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Physicochemical characterization of drug-loaded rigid and elastic vesicles

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ABSTRACT

Ketorolac loaded rigid and elastic vesicles were prepared by sonication and the physicochemical properties of the drug loaded-vesicle formulations were examined. Rigid and elastic vesicles were prepared from the double chain surfactant sucrose-ester laurate (L-595) and the single chain surfactant octaoxyethylene-laurate ester (PEG-8-L). Sulfosuccinate (TR-70) was used as a negative charge inducer. Evaluation of the prepared vesicle was performed by dynamic light scattering, extrusion and by ^{1}H NMR (T_2 relaxation studies). The vesicles mean size varied between 90 and 150 nm. The elasticity of the vesicles was enhanced with increasing PEG-8-L/L-595 ratio, while an increase in loading of ketorolac resulted in a reduction in vesicle elasticity. ^{1}H NMR measurements showed that the molecular mobility of ketorolac was restricted, which indicates that ketorolac molecules were entrapped within the vesicle bilayers. The T_2 values of the aromatic protons of ketorolac increased gradually at higher PEG-8-L levels, indicating that ketorolac mobility increased in the vesicle bilayer. The chemical stability of ketorolac was dramatically improved in the vesicle formulation compared to a buffer solution. The strong interactions of ketorolac with the bilayers of the vesicles might be the explanation for this increased stability of ketorolac.

1. Introduction

In dermal and transdermal drug delivery vesicle formulations are used to enhance the transport of drugs across the upper part of the skin, the stratum corneum. The stratum corneum acts as the barrier for diffusion of compounds across the skin. In previous studies liposomes (prepared from only phospholipids) as well as non-ionic surfactant vesicles (prepared from only surfactants) were used for enhancing the transport across the skin (Hofland et al., 1995; Cavalcanti et al., 2007; Wachter et al., 2008). Several studies have demonstrated that the vesicle composition and hence its physicochemical properties have a significant effect on drug permeation (Knepp et al., 1990; Cevc and Blume, 1992; Hofland et al., 1994a; Cevc et al., 2002). For example, permeation studies in vitro have revealed that *liquid-state* vesicle is more effective than *gel-state* vesicle in enhancing drug transport (Hofland et al., 1994a; Ogiso et al., 1996; Van Kuijk-Meuwissen et al., 1998a,b). More recently these results have been confirmed in vivo (Perez-Cullell et al., 2000). However, not only the thermodynamic state of the bilayer, but also other physicochemical properties such as size, charge, and elasticity may influence the effectiveness of vesicles as a (trans)dermal delivery vehicle (Cevc and Blume, 1992; Hofland et al., 1994b; Cevc et al., 2002).

In the beginning of 1990s, the first generation of elastic vesicles, Transfersomes®, was designed by Cevc et al. (Cevc and Blume, 1992, 2003; Cevc, 2003; Cevc and Gebauer, 2003; Wachter et al., 2008). Transfersomes® are composed of a phospholipid, such as soybean or egg phosphatidylcholine, in combination with a surfactant. Subsequent studies have demonstrated that Transfersomes® were more effective than conventional liposomes in enhancing the transport of lidocain, insulin and corticosteroids transport across the mouse and human skin in vivo (Planas et al., 1992; Cevc, 2003; Cevc and Blume, 2003).

Another class of vesicles prepared using phospholipids and ethanol have been reported by Touitou et al. (2000), referred to as ethosomes. They reported that ethosomes are highly efficient in the transport molecules across the skin, as demonstrated by in vitro and in vivo studies (Dayan and Touitou, 2000; Ainbinder and Touitou, 2005).

In previous studies we have developed surfactant based vesicles composed of a bilayer-forming surfactant (sucrose-laurate ester (L-595)), a micelle-forming surfactant (octaoxylene-laurate ester (PEG-8-L)) and a charge inducer (sulfosuccinate (TR-70)). Van den Bergh et al. studied the elasticity by electro spin reso-

Abbreviations: L-595, sucrose-ester laurate; PEG-8-L, octaoxyethylene-laurate ester; TR-70, sulfosuccinate; ^{1}H NMR, proton nuclear magnetic resonance; T_2, spin–spin relaxation time.

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nance and extrusion and reported an increase in vesicle elasticity when increasing the PEG-8-L/L-595 ratio (Van den Bergh et al., 1999a). Furthermore, using freeze fracture electron microscopy it was shown that after application of elastic vesicle formulation non-occlusively onto human skin in vitro and in vivo, vesicle structures were frequently observed in the stratum corneum. This was not observed after applying rigid vesicle formulations onto the skin (Honeywell-Nguyen et al., 2002a, 2004). No evidence was observed for vesicular structures into the viable epidermis (Honeywell-Nguyen et al., 2004). In subsequent studies the vesicles were loaded with drug molecules and an increase in the in vitro transport efficiency of small drug molecules such as pergolide, lidocaine and rotigotine across human skin was observed. Elastic vesicles were more efficient in enhancing the transport of drugs than rigid vesicles and no increase in drug transport was obtained when the drugs were not associated with the vesicles (Honeywell-Nguyen et al., 2002a,b, 2003a,b, 2004; Honeywell-Nguyen and Bouwstra, 2003; Dhanikula and Panchagnula, 2008). In recent years several review were published on the use of vesicular system for dermal and transdermal system (Sinico and Fadda, 2009; Elsayed et al., 2007; El Maghraby et al., 2006).

Although several studies reported on the transport enhancing properties of the surfactant based elastic vesicle formulations, the interactions between drug and vesicles have not yet been studied in detail. In several previous studies NMR, circular dichroism, DSC, fluorescence and FT-IR techniques were used to evaluate the interaction between the vesicle and encapsulated molecules (Ali et al., 2000; Campbell et al., 2001; Zhao and Feng, 2004; Dhanikula and Panchagnula, 2008). Especially, NMR is a very powerful tool to study the intermolecular interaction and molecular mobility. For example, Panicker reported the interaction between encapsulated molecules and phosphatidylcholine membranes (Panicker et al., 2008; Pentak et al., 2008). Information on the molecular motion can be achieved by the spin–lattice relaxation time (T_1) and the spin–spin relaxation time (T_2) (Fischer and Jardetzky, 1965; Katzhendler et al., 2000).

The aim of the present study was to characterize ketorolac loaded vesicle formulations prepared from L-595, PEG-8-L and TR-70. We chose for ketorolac, as this drug has been used in previous transport studies in vitro and in vivo (Honeywell-Nguyen et al., 2004). We measured the elasticity of ketorolac loaded vesicles and studied the effect of encapsulation on ketorolac mobility and chemical stability. The molecular mobility of ketorolac was examined using T_2 relaxation measurements of ketorolac. The elasticity was determined by extrusion.

2. Materials and methods

2.1. Material

Ketorolac tris salt was purchased from Sigma (Zwijndrecht, The Netherlands). L-595 (30% mono-, 40% di-, and 30%triesters, mean MW 734) was kindly supplied by Mitsubishi Kasei (Tokyo, Japan). PEG-8-L (mean MW 552) was a gift from Lipo Chemicals (Paterson, NJ, USA) and sodium bistridecyl sulfosuccinate (TR-70; mean MW 585) was a gift from Cytec BV (Rotterdam, The Netherlands). All other chemical reagents were of analytical grade and all buffer solutions were prepared with distilled water.

2.2. Extraction of ketorolac from tris salt

Ketorolac base was obtained from ketorolac tris salt using the precipitation method reported by Liu et al. (2005). Briefly, ketorolac tris salt was dissolved in distilled water and ketorolac base was precipitated by adding 1 N HCl. The precipitated ketorolac base was recrystallized from ethyl acetate. The purity of ketorolac was measured (>98%) by high pressure liquid chromatography (HPLC) as described below.

2.3. Preparation of rigid and elastic vesicle

Vesicles were prepared by a modified sonication method as described by Ding et al. (2008). Briefly, L-595, PEG-8-L and ketorolac were dissolved in ethanol. TR-70 was dissolve in 2-propanol. The solutions were mixed in appropriate molar ratios to achieve the required ratio of the three surfactants. The organic solvent was evaporated overnight in a vacuum centrifuge and the remaining film was rehydrated using 50 mM citric buffer at pH 3.0 and 5.0. For the preparation of vesicles used for NMR measurements the surfactant film was rehydrated with a citric buffer (pH 3.0) prepared with D_2O. Subsequently, the obtained vesicle formulations were sonicated with a Branson Sonifer 250 (Branson Ultrasonics, Danbury, UK). The elastic vesicles prepared from L-595, PEG-8-L and TR-70 were sonicated for 2×10 s, while the rigid vesicles prepared from L-595 and TR-70 were sonicated for 2×60 s. The micelle formulations consisted of only PEG-8-L and were prepared as described above, but without sonication. The final concentration of total surfactant in all formulations was 10% w/w.

2.4. Determination of saturated concentration of ketorolac loaded vesicles

To determine the saturated concentrations of ketorolac, samples of increasing ketorolac concentrations were prepared in each vesicle formulation and stored in the dark. Samples were observed by polarization microscope after 14 days from preparation. Saturated concentrations of ketorolac were determined as the highest ketorolac concentration at which ketorolac crystal formation was absent.

2.5. Particle size and zeta-potential measurement

The vesicle size and polydispersity index (PDI) of all formulations were measured by dynamic light scattering (DLS) using a Malvern Zetasizer 3000 HAS (Malvern Ltd, Malvern, UK). All size measurements were performed at 25 °C at an angle of 90° between laser and detector. Before measuring, all samples were diluted with their original buffer. PDI has values between 0 (mono-disperse) and 1 (very hetero-disperse). Formulations were measured at regular time intervals during a period of 2 weeks after preparation to check the colloidal stability.

For measurement of the surface charge of the vesicles, 25 μl of vesicle formulation was diluted in 1.00 ml of 10 mM citric buffer at same pH and the zeta-potential was measured by laser doppler electrophoresis using the same device.

2.6. Extrusion study

Extrusion studies were performed according to the method reported by Van den Bergh et al. (2001). Vesicle formulations were extruded through polycarbonate filter (Millipore, USA) of 30 nm pore diameter filters using a stainless steel pressure holder with 1 ml capacity barrel at a pressure of 10 bar. During 10 min the extruded volume of the suspension at various time intervals was monitored and plotted against time. In this way an extruded volume–time profile was obtained. All studies were performed in triplicate. Vesicle size was measured before and after the extrusion.

2.7. NMR studies

The ^{1}H NMR spectra of ketorolac loaded rigid and elastic vesicles were recorded at 400 MHz on a Bruker DMX-400 spectrometer. As reference 50 mg of ketorolac was dissolved in 0.6 ml of CD_3OD.

The $1/T_2$ can be obtained from the NMR peaks in the spectrum from the spectral line widths using Eq. (1):

$$\frac{1}{T_2} = \pi(\Delta\nu) \quad (1)$$

where $\Delta\nu$ is the peak width at half peak maximum (Fischer and Jardetzky, 1965; Fruttero et al., 1998). In the present studies, we focused on the peak broadening of suitable isolated proton spins of the ketorolac molecules. From the peak width at half peak maximum of aromatic proton of ketorolac ($\Delta\nu$), T_2 value was calculated from Eq. (1). All measurements were performed at 32 °C which is at skin surface temperature. To remove the effect of dissolved oxygen on the T_2 value, all samples were degassed with argon gas before measurement.

2.8. Chemical stability of ketorolac in vesicle formulations

Stability studies were performed in ketorolac citric buffer solution (pH 3.0) and ketorolac loaded rigid and elastic vesicles. Each vesicle formulation was filled in a glass vial and was stored at 25 °C in the dark for different time periods (1, 3, 7 and 14 days). To extract the ketorolac from the vesicle formulations, samples were diluted 10 times with methanol. The ketorolac in the diluted samples were measured by reverse-phase HPLC. The HPLC consisted of a Thermoseparation products Spectra system P4000 HPLC pump equipped with a Waters 717 plus automatic injector and a Thermoseparation products Spectra system UV 2000 UV detector set at a wave length of 313 nm. The initial ketorolac content was set at 100%. 10 μl of diluted sample solution was injected onto a Prontocil Eurobond C18 (125 × 4.0 mm, 5 mm) column and eluted at 1.0 ml/min with a mobile phase consisting acetonitril/10 mM potassium dihydrogen phosphate buffer (pH 2.5) at a ratio of 5:5 (v/v). This resulted in a retention time of 2.5 min for ketorolac. The calibration curves for all experiments were linear between the concentration range 10–200 μg/ml ($r^2 \geq 0.999$). The limit of detection of ketorolac was 300 pg.

2.9. Statistical analysis

Stability data was analyzed with two-way ANOVA with Bonferoni post-test. Other analyses were performed where suitable as indicated. Statistical analysis was carried out using SPSS and a p value less than 0.05 was considered to be significant.

3. Result and discussion

In this study, we focused particularly on the molecular interaction between drug and vesicle components. Previous studies showed that uncharged compounds are more easily encapsulated in the liposomes than charged compounds (Honeywell-Nguyen et al., 2003a), For the reason, we decided to select two pH values, Since it was reported that pK_a of ketorolac is 3.5, we selected pH 3.0 for our studies as at this pH most of the ketorolac is in acid form and therefore a high entrapment in the vesicles can be expected. To investigate the effect of pH on the drug loaded vesicular formulation, the formulations at pH 3 were compared to those at pH 5.0, which is above the pK_a of ketorolac. Table 1 shows the average size and zeta potential of the different ketorolac-loaded vesicle formulations. The mean diameters of all vesicles were in the range of 90–150 nm. All formulations exhibited negative zeta potential, which is caused by the negatively charged TR-70 surfactants having a negatively charged sulphonyl group. The charge of these surfactants is at least partly located on the surface of vesicles. No effect of ketorolac concentration on the particle size and zeta potential of vesicles were observed (data not shown). An increase in the PEG-8-L/L-595 molar ratio increased the saturation concentration of ketorolac in the vesicle formulations. The saturated concentration of ketorolac at pH 3.0 and pH 5.0 in the buffer solutions were 0.07 and 1.7 mg/ml, respectively. The saturated ketorolac concentration in the vesicle formulations were much higher, varying from 4.4 (rigid vesicles, pH 3) to 15.2 mg/ml (elastic vesicles, pH 5), see Table 1, demonstrating that the vesicles drastically increased the ketorolac solubility in the formulation. It should be stressed that these saturated concentrations are the total concentrations of ketorolac in the vesicular system and not the entrapment values. Therefore, within the vesicle formulation ketorolac could be associated with the vesicle bilayers as well as dissolved in the aqueous phase. Over the course of 14 days, none of the formulations showed a significant change in mean vesicle size or zeta potential of the vesicles (data not shown). In the formulation prepared with the surfactants PEG-8-L and TR-70 no particles could be detected as this formulation consisted of only micelles, the sizes of which are smaller than the detection limit of DLS equipment.

It has been reported that an increase in elasticity of vesicles enhances the drug transport across the skin (Cevc and Blume, 1992; Cevc et al., 2002, 2008; Honeywell-Nguyen et al., 2002a,b, 2003a,b, 2004; Honeywell-Nguyen and Bouwstra, 2003). For this reason we measured the elasticity of the vesicles as function of its composition in the absence of ketorolac. Fig. 1(a) shows the volume extruded through 30 nm pore membranes as function of time of empty vesicles. It is obvious that the extruded volume increases when increasing the PEG-8-L/L-595 ratio in the formulations, suggesting that an increase in the PEG-8-L/L-595 molar ratio increases the elasticity of the vesicles, in agreement with earlier reported data (Van den Bergh et al., 1999b).

To investigate the effect of ketorolac molecules on vesicle elasticity, extrusion studies were performed of ketorolac loaded vesicle formulations at pH 3.0. Fig. 1(b) shows the results of effect of ketorolac molecules on the vesicle elasticity. Adding ketorolac to the vesicle formulation to a saturated value decreased the extruded volume, but the elasticity of ketorolac loaded elastic vesicle formulation was still higher than that of ketorolac loaded rigid vesicles. The reduced elasticity is probably due to an interaction of ketorolac with the vesicle bilayer. To study this interaction, ^{1}H NMR measurements of the vesicle formulations were performed. Fig. 2 shows the ^{1}H NMR spectra of aromatic region in ketorolac loaded vesicle formulation together with the molecular structure of ketorolac. The peak assignment in the spectrum was in agreement with the stud-

Table 1
Particle characterization of ketorolac loaded vesicles.

Formulations (L-595/PEG-8L/TR-70/Ketorolac)	Ketorolac concentration (mg/ml)	pH	*Z*-Average (d nm)	PDI	ZP (mV)
100/0/5/10	4.4	3.0	108.7 ± 3.5	0.17 ± 0.01	−35.7 ± 1.8
70/30/515	5.4	3.0	116.7 ± 1.2	0.18 ± 0.02	−34.5 ± 1.2
50/50/5/15	5.7	3.0	94.3 ± 6.5	0.18 ± 0.01	−34.1 ± −0.7
30/70/5/15	6.0	3.0	142.7 ± 4.1	0.12 ± 0.01	−41.7 ± 0.6
0/100/5/20	8.8	3.0	n.m.	n.m.	n.m.
100/0/5/15	6.6	5.0	121.3 ± 2.5	0.18 ± 0.02	−23.2 ± −0.8
70/30/5/20	7.2	5.0	109.7 ± 0.6	0.18 ± 0.02	−23.5 ± −0.4
50/50/5/40	15.2	5.0	94.7 ± 0.6	0.18 ± 0.02	−31.1 ± −0.7

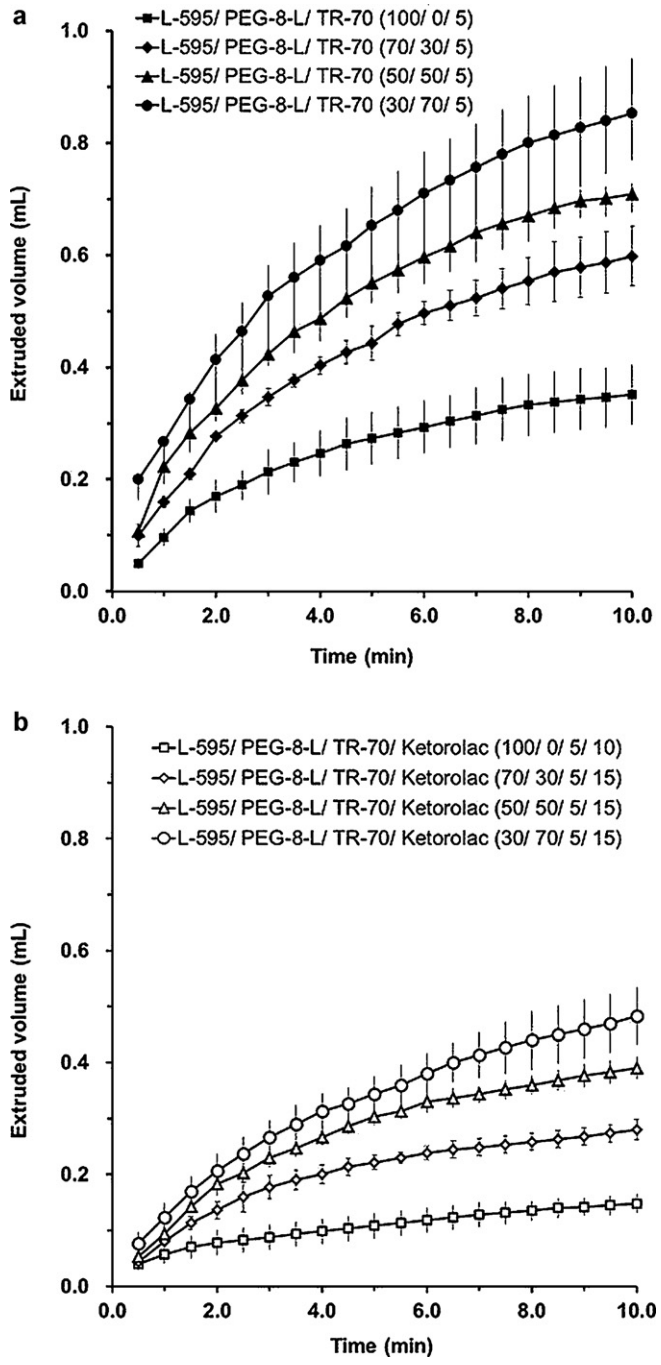

Fig. 1. The vesicle elasticity measured by extrusion method at 25 °C. Empty vesicle (a) and ketorolac loaded vesicle (b) formulations were extruded through polycarbonate filters with pore size of 30 nm under a constant pressure of 10 bar. The extruded volumes of these dispersions were recorded once per 30 s during 10 min. In ketorolac loaded vesicle system, ketorolac concentration in L-595/PEG-8-L/TR-70/ketorolac (100/0/5/10), (70/30/5/15), (50/50/5/15), and (30/70/5/15) are 4.4, 5.4, 5.7 and 6.0 mg/ml, respectively. In empty vesicle system, the elasticity was increased with increase of PEG-8-L concentration in the formulations. However, encapsulated in the ketorolac molecule, the vesicle elasticity was decreased. Data shown are mean ± SD of three batches.

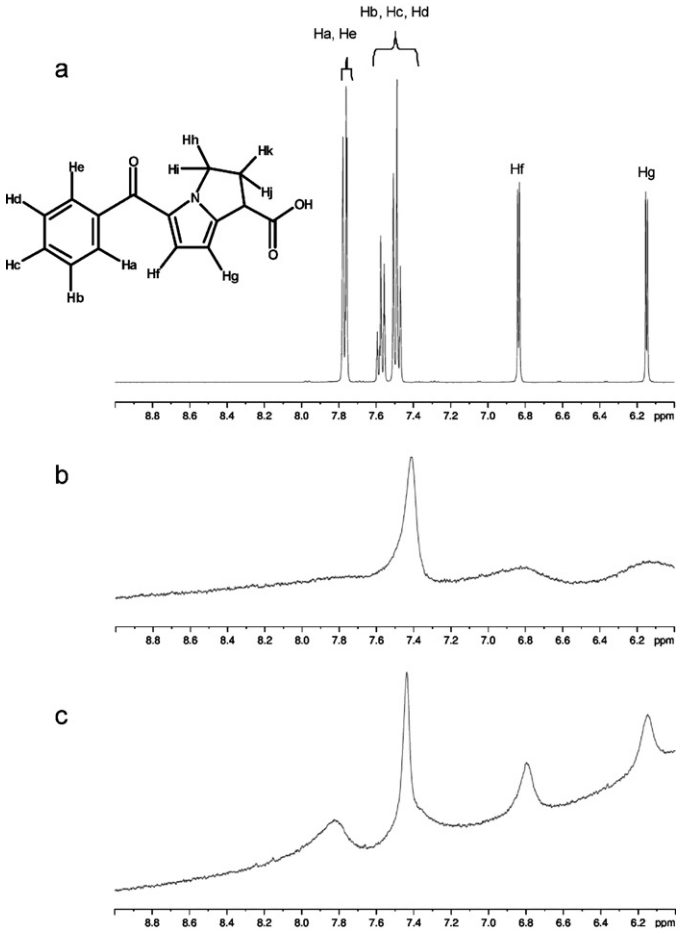

Fig. 2. ^{1}H NMR spectra of aromatic region in the ketorolac loaded vesicle system. (a) Ketorolac CD_3OD solution, (b) ketorolac loaded rigid vesicle [L-595/PEG-8-L/TR-70/ketorolac (100/0/5/10)], (c) ketorolac loaded elastic vesicle [L-595/PEG-8-L/TR-70/ketorolac (50/50/5/15)]. Ketorolac concentration in CD_3OD solution, rigid and elastic vesicle systems were 83, 4.4 and 5.7 mg/ml, respectively. In the ketorolac solution, aromatic proton of ketorolac showed sharp signal. On the other hand, broader signal were clearly observed in the both vesicle system.

ies of Nagarsenker et al. (2000). No ^{1}H NMR signals were observed in the ketorolac citric buffer solution at pH 3.0, suggesting that ketorolac concentration were below the detection limit for the ^{1}H NMR measurement (no data are shown). Therefore, it is expected that the ketorolac in the external phase of the vesicles does not contribute to the ^{1}H NMR signals. In the ketorolac CD_3OD solution, the chemical shift of aromatic protons of ketorolac results in sharp peaks in the spectrum. However, broader chemical shifts of aromatic protons of ketorolac in the ketorolac loaded rigid and elastic vesicle were observed. The peak broadening was stronger in the rigid vesicle formulation compared to the elastic vesicle formulations. Panicker showed that broadening of aromatic proton signal of propyl paraben was due to the entrapment of paraben in the dipalmitoyl phosphatidylcholine bilayers, which resulted in a reduction of the proton mobility (Panicker, 2008). Therefore, the results strongly indicate that protons of ketorolac reduce the mobility, which in turn indicates that ketorolac molecules are entrapped in the vesicle bilayer.

Urano et al. reported that T_2 values of ^{19}F labeled α-tocopherol fluorine in egg yolk phosphatidylcholine and dipalmitoyl phosphatidylcholine liposome dramatically decreased compared with those in $CDCL_3$ solution and their T_2 value is depending on the lipophylic environment of the liposome membrane (Urano et al., 1993). Since vesicle environment is changing by the PEG-8-L/L-595 ratio in our formulation, the T_2 relaxation of ketorolac aromatic protons at 32 °C were measured by NMR to investigate the effect of PEG-8-L/L-595 ratio in the formulation on the ketorolac mobility. Fig. 3 depicts the T_2 value of the doublet signals due to the protons Ha & He, Hf and Hg plotted against PEG-8-L/L-595 ratio. This figure clearly shows that the T_2 values of aromatic protons increased with the increase of PEG-8-L/L-595 ratio in the formulation. These

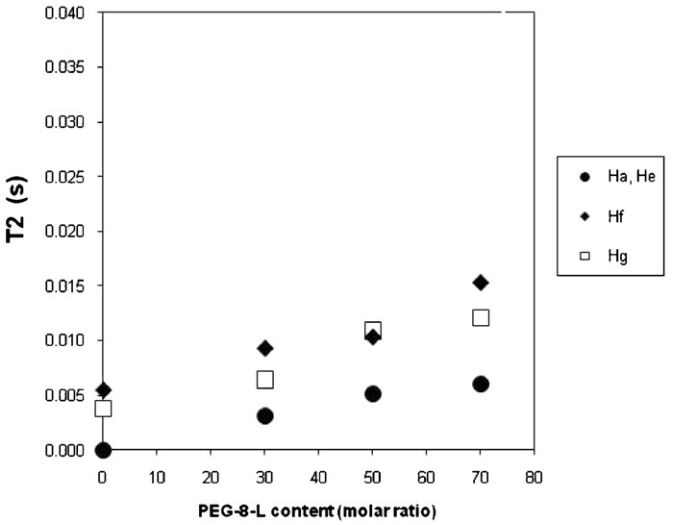

Fig. 3. Relationship between T_2 of aromatic proton of ketorolac and PEG-8-L content in the formulation at pH 3.0 condition. Each sample was measured at 32 °C in the degassed condition. T_2 of aromatic proton of ketorolac molecule were plotted against molar ratio of PEG-8-L. All T_2 values were increased with increase of PEG-8-L concentration in their formulations.

results suggest that molecular mobility of ketorolac in vesicle bilayer increases when increasing the PEG-8-L/L-595 ratio. Since the ketorolac mobility decreased in the vesicle formulation compared to the buffer solution, it was of interest to study the chemical stability of ketorolac in the vesicles formulation and the buffer control. The results are shown in Fig. 4. After a period of 14 days, the intact percentage of ketorolac in the buffer solution was 76.0%. However, when using a vesicle formulation of L-595/PEG-8-L/TR-70 100/0/5/10 (rigid vesicle) and 50/50/5/10 (elastic vesicles) the ketorolac stability was dramatically increased. After 14 days 97.1 and 91.6% of the ketorolac was intact in respectively rigid and elastic vesicles, respectively. These results demonstrate that ketorolac oxidation was decreased by the encapsulated in the vesicle formulation. Vesicular formulations such as liposomes are one of most widely studied drug carriers. It has been reported that encapsulation of drug molecule in liposome stabilized the drug molecule from photolysis, oxidation and hydrolysis (Fiore and Serhan, 1989; Ioele et al., 2005; Drummond et al., 2006, 2010). Similarly, our surfactant based vesicle could play a roll of protective carrier to encapsulate drug in the vesicle bilayer.

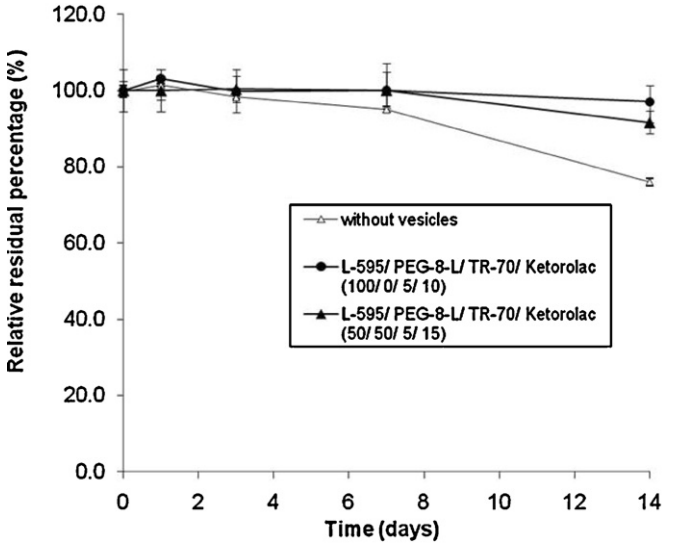

Fig. 4. Change in the relative residual percentage of ketorolac after storage in light shielding condition at 25 °C. Decomposition of ketorolac was suppressed in both vesicle systems, compared to ketorolac solution. Ketorolac concentration of rigid and elastic vesicle systems were 4.4 and 5.7 mg/ml, respectively. Data shown are mean ± SD of three batches.

Ketorolac loaded rigid and elastic vesicles were prepared by the modification of sonication method. Ketorolac was solubilized by the encapsulated in the vesicle formulations, which showed supersaturated solution. From the result of ^{1}H NMR results, it was suggested that ketorolac molecules was entrapped in the vesicle bilayer and molecular mobility of ketorolac was restricted by interacting with vesicle bilayer. PEG-8-L molecules affect the increase of the vesicle elasticity and molecular mobility of ketorolac in the vesicle formulations. Vesicle elasticity was influenced by the loading of ketorolac molecules, due to a strong interaction between the ketorolac and bilayer components, which might increase chemical stability of ketorolac in the formulation.

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