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Ionic liquid-in-oil microemulsion as a potential carrier of sparingly soluble drug: Characterization and cytotoxicity evaluation

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ABSTRACT

Pharmaceutical industries have posed challenges in the topical and transdermal administration of drugs which are poorly soluble or insoluble in water and most of organic solvents. In an approach to overcome this limitation, ionic liquid-in-oil (IL/o) microemulsions (MEs) were employed to increase the solubility of a sparingly soluble drug to enhance its topical and transdermal delivery. The formulation of MEs was composed of a blend of nonionic surfactants, polyoxyethylene sorbitan monooleate (Tween-80) and sorbitan laurate (Span-20), isopropyl myristate (IPM) as an oil phase, and IL [C_1mim] [$(CH_3O)_2PO_2$] (dimethylimidazolium dimethylphosphate) as a pseudophase. Among various weight ratios of Tween-80 to Span-20 investigated in the ME systems, the ratio 3:2 showed excellent solubility and skin permeation enhancing effect for acyclovir (ACV) used as a model sparingly soluble drug. The size and size distribution of the ME droplets with and without drug were determined by dynamic light scattering. The permeability study of ACV incorporated in IL droplets as well as other formulations was performed into and across the Yucatan micropig (YMP) porcine skin, and the use of IL/o MEs has been shown to dramatically increase ACV administration. Finally, the cytotoxicity of the new carrier was evaluated *in vitro* using the reconstructed human epidermal model LabCyte™ EPI-MODEL12. It was found that the cell viability of IL/o MEs containing 4 wt% IL was over 80% compared to Dulbecco's Phosphate-Buffered Salines, indicating low cytotoxicity of the carrier. Taken together these results, it can be assumed that IL-assisted nonaqueous ME could serve as a versatile and efficient nanodelivery system for insoluble or sparingly soluble drug molecules that require solubilizing agents for delivery.

1. Introduction

A topical and transdermal drug delivery method provides many advantages over traditional delivery strategies such as oral route and injections (Hadgraft, 1999; Prausnitz and Langer, 2008). However, in pharmaceutical fields, transdermal delivery of many poorly soluble drugs has been found to be difficult due to their insolubility or sparingly solubility in water and most pharmaceutical grade organic liquids. In the pharmaceutical industry, it is well known that many promising drugs which are sparingly soluble never enter a formulation stage due to their poor solubility as well as difficulties in delivery. To date, some methods have been employed to delivery such drugs to a given target cell or tissue. One conventional way to address the poor solubility is to use excipients such as ethanol and dimethyl sulfoxide (DMSO) and/or certain surfactants (Yalkowaky, 1981). However, in most of the cases, significant enhancement of permeation rates is obtained only at high concentrations of excipients, which can cause toxic and other undesirable side effects (Ray et al., 2003). Although acidic (*e.g.*, hydrochloride) or basic (sodium) salts facilitate the dissolution of poorly soluble drugs containing ionizable groups (Ansel et al., 1999), such salts can precipitate in the body when they encounter pH conditions. Some poorly soluble drugs were found to be soluble in specially designed liposomes (Jain et al., 2008) although the solubilization capacity varies for different drugs. Furthermore, iontophoresis facilitated transdermal delivery of poorly soluble drugs but this approach is limited by the undesirable side effects of applied electricity (Lasmar and Manger, 1994; Volpato et al., 1998). These observations indicate that sparingly soluble drugs need to be formulated with superior solvent systems and/or a delivery technology that can make topical and transdermal administration of such drugs potentially feasible.

To help meet the challenge of using sparingly soluble drugs, it was found that ionic liquids (ILs) and/or mixtures of ILs and/or ILs based solvent systems could be used for solubilization of

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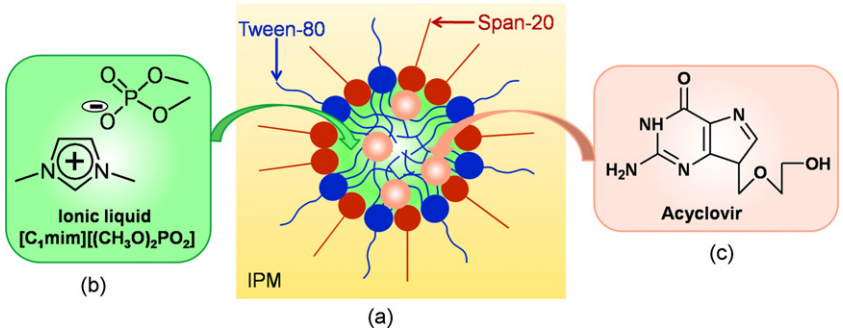

Fig. 1. (a) Schematic representation of ionic liquid-in-oil (IL/o) microemulsions containing drug molecules. Chemical structure of IL (b) and acyclovir (c).

such drugs (Anderson, 2003; Jaitely et al., 2008; Mizuuchi et al., 2008; Moniruzzaman et al., 2010a). Furthermore, ILs can also be used to synthesize active pharmaceutical ingredients (APIs) with modified solubility, increased thermal stability, and a significant enhancement in the efficacy of topical analgesia compared to their starting materials (Hough and Rogers, 2007; Hough et al., 2007; Stoimenovski et al., 2010). ILs represent nonvolatile, thermal stable, non-flammable and tunable designer “green” solvents that hopefully can replace the highly volatile organic solvents for a wide range of applications (Seddon, 1997; Welton, 1999; Earle and Seddon, 2000; Huddleston et al., 2001; Moniruzzaman et al., 2010b,c). ILs can be synthesized with different polarities and/or properties simply by changing the anion/cation combination (Brennecke and Maginn, 2001). This interesting property of ILs enables the dissolution of many sparingly soluble substrates, which leads to the concept of tailor-made solvents for a specific application. Although ILs have been used extensively in a wide range of fields, their use in pharmaceutics application is very limited due to the question whether ILs are “toxic” or “not” (Constable et al., 2007). However, considering the tunability of ILs, it is clear that nontoxic ILs can be designed by using nontoxic, biodegradable and pharmaceutically acceptable organic cations and inorganic anions. Some biocompatible ILs have already been synthesized (Pernak et al., 2004; Weaver et al., 2010). Hopefully, some studies have shown low/negligible toxicity of some common ILs towards Caco-2 cells (Jaitely et al., in press) or female Wistar rats (Pernak et al., 2004). Note that many useful and necessary chemicals are toxic: many pharmaceutical excipients such as DMSO and nonionic surfactants (*e.g.* polysorbate 80) display similar toxicities what was observed in many ILs. Hence, the toxicity of ILs does not preclude their use as pharmaceutical solvents.

Recently, it was found that hydrophilic ILs having coordinating anions are very effective in dissolution of acyclovir (ACV), which is practically insoluble in water, and its solubility in pharmaceutical grade solvents is very limited (Moniruzzaman et al., 2010a). Preliminary results suggested that although ACV-IL formulation was not suitable for drug administration, IL-in-oil microemulsions (IL/o MEs) could be used for ACV delivery. In a later study, the influence of ILs type on the formation IL/o MEs, formation mechanism and drugs encapsulation efficiency were studied (Moniruzzaman et al., 2010d). It was observed that such MEs are able to solubilize higher amount of poorly soluble drugs (*e.g.*, acyclovir, methotrexate and 1-[(5-(*p*-nitro-phenyl) furfurylidene) amino] hydantoin sodium) than their individual components and water. It is well recognized that conventional MEs, which are homogenous, transparent and thermodynamically stable dispersions of water and oil, stabilized by a surfactant or a blend of surfactants have emerged as prospective systems for drug delivery mainly due to their size, biocompatibility and straightforward preparation (Kogan and Gati, 2006; Narang et al., 2007; Gupta and Moulik, 2008; Heuschkel et al., 2008; Yuan et al., 2008). MEs with their supramolecular structure can increase not only the skin permeability, but also drug solubilization and drug partitioning into the skin. Oil-soluble drugs can be formulated in o/w MEs whereas, water-soluble ones are better suited to w/o systems. Consequently, stable IL/o MEs could lead to effective drug carriers for IL soluble drugs. However, this new IL/o systems need to be characterized in terms of drug solubility and delivery capacity. More importantly, the safety profile of the IL-assisted ME should be conducted to promote their application in pharmaceuticals.In this study, IL/o MEs were adopted to increase solubility and permeability of ACV (see Fig. 1c) used as a model sparingly soluble drug. The system was characterized by varying the weight ratio of Tween-80 to Span-20. Furthermore, the safety profile of the new carrier was evaluated *in vitro* (acute skin irritation test using a cytotoxicity assay) using LabCyte EPI-MODEL. For drug delivery study, Yucatan hairless micropig (YMP) porcine skin was used as a model skin.

2. Material and methods

2.1. Materials

Tween-80 (polyoxyethylene sorbitan monooleate) and Span-20 (sorbitan laurate) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO) and were of the highest purity commercially available. They were used without further purification. IL $[C_1mim][(MeO)_2PO_2]$ dimethylimidazolium dimethyl phosphate was obtained from Solvent Innovation GmbH (Germany) having purity ≥98%. According to company specification, this IL contains two notable impurities, *e.g.*, water (≤1.0%) and halides (≤0.1%). Frozen (−80 °C) porcine skin samples, excised from a 5 months old female Yucatan Micropig (YMP), were obtained from Charles River Japan Inc. (Tokyo, Japan). Acyclovir was obtained as a generous gift from ASPION Pharmaceuticals (Kobe, Japan). Dulbecco's Phosphate-Buffered Saline (D-PBS) was purchased from Invitrogen. The reconstructed human epidermal model LabCyte™ EPI-MODEL12 was obtained from Japan Tissue Engineering Co., Ltd. (Aichi, Japan). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) medium was obtained from Dojindo Co., Kumamoto, Japan. ACV topical cream was purchased from Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan). All other reagents used in the experiments were analytical grade.

2.2. Preparation of IL-in-oil microemulsions with drugs

To investigate the ME formation areas, phase diagrams were constructed by titration of a series of IPM and Tween-80/Span-20 mixtures with IL at 25 °C (Moniruzzaman et al., 2010d). To prepare a microemulsion, Tween-80 was blended with Span-20 at fixed weight ratios as shown in Table 1. Then, 20 wt% mixtures of surfactants were mixed with IPM and mixing thoroughly until a clear

Table 1
Contents of the microemusion formulations[a].

Formulations	Tween-80: Span-20 (mass ratio/molar ratio)	HLB[b]	Viscosity (mPa s)	Mean diameter of particles (nm)[c]	Polydispersity index[c]
ME1	3:1/1:1.3	13.4	32.5	26.5 ± 1.0	0.115 ± 0.033
ME2	3:2/1:2.5	12.4	28.8	25.0 ± 1.2	0.096 ± 0.026
ME3	1:1/1:3.8	11.7	26.7	16.3 ± 1.4	0.121 ± 0.030
ME4	1:2/1:7.6	10.6	24.2	15.6 ± 1.3	0.123 ± 0.042
ME5	1:3/1:11.3	10.1	22.6	13.5 ± 0.9	0.147 ± 0.063

[a] Microemulsions prepared with 20 wt% surfactant and 4 wt% IL in IPM.
[b] HLB values of surfactant mixture(HLB_{mix}) were calculated using the following equation: $HLB_{mix} = f_A\ HLB_A + f_B\ HLB_B$,where HLB_A, HLB_B are the HLB values of Tween-80 and Span-20, respectively, and f_A, f_B are the weight fractions of Tween-80 and Span-20, respectively.
[c] The data correspond as mean ± SD (standard deviation) from five experiments.

and optically transparent solution was obtained. Then, IL was added to produce IL/o microemulsions by vortexing the resultant mixture vigorously at 25 °C. For the preparation of water-in-oil MEs, water was added instead IL as stated above. Drug loaded microemulsions were obtained by injecting an appropriate amount of IL in which drugs (10–20 wt%) were solubilized in advance.

2.3. Drug solubility determination

The solubility of drugs was determined in ME as follows. Drug was added in excess to IL/o MEs as prepared as described in the above section. The sample was stirred for 24 h at 25 °C. The precipitated drug was removed by filtration through a 0.45 μm Millipore Millex-LG filter. The amount of ACV in the resulting clear filtrate was determined using a UV spectrophotometer at 252 nm after appropriate dilution with methanol keeping the respective blank.

2.4. Viscosity measurement

The viscosity of each formulation was measured using an Automated Microviscometer (AMVn, Anton Paar GmbH, Graz, Austria). The samples were introduced into a glass capillary in which a steel ball rolls. The viscosity of the tested sample is determined by measuring the rolling time of the steel ball.

2.5. Particle size determination

The size and size distribution of the ME droplets with and without drug were determined by dynamic light scattering (DLS) method using a computerized inspection system (a Zetasizer Nano ZS MARVERN Instruments, U.K.). All samples were equilibrated for more than 5 h before measurements, and no macroscopic heterogeneity was visible. Experiments were performed at 25 ± 0.1 °C. Samples were equilibrated for 15 min before data collection. The mean diameter of ME droplets is the average of five replicated experiments.

2.6. Stability of microemulsions

The physical stability of the MEs with loaded ACV during the prolonged storage, both as a function of storage time and temperature, was determined by size measurements with DLS technique on MEs and by visual inspection at regular time intervals. In addition, the centrifuge test was carried out to assess the physical stability of MEs. To this approach, MEs were centrifuged for 30 min at 15,000 rpm (Chen et al., 2004). In addition to physical stability, chemical stability of IL/o ME formulation was examined by observing degradation extent and encapsulation efficiency of ACV in the present ME carriers.

2.7. Skin permeation studies

Drug release was evaluated *in vitro*. The penetration of drug from the formulations into (topical delivery) and across (transdermal delivery) full thickness skin pieces of Yucatan hairless micropigs (YMPs) was performed using a hand-made Franz diffusion cells with a diameter of 10 mm. The porcine skin was prepared as reported in the literature (Piao et al., 2008; Tahara et al., 2008). A Franz diffusion cell consists of two compartments, a donor and a receiver compartment. The skin piece was clamped between the donor and receiver compartments of the diffusion cell. The stratum corneum faced the donor side of the cell whereas the dermal side of the skin was allowed to be in contact with PBS solution (pH 7.4). Note that skin pieces were hydrated with PBS for 1 h before permeation experiments. After setting the diffusion cell, 0.5 mL of the formulation containing drug molecule was placed in the donor compartment of the skin. The acceptor compartment contained 5 mL PBS solution that was thermostatically maintained at 32.5 ± 0.1 °C by a circulating water bath (NTT-20S, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) and was magnetically stirred at 600 rpm throughout the experiments. After a fixed time, 0.5 mL of the receptor phase was withdrawn and solutions were analyzed for drug content. Each sample removed was replaced by an equal volume of fresh PBS solution. To quantify the drug concentration in the skin after predetermined time, donor solutions were removed and the skin was washed twice with 0.1 M HCl acid before unclamping the diffusion cells. Finally, tested skin pieces were processed to determine the drug content inside the skin (Tahara et al., 2008).

2.8. Analysis assay

Acyclovir contents in the receptor solutions and the skin were detected using by HPLC with column Shiseido CAPCELL PAK C18 MG (4.6 × 250). It should be noted that the drug content of receptor solution could not be measured using UV spectroscopy (as stated in Section 2.3) because solutions contain some water soluble components (*e.g.*, proteins) extracted from the YMP skin during the permeability study. Consequently, various types of peaks in UV–vis spectra appeared around 252 nm, a selective wavelength for acyclovir quantification. The HPLC mobile phase consisting of 0.1 wt% trifluoroacetic acid (A) and acetonitrile (B) was adjusted as A:B = 1:99 with no gradient elution. Flow rate was 0.5 mL/min and the volume injected was 100 μL. The detection limit was 0.1 μg mL^{-1}. The concentration range of the standard curves was 0–0.1 mg mL^{-1} and the squared correlation coefficient of the standard curve was more than 0.99 ($R^2 > 0.99$).

2.9. Data analysis

To determine the transdermal flux, the cumulative amount of ACV permeated through the YMP skin was plotted as function of time. The slope and intercept of the linear portion of the plot was derived by regression. The transdermal flux (J, μg/cm^2/h) was calculated as the slope divided by the skin surface area. The skin permeability, P, can be calculated from the transdermal flux with the equation $P = J/\Delta C$, where ΔC is the concentration difference across the skin.

2.10. In vitro histological study

After the post-application of formulations (*e.g.*, IL/o and w/o MEs), the skin samples was placed immediately in 4% phosphate-buffered formalin (pH 7.4). Then, the skin was immersed in Histo PrepTM compound (Fisher Scientific, NJ, USA) and placed at −80 °C. Histo PrepTM embedded skin was sectioned using a cryostat microtome (CM1510; Leica, Wetzlar, Germany) and were mounted on glass slides. The slides were stained with hematoxylin (Muto Pure Chemicals Co. Ltd., Tokyo, Japan). The skin section was observed through a 20× objective using a microscope (Olympus, Tokyo, Japan).

2.11. In vitro cytotoxicity studies

The MTT cell viability assay was performed on the reconstructed human epidermal model LabCyte^{TM} EPI-MODEL 12 as described in the literature with some modifications (Katoh et al., 2009). In briefly, the tissues into 24-well plates (BD Biosciences, San Jose, CA, USA) with assay medium (1 mL) were incubated for over night at 37 °C in a 5% CO_2 environment. One hundred (100) μL formulations were placed on the tissue surface and the cultures were incubated for 24 h (37 °C, 5% CO_2, humidified atmosphere). At the end of the incubation period, the tissues were carefully rinsed with D-PBS ten times to remove any remaining formulations from the tissue surface. After removing the culture medium, the treated and control tissues were post incubated for overnight at the same conditions stated as previously. Then 1 mL of freshly prepared MTT medium (0.5 mg mL^{-1}) was added to the wells and the tissues were incubated for 3 h (37 °C, 5% CO_2, humidified atmosphere). Then the tissue fragment was transferred to the microtube containing 0.5 mL of 2-propanol, completely immersing the tissue, and extracted for over 2 h at room temperature in the dark. Subsequently, the extracted solution was transferred to 96-well plates and the optical density was measured at 570 and 650 nm as a reference absorbance, keeping 2-propanol as a blank. The tissue viability was calculated as percentage relative to the viability of negative control, where D-PBS was used as a negative control.

3. Results and discussion

3.1. Formation of microemulsions with pharmaceutically acceptable excipients

The selection of components for pharmaceutical MEs should be a balance between compounds, which are nontoxic and are able to form stable vehicle for drug delivery (*e.g.*, high drug solubility as well as permeation rate). In this study, a blend of two nontoxic surfactants, Tween-80 (having a high HLB = 15.0) and Span-20 (having a low HLB = 8.6), was used as the emulsifying agents whereas IPM (considered as a safe and a widely used organic solvent for drug formulation) was employed as the continuous oil phase because these components were extensively used to prepare the MEs for drug delivery (Schmalfuss et al., 1997; Neubert et al., 2005; Huang et al., 2008; Tsai et al., 2010). Ionic liquid [C_1mim][$(MeO)_2PO_2$] (Fig. 1b) was selected due to its greater solubility efficiency of acyclovir (Moniruzzaman et al., 2010a) and it facilitates to form ME droplets compared to other ILs (Moniruzzaman et al., 2010d). Since a low content of surfactant favors the dermal/transdermal administration, 20 wt% surfactant mixtures (generally more surfactants are used for the preparation of w/o or o/w formulations) was used for this investigation. According to the phase diagram, MEs prepared with this surfactant concentration can solubilize significant amount of IL. In the ME, the IL phase is the internal or dispersed phase, whereas the IPM makes up the external or continuous phase

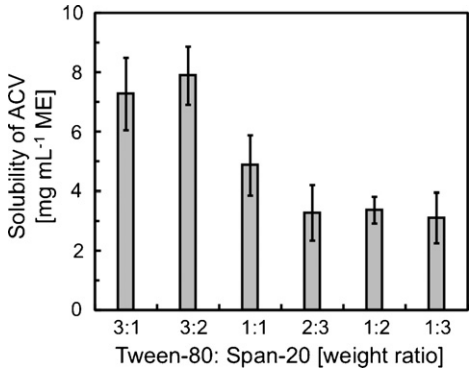

Fig. 2. Solubility of acyclovir in microemulsions made up of various surfactant mixtures. The overall surfactant concentration was 20 wt% and the IL content was 4 wt%. The error bars correspond to the standard deviation from three experiments.

(see Fig. 1a). In this study, IL/o MEs were formulated using various weight ratios of Tween-80 and Span-20 as shown in Table 1. It was observed that the droplet sizes of IL aggregates decreased with increasing of Span-20 in the surfactant mixtures. To explain this trend, the molar concentration of the individual surfactant in MEs can be considered (Table 1). At higher Tween-80 concentration at the interface, the POE (polyoxyethylene) chains on the head group experience steric interaction and are imposed a larger interfacial area (Lu and Rhodes, 2000). In addition, at higher Span-20 contents, micelles may form with this single surfactant and consequently, the size of such micelles decreased compared the droplets formed with a blend of surfactants. The viscosity of MEs increased with increase of the HLB value of surfactants mixture.

3.2. Solubility of ACV in IL/o microemulsions

To investigate and assess IL/o MEs as a delivery system for sparingly soluble drugs, their loading capacity was determined. The solubility of ACV into MEs prepared with different ratios of Tween-80 and Span-20 was investigated since drug solubility in MEs is highly dependent on the content of individual surfactant (Kim et al., 2005). The solubility of ACV in IL free Tween-80/Span-20/IPM micelles was found to be very low (about 0.1 mg mL^{-1}) (Moniruzzaman et al., 2010d). In the presence of IL to form IL/o MEs, a remarkably high amount of ACV can be incorporated into MEs as shown in Fig. 2 via the solubilization in the IL dispersed phase. The solubility of ACV was found to vary on the Tween-80 to Span-20 weight ratios. Higher Tween-80 to Span-20 ratios lead to the solubilization of ACV in the formulations. Particularly, when the weight ratios were above 1:1, the solubility of ACV was reduced significantly. This large increase in ACV solubility in ME1 and ME2 is thought to be caused by the formation of stable ME droplets with large interface compared to that of others MEs (Narang et al., 2007). Moreover, the relative solubility of the drug in Tween-80 and Span-20 would contribute to the drug entrapment in a given microemulsion.

3.3. Particle size determination with drug

The shape, sizes and size distribution of droplets in the ME were characterized by DLS. This system yields spherical ME droplets with a range of 8–34 nm in diameter (Moniruzzaman et al., 2010d). In this study, to observe the effect of loaded drug on the size of ME droplets, we measured sizes and size distributions of the droplets of IL/o ME2 with various drug concentrations at 25 °C. To this approach, ACV (0–5 mg mL^{-1}) was incorporated into the ME2 at 4 wt% IL content. No significant change in the size and size distributions was found when small amount of ACV was loaded into

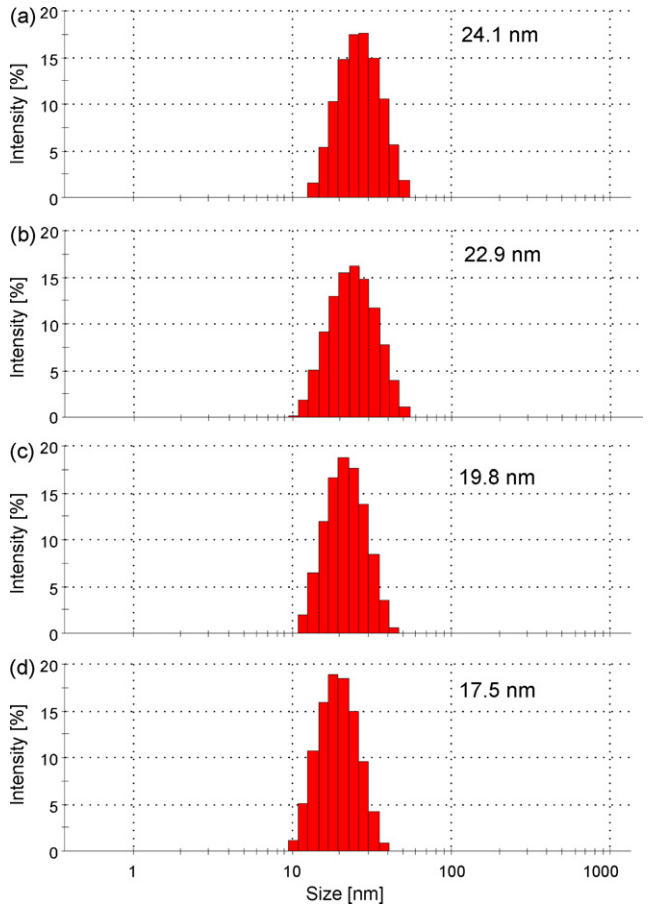

Fig. 3. Sizes and size distribution of the droplets of the IL-in-IPM microemulsions with various acyclovir (ACV) concentrations at 25 °C. The weight fraction of surfactants was 20 wt% (Tween-80: Span-20 = 3:2 (w:w)) and IL = 4 wt%. (a) ACV = 0, (b) 1 mg/mL, (c) 3 mg/mL and (d) 5 mg/mL.

MEs. However, incorporation of large amount of ACV into the MEs decreased the mean diameter gradually (Fig. 3). Such a trend has been previously observed for the incorporation of drug molecules in w/o and o/w ME systems (Park and Kim, 1999; Kantarci et al., 2009). By considering these studies, it can be concluded the following possible reason for this trend. At higher ACV concentration, a certain amount of undissolved drug could be deposited at the interface of ME and acted an emulsifying agent. Consequently, the size of drug loaded IL droplets decreased. Furthermore, the deposited ACV at the interface may reduce the mobility of surfactants which can reduce the droplets size of drug loaded MEs.

3.4. Stability of microemulsions

The stability of the carrier containing the drug molecules is very important in the design of ME for drug delivery. Therefore, the stability of ACV loaded ME1, ME2 and ME3 was evaluated via clarity and phase separation observation and droplet size determination over 2 months at 25 °C. No significant change of these properties of ME1 and ME2 was found during the observed time. For example, after 2 months of storage at 25 °C, the change in the size range of ME1 containing 4 mg mL^{-1} ACV was from 19.3 to 22.4 nm whereas the values for ME2 were from 18.6 to 20.1 nm. However, the size of ME3 was started to increase linearly from 30 days and finally it became turbid after 45 days, which again confirm the formation of stable MEs with higher Tween-80 content in the surfactant mixtures. Centrifugation of the samples was applied in order to assess the physical instabilities, like phase separation, phase inversion, aggregation, and cracking of the microemulsion formulations (Chen et al., 2004). The results showed that formulations had good physical stability. In addition, identical samples of ME2 containing ACV were stored in different temperatures (4, 25 and 37 °C) for studying the effects of storage temperatures on stability. Then these samples were subject to visual inspection and DLS monitoring on ME sizes at determined time intervals. It was found that storage temperature showed negligible impact on the long-term stability of drug containing IL/o MEs. According to the chemical stability study, ME1 and ME2 formulations showed excellent encapsulation efficiency of ACV. The encapsulation efficiency of ME1 and ME2 (both contained 4 mg mL^{-1} ACV at initially) was found to be 95.3% and 97.6%, respectively after 60 days, indicating no degradation.

Table 2
Acyclovir penetration into and across the skin using various formulations at 32.4 °C.[a] All data are presented as mean ± SD from three experiments.

Formulations	Topical delivery ($\mu g/cm^3$)	Transdermal delivery ($\mu g/cm^2$)
IL	ND	ND
IPM[b]	0.18 ± 0.34	ND
Surfactants + IPM[c]	3.86 ± 1.65	ND
w/o ME[b,c,d]	4.57 ± 3.17	ND
IL/o ME[c,e]	10.78 ± 3.12[g]	1.95 ± 1.30
ACV cream[f]	1.21 ± 0.10	ND

[a] All formulations contained 5 mg/mL ACV and penetration time 24 h.
[b] Almost all ACV remained as suspended powders in formulations.
[c] IL/o ME2 as mention in Table 1 in which the weight fraction of surfactants was 20 wt% (Tween-80: Span-20 = 3:2 (w/w).
[d] w/o emulsion contained 4 wt% water.
[e] The system containing 4 wt% IL.
[f] ACV herpecia topical cream (equivalent to 5 mg ACV) used for penetration study.
[g] There was statistically significant difference in the topical delivery between formulations IPM and IL/o ME ($P < 0.01$). Statistical significance was calculated using Student's *t*-test.

3.5. Skin permeation studies

The permeation of ACV from the formulations was performed *in vitro* using YMP full thickness skin as a model skin. The YMP porcine skin was selected because it has clinical, structural, immunohistochemical and reactivity features that are remarkably similar to those of human skin (Lavker et al., 1991). The permeation of ACV from IL/o MEs and others formulations considered as control experiments (*e.g.*, IL, IPM, surfactants + IPM, w/o MEs and commercially available ACV cream) was shown in Table 2. Note that ACV was suspended in IPM, surfactants+ IPM and w/o ME formulations whereas ACV was solubilized in IL and IL/o MEs. As shown in Table 2, the skin permeability of ACV into the skin was increased by several orders of magnitude, when IL/o MEs were used. More significantly, application of IL/o system induced significant transdermal permeation of ACV whereas other formulations showed essentially zero (below the detection level). These results are expected if the advantages of conventional w/o or o/w MEs (*e.g.*, high solubilization capacity, favorable drug transportation mechanism) as drug carriers take into consideration (Neubert et al., 2005; Kogan and Gati, 2006; Gupta and Moulik, 2008). In fact, drugs are able to enter the skin only in a dissolved state and IL/o MEs can solubilize a large ACV amount in the IL droplets which play the role of a drug reservoir. Besides, lipophilic components of IL/o MEs can easily disrupt the SC lipid structure to move nanometer size IL droplets containing solubilized drug into the skin. The drug can easily be diffused from the IL droplets into external phase when absorption (*in vitro*) takes place. In case of IL+ ACV formulation, although ACV was fully solubilized in the formulations, the diffusion of ACV into the skin was hindered by the hydrophobic barrier function of the skin, provided by the highly organized structure of the SC due to the highly hydrophilic nature of IL (Schmalfuss et al., 1997). Besides, the formulations con-

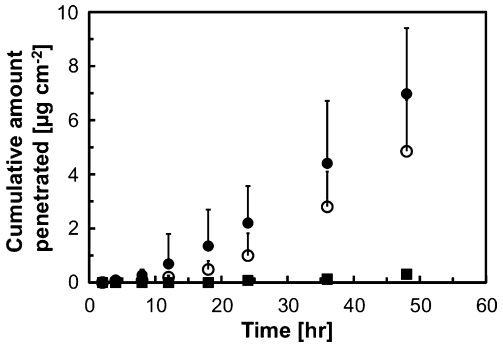

Fig. 4. Permeation profile of ACV across the skin from microemulsions containing different weight ratio of Tween-80 to Span-20 ($n = 3$). [Overall surfactants] = 20 wt% and [IL] = 4 wt%. Symbols: (●) 3:1; (○) 3:2; (■) 1:1. The error bars represent to the standard deviation from three experiments.

taining a large amount of IPM a well-known enhancer to overcome the barrier of SC, the presence of a large amount of suspended ACV probably obstructed the skin (Pattarino et al., 1994). Taking these results together, it is possible to conclude that the excellent skin permeation rate was obtained using IL/o MEs due to the following dual effects. Firstly, a large amount of ACV was totally dissolved in nanometer-sized IL droplets, which led to larger concentration gradient towards the skin (Kreilgaard et al., 2000; Sintov and Shapiro, 2004). Secondly, IPM and surfactants were used to overcome the barrier of SC lipid structure.

Next, ACV permeability was evaluated by varying the ratio of Tween-80 to Span-20 keeping the overall surfactant concentration fixed at 20 wt% as the molar ratios of the individual surfactant in ME formulations control the physicochemical properties of MEs which have a great effect on drug delivery (Peltola et al., 2003; Huang et al., 2008). The transdermal permeation profiles (Fig. 4) show the cumulative mass of ACV permeated across the skin as a function of time. As like as the drug solubility, ME1 and ME2 containing large amount of Tween-80 favor the transdermal delivery of ACV (Table 3). To explain the results, the interfacial area of the IL droplets can be considered. In general, a larger stable interfacial area of the dispersed phases at specific surfactant concentration favors the topical and transdermal delivery (Ktistis and Niopa, 1998; Sintov and Shapiro, 2004). As shown in Table 1, each aggregate of ME1 and ME2 provides a larger interfacial area compared to ME3. In addition to this, stability studies clearly indicated that the aggregates of ME1 and ME2 were more stable than that of ME3. Theses two factors are possibly reflected in the enhanced transdermal permeation. Furthermore, considering the HLB values of the surfactant mixtures, it can be said that thermodynamic activity of ME1 and ME2 in IPM is higher than that of ME3 because the blend of surfactants solubility of ME1 and ME2 in IPM is lower than that of ME3. These differences in thermodynamic activity among the various MEs may affect the drug permeation through the skin (Shah, 1994; Rhee et al., 2001). Again, ME2 (having Tween-80/Span-20 = 3/2) was found to be most suitable one as a transdermal drug carrier. These results suggest that an optimized amount of Tween-80 is beneficial for regulatoring the IL droplets interfacial area needed for preparing effective MEs for drug delivery, which is a common phenomenon observed in conventional MEs.

3.6. In vitro histological study

Histological studies were performed to make a preliminary assessment of the safety of IL/o ME as a transdermal carrier. For this purpose, the YMP skins exposed to ME formulations as well as to other control formulation were stained with hematoxylin.

Table 3
Effects of microemulsion formulations containing different surfactant mixtures on acyclovir skin permeation parameters.

Formulations	J ($\mu g\,cm^{-2}\,h^{-1}$)	P ($\times 10^{-5}\,cm\,h^{-1}$)
ME1	0.162	4.04
ME2	0.182	4.64
ME3	0.010	0.27

The samples were later observed under light microscope (20-fold magnification) to be assessed for possible structural damage. The preliminary studies indicated that no significant change has been detected in the skin structure after treating for 24 h with IL/o ME compared with the untreated one as well as treated with other control solutions (PBS and w/o MEs).

3.7. In vitro cytotoxicity studies

To evaluate the relative safety of the IL/Tween-80/Span-20/IPM MEs, we compared their cytotoxic effects to those of water/Tween-80/Sapn-20/IPM (w/o) MEs and D-PBS. Note that the only difference between IL/o and w/o MEs is the substitutions of 4 wt% IL by 4 wt% water. As shown in Fig. 5, the preliminary results clearly demonstrated that a significant decrease in cell viability was observed when pure IL was used as medium. Such trend of toxicity of bulk ILs is generally observed in case of most common ILs, particularly having imidazolium cations. However, ME containing 4 wt% IL showed over 80% in cell viability compared to control experiment (D-PBS). It was also found that the cell viability of IL/o ME is comparable to w/o ME and IPM. This trend is consistent for many useful and necessary chemicals being used in pharmaceutical industry. For an example, DMSO, a well-known chemical enhancer for transdermal drug delivery, is safe when they use at low concentration (up to 10%) although the presence of high concentrated DMSO increases the system cytotoxicity significantly which causes erythema and wheals of the SC (Afouna et al., 1998; Williams and Barry, 2004). Although cytotoxicity effects of this IL/o MEs are encouraging, biodegradation study is required to develop these nonaqueous MEs as a drug delivery carrier.

In fact, to fulfill the requirements as drug delivery solvent, ILs should preferentially exhibit good biodegradability with their low toxicity. Generally, ILs with a short alkyl chain appended in the imidazolium cation and also with hydrophilic anions have been shown to exhibit low toxicity (Stolte et al., 2007). Considering the toxicity point, the use of IL $[C_1mim][(CH_3O)_2PO_2]$ (see Fig. 1b) is beneficial for developing drug delivery systems, which

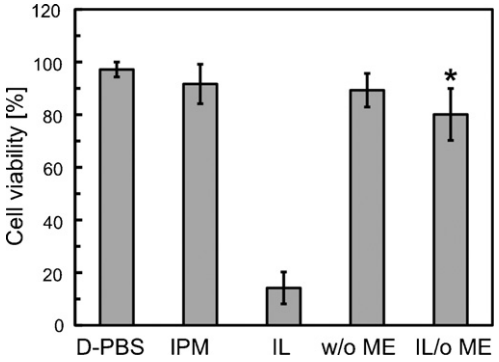

Fig. 5. Cytotoxicity of different formulations according to the MTT assay ($n = 3$). Microemulsions contained 20 wt% surfactants (Tween-80: Span-20 = 3:2), whereas 4 wt% water and IL used in w/o and IL/o microemulsions, respectively. Note that IL/o ME is the identical to ME1 as shown in Table 1. The data represents the average of the three experiments, and the error bars represent the standard deviation. Statistical significance was calculated using Student's t-test. $^*P < 0.05$ *vs.* ionic liquid (IL).

also reflected the present study. Unfortunately, imidazolium based ILs having short alkyl chain have proven resistant to biodegradation (Gathergood et al., 2004). Hopefully, very recently, a comprehensive tutorial review of biodegradable studies of ILs was published by Coleman and Gathergood (2010) where they reported different routes for the preparation of ILs that are nontoxic as well as biodegradable. Investigations to develop more biocompatible MEs with nontoxic as well as biodegradable ILs are now being carried out.

4. Conclusions

The results of the present study demonstrated that IL/o MEs can be used for transdermal delivery of acyclovir which is insoluble or sparingly soluble in water and most pharmaceutically accepted organic solvents. Microemulsion with their high solubility of ACV, excellent formulation stability and enhanced permeation to the YMP skin were obtained from a blend of surfactant composed of 3:2 weight ratio of Tween-80 to Span-20. The enhancement of ACV penetration using this new IL/o ME is thought to be due to the combination of factors including the high ACV solubility in IL disperse phase and the effect of lipophilic components. Stability studies clearly indicated the excellent physical and chemical stability of IL-based MEs. The cell viability study showed very low cytotoxicity of IL/o microemulsions. This result will certainly help to change the perception that the toxicity issue of ILs is not a barrier for employing them in the field of drug delivery. Hopefully, the biocompatibility of this formulation can easily be improved by tuning the tailor-made property of ILs. Finally, this study allowed us to conclude that IL/o microemulsions create a new opportunity for the controlled delivery of sparingly soluble drugs that have problems of administration by other routes.

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