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ScienceDirect
International Journal of Pharmaceutics 349 (2008) 108–116
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Cremophor-free intravenous microemulsions for paclitaxel I: Formulation, cytotoxicity and hemolysis

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Received 8 April 2007; received in revised form 20 July 2007; accepted 24 July 2007
Available online 7 August 2007

Abstract

Two cremophor-free microemulsions, lecithin:butanol:myvacet oil:water (LBMW) and capmul:myvacet oil:water (CMW) for paclitaxel (PAC) were developed for intravenous (i.v.) administration. Six surfactants and four oils were screened with various combinations for maximal water incorporation and PAC solubility. Microemulsion regions were subsequently determined in ternary phase diagrams. Cytotoxicity in an MDA-M231 human breast cancer cell line and hemolytic potential were assessed in these systems compared to Taxol® (cremophor EL:ethanol, 1:1, 6 mg PAC/ml). The maximal water incorporation into the lecithin:butanol surfactant blend was greater than that incorporated into capmul when combined with the oils screened. PAC solubility in myvacet oil was increased 1389-fold over its aqueous solubility. LBMW had a larger microemulsion region (46.5% of total ternary phase diagram) than that seen with CMW (18.6%). The droplet size of the dispersed phase was 111.5 (4.18) nm for LBMW and 110.3 (8.09) nm for CMW. Cytotoxicity of PAC was in decreasing order of: Taxol® > LBMW > CMW. The IC_{50} values for LBMW and CMW ranged from 4.5 to 5.7 and >10 μM, respectively, as compared to that of Taxol® (1.3 to 1.8 μM). Eighty-three percent, 68%, and 63% of red blood cells remain unlysed at a formulation volume to blood ratio of 0.035 in LBMW, CMW and Taxol®. Promising microemulsions, LBMW and CMW were developed that can incorporate approximately 12 mg/g of PAC, substantially higher than its aqueous solubility (10.8 μg/ml) and that in the Taxol® vehicle (6 mg/ml). PAC retained its cytotoxicity in the LBMW and CMW and was less likely to cause hemolysis compared to Taxol®. This higher drug loading results in a smaller vehicle volume in required doses of these formulations and potentially less vehicle-related side effects are anticipated.

Keywords: Paclitaxel; Microemulsion; Intravenous; Hemolysis; Cytotoxicity

1. Introduction

Paclitaxel (PAC) is an antineoplastic agent derived from the needles and branches of the Himalayan Yew tree. It demonstrates impressive clinical activity against ovarian, breast, non-small cell lung carcinomas and AIDS-related Kaposi's sarcoma and it acts by stabilizing abnormal microtubule structures (Wall and Wani, 1996). Due to its poor oral systemic availability as a result of its limited aqueous solubility (10.8 μg/ml) and high lipophilicity ($K_{o/w} = 311$), it is administered as an intravenous (i.v.) infusion to patients as Taxol® (Trissel, 1997).

Taxol® is a clear to colorless formulation in 5 ml vials containing PAC (6 mg/ml) in cremophor EL (polyoxyethylated castor oil):ethanol (1:1, v/v) (Vyas, 1995). Upon dilution in dextrose saline, a slightly hazy dispersion is observed. This dispersion is stable for 27 h at room temperature (Taxol® (Paclitaxel) Injection Patient Information, 2003). However, since PAC is not actually in solution, precipitation of particles with clinically unacceptable sizes eventually occurs. This necessitates the use of in-line filters with infusion sets when PAC is infused for over 24–96 h (Trissel et al., 1994). In addition, components in the Taxol® formulation have the tendency to leach the plasticizer diethylhexyl phthalate (DEHP) from polyvinylchloride containers, administration sets and extension sets (Mass et al., 1996). As a result, glass containers or containers composed of nonplasticized plastic such as the polyolefins are used. In animals, changes in hepatocellular structure and liver function,

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doi:10.1016/j.ijpharm.2007.07.042

and the development of hepatocellular carcinoma occurred with DEHP exposure. In humans however, prolonged exposure to DEHP products leached into blood has not yet been associated with specific toxicities (Mass et al., 1996).

Several PAC formulations for i.v. and oral delivery have been developed in an attempt to address the aforementioned drawbacks of Taxol®. These strategies include (but are not limited to) increasing the aqueous solubility of PAC by cosolvency, emulsification, microemulsification, nanoemulsification, drug complexation with cyclodextrins, carrier mediation using liposomes, microspheres, nanoparticles, structural modification to form analogues and prodrugs (Singla et al., 2002; Hennenfent and Govindan, 2006; Tiwari and Amiji, 2006). Microemulsions stand out as an appropriate system to deliver PAC intravenously. They form spontaneously and are composed of a surfactant and/or surfactant mixture, co-surfactant, an oil and water with a dispersed phase of less than 110 nm in diameter (Gillberg et al., 1970; Lawrence and Rees, 2000; Bagwe et al., 2001). The combination of surfactants with oils to form microemulsions offers an advantage over a micellar or co-solvent system in terms of drug solubilization capacities for lipophilic compounds, because of the extra locus for solubilization provided by the oil phase (Lawrence and Rees, 2000). Also, because of the small droplet diameter, microemulsions can be sterilized by filtration (Groves, 1989), as the non-ionic surfactants contained in the formulation are unable to withstand high temperatures used in autoclaving. However, certain potential drawbacks of microemulsions as parenteral dosage forms have been recognized such as the limited availability of biocompatible ingredients and an excessive amount of co-surfactant and surfactant required in forming microemulsions.

The obvious benefits of microemulsions as drug delivery systems have led to the development of several systems for i.v. and oral administrations of PAC over the past 5 years (Gursoy et al., 2003; He et al., 2003; Kang et al., 2004; Yang et al., 2004; Zhang et al., 2005). These systems all contain cremophor, except one (Gursoy et al., 2003). Cremophor EL contained in Taxol® has been implicated in anaphylactic reactions in certain individuals (Taxol® Solution for Injection, Drug Information, 2007), making it necessary to pre-medicate patients with antihistamines and corticosteroids to reduce the frequency of the reactions to an acceptable level. Also, cremophor may affect red blood cells (RBC) because they penetrate biological membranes to cause an increase in permeability and cell damage (Bielawski, 1990). These effects depend on the quantitative ratio of the surfactant to the membrane. At low surfactant:cell (formulation:blood) ratios, the erythrocytes are protected against osmotic shock. At higher ratios, hemolysis is induced and a further increase causes solubilization of the membrane. Rouleaux formation of RBC and a change in the shape of white blood cells were observed in blood smears in a study of the effect of Taxol® on human RBC (Shimomura et al., 1998).

Although one other cremophor-free self-emulsifying drug delivery system (SEDDS) has been developed (containing tyloxapol as a surfactant and co-solvent, sodium deoxycholate (DOC-Na) and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) as surfactants, ethanol as a co-surfactant and vitamin E as the oil phase), this system containing 3% (w/w) of PAC (SEDDS diluted with water) was unstable after 24 h at 37 °C losing 20–25% of its chemical integrity (Gursoy et al., 2003). The authors stated that a ready-to-use SEDDS formulation could not be anticipated in the future.

Therefore, the goal of this study was to develop biocompatible, cremophor-free microemulsions containing PAC for i.v. administration. Formulation development entailed the screening of six surfactants and four oils, determining the solubility of PAC in the oils, and constructing ternary phase diagrams. The optimal microemulsions were characterized by droplet size, cytotoxic activity in a breast cancer cell line, MDA-M231, and in vitro hemolytic potential using Taxol® as a reference.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

PAC [Hande Technology Development Co. USA Inc., Houston, TX], *n*-benzylbenzamide [Aldrich Chemical Co., Inc., Milwaukee, WI], methanol, acetonitrile HPLC grade [VWR Scientific Products, Sugarland, TX], Taxol® containing 30 mg PAC in 5 ml [Bristol-Myers Squibb Co., Princeton, NJ]. Each millilitre contains 6 mg of PAC, 527 mg Cremophor® EL (polyoxyethylated castor oil) and 49.7% (v/v) dehydrated alcohol USP.

2.1.2. Six surfactants

Capmul MCM® (mono/diglycerides of caprylic/capric acid in glycerol) [Abitec Corp., Janesville, WI], Labrafac CM10® (polyglycolized glycerides), Labrafil M1944 CS® (PEG-6 glyceryl monoleate), Labrafil M2125 CS® (PEG-6 glyceryl linoleate), Labrasol® (PEG-8 glyceryl caprylate/caprate) [Gattefosse S.A., Saint-Priest, Cedex, France], and L-α-phosphatidylcholine (L-α-lecithin) from soybean [Sigma Chemical Co., St. Louis, MO].

2.1.3. Four oils

Captex 200® (mixed diesters of caprylic/capric acids in propylene glycol), Captex 355® (triglycerides of caprylic/capric acids) [Abitec Corp.], Miglyol 812® (glycerine plus mixed triester with capric/caprylic acids) [Sasol North America, Westwood, NJ], and Myvacet 9–45® (distilled acetylated monoglycerides) [Eastman Chemical Co., Kingsport, TN].

2.2. Methods

2.2.1. HPLC assay for PAC samples

Concentrations of PAC in the four oils was determined using a modified high-performance liquid chromatography (HPLC) method (Wang et al., 2003). The method was modified to include a different flow rate, quantity of organic phase in the mobile phase and column length. The chromatographic system consisted of a Waters 600 controller and pump, a Waters 717 plus autosampler, and a Waters 996 photodiode array detector [Waters Corp., Milford, MA]. Chromatographic separa-

tions were achieved using a Synergi Hydro reversed-phase C18 (150 mm × 4.6 mm, 4 μm) column and a Synergi Hydro C18 guard column (4 mm L × 3.0 mm i.d.;) [Phenomenex, Torrance, CA]. The mobile phase consisting of 48% acetonitrile in water was pumped through the column at a flow rate of 1.2 ml/min at room temperature. The UV detection wavelength for PAC was 228 nm using *N*-benzylbenzamide (*N*-BB) as an internal standard.

One-hundred micrograms per millilitre stock solutions of PAC and *N*-BB were prepared in methanol. These stock solutions were diluted with methanol to obtain 0.45, 0.9, 1.8, 2.7, 4.5 and 9 μg/ml working standards for PAC and 1 μg/ml for *N*-BB. Linearity was determined by plotting the peak height ratio of PAC to *N*-BB versus PAC concentrations. Precision was determined using three PAC standards (0.9, 2.7 and 9 μg/ml). The mean, standard deviation and relative standard deviation (R.S.D.), or correlations of variation of the determined concentrations were recorded. Within- and between-day variations of PAC concentrations were determined by constructing calibration curves on the same day (within-day) and on different days over a 3-month period (between-day). The mean, standard deviation and relative standard deviation of the slopes were recorded.

2.2.2. Microemulsion formulation development

2.2.2.1. Selection of surfactant and oil blends. Components for the microemulsion were selected based on their known usefulness in formulating microemulsions and applications in food and i.v. products. The oils and surfactants were initially screened by combining these components at a 50:50 ratio. Water was then added dropwise until the mixture changed from translucid to turbid or milky. Promising blends were further screened at oil to surfactant ratios of 25:75, 70:30 and 75:25. The maximum amount of water that could be incorporated was determined. The extent of water incorporation into the oil:surfactant mixture served as one of the criteria in the final selection of the surfactants and oils in the microemulsion development.

2.2.2.2. PAC solubility in oil. The lipophilic PAC was anticipated to dissolve in the oil phase of the microemulsion; hence the solubility of PAC was determined in myvacet, miglyol and captex 355 oils. The solubility of captex 200 was not determined because of its lowest water incorporation when combined with the surfactants screened. PAC was added in 1 mg increments to 1 g of oil. The concentration of PAC was determined by dilution in methanol (1:18000) and subsequent injection onto the HPLC. The oil with the highest solubility in mg/g oil was chosen for the final formulation.

2.2.2.3. Phase diagram construction. In order to aid successful microemulsion development, it is essential to study the phase behavior of potential combinations of water, surfactant and oil. Phase diagrams were therefore constructed to determine regions of microemulsion formation, from which a large number of potential microemulsions were identified. The oils and surfactants were combined at ratios from 95:5 to 5:95 with 5% increments of the surfactant and the maximum amount of water that could be incorporated was determined for each oil:surfactant combination. The phase diagram was constructed by connecting the ratios of oil:surfactant:water at each transition point on a ternary phase diagram. Phase diagrams were constructed for two systems: lecithin:butanol:myvacet oil:water (LBMW) and capmul:myvacet oil:water (CMW). These optimal microemulsions were selected based on the water incorporation and the highest solubility of PAC in the previously chosen oil.

2.2.2.4. Preparation of microemulsions containing PAC for evaluation. The microemulsions containing PAC were prepared by dissolving an appropriate amount of PAC in methanol. PAC was first dissolved in methanol to expedite the dissolution process and ensure that PAC is completed solubilized in the oil. Myvacet oil was added to the drug solution and the methanol evaporated under vacuum. The surfactants and water were then added and shaken to form the microemulsion.

2.2.3. Microemulsion droplet size characterization

The droplet size of the dispersed phase of the microemulsion was determined using a dynamic light scattering technique. Measurements were performed using a BI-200 SM goniometer with a custom-selected Ti-Sapphire laser and Hamamatsu photomultiplier tube [Brookhaven Instruments, Inc., Holtsville, NY]. The mean droplet size for each microemulsion was determined at room temperature at an angle of 90° and wavelength of 678 nm. The viscosity was set at 0.955 cP and refractive index at 1.33. Microemulsions were diluted 1:100 with water before measurements.

2.2.4. Cytotoxicity studies

The ability of PAC in the microemulsions of LBMW (lecithin:butanol:myvacet oil:water, 20:10:68:2, by weight) and CMW (capmul:myvacet oil:water, 24:74:2, by weight) with 12 mg/g PAC to inhibit cell proliferation was determined as compared to Taxol®. Cytotoxicity was determined in the MDA-M231 breast cancer cell line [MD Anderson Cancer Center, Houston, TX] using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega, WI] reduction assay. Cells were grown in Dubelco's Modified Eagle's medium (DMEM) at pH 7.2 (supplemented with 2mM-glutamine, 0.08% (w/w) sodium bicarbonate, 1% penicillin–streptomycin, and 10% (v/v) fetal bovine serum) [Gibco-BRL, Life Technologies, NY]. Control cultures represented cells without treatment. Cells were plated at a density of 6000 cells/100 μl in each well of the 96-well plate and incubated overnight at 37 °C. The medium was replaced with 100 μl of the diluted PAC formulation in culture media. The concentration of the diluted PAC standards ranged from 0.1 nM to 10 μM. The cells were incubated with PAC solutions for incubation durations of 2, 18, 24 and 48 h, after which 20 μl of the MTS reagent was added to each well. The plates were shaken for 10 min and read at 490 nm on a microplate reader [Molecular Devices, Sunnyvale, CA]. The optical densities obtained from viable cells for drug-treated and untreated cells were measured. The optimal duration of incubation was determined from a plot of optical density of viable cells versus duration of incubation

at the maximum concentration of 10 μM. A graph of percent inhibition versus PAC concentration incubated with cells was constructed. The reduction in proliferation by 50% (IC_{50}) was measured for each formulation. The extent of inhibition at maximal test concentration of 10 μM was also compared amongst the formulations.

2.2.5. Hemolytic potential studies

The hemolytic potential of the microemulsions LBMW and CMW with and without 12 mg/g PAC as compared to Taxol® were determined using a modified method of Reed and Yalkowsky (1987).

2.2.5.1. Volume of water for complete hemolysis. The amount of water for complete hemolysis of RBC from male Sprague Dawley rats (180–200 g) [Charles River, Inc., Indianapolis, IN] was determined by combining different volumes of water with 0.1 ml of fresh heparinized (heparin sodium injection, USP, 1000 units/ml) [Gibco Ivenex Division, Chagrin Falls, OH] whole rat blood in test tubes to obtain volume ratios of 0.1, 0.2, 0.5, 1, 5 and 10. The mixtures were centrifuged at 1000 × *g* for 5 min in a refrigerated centrifuge [Beckman Instruments Inc., Fullerton, CA], after mixing for 10 s and incubating for 2 min at room temperature. The supernatant was discarded and the packed cells were washed with 5 ml heparinized normal saline to stop the hemolysis. The intact cells left over were lysed with 1 ml of water. The sample was diluted 1:9 with distilled water and the absorbance measured at 540 nm [Du®-70 Spectrophotometer, Beckman, Fullerton, CA]. The water:blood ratio that produced insignificant absorbance was identified as the ratio at which complete hemolysis occurs. From this ratio, the amount of water needed for complete hemolysis of RBC was determined.

2.2.5.2. Construction of standard curve. One-millilitre solutions containing healthy cell fractions of 0, 0.2, 0.5, 0.8, and 1 were made up by combining water with fresh heparinized blood. Hemolysis was stopped and the solutions centrifuged according to the aforementioned protocol. One millilitre of water was then added for every 0.1 ml of blood used to lyse the remaining intact cells. After centrifugation, the supernatant was diluted 1:9 with water before the absorbance was measured at 540 nm. A standard curve of healthy cell fraction versus absorbance was constructed.

2.2.5.3. Hemolytic potential. The hemolytic potential of the formulations with and without PAC was evaluated by mixing fresh heparinized rat blood with the formulations at ratios of 0.025, 0.05, 0.1, 0.125 and 0.2. The samples were then processed according to the standard curve protocol. The fractions of unhemolyzed cells were calculated from absorbance and the standard curve. The ratio at which 50% hemolysis (H_{50}) occurred for each formulation was determined from plots of fraction of healthy cells versus the log volume ratio (formulation:blood). The maximal tolerable dose of the formulations was determined for future i.v. administration to rats in subsequent pharmacokinetic experiments.

3. Results and discussion

3.1. HPLC assay for PAC samples

Baseline resolution between PAC and *N*-BB peaks was achieved with retention times of approximately 13.0 and 5.8 min, respectively. No peaks in the blank methanol chromatogram interfered with either of the PAC or *N*-BB peaks. Linearity was demonstrated over a PAC concentration range of 0.45–9 μg/ml ($R^2 = 0.9997$). The method was precise in the sample analysis with coefficient of variations of 1.33, 3.53 and 1.79% at PAC concentrations of 0.9, 2.7 and 9 μg/ml, respectively. The assay was reproducible with within- and between-day variations of 5.75 and 5.97%, respectively.

3.2. Microemulsion formulation development

The selection of potential surfactants and oils as components in the microemulsion was based on their suitability for i.v. microemulsions, their ability to incorporate large amounts of water into the surfactant:oil mixture and the solubility of PAC in the oil phase. Based on the criteria, six surfactants and four oils were selected for the initial screening. The surfactant/co-surfactant blends lecithin:butanol (67:33) and capmul were able to incorporate the largest amounts of water out of all the surfactants screened when combined with the four oils (Table 1). The other surfactants screened only incorporated less than 5% of water, while some were cloudy initially and remained cloudy upon further addition of water. Lecithin:butanol when combined with the four oils incorporated 28.6–48.2% of water; the highest was seen with miglyol oil. Capmul when mixed with the four oils incorporated 4.6–8.9%, the highest in this case was seen with myvacet oil. Hence two promising surfactants from the initial screening, capmul and lecithin:butanol (67:33), were combined with captex 355, miglyol and myvacet oils at surfactant/oil ratios of 25:75, 50:50 and 75:25 for campul and 30:70, 50:50 and 70:30 for lecithin/butanol, respectively, for further screening (Fig. 1).

Increasing the concentration of surfactant in the surfactant:oil mixture results in a corresponding increase in water incorporation (Fig. 1). Water incorporation seen with capmul:oil mixtures were generally lower than that with lecithin:butanol:oil mixtures. Capmul showed comparable effectiveness in terms of water incorporation amongst the three oils at all ratios, except that a slightly higher value was observed with myvacet oil at a surfactant:oil ratio of 75:25. The highest water incorporation was observed when lecithin:butanol was combined with captex 355 and miglyol at ratios of 30:70 and 50:50, but was lowest when combined with myvacet. However, when the lecithin:butanol concentration was increased to 70% all three oils were equally effective in incorporating water. Subsequently, PAC solubility in the three oils was determined to further streamline the selection of oils for the optimal microemulsion. The solubility of PAC increased 1389-fold in myvacet oil (15 mg/g oil), 537-fold in captex 355 oil (5.8 mg/g oil) and 111-fold in the miglyol oil (1.2 mg/g oil), as compared with its aqueous solubility (10.8 μg/ml). Myvacet oil was thus selected as the oil

Table 1
Water incorporation into five surfactants and one surfactant blend when combined with four oils at surfactant:oil ratios of 50:50

Surfactants	HLB[b]	Water incorporation (%)[a]			
		Oils			
		Captex 200	Captex 355	Miglyol	Myvacet
Capmul MCM	5.5–6	4.6	7.6	7.9	8.9
Labrafac CM 10	10	4	C	2.5	2.9
Labrafil M1944 CS	3–4	C[c]	1.9	C	1.9
Labrafil M2125 CS	3–4	C	2.9	C	C
Labrasol	14	C	C	C	4.9
Lecithin:butanol (67:33)	5	28.6	41.9	48.2	32.0

[a] Water incorporated (%) = [weight of water added/(weight of mixture + weight of water added)] × 100.
[b] HLB: Hydrophilic–lipophilic balance.
[c] C: Surfactant:oil mixture was cloudy initially and remained cloudy with water addition.

phase in the phase diagram construction based on its highest PAC solubility.

The selection of appropriate oils and surfactants is critical to the functionality of parenteral microemulsions. Vehicle selection should consider toxicity, biocompatibility and reproducibility (Groves, 1989; El-nokaly and Cornell, 1991). To this end, the oils used in this study (captex 200, captex 355, miglyol 812 and myvacet oils) were low molecular weight, medium chain triglycerides (MCT) which are particularly attractive for formulating microemulsions for i.v. administration. They are (i) stable food grade products and generally regarded as safe (GRAS) by the Food and Drug Administration, and (ii) microemulsions incorporating these low viscosity oils can be formulated over a wide range of compositions at ambient temperatures (El-nokaly and Cornell, 1991). The toxicity and biocompatibility of non-ionic surfactants were a consideration during the selection process. Hence, the selection of biocompatible phospholipids like lecithin has been widely investigated as a surfactant in microemulsions (Kahlweit and Strey, 1987; Shinoda et al., 1991; Moreno et al., 2003).

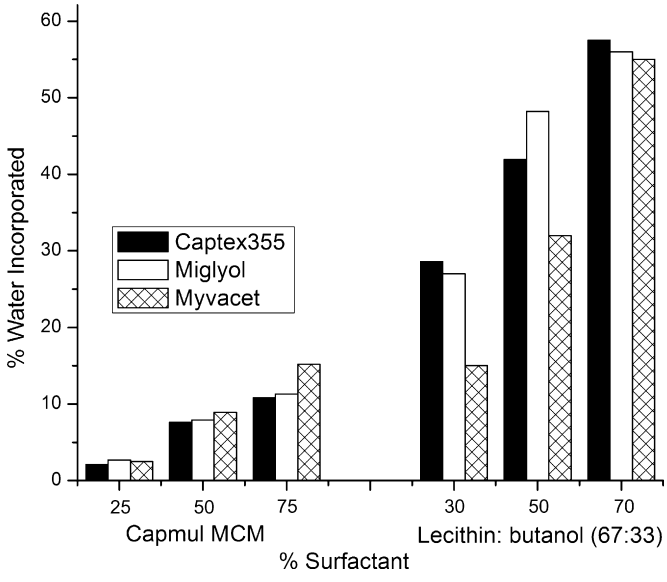

Fig. 1. Water incorporation into the surfactants capmul MCM and lecithin: butanol (67:33) when mixed with the oils captex 355, miglyol and myvacet.

Drug-containing microemulsions should be dilutable with water without causing precipitation of the drug incorporated (Kiepert, 1989; Lawrence and Rees, 2000) during clinical use. Hence in the second stage of formulation development, the amount of water that a surfactant:oil mixture could incorporate was determined. From these experiments, it was demonstrated that the amount of water incorporated into these systems depended on both the surfactant concentration and the nature of the oil (Fig. 1). The higher the surfactant concentration, the higher the water incorporation. Also, the water incorporation differed with oil type used. This was most obvious with the lecithin:butanol surfactant blend which incorporated up to 48.2% of water when combined with miglyol and 32% when combined with myvacet (Table 1).

Phase diagrams were utilized in the third stage of development to determine the macroscopic phase behavior (Gillberg et al., 1970; Kiepert, 1989) of the microemulsion and to compare the efficiency of different surfactants in terms of water incorporation. The goal in the formulation of a microemulsion is to have the lowest possible surfactant content with an optimal solubilization of the hydrophilic and lipophilic components. However, relatively high surfactant contents are often needed to form microemulsions. In this study, only the boundary between the single and multi-phase region were identified. Phase diagrams were constructed for two surfactants lecithin:butanol (67:33) and capmul (Fig. 2), when combined with myvacet oil to further characterize these microemulsion systems. The regions to the right of the boundary lines were single-phase microemulsion regions, and to the left multi-phase regions. The microemulsion region was larger for the LBMW system (lecithin/1-butanol/myvacet oil/water, solid line) which covered approximately 46.5% of the ternary phase diagram. The CMW system (capmul/myvacet oil/water, dashed line) on the other hand covered 18.6%. In this study an alcohol, *n*-butanol was combined with lecithin, based on the information gathered from literature reports (Shinoda et al., 1991; Moreno et al., 2003). When the short chain alcohol, *n*-butanol is introduced; the polar solvent (water) becomes less hydrophilic. Also, because the *n*-butanol will partially be incorporated in the polar parts of the lipid layers, there is an increase in the area of each lipid polar head group. As a result, the spontaneous curvature of the lipid layers towards the oil changes, thereby decreasing the stability of the lamellar liq-

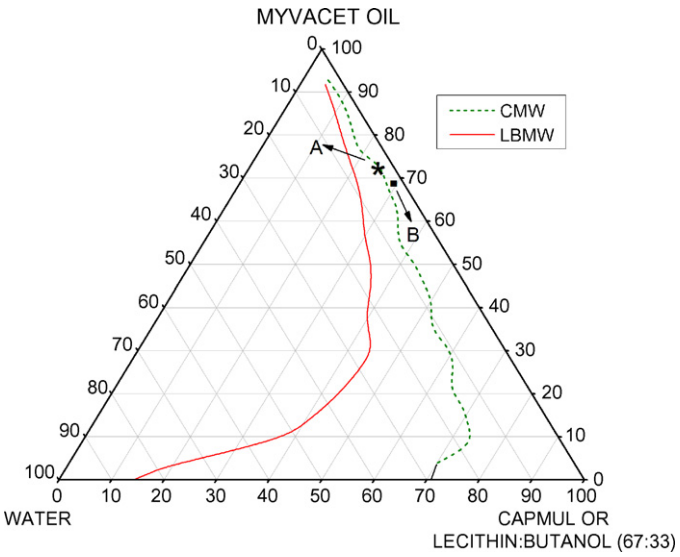

Fig. 2. Ternary phase diagram of capmul/myvacet oil/water system (CMW) and pseudo-ternary phase diagram of lecithin:1-butanol/myvacet/water system (LBMW). The area to the right of the solid line represents the single-phase microemulsion region. Points A (*) and B (■) represents ratios of 24:74:2 for CMW and 20:10:68:2 for LBMW (surfactant/surfactant blend:myvacet:water).

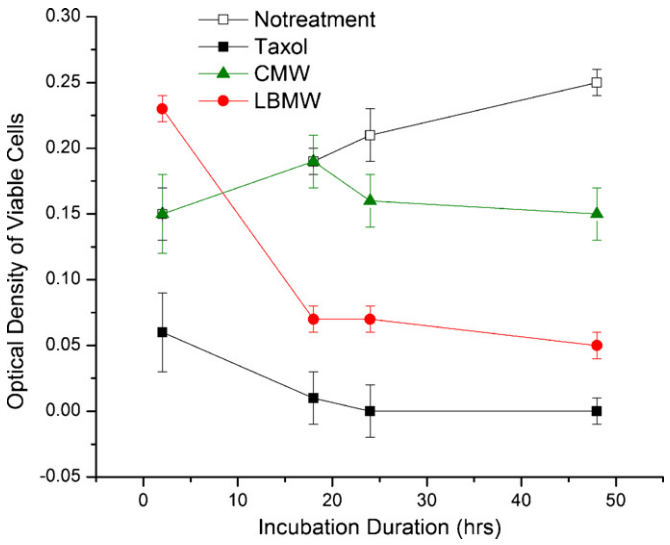

Fig. 3. Percent cell viability versus incubation duration at 10 μ M of PAC. Cells were incubated at 37 °C with PAC in Taxol®, LBMW and CMW for 2, 18, 24 and 48 h. An MTS assay was used to determine cell viability.

uid crystalline phase and increasing the isotropic single phase region (Shinoda et al., 1991) that is seen with systems containing lecithin:butanol. The foregoing discussion could explain the larger microemulsion region seen with LBMW compared to CMW. Several microemulsions can be identified in these regions for both LBMW and CMW systems. These microemulsions are to be used as drug delivery systems for PAC to be administered i.v. Hence, an optimal surfactant ratio necessary to form a microemulsion with limited toxicity was chosen. As a result, the ratios of the components that were used in the two microemulsions, CMW and LBMW, to determine the cytotoxicity and hemolytic potential were identified as point A, 24:74:2, by weight and B, 20:10:68:2, by weight, respectively.

In conclusion, promising cremophor-free microemulsions were developed in this study into which PAC could be incorporated. These microemulsions utilized biocompatible surfactants such as lecithin and oils such as the medium chain triglyceride myvacet oil which have been employed in parenteral formulations for i.v. infusion (El-nokaly and Cornell, 1991).

3.3. Microemulsion droplet size characterization

Biopharmaceutical characteristics and preparation stability are directly related to the physical properties of microemulsions, including the microstructure, droplet size, and location of the drug molecule in the microemulsion (Lawrence and Rees, 2000). The droplet sizes of the formulated microemulsions were in the 110 nm range (Table 2). With oil in water microemulsions administered orally there is a known relationship between droplet size and bioavailability (Constantinides and Wasan, 2007). However, based on the HLB values of the surfactants, water in oil (w/o) microstructures are anticipated and various authors suggest that w/o microemulsions show no obvious correlation between droplet size and the bioavailability of a drug when given orally, rather the type of surfactant becomes a more important factor.

3.4. Cytotoxicity of paclitaxel

The MTS cell proliferation assay used to measure the cytotoxicity of PAC is a colorimetric method to determine the number of viable cells (CellTiter 96Aqueous One Solution Cell Proliferation Assay, 2001). The optical density of the viable cells treated with various formulations was plotted against the incubation duration of 2, 18, 24 and 48 h at a maximal PAC concentration of 10 μM (Fig. 3). The optical density after 2 h of incubation was 0.15, 0.06, 0.23 and 0.15 for no treatment, Taxol®, LBMW and CMW, respectively. It appears that there was initial growth of the cells with all the treatments except the cells treated with Taxol®. After this initial growth there was a rapid decline in cell number reflecting their cytotoxic activity from 2 to 18 h in the Taxol® and LBMW treated cells. However,

Table 2
Hydrophilic–lipophilic balance of surfactants, microstructure of microemulsions and droplet size

	HLB of surfactant	Anticipated structure (based on HLB)	Mean diameter (nm)	Microemulsion region (% of ternary phase diagram)
LBM (lecithin:butanol (67:33):myvacet oil)	5	w/o	111.50 (4.18)	46.5
CMW (capmul MCM:myvacet oil)	6	w/o	110.03 (8.09)	18.6

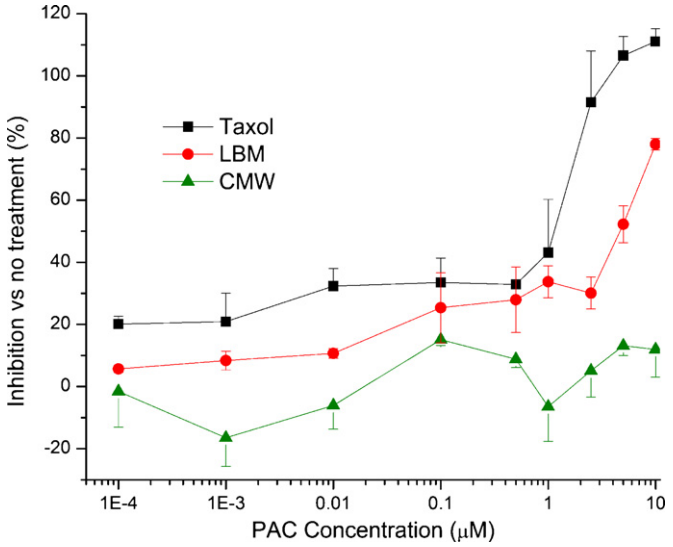

Fig. 4. Percent inhibition versus concentration of PAC after incubation for 48 h. Cells were incubated at 37 °C with 0.01 nM to 10 μM PAC in Taxol®, LBMW and CMW. An MTS assay was used to determine cell viability.

there was continuous growth of the cells in the no treatment and CMW groups. The cytotoxic effect of CMW only becomes obvious at incubation periods longer than 20 h. The cytotoxic effect of PAC in all treatment groups appears to reach a plateau after 24 h as a further increase in incubation duration did not lead to a corresponding decrease in cell number. The magnitude of cytotoxicity was the highest with Taxol® and lowest with CMW.

The percents of inhibition (against no treatment) of the cell growth by the various formulations were plotted versus the logarithm of the PAC concentrations in the formulations after 2, 18, 24 (data not shown) and 48 h of incubation (Fig. 4). The dose–response curves for Taxol® and LBMW were sigmoidal in shape. As the concentration of PAC increased the percent inhibition sharply increased until a maximum was reached, after which no further inhibition occurred with an increase in concentration of PAC (Fig. 4). At concentrations of 10^{-4}–1 μM of PAC the magnitude of inhibition was minimal; however, at concentrations greater than 1 μM there was a drastic increase in percent of inhibition for both the Taxol® and LBMW, but not for the CMW. The extent of inhibition was determined from the plateau of the dose–response curves and the IC_{50} was determined as the concentration that corresponded to 50% of the maximal response. The extent of inhibition and IC_{50} values for each of the three formulations after 2, 18, 24 and 48 h of incubation are summarized in Table 3. The extent of inhibition of the cells by PAC in Taxol® increased from 67.96 to 111.11% with an increase in incubation duration from 2 to 48 h; however, the IC_{50} of PAC did not change significantly (1.5–1.3 μM). The extent of inhibition for LBMW increased from 60.99 to 78.0% from 18 to 48 h of incubation. There was an initial growth at 2 h. The IC_{50} values did not significantly change with an increase in incubation duration from 18 to 48 h (5.7 to 4.5 μM). The extent of inhibition for CMW did not follow any specific trend and the IC_{50} values were greater than 10 μM for all incubation durations. These results indicated that PAC retained its cytotoxic activity in the PAC microemulsions as evidenced by the decrease in cell number when treated with these formulations. However, to observe a magnitude of effect comparable to that of Taxol®, a three-fold and greater than six-fold increase in concentration of PAC will be needed for LBMW and CMW, respectively.

3.5. Hemolytic potential of formulations

Surfactants are known to cause hemolysis of red blood cells. In order to determine that the formulation is safe for i.v. administration, the hemolytic potential of the formulations were evaluated using a modified method of Reed and Yalkowsky (1987). Drug-induced hemolysis in vitro is considered to be a simple and reliable estimation of membrane damage caused by drugs in vivo (Ogiso et al., 1978). This method takes into account the in vivo situation of an i.v. administered formulation especially by infusions, which are rapidly diluted by the blood. A wide range of formulation:blood ratios were tested in the assay, which is a more realistic model for i.v. formulations. The fraction of healthy cells decreased significantly as the formulation:blood ratio increased from 0.025 to 0.2 for all the formulations (Fig. 5). The LBMW PAC and formulation without PAC, exhibited a lower hemolytic potential at the volume ratios tested compared to Taxol®. The formulation:blood ratio at which 50% hemolysis (H_{50}) occurs was 0.085, 0.12 and 0.055 for Taxol®, LBMW and CMW containing PAC, respectively (Table 4). There was a difference in the hemolytic potential of the CMW formulation without PAC ($H_{50} = 0.07$) and CMW formulation containing PAC ($H_{50} = 0.055$), indicating that PAC

Table 3
Concentrations of PAC for inhibition (IC_{50}) and extent of inhibition with three formulations

Time (h)	Formulations					
	Taxol®		LBMW[a]		CMW	
	IC_{50}[b] (μM)	Extent (%)[c]	IC_{50} (μM)	Extent (%)	IC_{50} (μM)	Extent (%)
2	1.5	67.96	>10	−51.01	>10	17.41
18	1.8	94.93	5.7	60.99	>10	8.5
24	1.7	102.45	5.5	68.6	>10	−0.62
48	1.3	111.11	4.5	78.0	>10	11.96

[a] LBMW: lecithin:butanol:myvacet oil:water (20:10:68:2); CMW: capmul:myvacet oil:water (24:74:2).
[b] IC_{50}: Concentration of PAC at 50% of inhibition.
[c] Extent: The percent inhibition at 10 μM.

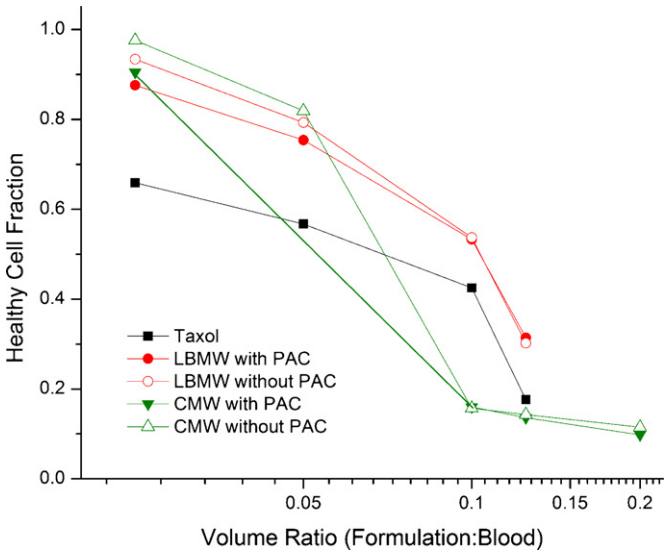

Fig. 5. Hemolytic potential of Taxol®, LBMW and CMW with and without PAC 12 mg/g. CMW: Capmul:myvacet oil:water (24:74:2); LBMW: lecithin:butanol:myvacet oil:water (20:10:68:2); Taxol®: cremophor:ethanol with 6 mg/ml PAC. Healthy cell fractions were calculated from the hemolysis standard curve.

contributes to the overall hemolytic potential of CMW. This difference was not apparent in the LBMW formulations. The results showed that the hemolytic potential was the lowest for the LBMW (with and without PAC); followed by Taxol® and lastly CMW. Comparable results in this study were reported in a study that showed the interaction between erythrocytes from various animals and emulsions stabilized with various lecithins. In rats' blood, the percentage of hemolysis induced by emulsions stabilized with soybean lecithin was 0.7% (Ishii et al., 1989). Based on the results of this study, the quantity of the formulation to be delivered to a rat during pharmacokinetic studies could be rationally selected. Blood volume in a 250 g rat is approximately 10 ml and the volume of formulation needed for effective dosing should not exceed 250 μl, resulting in a formulation:blood ratio of 0.02. Fig. 5 shows that 83, 68 and 63% of cells will remain unlysed at a volume ratio of 0.035 in LBMW, CMW and Taxol®.

Table 4
Hemolytic potential expressed as the fraction of healthy cells of two microemulsions CMW and LBMW compared to Taxol®

	H_{50}[b] (ratio)	Volume (μl)[c]
Taxol®	0.085	85
LBMW[a]		
Sham	0.12	120
PAC	0.12	120
CMW[a]		
Sham	0.07	70
PAC	0.055	55

[a] CMW: Capmul:myvacet oil:water (24:74:2); LBMW: lecithin:butanol:myvacet oil:water (20:10:68:2).

[b] H_{50}: Volume ratio at which 50% hemolysis occurs, interpolated from the graph (Fig. 5).

[c] Volume: The corresponding volume of formulation that will cause 50% hemolysis, calculated based on 10 ml of blood volume in a 250 g rat.

4. Summary and conclusion

In conclusion, promising cremophor-free microemulsions LBMW (lecithin:butanol:myvacet:water, 20:10:68:2) and CMW (capmul:myvacet:water, 24:74:2) were developed with droplet sizes in the 110 nm range, into which 12 mg/g of PAC could be incorporated with significantly enhanced solubility. The solubility of PAC was increased at least 1389-fold in the microemulsion over its aqueous solubility. These microemulsions utilized biocompatible surfactants such as lecithin and capmul that are used in parenteral emulsions. The presence of a surfactant in the formulation will aid in the even mixing of the formulation with the blood to facilitate the controlled and extended release of the drug without precipitation which is an advantage over a pure oil formulation. The cytotoxic activity of PAC was retained in PAC microemulsions as evidenced by the decrease in cell number when treated with these formulations. However, to observe a magnitude of effect in the cell culture comparable to that of Taxol®, a three-fold and greater than six-fold increase in concentration of PAC will be needed for LBMW and CMW, respectively. Insignificant hemolysis was observed at formulation to blood ratios less than 0.025 and the hemolytic potential of the LBMW formulation was lower than the commercially available Taxol®. At the volume ratio of 0.025, 87.6%, 72% and 61% of cells will remain unlysed with LBMW, CMW and Taxol®.

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