecithin have been widely used for parenteral nutrition for more than 30 years and they have no particular side effects even at a dose of more than 100 ml per person.[36]

Physico-Chemical Properties of Egg Yolk Lecithin as Emulsifier The first investigation was to characterize the physical properties of lecithin as a primary emulsifier. The egg yolk lecithin normally used in the parenteral emulsion contains PC as a main component and minor components such as phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylinositol (PI). The lipid emulsions used for intravenous nutrition are commonly composed of soybean oil and egg yolk lecithin at a ratio of 1 : 0.12, the weight ratio.[36] We investigated the effect of lecithin content on the particle size of the emulsion, where the concentration of soybean oil was kept constant at 50 mg/ml. Fig. 3 shows a plot of particle size against the emulsion composition. The mean particle size of the conventional emulsion (oil/lecithin=1 : 0.12, the weight ratio) was about 220 nm, which is similar to that of the lipid emulsions in commercial use.

Increasing the lecithin to oil ratio decreases the particle size of the emulsion (Fig. 3). At a ratio of oil/lecithin=1 : 1,

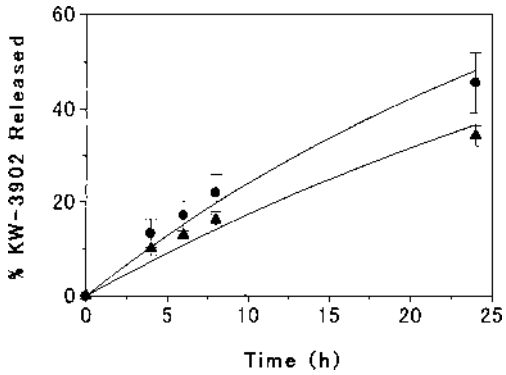

Fig. 4. Release Profiles of KW-3902 from the Lipid Emulsions Particles with Different Mean Particle Sizes into Aqueous Solution Containing BSA at 37 °C

●, 0.13 μm; ▲, 0.22 μm. Each emulsion contains 0.5 mg/ml of KW-3902. The solid lines indicate the theoretical curves of Eq. 3. Each point represents the mean±S.D. of three experiments.

the weight ratio, the particle size of the emulsion was found to be about 130 nm. Further increasing this ratio, however, resulted in a plateau in particle size. Handa *et al.*[37] reported the phase diagram of triolein and PC mixtures in saline. In their study, the liposome fraction coexisted with the lipid emulsion, and the fractions of PC participating in the liposome increased with an increase in the mole fraction of PC in the triolein-PC mixtures. This might explain why the concentration effect of lecithin on the particle size had reached saturation.

Drug Release We investigated the release of KW-3902 from lipid emulsion into aqueous media. If Fick's theory is applied to explain the mass transport phenomena at a interfacial membrane of lipid emulsion, the amount, M, of material flowing through a surface area, S, of the interfacial membrane in unit time, t, can be derived as Eq. 3 from Fick's first law,[35]

$$\frac{dM}{dt} = PSC_{O} \tag{3}$$

where P is the permeability, C_O is the concentration of a drug in the oil phase. On the assumption that the receptor compartment is applied to sink to simplify the equation, Eq. 4 is derived:

$$\frac{C_{Ot}}{C_{Oi}} = \exp\left(-\frac{PS}{V_O}\times t\right) \tag{4}$$

where V_O is the volume of oil, C_{Oi} is the initial concentration of drug in the oil phase and C_{Ot} represents the concentration at time t. The release ratio (%) is thus given by Eq. 5:

$$\%\ \text{released} = 100 - 100\times\exp\left(-\frac{PS}{V_O}\times t\right) \tag{5}$$

Figure 4 shows the *in vitro* release of KW-3902 from the conventional emulsion (oil/lecithin=1 : 0.12, the weight ratio) and the small emulsion (oil/lecithin=1 : 1, the weight ratio) into an aqueous solution containing BSA kept at 37 °C as a function of time. The plots show the percentages of KW-3902 released at appropriate intervals, and the full lines are the theoretical curves obtained from Eq. 5 by non-linear regression. As shown in Fig. 4, the experimental results agree well with those calculated by Eq. 5. The release rate of KW-3902 from the small emulsion after 24 h was 45% and that from the conventional emulsion was 35%.

Table 1. Physicochemical Property of KW-3902

Item	
Solubility (at 20 °C)	
pH 2	0.00080 mg/ml
pH 7	0.00042 mg/ml
pH 10	0.00079 mg/ml
Methanol	12 mg/ml
Ethanol	18 mg/ml
n-Hexane	3.4 mg/ml
n-Octane	4.2 mg/ml
Soybean oil	7 mg/ml
Partition coefficient (pH 7)	log P=4.7
Dissociation constant	$pK_{a1'}$=1.4 (N of imidazole)
	$pK_{a2'}$=9.9 (OH of enol form)
UV absorption	λ_{max}=277 nm
Melting point	190 °C

Yamaguchi *et al.*[38] studied the distribution of prostaglandin E_1 in lipid emulsion. In their study, the distribution style of prostaglandin E_1 in injectable emulsion was analyzed from the equilibrium concentrations calculated by the release profiles using an equation derived from Fick's first law. In this study, we focused on the permeability, P, of Fick's theory and discussed the distribution mode of KW-3902 in the lipid particles. Assuming that all particles have a mean particle size, d, measured by dynamic light scattering, a term of S/V_O in Eq. 5 can be represented as $6/d$. Hence, rearrangement of Eq. 5 yields

$$\%\ \text{released} = 100 - 100\times\exp\left(-\frac{6P}{d}\times t\right) \tag{6}$$

The apparent permeability, P, at the interfacial membrane of the small emulsion was calculated as 1.78×10^{-11} cm/s and that of the conventional emulsions was 1.76×10^{-11} cm/s from Eq. 6 by non-linear regression. It should be noted that the apparent permeabilities of KW-3902 at the interfacial membrane from the small emulsion and the conventional emulsion are identical and are not affected by the particle size. This suggests that KW-3902 would be distributed mainly in the oil/water interface of the lipid emulsion. In general, the drug in smaller particles has higher chemical potential than that in larger particles, because of the increased internal pressure due to the highly curved particle surface, which is quantified by the Kelvin equation.[21,35] Distributing to the peripheral part of the lipid emulsion, the expected increased chemical potential of KW-3902 is cancelled out. In addition, the permeability barrier to flow through into the water is the lipid monolayer, in which thickness does not depend on the particle size of the lipid emulsion. This idea would be supported by the finding that KW-3902 has a good affinity to the polar organic solvents such as alcohols rather than the hydrocarbons (Table 1). In addition, KW-3902 is miscible with egg lecithin; about 10 mg of KW-3902 is miscible with egg lecithin (1 g). Therefore, it is possible to argue that KW-3902 has a tendency to distribute in the oil/water interface. The detailed distribution mode of KW-3902 in the

lipid emulsion will be analyzed elsewhere using instrumental methods. In view of the formulation study, the smaller particle size of KW-3902 lipid emulsion (oil/lecithin=1 : 1, the weight ratio) has favorable properties to be filter-sterilized due to its particle size of less than 0.22 μm. Intravenous lipid emulsions must be sterile and terminal steam sterilization has been generally used for this purpose. However, it was reported that the exposure to the high temperatures required for the steam sterilization (121 °C) may cause the physical and chemical instability of the lipid emulsion.[39—41] In this respect, the KW-3902 lipid emulsion with the smaller particle size has an advantage.

Intravenously injected drugs[42] and emulsions[43—45] go through many impacts with plasma components such as lipoproteins due to the well agitated state in the systemic circulation. Under these conditions, drugs incorporated in emulsion would be extracted out of the oil phase because the partitioning of the drugs away from the oil discontinuous phase into the serum components is rapid.[46] In this case, the performance would be lowered when a lipid emulsion was designed as a carrier. Considering the DDS, the tendency of the conventional emulsion to be captured by the RES is another problem. In contrast, if the incorporated drugs are rapidly and completely dissociated from the emulsion after injection, the lipid emulsion will simply act as a solvent and the drug itself will show its intrinsic pharmacokinetics parameter instead of that of the lipid emulsion. The fate of the KW-3902 lipid emulsion in the systemic circulation after injection will be reported elsewhere.

Formulation of KW-3902 Lipid Emulsion Intravenous lipid emulsions are stabilized by a combination of forces such as electrostatic, hydration and steric repulsive forces, that is, those of the Derjaguin-Landau-Verway-Overbeek (DLVO) theory.[35] Due to its natural origin and biocompatibility, egg yolk lecithin is normally used as a primary emulsifier for parenteral lipid emulsion, although it has poor emulsification capability.

Therefore, many studies about the ability of the secondary emulsifiers have been carried out to improve the emulsification efficacy of lecithin. Attempts to improve the thermal stability of lipid emulsions during autoclaving by increasing in the electrostatic repulsive force between oil droplets produced by formulation modification have been reported by Washington and Davis.[47] using the oleic acid, and by Chansiri *et al.*[39] using PA, PG and PI. Krishna *et al.*[48] reported that supplementing the hydrophilic attributes with synthetic hydrophobic surfactants such as Span 20 improved the emulsification property of lecithin.

Oleic acid is suggested to be biocompatible, because it is a typical product of phospholipid and oil metabolism. Therefore, we used the oleic acid as a secondary emulsifier to improve the property of lecithin. Figure 5 shows the ζ potential of lipid emulsion at pH 7.4 as a function of the amount of oleic acid. Without oleic acid, the ζ potential of emulsion was almost neutral (−0.2 mV), because we used the egg lecithin containing more than 99% of PC, which is a neutral lipid.

The ζ potential of lipid emulsion decreases as the amount of oleic acid in the emulsion increases. When oleic acid is added at 0.24% w/v in the emulsion, the emulsion shows a ζ potential of −22 mV (Fig. 5). In contrast, the conventional

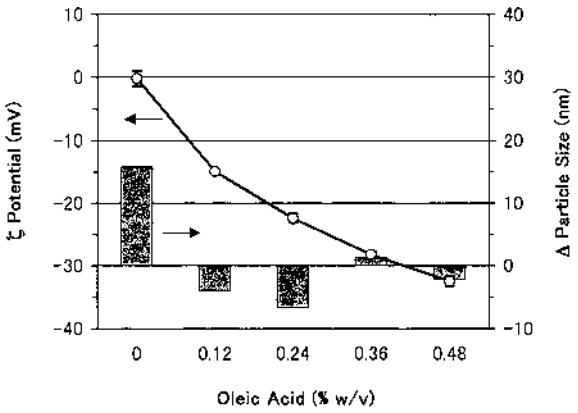

Fig. 5. Zeta Potential of Lipid Emulsions Containing Various Amounts of Oleic Acid at pH 7.4 and the Change in the Mean Particle Size after Thermal Stress

The concentrations of soybean oil and egg yolk lecithin are 50 mg/ml each. Lipid emulsions were stressed at 60 °C for 7 d.

Δparticle size=mean particle size (after thermal stress)−mean particle size (before thermal stress)

Each point represents the mean±S.D. of four experiments.

Table 2. Formulation of KW-3902 Lipid Emulsion

Ingredient	Content
KW-3902	0.5 mg
Soybean oil	50 mg
Egg yolk lecithin	50 mg
Concentrated glycerin	22.1 mg
Oleic acid	2.4 mg
Sodium hydroxide	A sufficient quantity (pH 7)
Water for injection	A sufficient quantity To make 1 ml

lipid emulsion in commercial use had a ζ potential of about −20 mV when measured in the same way. The egg lecithin used in the manufacturing of the conventional emulsion is containing many anionic components such as PA, PG and PI and so on. The absolute value of the ζ potential of this small emulsion containing oleic acid (0.24% w/v) is very similar to that of the conventional emulsion currently in commercial use.

The thermal stability of this emulsion was assessed to verify the effect of a negatively increased ζ potential (Fig. 5). The mean particle size of the emulsions with an increased negative ζ potential was unchanged after thermal stress at 60 °C for a week. In contrast, the emulsion with a neutral ζ potential, *i.e.* without oleic acid, showed an increase in the mean particle size from 130 to 160 nm. We could attribute this stability of the emulsion to its increased electrostatic repulsive force. This finding agrees with that obtained by Chansiri *et al.*[39] and Yamaguchi *et al.*[40]

Table 2 shows the formulation of KW-3902 lipid emulsion. The pH of KW-3902 lipid emulsion was adjusted by about 7 and sealed with nitrogen gas to prevent hydrolyzation or oxidization.[47,49,50] The stability of this emulsion is summarized in Table 3. No separation nor change in particle size under experimental conditions (at 10 and 25 °C for 12 months and at 40 °C for 3 months) was observed. The pH of KW-3902 lipid emulsion was lowered when it was stored at 25 °C and 40 °C, but it was a slight alteration in pH and this would not

Table 3. Stability of KW-3902 Lipid Emulsion

Storage condition		KW-3902 (mg/ml)	pH	Mean particle size (nm)
Initial		0.492±0.003	6.7	126±4
10 °C	3 months	0.506±0.002	6.6	115±2
	6 months	0.503±0.001	6.5	128±2
	12 months	0.487±0.003	6.6	122±4
25 °C	3 months	0.504±0.002	6.4	116±2
	6 months	0.507±0.001	6.3	136±4
	12 months	0.485±0.002	6.2	129±2
40 °C	3 months	0.505±0.002	6.1	125±3

Values represent the means±S.D. of three experiments.

affect the safety and performance of this emulsion. This is probably due to the lesser amount of the fatty acid generated by the hydrolysis of the lecithin and oil.[47] Moreover, there was no major change in the chemical stability of KW-3902 in the emulsion under these storage conditions.

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