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Research Article

Development and Characterization of Lyophilized Diazepam-Loaded Polymeric Micelles

Jiraphong Suksiriworapong,[1,2] Tanaporn Rungvimolsin,[1] Atitaya A-gomol,[1] Varaporn Buraphacheep Junyaprasert,[1] and Doungdaw Chantasart[1]

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Abstract. Polymeric micelles were studied as delivery carriers of diazepam, a practically insoluble drug in water, for rectal administration. The diazepam-loaded polymeric micelles were developed by using poloxamer 407 (P407), poloxamer 188, and D-α-tocopheryl poly(ethylene glycol) 1000 succinate (TPGS). Among the used polymers, TPGS resulted in polymeric micelles with good characteristics for encapsulation of diazepam which had the small particle size of 8–12 nm and narrow size distribution (PI 0.053–0.275). Additionally, 7.5% *w/v* of TPGS could entirely entrap the desired concentration of diazepam (5 mg/mL). To improve the physical stability upon lyophilization, an addition of P407 of 1% *w/v* prevented aggregation, increased physical stability, and maintained chemical stability of the lyophilized powders of diazepam-loaded polymeric micelles for 3 months storage at 4°C. The rate and amount of diazepam release from TPGS polymeric micelles mainly depended on the concentration of TPGS. The release data were fitted to Higuchi's model suggesting that the drug release mechanism was controlled by Fickian diffusion. In conclusion, 10% *w/v* TPGS and 1% *w/v* P407 were the optimum formulation of lyophilized diazepam-loaded polymeric micelles.

KEY WORDS: diazepam; lyophilization; poloxamer 407; polymeric micelles; D-α-tocopheryl poly(ethylene glycol) 1000 succinate (TPGS).

INTRODUCTION

Seizures are a common neurological disorder with considerable negative impacts on clinical, economic, and humanistic outcomes. Diazepam is one of the benzodiazepines typically used for the treatment of seizures (1,2). During an acute attack of seizures, most patients are unconscious so that an oral administration of diazepam is frequently impractical. Intravenous injection of diazepam is considered an important treatment option since its onset of effect usually occurs within a few minutes. However, this route of administration required skilled medical personnel and can result in serious adverse effects. Alternatively, the rectal administration of diazepam has been shown to be clinically effective in the treatment of seizures and the prevention of febrile recurrent convulsions (1,3). Due to its practical insolubility in water, a major obstacle of the rectal formulation generally is a limited solubility of drug in a very small volume of rectal fluid.

Recently, polymeric micelles have received much scientific attention and introduced a new paradigm of drug delivery systems due to their very small size (<100 nm), easy production, longevity in blood circulation, and ability of incorporating and protecting poorly water-soluble drugs (4–6). Polymeric micelles can self-assemble from amphiphilic copolymers and typically are composed of a spherical hydrophobic inner core surrounded by a hydrophilic outer shell (7). Hydrophobic drugs can physically be entrapped into the micelle core resulting in high drug loading of polymeric micelles by means of hydrophobic and π–π interactions (8). Moreover, micelles reportedly could enhance the absorption of lipid-soluble molecules from the gastrointestinal tract (9–11). As a consequence, the solubility and bioavailability of hydrophobic drugs can be enhanced (12–14). A few studies have attempted to fabricate carriers of diazepam by means of PEG-phospholipid-based polymeric micelles, including the classical mixed micelles as well as the sterically stabilized mixed micelles (15,16). Nevertheless, no polymeric micelle formulation of diazepam has been achieved and available in the pharmaceutical market up to now.

Poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO) is well known as a nontoxic, non-immunogenic, and non-antigenic water-soluble polymer. The derivatives of PEG/PEO have been proven to be useful and versatile in a variety of drug delivery carriers, especially polymeric micelles. Most copolymers used to fabricate polymeric micelles consist of PEG/PEO chain embedded in copolymer backbone (6). The PEG/PEO segment tethers into an aqueous environment possessing "stealth" property in blood circulation. Owing to its steric hindrance, the hydrophilic anchor of micelles can prevent opsonization of proteins or blood components and thus prolong the micelle circulation time in the blood.

[1] Excellent Center of Innovative Drug Delivery and Nanomedicine, Department of Pharmacy, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayutthaya Road, Rajthevi, Bangkok 10400, Thailand.
[2] To whom correspondence should be addressed. (e-mail: jiraphong.suk@mahidol.ac.th)

Poloxamers are one of the extensively studied examples for drug delivery and consist of various units of propylene oxide (PO) and ethylene oxide (EO) rendering numerous PO/EO block lengths (17,18). Poloxamer 407 (P407, critical micelle concentration (CMC) of 2.8×10^{-6} M) and poloxamer 188 (P188, CMC of 4.8×10^{-4} M) have been widely investigated for drug delivery vehicles including polymeric micelles (6,19). The core formed by PO chains is separated from the aqueous compartment by EO chain corona. Recently, D-α-tocopheryl poly(ethylene glycol) 1000 succinate (TPGS), a water-soluble derivative of vitamin E and PEG, has been introduced and gained much attention in drug delivery research. It is an excellent solubilizer, emulsifier, and bioavailability enhancer. It has been approved by USFDA for use as a water-soluble vitamin E nutritional supplement and drug delivery vehicle. Above its CMC of 1.3×10^{-6} M, the low viscous solution of polymeric micelles was generated (20,21). Moreover, its hydrophobic compartment is relatively bulky, which possibly facilitates high drug solubilization in the micelle core (22).

In spite of many attractive advantages of polymeric micelles as promising drug delivery systems, one drawback is their physical instability. As micelles are in the nanosize range and more dynamic than polymeric nanoparticles, they exhibit greater aggregation tendency due to kinetic motion. Upon storage and transportation, drug leakage may take place due to diffusion of the drug outward the micelles during fluctuation of temperature (23). One approach to overcome such problems is complete elimination of water by lyophilization into a dried powder form. Consequently, their shelf-life can be extended by preventing aggregation of the system and leakage of the encapsulated drug. Therefore, this study aimed to develop lyophilized diazepam-loaded polymeric micelles using TPGS, P407 and P188 as polymers. The physicochemical characteristics of the polymeric micelles were investigated in terms of particle size, polydispersity index, zeta potential, %entrapment efficiency, %drug loading, and redispersibility. Furthermore, their physical and chemical stabilities after 3 months storage at 4°C were evaluated. Finally, an effect of composition of the selected formulations on the release kinetics was also determined.

MATERIALS AND METHODS

Materials

Diazepam was supplied by A.N.H. Trading Limited Partnership, Bangkok, Thailand. TPGS, P188, and P407 were kindly gifted from BASF, Ludwigschafen, Germany. Methanol (Honeywell Burdick & Jackson, USA) was of high-performance liquid chromatography (HPLC) grade. Acetone (Honeywell Burdick & Jackson, USA), potassium dihydrogen phosphate (Carlo ERBA reagents, Val de Reuil, France), and sterile water for irrigation (Thai Otsuka Pharmaceutical Co. Ltd., Samutsakorn, Thailand) were used as received.

Screening a Suitable Polymer for Diazepam-Loaded Polymeric Micelles

To screen for a suitable polymer which entirely entrapped diazepam at the desirable concentration, three polymers were chosen, namely, TPGS, P188, and P407. The percentages of polymers were varied over a range of 1–20% *w/v*. A concentration of the polymer at which could completely entrap 5 mg/mL of diazepam was selected for further experiment. The polymeric micelles were prepared by solvent diffusion evaporation method according to the previously published procedure with minor modification (24). Briefly, various amounts of polymer were weighed and dissolved in 4 mL of acetone. In case of drug-loaded polymeric micelles, 50 mg of diazepam was added in acetone containing polymer. Subsequently, the polymeric solution was added drop-by-drop in 10 mL of water under magnetic stirring. The solvent was allowed to evaporate for an hour at room temperature under continuously magnetic stirring. Afterwards, the volume of the obtained solution was adjusted to an initial volume with water and then centrifuged at 4,500 rpm for 15 min to remove any aggregates. Finally, the supernatant was collected for further analysis.

Formulation and Optimization of Lyophilized Diazepam-Loaded Polymeric Micelles

To formulate the dried powder form of diazepam-loaded polymeric micelles, the suitable polymer concentration of diazepam-loaded polymeric micelles was chosen to formulate lyophilized diazepam-loaded polymeric micelles. Either P188 or P407 was added in an aqueous phase as a stabilizer. The concentrations of stabilizer were varied at 1%, 3%, and 5% *w/v*. The diazepam-loaded polymeric micelles were prepared as previously described in the screening study by dissolving the stabilizer in 10 mL of water prior to the addition of polymeric solution. After centrifugation, 2 mL of supernatant was pipetted into a 5-mL vial and frozen at −75°C overnight. Subsequently, a vial containing polymeric micelles was submitted to lyophilizer (Christ Alpha1-4, SciQuip, UK) for at least 24 h. The temperature of the condenser was −40°C and the pressure was 0.120 mbar. The lyophilized diazepam-loaded polymeric micelles were finally collected and kept in the refrigerator until used.

Particle Size and Polydispersity Index Analysis

The particle size (z-ave) and polydispersity index (PI) were measured by Zetasizer NanoZS (Malvern Instrument, Malvern, UK). The sample was measured with He–Ne laser at a wavelength of 633 nm, 173° angle through a quartz cell without any dilution. All measurements were performed in triplicate at 25°C.

Zeta Potential Analysis

The surface charge of polymeric micelles was evaluated in terms of zeta potential (ZP) by Zetasizer NanoZS. According to the electrophoretic mobility of micelles, the ZP value was calculated by the Helmholtz–Smoluchowsky equation. All measurements were performed in triplicate at 25°C.

Percentages of Entrapment Efficiency and Drug Loading

The percentages of entrapment efficiency (%EE) and drug loading (%DL) were determined by direct method (24). Two milliliters of methanol were added into the vial containing the lyophilized cake. The sample was mixed and

sonicated for 30 min. The methanolic solution was pipetted and diluted with a mobile phase prior to HPLC analysis. The %EE and %DL were calculated according to Eqs. (1) and (2), respectively.

$$\%EE = \frac{\text{Analyzed amount of diazepam}}{\text{Theoretical amount of diazepam}} \times 100 \quad (1)$$

$$\%DL = \frac{\text{Analyzed amount of diazepam}}{\text{Theoretical amount of solid content}} \times 100 \quad (2)$$

Quantitative Analysis of Diazepam by HPLC

The amount of diazepam was quantified by HPLC assay. The sample was injected to HPLC instrument (1200 series, Agilent Technologies, Santa Clara, USA) equipped with a diode array detector at a wavelength of 230 nm. The drug was eluted through a reverse phase Luna C18 column, 5 μm particle size, 150×4.6 mm, plus a C18 guard column (Phenomenex, Torrance, USA) at a flow rate of 1 mL/min. The mixture of methanol and water (65:35% *v*/*v*) was used as a mobile phase. The HPLC performance was validated in terms of linearity, precision, and accuracy. The amount of drug was calculated from a calibration curve over the concentration range of 0.5–100 μg/mL with R^2 of at least 0.9995. The relative standard deviation of inter- and intraday precisions was less than 2%. The %recovery of diazepam was in the acceptable range of 90–110% (25).

Redispersibility Study of Lyophilized Diazepam-Loaded Polymeric Micelles

The redispersibility of lyophilized polymeric micelles was evaluated according to the previously published method (26,27). Sterile water (2 mL) was slowly added in a lyophilized vial. The lyophilized sample was rehydrated for 2 min and then continuously mixed by a vortex mixer (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) for 5 min. Subsequently, the particle size of the sample was characterized by Zetasizer NanoZS. The results are expressed as the particle size ratio (S_2/S_1) according to Eq. (3).

$$S_2/S_1 \text{ ratio} = \frac{\text{Particle size after reconstitution}}{\text{Particle size before lyophilization}} \quad (3)$$

Physical Stability Study of Lyophilized Diazepam-Loaded Polymeric Micelles

The physical stability of lyophilized polymeric micelles was evaluated after 3 months storage at 4°C. The height of lyophilized cake was compared with that at an initial time. The redispersibility of lyophilized polymeric micelles was conducted according to the aforementioned method. The height of lyophilized cake, particle size, polydispersity index, and zeta potential were recorded after 3 months compared with those at the initial time. The changes in height of lyophilized cake and particle size and polydispersity index of polymeric micelles are expressed as the height (H_3/H_0), particle size (S_3/S_0), and PI (PI_3/PI_0) ratios, respectively. The subscript numbers 0 and 3 denote 0 and 3 months after storage, respectively.

Chemical Stability Study of Lyophilized Diazepam-Loaded Polymeric Micelles

Based on the physical stability results, the formulations stabilized with 1% *w*/*v* P407 were chosen to evaluate their chemical stability in comparison with those without P407. The chemical stability of the selected lyophilized polymeric micelles was evaluated after 3 months storage at 4°C. The lyophilized powder was dissolved in 2 mL of methanol and then sonicated for 30 min. The methanolic solution was diluted with a mobile phase and analyzed by HPLC method. The remaining amount of diazepam was expressed as %diazepam remaining relative to an amount of drug at an initial time.

Morphological Examination of Polymeric Micelles

The morphology of polymeric micelles was examined by transmission electron microscopy (TEM, TECNAI^{TM} T20 G^2, FEI, Hilsboro, USA) and scanning electron microscopy (SEM, Hitachi S4500, Hitachi, Tokyo, Japan). In case of TEM, an aliquot of samples was air-dried on a carbon-coated grid and then visualized by TEM at an accelerating voltage of 120 kV. For SEM measurement, the sample was air-dried and sputtered with gold prior to observation by SEM equipped with field-emission-cathode using a 15-kV upper detector.

In vitro Drug Release Study

The release of diazepam from polymeric micelles was conducted by dialysis method (28,29). A milliliter of diazepam-loaded polymeric micelles was filled in a dialysis bag (MWCO 1,000 Da, Spectra/Pore®, Spectrum Laboratories Inc., USA). The tightly sealed dialysis bag was then immersed in a release chamber containing 500 mL of phosphate buffer solution pH 7.4. The experiment was performed at 37°C under continuously magnetic stirring at 100 rpm. One milliliter of sample was withdrawn at predetermined time intervals and immediately replaced with an equal volume of fresh release medium. The amount of diazepam in the sample was analyzed by HPLC. The release study of diazepam solution was also performed for comparison.

In vitro Release Kinetics

To evaluate the release mechanism of diazepam from TPGS polymeric micelles, the drug release profile was fitted by zero-order, first-order, Higuchi's, Hixson–Crowell's, and Korsmeyer–Peppas's models (30–32). The first 60% of the drug release was fitted to the models. The linear regression analysis was applied for the calculation of R^2. To compare among all models, the adjusted coefficient of determination (R^2_{adjusted}) was applied. The Akaike Information Criterion (AIC) and sum of squares of residues (SSR) were further applied for the determination of the best fit model to the release profiles. The model that gives the smallest values of SSR and AIC describes the best drug release mechanism (33,34).

Statistical Analysis

The data are expressed as mean±S.D. from at least three measurements. The *t* test or one-way ANOVA with the Scheffe test applied post hoc for paired comparisons were performed to compare two or multiple groups, respectively. All analyses were determined using the SPSS program (SPSS 13.0 for Windows) and differences were considered to be significant at a level of p value<0.05. To compare the release profiles, the difference and similarity factors (f_1 and f_2, respectively) were calculated (35). In general, the f_1 values closed to 0 (0–15) and f_2 values more than 50 (50–100) consider that two release profiles are similar.

RESULTS AND DISCUSSION

Screening a Suitable Polymer for Diazepam-Loaded Polymeric Micelles

In general, a usual rectal dose of diazepam for pediatrics is 0.2–0.5 mg/kg for children older than 2 years of age (2). By calculation based on body weight, age, and dose of patients, the smallest rectal dose of diazepam was 5 mg (36). Therefore, the designed concentration of diazepam in this study was 5 mg/mL at which 1 mL of micelles can be easily administered to the rectum of children and would not cause drug leakage. Thus, in this study, the concentration of drug was fixed at 5 mg/mL while the polymer concentration was varied at 1%, 3%, 5%, 7.5%, 10%, 15%, and 20% *w/v*. The optimum concentration of polymers that enabled complete entrapment of 5 mg/mL diazepam was chosen. Three commercially available polymers (TPGS, P188, and P407) were used in this study since they have been approved by USFDA for the internal use in human and their CMC values have been well established (37–39). The physicochemical characteristics of blank and diazepam-loaded polymeric micelles are illustrated in Fig. 1. The z-ave and PI values of TPGS polymeric micelles remained almost constant at all polymer concentrations even after drug loading (8–12 nm and 0.053–0.275, respectively). However, the z-ave of all P407 polymeric micelles was found in the range of 20–35 nm except for the formulation containing 20% *w/v* of P407 whose particle size could not be evaluated

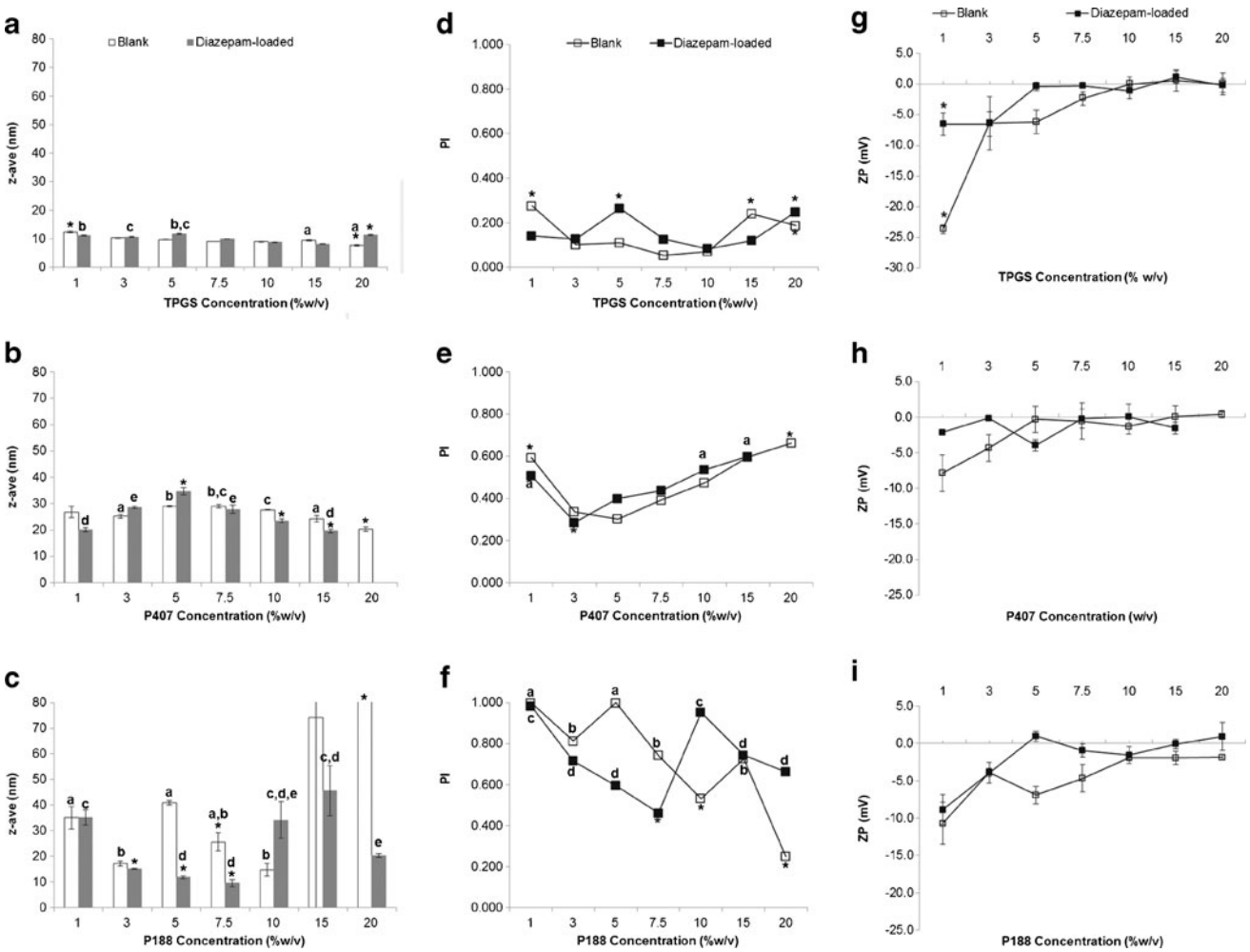

Fig. 1. Particle size (z-ave, *left column*), polydispersity index (PI, *middle column*), and zeta potential (ZP, *right column*) of the blank (*white column and opened square*) and diazepam-loaded (*black column and closed square*) polymeric micelles prepared by TPGS (*top row*), P407 (*middle row*), and P188 (*bottom row*). Mean±S.D, $n \geq 3$. *Statistically significant difference comparing among all formulations prepared by the same polymer type but the different polymer concentrations. No significant difference comparing between the formulations labeled with the same *lowercase letter*

due to the gelation after drug incorporation. The PI values increased with an increase in polymer concentration. In contrast, P188 micelles provided various particle sizes and large PI values which were unrelated to the polymer concentration. Among all the polymers used, the largest particle of polymeric micelles (737±17 nm) was obtained from 20% *w/v* P188. As illustrated in the size distribution curves (Fig. 2), two distinct peaks were observed in case of P407 and P188 micelles. The first peak was associated with the micelles and the latter contributed to the micellar aggregates. In addition, P188 micelles exhibited larger aggregates as compared to P407 micelles. These results are consistent with the previous reports demonstrating the coexistence of unimer, micelles, and micellar clusters (40,41).

All formulations showed the negative ZP and these values approached zero when increasing the polymer concentration. The reduction in absolute ZP was possibly attributed to the adsorption of polymers on the surface of particles (42,43). The residual unimers of polymers which do not form the micelles could form an adsorption layer on the surface of particles. The adsorption layer of polymers shifts the plane of shear to a larger distance from the particle surface. The results showed that increasing P188 and P407 concentrations reduced the absolute ZP closed to zero. In the meantime, incrementing TPGS concentration demonstrated more dramatic reduction in absolute ZP particularly that of the blank TPGS micelles. These results are in agreement with the previous reports (42,43). It has been found that P188 and P407 exhibited the

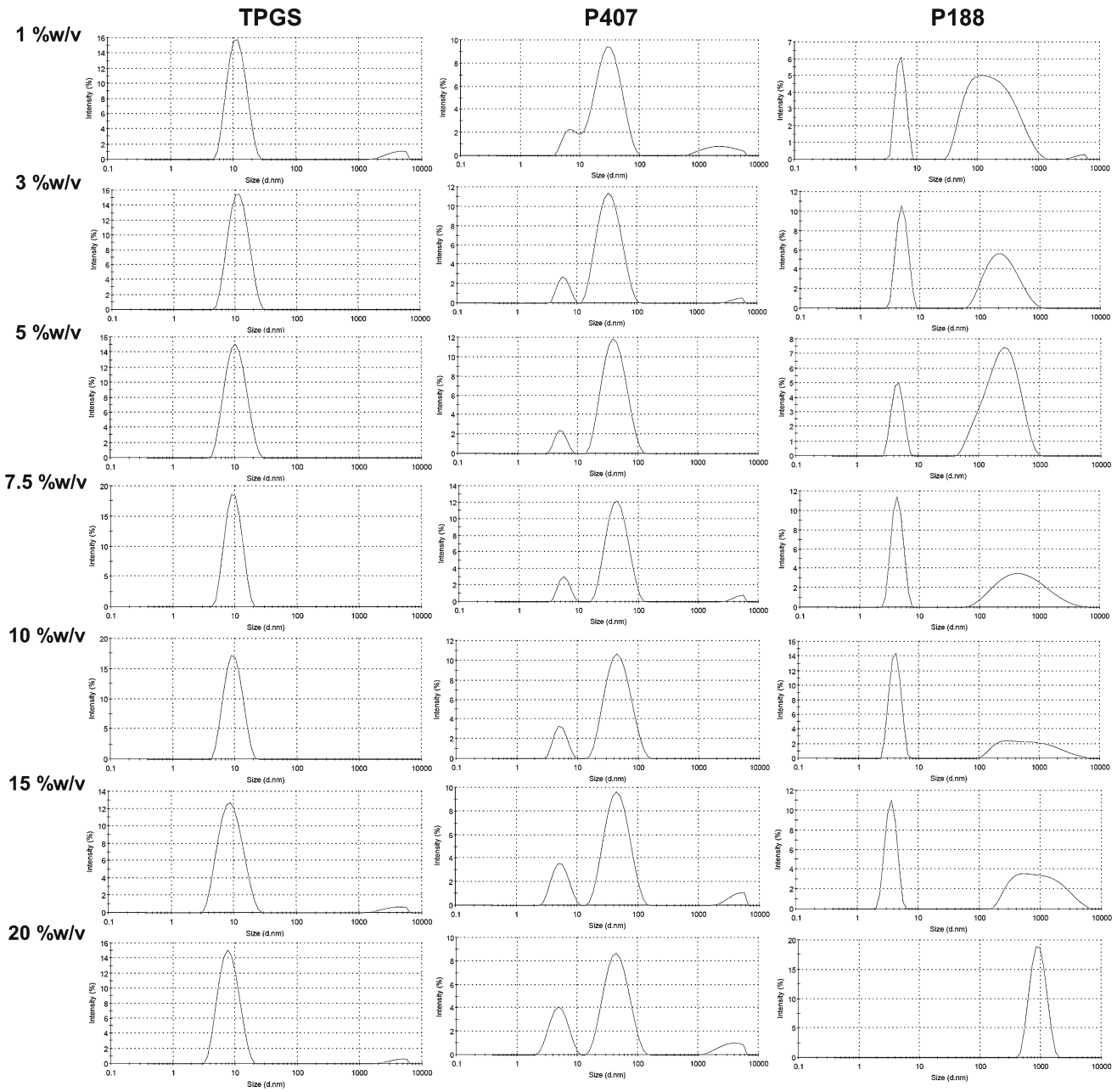

Fig. 2. Size distribution curves of the blank TPGS (*left column*), P407 (*middle column*), and P188 (*right column*) polymeric micelles at various concentrations of polymers

general tendency of a further decrease of ZP with increasing polymer concentration especially for P407. Likewise, TPGS showed even more notable reduction in absolute ZP with incrementing polymer concentration compared to P188 and P407.

Concerning the entrapment efficiency and drug loading of polymeric micelles as shown in Fig. 3, %EE of TPGS and P407 polymeric micelles was related to an increment of polymer concentration. The TPGS concentrations at 7.5%, 10%, 15%, and 20% *w/v* yielded %EE beyond 85%. %DL of TPGS gradually increased with TPGS concentration and the maximum %DL was obtained at 7.5% *w/v* of TPGS. After this point, a decrease in %DL was due to the calculation of that value based on the total solid content at initial feeding which increased with an increase in TPGS concentration at a constant loaded drug. The highest loading capacity of TPGS was probably due to the bulky inner core facilitating the retention of drug inside the micelle core (22). In case of P407, %DL of P407 polymeric micelles did not depend on the polymer concentration until it reached 20% *w/v*. At this level, the greatest %EE (99.13%) and %DL (2.39%) were attained; however, the formulation became a gel and could not be used. Furthermore, the use of P188 led to unfavorable loading capacity of diazepam-loaded polymeric micelles. The maximum %EE and %DL of P188 polymeric micelles were 5.06% and 1.12%, respectively. The increasing concentration of P188 up to 20% *w/v* did not improve %EE and %DL. Lower loading capacity of P188 as compared to that of P407 was possibly due to higher CMC, shorter hydrophobic chain, and smaller molecular weight of P188 (17). Of these results, the maximum loading capacity and the drug entrapment at the desired concentration were achieved from the formulation composed of at least 7.5% *w/v* TPGS. Therefore, TPGS polymeric micelles comprising 7.5%, 10%, and 15% *w/v* were selected for further study.

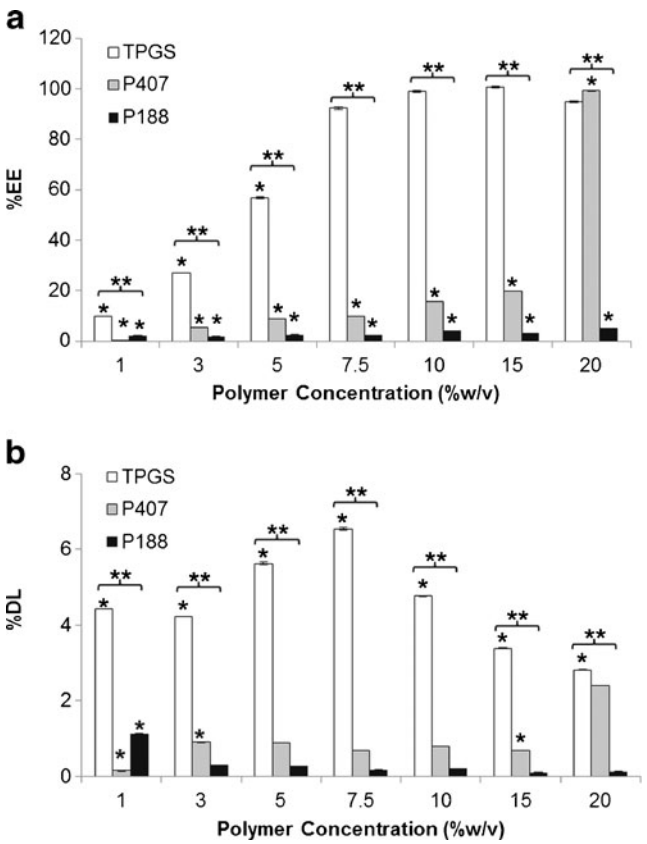

Fig. 3. The percentages of entrapment efficiency (%EE) and drug loading (%DL) of diazepam-loaded polymeric micelles prepared by TPGS (*white column*), P407 (*gray column*), and P188 (*black column*). Mean±S.D, $n \geq 3$. *Statistically significant difference comparing among all formulations prepared by the same polymer type but the different polymer concentrations. **Statistically significant difference comparing the formulations prepared by the same polymer concentration but the different polymer types

After lyophilization, the lyophilized powder of polymeric micelles consisting of 7.5%, 10%, and 15% *w/v* of TPGS was reconstituted and the particle size was then measured. The results are expressed as S_2/S_1 ratio as illustrated in Table I. It was found that S_2/S_1 ratios of all selected formulations were in the range of 1.3–1.8. The largest ratio was found in case of blank polymeric micelles and the increasing TPGS concentration did not affect this ratio. Meanwhile, in case of drug-loaded polymeric micelles, the addition of drug and the increment of TPGS concentration tended to reduce S_2/S_1 ratio. Nevertheless, the particle size of lyophilized polymeric micelles after reconstitution was still larger than that after fresh preparation by at least 1.3 times. Hence, it was necessary to further study a suitable stabilizer added in the formulation to prevent the aggregation of polymeric micelles during the lyophilization process.

Formulation and Optimization of Lyophilized Diazepam-Loaded Polymeric Micelles

Effect of Poloxamers (P188 and P407) as Stabilizers

From the preliminary experiment, 1% *w/v* of P188 or P407 was chosen as a stabilizer for lyophilization of TPGS polymeric micelles. The physicochemical characteristics of TPGS polymeric micelles stabilized with P188 or P407 are presented in Figs. 4 and 5. As shown in Fig. 4, the addition of P188 had an impact on the particle size of blank polymeric micelles (p value<0.05) but did not significantly affect the particle size of diazepam-loaded polymeric micelles as compared to those without a stabilizer (p value>0.05). The size distribution of blank polymeric micelles tended to become larger but that of diazepam-loaded formulations remained almost unchanged except for 15% *w/v* TPGS micelles. In case of P407, the addition of 1% *w/v* P407 significantly reduced the size of particles before and after drug loading. Moreover, it

Table I. Particle Size Ratios (S_2/S_1 ratios) of the Blank and Drug-Loaded Polymeric Micelles After Reconstitution Compared with Those After Fresh Preparation

TPGS concentration	S_2/S_1 ratio	
(% *w/v*)	Blank polymeric micelles	Drug-loaded polymeric micelles
7.5	1.8±0.3	1.6±0.4
10	1.8±0.2	1.5±0.1
15	1.8±0.0	1.3±0.0

Mean±S.D. (n=3)

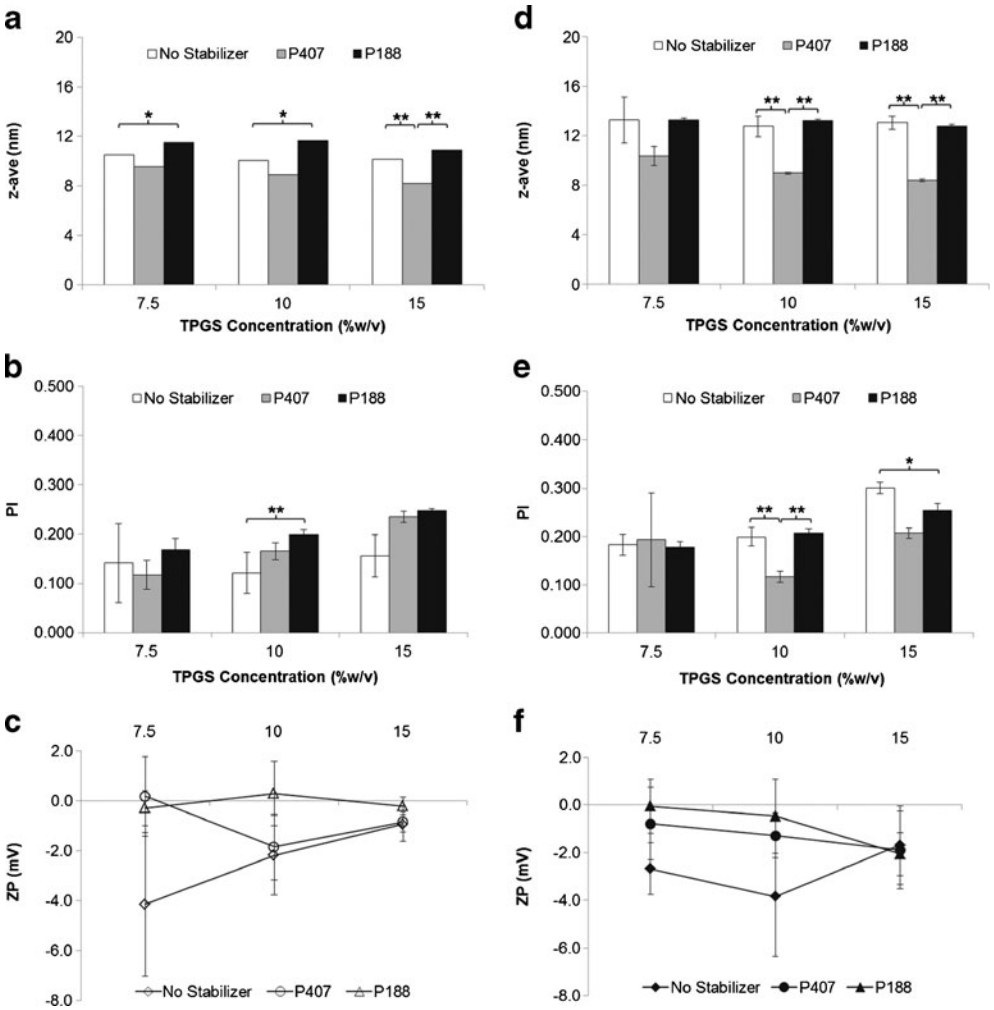

Fig. 4. Particle size (z-ave, *top row*), polydispersity index (PI, *middle row*), and zeta potential (ZP, *bottom row*) of the blank (*left panel*) and diazepam-loaded (*right panel*) TPGS polymeric micelles with or without a stabilizer (P407 or P188). Mean±S.D, $n \geq 3$. *Statistically significant difference comparing among all formulations prepared by the same TPGS concentration but the different stabilizer type. **Statistically significant difference between the compared formulations

decreased the PI values of diazepam-loaded polymeric micelles to less than 0.200. In addition, after adding either P188 or P407, the ZP values approached zero due to the adsorption layer of P188 or P407 on the particle surface as described previously. In Fig. 5, higher %EE was attained when P407 was used as a stabilizer as compared to P188. The greatest

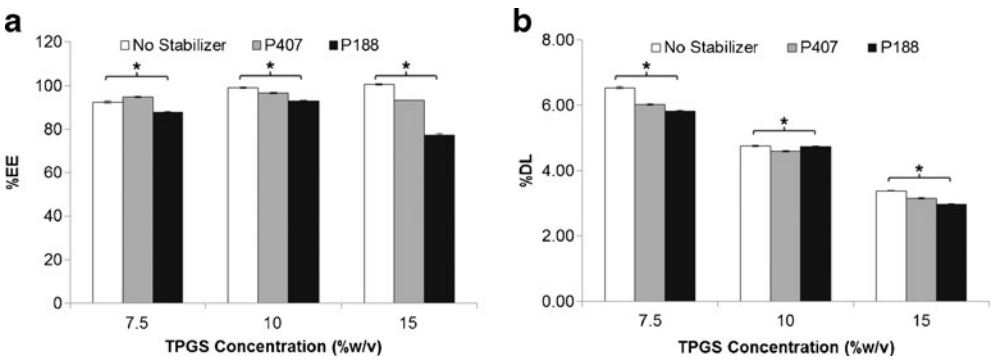

Fig. 5. The percentages of entrapment efficiency (%EE, **a**) and drug loading (%DL, **b**) of diazepam-loaded TPGS polymeric micelles with or without a stabilizer (P407 or P188). Mean±S.D, $n \geq 3$. *Statistically significant difference comparing among all formulations prepared by the same TPGS concentration but the different stabilizer type

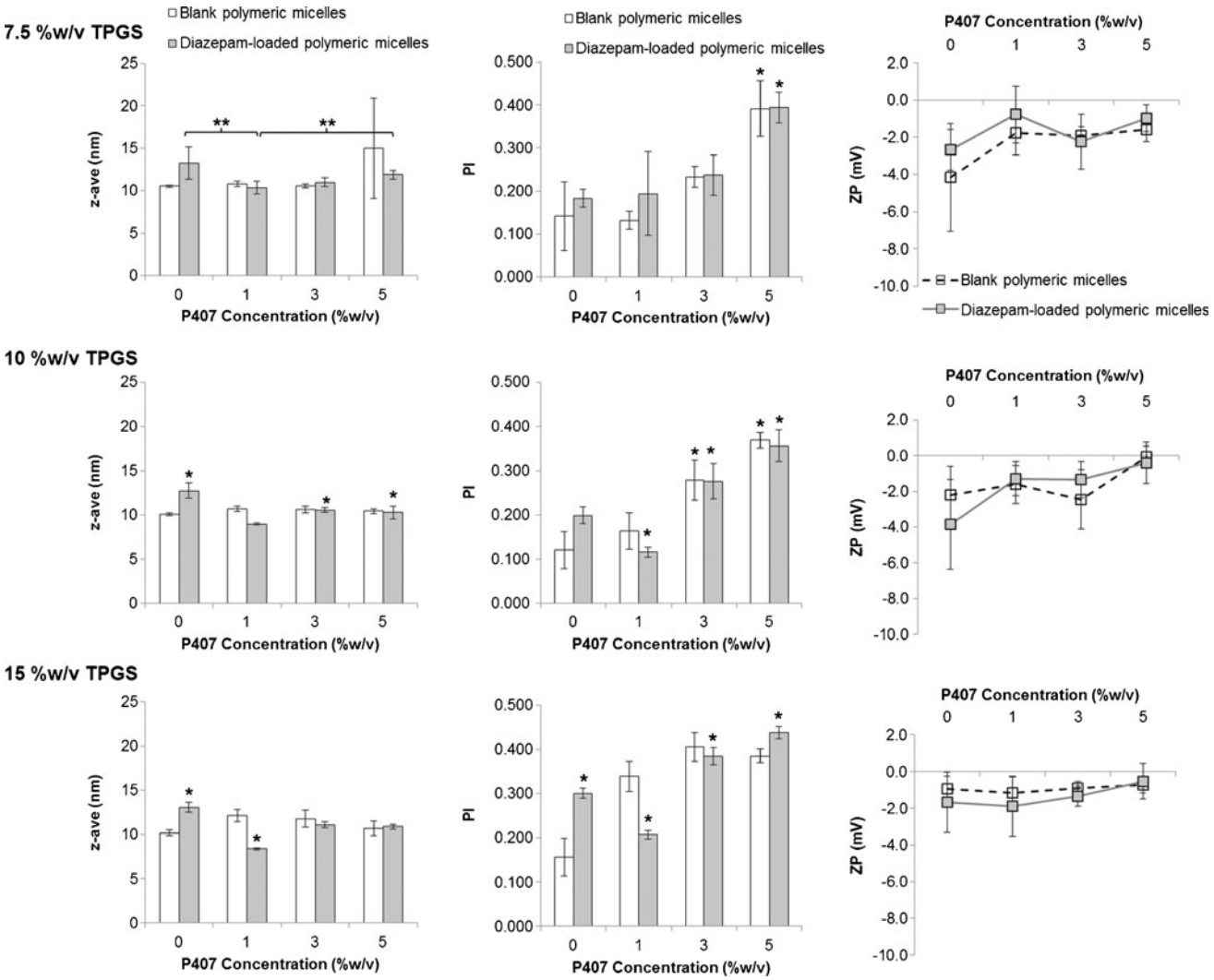

Fig. 6. Particle size (z-ave, *first panel*), polydispersity index (PI, *second panel*), and zeta potential (ZP, *third panel*) of polymeric micelles composed of 7.5, 10, and 15% *w/v* of TPGS and various concentrations of P407 acting as a stabilizer. Mean±S.D, $n \geq 3$. *Statistically significant difference comparing either the blank or diazepam-loaded formulations prepared by the same TPGS concentration but the different P407 concentrations. **Statistically significant difference between the compared formulations

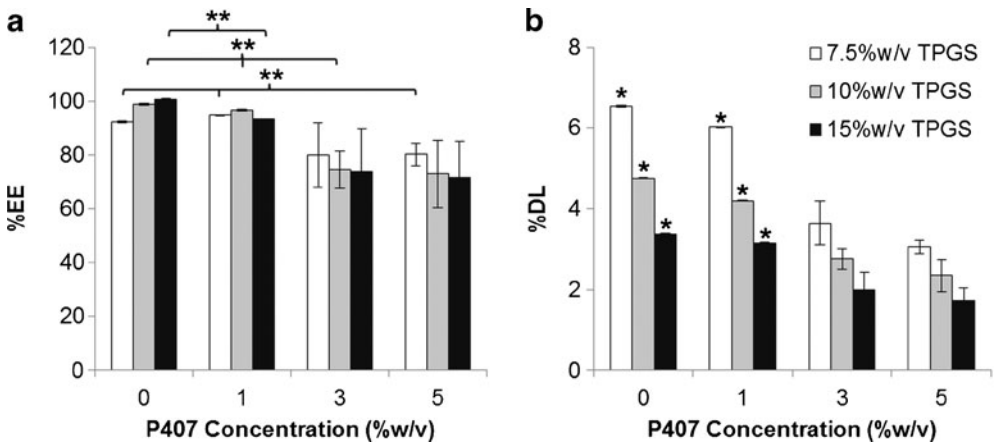

Fig. 7. The percentages of entrapment efficiency (%EE, **a**) and drug loading (%DL, **b**) of diazepam-loaded polymeric micelles composed of 7.5%, 10%, and 15% *w/v* of TPGS and various concentrations of P407. Mean±S.D, $n \geq 3$. *Statistically significant difference comparing among all formulations prepared by the same TPGS concentration but the different P407 concentrations. **Statistically significant difference between the compared formulations

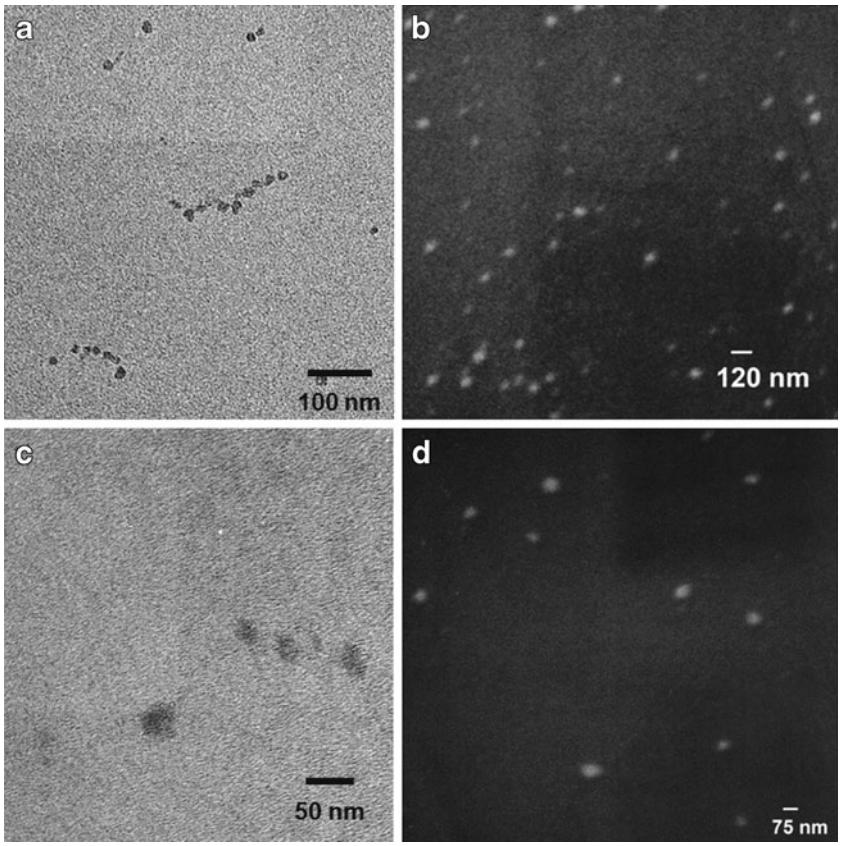

Fig. 8. Transmission electron microscopy (TEM, *left panel*) and scanning electron microscopy (SEM, *right panel*) of the blank (**a** and **b**) and diazepam-loaded (**c** and **d**) 10% *w/v* TPGS polymeric micelles containing 1% *w/v* P407

decrease of %EE was observed when P188 was used in the formulation. However, %DL slightly decreased after adding both stabilizers. From these results, P407 was chosen as a stabilizer for the lyophilization since it did not change the particle size and %EE of TPGS polymeric micelles.

Effect of Concentration of P407 Serving as a Stabilizer

Three concentrations (1%, 3%, and 5% *w/v*) of P407 were investigated to determine the minimum P407 concentration for stabilization of the lyophilized polymeric micelles. The

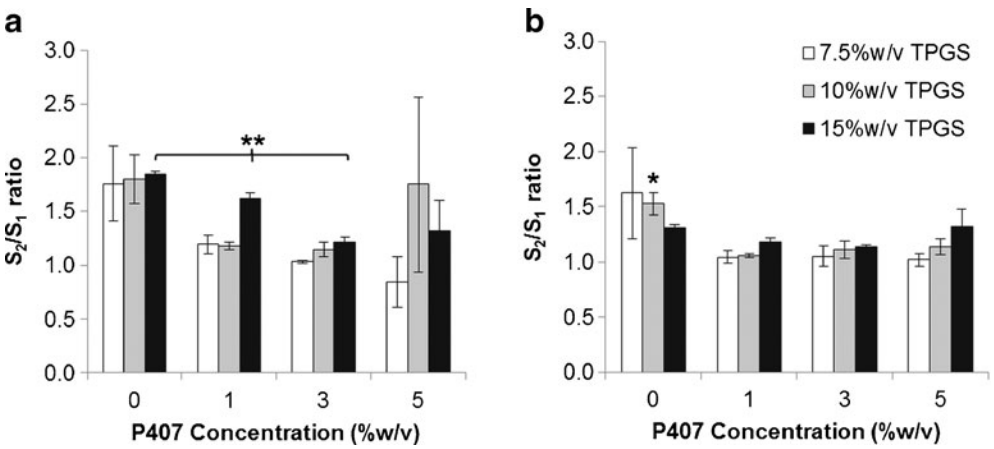

Fig. 9. Particle size (S_2/S_1) ratio of the blank (**a**) and diazepam-loaded (**b**) polymeric micelles composed of 7.5%, 10%, and 15% *w/v* of TPGS and various concentrations of P407. Mean±S.D, $n \geq 3$. *Statistically significant difference comparing among all formulations prepared by the same TPGS concentration but the different P407 concentrations. **Statistically significant difference between the compared formulations

Table II. The Measured Parameters of the Blank and Diazepam-Loaded Polymeric Micelles After 3 Months Storage at 4°C Compared with the Initial Time

Concentration (% *w/v*)		H_3/H_0[a]	S_3/S_0[b]	PI_3/PI_0[c]	ZP[d] (mV)	
TPGS	P407	Ratio	Ratio	Ratio	0 month	3 months
Blank polymeric micelles						
7.5	0	0.90	2.3	4.8	−2.28	−1.56
	1	0.88	1.3	1.3	−2.01	−2.76
	3	0.89	1.6	1.2	−1.78	−2.68
	5	0.87	1.0	1.0	−1.33	−1.03
10	0	1.03	2.0	3.5	−0.83	−1.13
	1	0.99	1.4	1.6	−0.81	−0.88
	3	1.04	1.0	0.9	−4.61	2.32
	5	1.06	1.1	1.0	−0.28	−1.41
15	0	0.91	2.2	2.2	−1.23	−0.40
	1	0.92	1.5	1.2	−0.38	−0.78
	3	0.94	1.1	1.0	−0.66	−0.77
	5	0.96	1.0	0.9	−0.88	−0.68
Diazepam-loaded polymeric micelles						
7.5	0	0.88	6.0	1.0	−3.12	−1.89
	1	0.93	458.1	4.1	−3.05	−2.21
	3	0.98	751.8	2.3	−3.96	−1.80
	5	1.02	1104.2	2.0	−1.18	−1.05
10	0	0.92	1.5	1.2	−5.06	−0.73
	1	0.99	1.0	0.9	−3.02	−0.19
	3	0.99	1.0	0.9	−2.39	0.76
	5	0.99	1.1	0.9	−0.62	−0.12
15	0	1.01	1.5	1.2	−0.61	−0.98
	1	0.97	1.0	0.9	−3.29	−0.66
	3	0.98	1.3	1.3	−1.76	−0.71
	5	0.97	1.1	1.0	−0.54	−0.36

[a] The ratio of height of lyophilized cake
[b] The ratio of particle size of polymeric micelles
[c] The ratio of polydispersity index of polymeric micelles
[d] Zeta potential, the subscript numbers 0 and 3 denote 0 and 3 months after storage, respectively

results are shown in Figs. 6 and 7. The addition of all concentrations of P407 reduced the particle size of all diazepam-loaded polymeric micelles; however it insignificantly altered the particle size of the blank micelles (Fig. 6). Meanwhile, the PI values of all polymeric micelles obviously increased with the P407 concentration but their ZP values were not significantly changed (p value > 0.05). In Fig. 7, the increasing P407 concentration gradually decreased %EE and %DL. The morphology of the blank and diazepam-loaded TPGS micelles was graphically exemplified in Fig. 8. TEM and SEM micrographs indicated that TPGS micelles were spherical in shape.

Redispersibility Study of Lyophilized Polymeric Micelles

To assess the efficiency of P407 acting as a stabilizer, the redispersibility of lyophilized polymeric micelles was investigated. As shown in Fig. 9, the absence of P407 in the blank and diazepam-loaded polymeric micelles caused the S_2/S_1 ratio higher than 1.3. The addition of P407 (1%, 3%, and 5% *w/v*) greatly decreased such ratio to nearby 1.0 especially in case of diazepam-loaded polymeric micelles at all TPGS concentrations. The result indicated that 1% *w/v* P407 was sufficient to stabilize diazepam-loaded polymeric micelles upon the lyophilization process.

Physical Stability of Lyophilized Diazepam-Loaded Polymeric Micelles

After storing at 4°C for 3 months, the physical stability of lyophilized polymeric micelles was evaluated in terms of the changes in particle size, polydispersity index, zeta potential, and height of lyophilized cake. The results are compiled in Table II. The height of lyophilized cake and particle size and polydispersity index of polymeric micelles are expressed as the

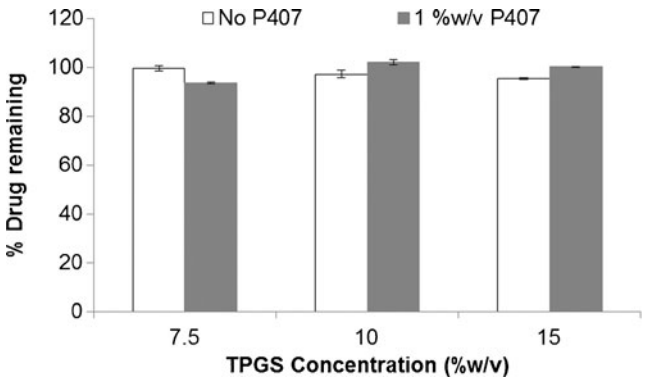

Fig. 10. Percent diazepam remaining of polymeric micelles comprising 7.5%, 10%, and 15% *w/v* of TPGS and 1% *w/v* of P407 in comparison with those without P407 after 3 months storage at 4°C. Mean ± S.D, $n \geq 3$

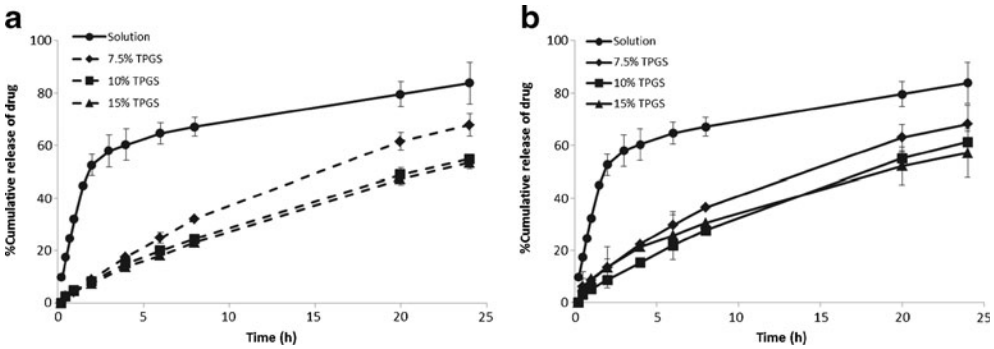

Fig. 11. Release profiles of diazepam-loaded polymeric micelles without (**a**) and with (**b**) 1% *w/v* of P407 as a stabilizer in comparison with that of diazepam solution. Mean±S.D, $n \geq 3$

ratios after 3 months storage compared with an initial time. The height ratio of most formulations after 3 months storage was unchanged. However, the formulation without a stabilizer could not be redispersed to obtain the same size and size distribution to the original ones. The physical stability of the blank directly depended on the concentration of P407. The highest concentration of P407 showed the maximum physical stability without any change in particle size and polydispersity index ratios. Meanwhile, the diazepam-loaded 7.5% *w/v* TPGS polymeric micelles could not be physically stabilized by P407. The inverse relation of physical stability and P407 concentration was observed. The increasing P407 concentration led to larger particles after reconstitution. The particle size of diazepam-loaded 7.5% *w/v* TPGS polymeric micelles greatly increased after storage for 3 months probably due to the precipitation of drugs during storage. Nevertheless, the TPGS concentrations at 10% and 15% *w/v* could prevent the physical instability of diazepam-loaded polymeric micelles. The formulations without P407 showed 1.5 times larger particles than the initial size. The addition of P407 at the lowest concentration (1% *w/v*) resulted in the diazepam-loaded polymeric micelles with unchanged particle size and size distribution as compared to those at the initial. The ZP values of the blank and drug-loaded polymeric micelles were not affected by storage at 4°C for 3 months. From these results, the concentration of TPGS could affect the physical stability of diazepam-loaded polymeric micelles higher than that of P407. The minimum concentrations of TPGS and P407 were 10% and 1% *w/v*, respectively.

Chemical Stability of Lyophilized Diazepam-Loaded Polymeric Micelles

From the physical stability results, 1% *w/v* of P407 was sufficient to stabilize the system. Hence, the formulations containing 1% *w/v* P407 were selected to evaluate their chemical stability in comparison with those without P407. The results are demonstrated in Fig. 10. The %diazepam remaining of all selected formulations was higher than 90% as compared to that at the initial time. According to the %labeled amount stated in USP 35/NF 30, the %diazepam remaining was in the range of 90–110% indicating that all formulations were chemically stable upon 3 months storage at 4°C (25). Combining the results of physical and chemical stability tests, it can be concluded that 10% and 15% *w/v* TPGS micelles with 1% *w/v* P407 were suitable for further development.

In vitro Drug Release Study

In order to investigate the release characteristics of diazepam from polymeric micelles, the *in vitro* release study was performed in phosphate buffer solution pH 7.4 at 37°C. The diazepam-loaded polymeric micelles composed of 7.5%, 10%, and 15% *w/v* TPGS and 1% *w/v* P407 were used to investigate in comparison with those without P407. In addition, the release of diazepam solution was also evaluated to assure that the released drug could freely pass through the dialysis bag. The release profiles are illustrated in Fig. 11. It was discovered that the diazepam solution showed the fast release from dialysis bag by over 50% within 2 h and more than 80% within

Table III. The Coefficient of Determination (R^2) and the Adjusted Coefficient of Determination ($R^2_{adjusted}$) Based on the Release Data Fitted to Different Kinetic Equations

Formulations		Zero-order		First-order		Higuchi's		Hixson–Crowell's		Korsmeyer–Peppas's	
TPGS (% *w/v*)	P407 (% *w/v*)	R^2	$R^2_{adjusted}$	R^2	$R^2_{adjusted}$	R^2	$R^2_{adjusted}$	R^2	$R^2_{adjusted}$	R^2	$R^2_{adjusted}$
7.5	–	0.978	0.980	0.701	0.701	0.994	0.994	0.725	0.725	0.980	0.977
10	–	0.983	0.987	0.765	0.765	0.991	0.991	0.713	0.713	0.987	0.986
15	–	0.988	0.992	0.799	0.799	0.986	0.986	0.731	0.731	0.992	0.991
7.5	1	0.958	0.958	0.786	0.786	0.998	0.997	0.651	0.651	0.971	0.967
10	1	0.985	0.985	0.786	0.786	0.989	0.989	0.724	0.724	0.989	0.988
15	1	0.949	0.949	0.786	0.786	0.999	0.999	0.613	0.613	0.970	0.965

Table IV. Comparison of the Sum of Squares of Residues (SSR) and the Akaike Information Criterion (AIC) Values Attained From the Experimental Release Data Fitted to the Kinetic Equations

Formulations		Zero-order			Higuchi's			Korsmeyer–Peppas's			
TPGS (% *w/v*)	P407 (% *w/v*)	SSR×10^2	AIC	k^a (mg/h)	SSR×10^2	AIC	k^a (h^{-1})	SSR×10^2	AIC	k^a (h^{n-1})	n[b]
7.5	–	1.03	−59.00	2.79	0.45	−66.46	16.30	1.02	−57.01	0.96	0.928
10	–	0.57	−64.33	2.19	0.27	−70.91	12.71	0.57	−62.31	0.79	0.783
15	–	0.38	−68.04	2.14	0.41	−67.32	12.38	0.38	−66.03	0.77	0.771
7.5	1	1.79	−53.98	2.72	0.10	−79.65	15.34	2.04	−50.80	0.64	0.642
10	1	1.73	−54.30	2.14	0.47	−66.06	14.33	0.58	−62.07	0.78	0.784
15	1	1.53	−55.37	2.21	0.10	−79.68	12.31	1.77	−52.07	0.58	0.582

[a] k denotes a release rate constant of the respective model
[b] Release exponent

24 h. In case of TPGS micelles, the total amount of released diazepam over 24 h was within the range of 53.41–68.10% for all formulations. The increasing TPGS concentration slightly decreased the total drug release over 24 h. The addition of 1% *w/v* P407 did not affect the rate and extent of drug release from TPGS polymeric micelles (f_1 and f_2 values = 13.2 and 70.7, 10.4 and 73.8, and 26.2 and 62.7 for 7.5%, 10%, and 15% *w/v* TPGS micelles with 1% *w/v* P407 compared to those without P407, respectively). The fastest release rate was obtained from the formulations containing 7.5% *w/v* TPGS with and without P407. In the meantime, 10% and 15% *w/v* TPGS polymeric micelles possessed somewhat similar release rate and amount of diazepam regardless of the presence of P407 in the formulation.

In vitro Drug Release Kinetics

To describe the release mechanism of diazepam from TPGS polymeric micelles, various kinetic equations (zero-order, first-order, Higuchi's, Hixson–Crowell's, and Korsmeyer–Peppas's models) were applied to fit with the release data. Table III demonstrates R^2 and $R^2_{adjusted}$ values from the model fitting of the release profiles. As can be seen by the highest R^2 and $R^2_{adjusted}$ in Table III, all formulations were best fitted with Higuchi's, zero-order, and Korsmeyer–Peppas's models. Thus, the experimental release data were further evaluated by using the AIC and SSR values. As compiled in Table IV, the smallest AIC and SSR values of most formulations were obtained when the release profiles were fitted to Higuchi's model. In contrast, the release data of 15% *w/v* TPGS micelles without P407 was properly fitted to zero-order model. Of these results, it can be concluded that the diazepam release from TPGS polymeric micelles was described by Fickian diffusion.

CONCLUSION

From the obtained results, it can be concluded that the diazepam-loaded polymeric micelles were successfully developed by using TPGS as a main composition. The minimum concentration of TPGS at 7.5% *w/v* could entrap an entire amount of diazepam in the polymeric micelles. The 1% *w/v* P407 properly acted as a good stabilizer in the formulation to maintain the micelle characteristics without a significant reduction in entrapment efficiency. The formulations containing 10% and 15% *w/v* TPGS and 1% *w/v* P407 were chemically and physically stable after storage at 4°C for 3 months. The release rate and extent of diazepam-loaded TPGS polymeric micelles were mainly dependent on TPGS concentration but independent of the presence of P407 in the formulation. The release of diazepam follows Fickian diffusion. From all results, the minimum concentrations of TPGS and P407 for diazepam-loaded polymeric micelles were 10% and 1% *w/v*, respectively.

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REFERENCES

1. Milligan NM, Dhillon S, Griffiths A, Oxley J, Richens A. A clinical trial of single dose rectal and oral administration of diazepam for the prevention of serial seizures in adult epileptic patients. J Neurol Neurosurg Psychiatry. 1984;47(3):235–40.
2. Taketomo CK, Hodding JH, Kraus DM. Pediatric & neonatal dosage handbook with international trade names index. 18th ed. Ohio: Lexi-Comp, Inc.; 2011–2012.
3. Hirabayashi Y, Okumura A, Kondo T, Magota M, Kawabe S, Kando N, et al. Efficacy of a diazepam suppository at preventing febrile seizure recurrence during a single febrile illness. Brain Dev. 2009;31(6):414–8.
4. Otsuka H, Nagasaki Y, Kataoka K. Self-assembly of poly(ethylene glycol)-based block copolymers for biomedical applications. Curr Opin Colloid Interface Sci. 2001;6(1):3–10.
5. Miyata K, Christie RJ, Kataoka K. Polymeric micelles for nanoscale drug delivery. React Funct Polym. 2011;71(3):227–34.
6. Aliabadi HM, Lavasanifar A. Polymeric micelles for drug delivery. Expert Opin Drug Deliv. 2006;3(1):139–62.
7. Gao Z, Eisenberg A. A model of micellization for block copolymers in solutions. Macromolecules. 1993;26(26):7353–60.
8. Yamamoto T, Yokoyama M, Opanasopit P, Hayama A, Kawano K, Maitani Y. What are determining factors for stable drug incorporation into polymeric micelle carriers? Consideration on physical and chemical characters of the micelle inner core. J Control Release. 2007;123(1):11–8.
9. Hoffman NE, Hofmann AF. A comparison of the rate of absorption micellar and nonmicellar oleic acid. A jejunal perfusion study in man. Am J Dig Dis. 1973;18(6):489–92.

10. Woollett LA, Wang Y, Buckley DD, Yao L, Chin S, Granholm N, et al. Micellar solubilisation of cholesterol is essential for absorption in humans. Gut. 2006;55(2):197–204.
11. Watt SM, Simmonds WJ. The specificity of bile salts in the intestinal absorption of micellar cholesterol in the rat. Clin Exp Pharmacol Physiol. 1976;3(4):305–22.
12. Lavasanifar A, Samuel J, Kwon GS. The effect of fatty acid substitution on the in vitro release of amphotericin B from micelles composed of poly(ethylene oxide)-block-poly(*N*-hexyl stearate-L-aspartamide). J Control Release. 2002;79(1–3):165–72.
13. Aliabadi HM, Mahmud A, Sharifabadi AD, Lavasanifar A. Micelles of methoxy poly(ethylene oxide)-b-poly(epsilon-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. J Control Release. 2005;104(2):301–11.
14. Nishiyama N, Okazaki S, Cabral H, Miyamoto M, Kato Y, Sugiyama Y, et al. Novel cisplatin-incorporated polymeric micelles can eradicate solid tumors in mice. Cancer Res. 2003;63(24):8977–83.
15. Ashok B, Arleth L, Hjelm RP, Rubinstein I, Onyuksel H. In vitro characterization of PEGylated phospholipid micelles for improved drug solubilization: effects of PEG chain length and PC incorporation. J Pharm Sci. 2004;93(10):2476–87.
16. Rupp C, Steckel H, Muller BW. Solubilization of poorly water-soluble drugs by mixed micelles based on hydrogenated phosphatidylcholine. Int J Pharm. 2010;395(1–2):272–80.
17. Schmolka I. A review of block polymer surfactants. J Am Oil Chem Soc. 1977;54(3):110–6.
18. Kabanov AV, Batrakova EV, Miller DW. Pluronic block copolymers as modulators of drug efflux transporter activity in the blood–brain barrier. Adv Drug Deliv Rev. 2003;55(1):151–64.
19. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic® block copolymers as novel polymer therapeutics for drug and gene delivery. J Control Release. 2002;82(2–3):189–212.
20. Mu L, Feng SS. Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol). J Control Release. 2002;80(1–3):129–44.
21. Krasavage WJ, Terhaar CJ. D-alpha-Tocopheryl poly(ethylene glycol) 1000 succinate. Acute toxicity, subchronic feeding, reproduction, and teratologic studies in the rat. J Agric Food Chem. 1977;25(2):273–8.
22. Mu L, Elbayoumi TA, Torchilin VP. Mixed micelles made of poly(ethylene glycol)-phosphatidylethanolamine conjugate and d-alpha-tocopheryl polyethylene glycol 1000 succinate as pharmaceutical nanocarriers for camptothecin. Int J Pharm. 2005;306(1–2):142–9.
23. Moynihan H, Crean A. The theory of disperse systems. In: Moynihan H, Crean A, editors. The physicochemical basis of pharmaceuticals. New York: Oxford University Press Inc.; 2009. p. 156–92.
24. Suksiriworapong J, Sripha K, Kreuter J, Junyaprasert VB. Functionalized (poly(epsilon-caprolactone))$_2$–poly(ethylene glycol) nanoparticles with grafting nicotinic acid as drug carriers. Int J Pharm. 2012;423(2):562–70.
25. The United State Pharmacopeia Convention. The United States Pharmacopeia (USP) 35/The National Formulary (NF) 30. Baltimore: United Book Press, Inc.; 2012.
26. Anhorn MG, Mahler HC, Langer K. Freeze drying of human serum albumin (HSA) nanoparticles with different excipients. Int J Pharm. 2008;363(1–2):162–9.
27. Holzer M, Vogel V, Mantele W, Schwartz D, Haase W, Langer K. Physico-chemical characterisation of PLGA nanoparticles after freeze-drying and storage. Eur J Pharm Biopharm. 2009;72(2):428–37.
28. Kamal BA. The release of diazepam from different conventional and hollow type suppository bases. Iraqi J Pharm Sci. 2007;16(2):21–6.
29. Kaewnopparat N, Oungpho K, Kaewnopparat S, Rittirod T, Rojanarat W, Tessiri T. Preparation of extemporaneously diazepam suppositories: in vitro release and aging study. IJPS. 2006;2(1):26–34.
30. Siepmann J, Peppas NA. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). Adv Drug Deliv Rev. 2001;48(2–3):139–57.
31. Aydın RST, Pulat M. 5-Fluorouracil encapsulated chitosan nanoparticles for pH-stimulated drug delivery: evaluation of controlled release kinetics. J Nanomater. 2012;2012(313961):10.
32. Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. Acta Pol Pharm. 2010;67(3):217–23.
33. Costa P, Sousa Lobo JM. Modeling and comparison of dissolution profiles. Eur J Pharm Sci. 2001;13(2):123–33.
34. Serra L, Doménech J, Peppas NA. Drug transport mechanisms and release kinetics from molecularly designed poly(acrylic acid-g-ethylene glycol) hydrogels. Biomaterials. 2006;27(31):5440–51.
35. Guidance for industry: Dissolution testing of immediate release solid oral dosage forms [database on the Internet]. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). 1997 [cited September 1, 2011]. Available from: http://www.fda.gov/cder/guidance/1713bp1.pdf.
36. Wickersham RM, Novak KK. Drug facts and comparisons. 2012th ed. St. Louis: Kluwer; 2012.
37. Sezgin Z, Yüksel N, Baykara T. Preparation and characterization of polymeric micelles for solubilization of poorly soluble anticancer drugs. Eur J Pharm Biopharm. 2006;64(3):261–8.
38. Yu L, Bridgers A, Polli J, Vickers A, Long S, Roy A, et al. Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability. Pharm Res. 1999;16(12):1812–7.
39. Ismailos G, Reppas C, Macheras P. Enhancement of cyclosporin A solubility by d-alphatocopheryl-polyethylene-glycol-1000 succinate (TPGS). Eur J Pharm Sci. 1994;1(5):269–71.
40. Alexandridis P, Alan Hatton T. Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) block copolymer surfactants in aqueous solutions and at interfaces: thermodynamics, structure, dynamics, and modeling. Colloids Surf Physicochem Eng Aspects. 1995;96(1–2):1–46.
41. Brown W, Schillen K, Almgren M, Hvidt S, Bahadur P. Micelle and gel formation in a poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) triblock copolymer in water solution: dynamic and static light scattering and oscillatory shear measurements. J Phys Chem. 1991;95(4):1850–8.
42. Abdelbary AA, Li X, El-Nabarawi M, Elassasy A, Jasti B. Effect of fixed aqueous layer thickness of polymeric stabilizers on zeta potential and stability of aripiprazole nanosuspensions. Pharm Dev Technol. 2013;18(3):730–5.
43. Keck CM. Cyclosporine nanosuspensions: optimised size characterisation & oral formulations. Berlin: Freie Universität; 2006.