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Anticancer efficacy, tissue distribution and blood pharmacokinetics of surface modified nanocarrier containing melphalan

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ABSTRACT

The objectives of the present study were to circumvent the moisture-associated instability, enhance bioavailability and achieve enhanced passive targeting of melphalan to the ovaries. Solubility of the drug was determined in various excipients to select the components of nanoemulsion. Pseudoternary phase diagrams were constructed using aqueous titration method. Formulations selected from the pseudoternary phase diagram were subjected to thermodynamic stability and dispersibility studies to select the final test formulations which were characterized for average globule size, polydispersity index (PDI), zeta potential, viscosity, refractive index, *in-vitro* drug release and percentage transmittance to optimize the final formulation. Pharmacokinetic and biodistribution studies of the optimized formulation in comparison to the pure drug suspension were done using γ-scintigraphy on female Balb/c mice. *In-vitro* cytotoxicity study on Hela cervical cancer cell lines was also done to compare the anticancer activity of the developed formulation with respect to the pure drug solution. *In vitro–in vivo* correlation was established for the amount of drug released and the amount of drug absorbed using suitable deconvolution. Stability studies on the final formulation were performed at 40 ± 2 °C and 75 ± 5% RH for 3 months and the shelf life was determined. Capmul MCM, Tween 80 and Transcutol P® (S_{mix}) were selected as the oil, surfactant and co-surfactant respectively on the basis of solubility studies. Out of 17 formulations prepared, six formulations were selected as the final test formulations on the basis of thermodynamic stress and dispersibility tests. The optimized formulation composed of oil (10%, v/v), S_{mix} (35%, v/v), and double distilled water (55%, v/v). Bioavailability studies revealed 4.83 folds enhancement in bioavailability of the drug from nanoemulsion as compared to that from suspension. Biodistribution studies revealed more than 2 folds increase in uptake of the drug from nanoemulsion by ovaries as compared to that from the suspension. *In vitro* cytotoxicity studies demonstrated augmented anticancer potential of the drug in the form of nanoemulsion formulation in comparison to the drug solution. Level A correlation was established between the amount of drug released and the amount of drug absorbed. The shelf life of the formulation was found to be 1.30 years. The results demonstrate surface modified nanoemulsion to be a promising approach so as to increase stability, bioavailability and cellular uptake of the drug.

1. Introduction

Oral delivery of anticancer drugs offers certain advantages over the current regimen of chemotherapy by injection or infusion. It can provide a continuous and prolonged exposure of the anticancer drugs to cancer cells at a relatively lower and thus safer concentration. Despite these merits, oral delivery of anticancer drugs has been a challenge since most anticancer drugs have poor bioavailability either due to their poor aqueous solubility, stability, and/or permeability. Therefore, orally administered anticancer drugs have little chance to get into the blood system and thus reach the tumor site (Karsulu et al., 2007). Lipid based formulations have been reported to improve the bioavailability of poorly water-soluble drugs and reduce the variability in their absorption, which is beneficial for drugs with low therapeutic index (Hauss, 2007). Such formulations have been reported to increase the bioavailability of drugs by various mechanisms including, lymphatic transport of drugs which decreases the hepatic first pass effect (Humberstone and Charman, 1997), improvement of aqueous solubility (Hoeller et al., 2009), prevention of P-glycoprotein efflux (Cornaire et al., 2004), inhibition of pre-systemic degradation of drugs by enzymes (Wandel et al., 2003), and by increasing the gut permeability (Kang et al., 2004). Nanoemulsions are dispersions of oil and water in which the dispersed phase droplets are stabilized by a surface active film comprised of surfactant and co-surfactant molecules. They are transparent or translucent systems

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that have dispersed phase droplet size range of typically 20–500 nm (Wang et al., 2007). The terms sub-micron emulsion (SME) and miniemulsion are used as synonyms (Tadros et al., 2004). The physical stability of nanoemulsion can be significantly improved with the help of suitable emulsifiers that are capable of forming a mono- or multi-layer coating around the dispersed liquid droplets in such a way as to reduce interfacial tension or increase droplet–droplet repulsion (Tamilvanan, 2009). Nanoemulsions with smaller droplet size provide large surface area for fast and uniform absorption of drugs (Kang et al., 2004).

Melphalan is an anticancer drug used for the treatment of ovarian cancer, breast cancer and multiple myeloma. It is available commercially as a tablet and injection dosage form (Brightman et al., 1999). The tablet dosage form of melphalan suffers from limitations like: first pass hepatic metabolism to give monohydroxy- and dihydroxy-inactive metabolites; variable and incomplete absorption; reduction in bioavailability of drug on repeated dosing, problem of dose adjustment. Moreover, owing to hydrolysis of the drug in presence of moisture, tablets are instructed not to be crushed. It was hypothesized that formulating a nanoemulsion based drug delivery system would help to overcome these limitations since, nanoemulsions have been reported to act as a super-solvent vehicle capable of solubilizing both hydrophilic as well as lipophilic drugs. A nanoemulsion based system is capable of bye passing the hepatic metabolism up to some extent as it has been reported that nanosized particles can be absorbed directly into the blood through paracellular pathway and enter the blood circulation, thus demonstrating a systemic drug effect (Vyas and Khar, 2005). Nanoemulsions have also been reported to make plasma concentration time profile more reproducible (Kommuru et al., 2001). Moreover, formulating a nanoemulsion of melphalan would help to prevent contact of the drug with the moisture by solubilizing the drug in the lipophilic core of the nanoemulsion and thus, circumventing the moisture associated instability problem of the drug. Emulsion droplet surface modification using a surfactant with highly hydrophilic chain like polyoxyethylene (POE) is one of the techniques being investigated to augment the nanoemulsion half-life in blood circulation (Tamilvanan, 2009). Taking a cue from this, Tween 80 was employed as a surfactant in the present study to formulate a surface modified nanoemulsion. Furthermore, literature survey reveals that there are no research reports on nanoemulsion based system of melphalan and hence, the developed nanoemulsion is expected to be a potential carrier for the lipophilic antitumor agent melphalan so as to enhance its delivery to the tumors. It was also one of the objectives to evaluate shelf life of the developed nanoemulsion formulation.

2. Materials and methods

2.1. Materials

Melphalan was a gift sample from GlaxoSmithKline (Feucht, Germany). Medium chain triglycerides (Labrafac CC®), caprylocaproyl macrogol-8-glyceride (Labrasol®), polyglyceryl-6-dioleate (Plurol oleique®), diethylene glycol monoethyl ether (Transcutol P®), macrogol glycerol oleate (Labrafil M®), propylene glycol monolaurate type II (Lauroglycol 90), linoleoyl polyoxyglycerides (Labrafil M 2125 CS) and glyceryl monooleate (Peceol) were gift samples from Gattefosse (Saint Priest, Cedex, France). Medium chain mono- and diglyceride (Capmul MCM) and Propylene glycol dicaprate ester (Captex 100) were kindly gifted by Abitec Corporation (Janesville, USA). Double distilled water was obtained using Hicon water filter (New Delhi, India). All other reagents were of analytical grade and were used as received.

2.2. Selection of components for formulation of nanoemulsion

The selection of component was done on the basis of saturation solubility studies. Solubility of melphalan in each vehicle was determined by using shake flask method whereby excess amount of drug was added to 1 mL of each of the vehicle including oils (Capmul MCM, Captex 100, Labrafil M, Labrafac CC), surfactant (Tween 80) and co-surfactants (Transcutol P®, Labrafil M2125 CS, Peceol, Lauroglycol 90, Labrasol) and kept in an isothermal shaker (Hicon, New Delhi, India) at 25 ± 1°C for equilibration for 72 h. The equilibrated samples were then centrifuged (Remi Pvt. Ltd., Vasai, India) at 3000 rpm for 15 min, 10 μL of the sample was taken and diluted 10,000 times with methanol. It was then filtered through a 0.5 μm filter and the drug content was determined using HPLC at 261 nm. Oily solutions were suitably diluted with methanol before being injected into the HPLC.

2.3. Preparation of pseudoternary phase diagram

The pseudoternary phase diagrams of oil, surfactant: co-surfactant and water were developed using water titration method. The mixtures of oil and surfactant/co-surfactant at certain volume ratios were diluted with water in a drop wise manner. For each phase diagram at a specific ratio of surfactant/co-surfactant 1:0, 1:1, 1:2, 1:3, 2:1, 3:1, and 4:1 (v/v), transparent and homogeneous mixture of oil and S_{mix} was formed under mixing by Vortex mixer (Hicon, New Delhi, India). Then each mixture was titrated with double distilled water and visually observed for phase clarity and flow ability. Sixteen different combinations of oil and S_{mix}, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 1:2, 1:3, 1:3.5, 1:5, 1:6, 1:7, and 1:8 were made so that maximum ratios were covered for the study to demarcate the boundaries of phases precisely formed in the phase diagrams.

2.4. Selection of formulations from phase diagrams

Different formulations were selected from the region of nanoemulsion in each phase diagram so that the drug could be incorporated into the oil phase on the following basis:

(a) The oil concentration should be such that it solubilizes the drug (single dose) completely depending on the solubility of the drug in the oil.
(b) There should be no effect of drug on the phase behavior and nanoemulsion area of the phase diagram.
(c) The minimum concentration of the S_{mix} used for that amount of oil was taken.

2.5. Selection of final test formulations

A total of seventeen (N1–N17) formulations were developed and were subjected to thermodynamic stability and dispersibility studies for the selection of final test formulations.

2.5.1. Thermodynamic stability studies

2.5.1.1. Centrifugation. Each of the developed nanoemulsion was centrifuged at 3500 rpm for 30 min. The nanoemulsion was then observed visually for creaming, cracking or phase separation.

2.5.1.2. Heating cooling cycle. Six cycles involving storage of nanoemulsion between temperature of 4 °C and 45 °C for not less than 48 h were performed.

2.5.1.3. Freeze thaw cycle. Three cycles of storage between −21 °C and +25 °C for not less than 48 h were performed.

2.5.2. Dispersibility test

One milliliter of each nanoemulsion was added to 500 mL of double distilled water maintained at 37 ± 1 °C, in USP XXII dissolution apparatus II (Grover Enterprises, Delhi, India) (Pouton, 1997; Khoo et al., 1998).

At each step, the formulations were rejected on the basis of visual assessment and the passed formulations were subjected to the next test. The efficiency of formulations for self-emulsification was assessed by dispersibility test on the basis of following grading system:

Grade A: Rapidly forming (within 1 min) nanoemulsion, having a clear bluish appearance.
Grade B: Rapidly forming, slightly less clear emulsion, having bluish white appearance.
Grade C: Fine milky white emulsion formed within 2 min.
Grade D: Dull, grayish white emulsion having slightly oily appearance that is slow to emulsify (longer than 2 min).
Grade E: Formulation, exhibiting either poor or minimal emulsification with large oil globules present on the surface.

Formulations that passed the above tests were selected as final test formulations for drug loading and further studies.

2.6. Preparation of drug loaded nanoemulsion

Two milligrams was chosen as the dose of melphalan as the recommended minimum oral dose of melphalan is 2 mg/day (Tripathi, 2003). The selected test formulations were produced as drug loaded nanoemulsions by dissolving melphalan in Capmul MCM (4 mg/mL). Then S_{mix} was added. Double distilled water was eventually added drop-wise in pre-calculated volume and shaken on a vortex shaker until clear, transparent nanoemulsions were formed. Each of the developed nanoemulsion was then subjected to characterization in terms of globule size, zeta potential, polydispersity index, viscosity, surface morphology and *in-vitro* drug release.

2.7. Characterization of nanoemulsion

2.7.1. Globule size, zeta potential and polydispersity index (PDI) determination

The nanoemulsion (0.1 mL) was diluted to 100 mL with double distilled water and gently mixed for the determination of globule size, zeta potential and PDI. All the three parameters of nanoemulsion were determined using Zetasizer Ver. 6.01 (Malvern Instruments, UK).

2.7.2. Viscosity

Viscosity of the formulations was determined without dilution at 25 ± 0.5 °C by Brookfield Viscometer DV-II$^+$ PRO (Brookfield Engineering Laboratories, Inc., MA), using S-93 spindle at 100 rpm. Refractive index of the formulations was determined using Abbe type refractometer. The nanoemulsions were diluted 10 times with distilled water and percentage transmittance was checked against distilled water at 630 nm using UV–visible spectrometer (Shimadzu, Tokyo, Japan).

2.7.3. Surface morphology and structure

Morphology of the oil droplets in the nanoemulsion formulations was visualized with a Transmission Electron Microscope [TEM] (Hitachi, H-7500, Tokyo, Japan), operated at 80 kV and at 50,000 times magnification. The TEM analysis was also performed to visualize any precipitation of the drug upon addition of the aqueous phase. A drop of nanoemulsion after suitable dilution was allowed to deposit directly on the microscope gold coated grid and observed after drying.

2.8. In vitro drug release

In vitro release of melphalan from nanoemulsion was performed in triplicate and compared with pure melphalan suspension. Suspension used for comparison in the present *in vitro* drug release experiment was prepared using 0.5% carboxy methyl cellulose as the suspending agent. The study was performed in 500 mL of phosphate buffer (pH 7.4) using USP XXIV dissolution apparatus #2, at 50 rpm. The release media was maintained at 37 ± 0.5 °C (Kang et al., 2004). Five milliliters of nanoemulsion formulation was placed in dialysis bag (MWCO 12–14 kD, Himedia, Mumbai, India). Five milliliters of sample was withdrawn at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h and replaced with same aliquots of phosphate buffer (pH 7.4). The samples were then analyzed for the drug content using HPLC at 261 nm. *In vitro* release data was analyzed using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

2.9. HPLC analysis of melphalan

The system consisted of CECIL-CE 4201 HPLC coupled with a UV detector (Shimadzu, Tokyo, Japan). The chromatographic column was a Microsorb MV-100.5, C_{18} (250 mm × 4.6 mm) with 10 μm particle size. The mobile phase consisted of methanol, purified water and acetic acid (49.5:49.5:1, v/v) which was run at a flow rate of 2 mL/min and the run time was 10 min. The eluents were analyzed at 261 nm (Pinguet et al., 1996). The calibration curve was found to be linear in the range of 5–25 μg/mL. r^2 value was found to be 0.9998.

2.10. Pharmacokinetic and biodistribution studies

All animal experiments were carried out according to the principles of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Chennai, India. The protocol for the animal studies was approved by the Institutional Animal Ethical Committee (Ref No-IAEC/RAP/2908/2010) of Rajiv Academy for Pharmacy, Mathura, India. Balb/C mice weighing between 25 and 30 g were obtained from the Animal Experimental Center of Institute of Nuclear Medicines and Allied Sciences (INMAS), Delhi, India and housed at temperature of 22 ± 2 °C and 45–50% relative humidity. All the animals were divided randomly into two groups comprising seven animals in each group and fasted overnight but were allowed free access to water *ad libitum*. Melphalan formulations (nanoemulsion and drug suspension) were labeled with reduced ^{99m}Tc (Kumar et al., 2008). The reduction was carried out with stannous chloride ($SnCl_2$) as the reducing agent. Briefly, 0.5 mL of formulation was mixed with 1.5 mCi of reduced technetium and incubated for 5 min at room temperature and labeling yield was measured by ascending (silica gel as the stationary and glacial acetic acid as the mobile phase) paper chromatography. Each mice in one group received (100 μL, 2 mCi) melphalan suspension (MS) and in another group received (100 μL, 2 mCi) melphalan nanoemulsion (MNE) orally. For pharmacokinetic evaluation, the mice were anesthetized using chloroform at 0 (predose), 0.5, 1, 2, 4, 6, 12, 18 and 24 h post administration and blood was collected via cardiac puncture. For biodistribution study, mice were sacrificed at 1, 2, 4 and 24 h and different organs (heart, lungs, liver, spleen, kidneys, stomach, intestine and ovaries) were removed. All the samples (blood and organs) were analyzed for radioactivity by gamma ray counter (Capintech, CAPRAC-R, Columbia, USA). Radioactivity in each sample was reported as counts per minute (cpm) and % uptake per gram of organ weight

was calculated by using the following formula (Eq. (1))

$$\%\ \text{uptake/gram blood or organ weight} = \left\{ \frac{[\text{counts in cpm/weight in g}]}{\text{total counts administered orally (cpm)}} \times 100 \right\} \quad (1)$$

Various pharmacokinetic parameters were calculated with the help of pharmacokinetic program Quickcal software (developed by Dr. Shivprakash, Plexus, Ahmadabad, India). Relative bioavailability of nanoemulsion was calculated using the following formula (Eq. (2)):

$$F_r(\%) = \frac{\text{AUC}_{\text{MNE}}(\text{in \% A/g} \times \text{h})}{\text{AUC}_{\text{MS}}(\text{in \% A/g} \times \text{h})} \times 100 \quad (2)$$

where F_r is the relative bioavailability, AUC is the area under the % radioactivity per gram of tissue/blood-time curve.

3. *In vitro–in vivo* correlation (IVIVC)

To establish IVIVC for nanoemulsion (F6), its *in vitro* drug release curve was compared to its *in vivo* input performance *i.e.*, the curve produced by suitable deconvolution of the plasma level data using mass balance model-independent technique (USP, 2004).

4. *In vitro* cytotoxicity studies

4.1. Cell cultures used

HeLa (human cervical cancer) cell lines were obtained from the National center for cell sciences, Pune, India. HeLa cells were grown in Earl's minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, penicillin (100 μg/mL), streptomycin (100 μg/mL) and amphotericin B (5 μg/mL). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and subcultured twice a week.

4.2. Cytotoxicity assay involving determination of total cell protein content by sulforhodamine B (SRB) assay

SRB is a dark pink amino xanthene dye with sulfonic groups. Under mild conditions, SRB binds to protein basic amino acid residues of protein in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude. Color development in SRB assay is rapid, stable and visible. The developed color can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96-well plate reader. When TCA-fixed, SRB stained samples are air-dried, they can be stored indefinitely without deterioration. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/mL using medium (MEM) containing 10% new born calf serum. To each well of the 96-well microtitre plate, 0.1 mL of diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and the monolayer was washed once. Hundred microliters of the medium and different drug concentrations were added to the culture in microtitre plates. The plates were then incubated at 37 °C for 3 days in 5% CO_2 atmosphere. Microscopic examination was carried out and observations were recorded every 24 h. After 72 h, 25 μL of 50% TCA was added to the wells gently such that it formed a thin layer over the drug dilution to form an over all concentration of 10%. The plates were incubated at 4 °C for 1 h. The culture plates were flicked and washed five times with water to remove traces of medium, drug and serum, and were then air-dried. The air-dried plates were stained with SRB for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. Hundred microliters of 10 mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula (Eq. (3)):

$$\%\ \text{growth inhibition} = 100 - \left\{ \left(\frac{\text{mean OD of individual test group}}{\text{mean OD of control group}} \right) \times 100 \right\} \quad (3)$$

The results were reported in terms of CTC_{50} (cytotoxic concentration for 50% cells).

4.3. Preparation of samples

Ten milligrams of drug was accurately weighed and dissolved in 1 mL of dimethyl sulfoxide. Volume was made up to 10 mL by adding double distilled water so as to achieve a final concentration of 1000 μg/mL. Similarly, 1 mL of nanoemulsion was diluted to 10 mL with double distilled water.

5. Stability studies

Stability studies as per ICH Q1A (R2) guidelines were performed on the formulation F6 for three months. The formulation was kept at temperature of 40 ± 2 °C and 75 ± 5% RH. Samples were withdrawn at the end of 0, 30, 60, and 90 days. The samples were then checked for any change in refractive index, viscosity, droplet size and remaining drug content using HPLC at 261 nm. The results obtained were presented in the form of plot between percentage label claim (% drug remaining) *versus* time in months to determine shelf life. Shelf life was determined as the time at which the 95% one-sided confidence limit for the mean curve intersected the acceptance criterion of 90% percentage label claim. The data was evaluated using Sigmaplot™ software (Cranes Software International, Bangalore, India).

6. Results and discussion

6.1. Selection of components

Important criteria for selection of the components were their pharmaceutical acceptability and low toxicity. Safety is major determining factor with regards to surfactants as large amount of surfactants have been reported to cause gastrointestinal irritation. Non-ionic surfactants are less toxic than ionic surfactants. An important criterion for selection of surfactants is that the required HLB value to form an oil-in-water submicron emulsion should be greater than 10. The right blends of low and high HLB surfactants lead the formation of a stable submicron emulsion formulation (Kommuru et al., 2001). In the present study Tween 80 was selected as the surfactant having HLB value of 15. Tween 80 is 'Generally Recognized As Safe' (GRAS) listed, has a very low toxic potential, and is recommended safe for use in oral and parenteral drug delivery by WHO with acceptable daily intake of 25 mg/kg body weight of adult human. Negative interfacial tension and fluid interfacial film required for achieving curved surfaces is rarely achieved by using single surfactant, so the use of a co-surfactant becomes necessary. The co-surfactant decreases the bending stress at interface and provides the interfacial film sufficient flexibility to take up different curvatures required for the formation of nanoemulsion over a wide range of composition (Philip and Pathak, 2008). Transcutol P® with HLB value of 4.2 was selected as the co-surfactant in

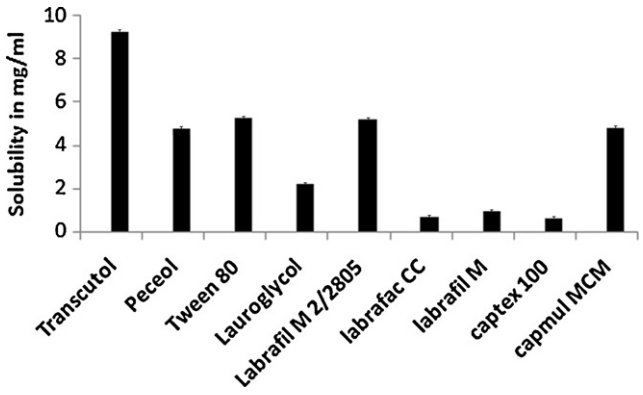

Fig. 1. Solubility of melphalan in different vehicles.

the present study. It is also a non-ionic surfactant and reported to increase the permeability of drugs (Lawrence and Rees, 2000). The HLB value of the system when calculated was found to be 12.3.

6.2. Screening of components

The most important criterion for the screening of components was the solubility of the poorly water-soluble drug in oil, surfactant and co-surfactants. The high solubility of drug in oil is important because more is the solubility of drug in oil, lesser is the amount of oil used for the formulation of nanoemulsion. Thus, the amount of surfactant and co-surfactant required for emulsification of droplets would also be less. Since high amount of surfactants are known to be toxic, there are less chances of toxic effects being caused by the surfactant and co-surfactant on selection of an oil providing the maximum solubility of drug in it. The solubility of drug in various vehicles was determined by using shake flask method. The solubility data of melphalan in different vehicles is shown in Fig. 1. The solubility of melphalan was found to be maximum in Capmul MCM (4.8 ± 1.02 mg/mL) among the oils. Solubility in Tween 80 as the surfactant was found to be 5.27 ± 1.14 mg/mL. The solubility of melphalan was found to be maximum in Transcutol P® (9.22 ± 1.72 mg/mL) among the co-surfactants. Hence, Capmul MCM, Tween 80 and Transcutol P® were selected as oil, surfactant and co-surfactant respectively for the development of the nanoemulsion formulation.

6.3. Construction of pseudoternary phase diagram

Phase diagram was constructed with the objective of studying the relationship between the phase behavior and the composition of the components (Bali et al., 2011). Pseudoternary phase diagrams also help to find out the concentration range of components for the formation of nanoemulsion. Pseudoternary phase diagrams were constructed for various S_{mix} ratios (Fig. 2), so as to identify the o/w nanoemulsion region for optimization of nanoemulsion formulations. In Fig. 2(a) [S_{mix} ratio 1:0] it was observed that when Tween 80 was used alone, 45.45% (v/v) of oil could be emulsified and the zone of nanoemulsion was quiet considerable. As Transcutol P® was introduced in the ratio 1:1 [Fig. 2(b)], in S_{mix}, there was an increase in the zone of nanoemulsion in comparison to that of Tween 80 alone. This could be attributed to the presence of co-surfactant which along with surfactant helped in reducing the interfacial energy as well as provided a mechanical barrier to coalescence. This eventually led to a decrease in the free energy required for the formation of nanoemulsion (Shafiq et al., 2007). When the ratio of co-surfactant was further increased to 1:2 [Fig. 2(c)], in S_{mix}, the zone of nanoemulsion was further increased and the maximum amount of oil that could be emulsified was found to be 27.27% (v/v). As the proportion of co-surfactant in S_{mix} was further increased to 1:3 [Fig. 2(d)], there was a decrease in the region of nanoemulsion and the least amount of oil (27.27%, v/v) could be emulsified. On increasing the proportion of Tween 80 in the S_{mix} to 2:1 [Fig. 2(e)], the zone of nanoemulsion was found to be similar to that observed with the ratio of 1:1 and the maximum amount of oil that could be emulsified was found to be (81.08%, v/v).When Tween 80 was further increased in the S_{mix} to 3:1 [Fig. 2(f)], the zone of nanoemulsion was highly increased. When the Tween 80 was further increased to S_{mix} with ratio 4:1 [Fig. 2(g)], the zone of nanoemulsion was slightly decreased but was still comparable to that of 3:1. Since the zone of nanoemulsion formation was found to be maximum in the S_{mix} ratio 3:1 followed by 4:1, both the ratios were chosen for the formation of nanoemulsion formulations.

6.4. Selection of formulations from phase diagrams

It is essential to use minimum amount of surfactant in the nanoemulsion because it is reported that large amount of surfactants cause GI irritation (Lawrence and Rees, 2000; Bali et al., 2010), so the formulations which consumed minimum amount of surfactant for a particular concentration of oil in nanoemulsion were selected from the phase diagrams. Among all the pseudoternary phase diagrams the S_{mix} (Tween 80: Transcutol P®) ratio of 3:1 gave the maximum region of nanoemulsion followed by S_{mix} ratio of 4:1in which the nanoemulsion region was comparable to that of S_{mix} ratio 3:1. Hence, both of the S_{mix} ratios were chosen for the selection of nanoemulsion formulation. In all, 17 formulations were selected taking care to keep the proportion of oil as low as possible so as to keep the corresponding proportions of surfactants required for the emulsification of oil also low owing to the toxicity of surfactants. No effect was observed in the phase behavior and nanoemulsion area in the phase diagrams when melphalan was loaded in the formulations. This could be the reason that containing non-ionic surfactant formation and stability of nanoemulsion has been reported to be unaffected by the pH or ionic strength changes (Ping et al., 2005).

6.5. Selection of final test formulations

All the developed formulations were subjected to Thermodynamic stress tests followed by dispersibility test. Thermodynamic stress tests were conducted to ascertain thermodynamic stability of the selected formulations where by effect of high shear and sharp variations in temperatures on the behavior of nanoemulsions were studied. The results of thermodynamic stress test are given in Table 1. All the formulations were subjected to dispersibility test for the assessment of self-emulsification ability. When the formulation undergoes infinite dilution in gastrointestinal fluids, it is likely that the drug may precipitate owing to the poor aqueous solubility of the drug to rule out the possibility of precipitation of drug *in-vivo*, dispersibility studies were paramount. Eventually, six formulations were selected for characterization after thermodynamic stress test and dispersibility studies. The formulation N2 was renamed as F1, N4 as F2, N7 as F3, N8 as F4, N16 as F5 and N17 as F6.

6.6. Characterization of nanoemulsion

6.6.1. Globule size analysis, PDI and zeta potential

It has been reported that droplet size have an effect on drug absorption, smaller is the size of droplets larger is the interfacial surface area available for the drug absorption (Kang et al., 2004). Polydispersity index measurement is the parameter which shows the uniformity in the globule size distribution. The minimum is the PDI the maximum is the uniformity in the globule size distribution. Nanoemulsion droplets posses some repulsive charge on their

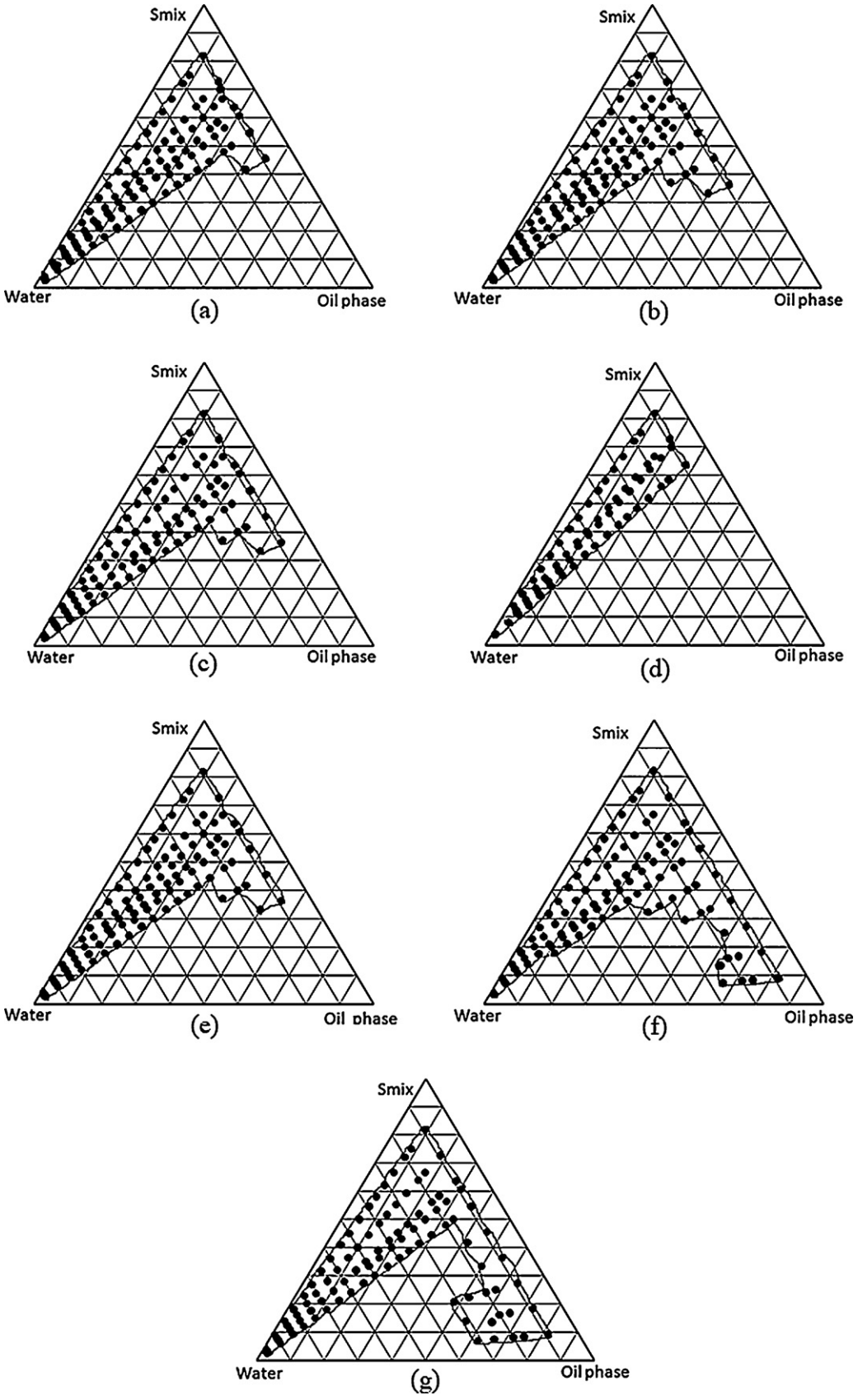

Fig. 2. Pseudoternary phase diagrams of system containing the following components: Capmul MCM as oil, Tween 80 as surfactant, Transcutol® P as co-surfactant. Ratio of surfactant to co-surfactant in (a) is 1:0, (b) is 1:1, (c) is 1:2, (d) is 1:3, (e) is 2:1, (f) is 3:1, (g) is 4:1. Dotted area shows oil in water nanoemulsion region.

Table 1
Observation table for thermodynamic stability studies and dispersibility studies.

Formulation code	S_{mix} ratio	Percentage (v/v) of formulation components			Observation of thermodynamic stability studies and dispersibility studies				Results
		Oil	S_{mix}	Water	Cent.[a]	HCC[b]	FTC[c]	DT[d]	
N1	4:1	5	45	50	√	×	–	Grade C	Failed
N2	4:1	10	35	55	√	√	√	Grade A	Passed
N3	4:1	5	20	75	√	×	–	Grade D	Failed
N4	4:1	10	25	65	√	√	√	Grade B	Passed
N5	4:1	10	20	70	×	–	–	Grade D	Failed
N6	4:1	5	10	85	×	–	–	Grade E	Failed
N7	4:1	10	30	60	√	√	√	Grade A	Passed
N8	4:1	10	35	55	√	√	√	Grade A	Passed
N9	4:1	10	50	40	√	√	×	Grade D	Failed
N10	4:1	5	25	70	√	√	√	Grade C	Failed
N11	4:1	5	30	65	√	√	×	Grade C	Failed
N12	4:1	5	40	55	√	√	×	Grade C	Failed
N13	3:1	10	15	75	×	–	–	Grade E	Failed
N14	3:1	10	10	80	√	√	√	Grade D	Failed
N15	3:1	10	20	70	√	√	√	Grade C	Failed
N16	3:1	10	30	60	√	√	√	Grade B	Passed
N17	3:1	10	35	55	√	√	√	Grade A	Passed

√ Indicates the formulation passed the test.
× Indicates the formulation failed the test.
[a] Cent. = centrifugation cycle.
[b] HCC = heating cooling cycle.
[c] FTC = freeze thaw cycle.
[d] DT = dispersibility test.

surface termed as Zeta potential which ensures the stability of nanoemulsion. For small molecules the stability is considered to be high if the zeta potential value is high, whereby the droplets will oppose the aggregation. The charge range of −30 to +30 mV is reported typically as high zeta potential. The negative sign of zeta potential indicated the formulations were negatively charged, and the magnitude of charge showed the stability of the formulation. The presence of high negative charge on the nanoemulsion formulations could be because of the anionic groups of the fatty acids and glycols present in the oil, surfactant and co-surfactant. Thus, there are least chances of flocculation or coagulation of the system in the biological environment and also during its shelf life (Bali et al., 2011). The results show that the average globule size of formulations F2 (89 ± 7.46), F3 (48.64 ± 12.24) and F6 (97.69 ± 10.24) are comparable. Among these three formulations F3 showed minimum globule size and there is significant difference in the average globule size of nanoemulsion formulation F6 and F3 ($p < 0.01$), and no significant difference in the average globule size of F2 and F6 ($p > 0.05$), but the PDI of formulation F6 is minimum, which indicates the uniformity in globule size distribution, there is significant difference ($p < 0.01$), in the PDI value of F6 and other formulations, however, the PDI values of F6 and F5 are comparable ($p > 0.05$), but the zeta potential that indicates the stability of formulation is lower for F5, (−12.70 ± 1.80) as compared to the zeta potential (−23.60 ± 2.46 mV) of formulation F6 so, the formulation was found to be stable and F5 is not as stable as F6. The zeta potential of formulation F2 (−17.50 ± 2.24) follows the zeta potential of F6, followed by F3, so, on the basis of average globule size, PDI and zeta potential formulation F2, F3 and F6 were found to be promising. Table 2 shows the globule size distribution, zeta potential and PDI, of the final six formulations.

6.6.2. Viscosity, refractive index and percentage transmittance

It is not easy to administer a highly viscous liquid formulation orally to a cancer patient since the highly viscous nature of the formulation may limit its clinical application due to inconvenience in use (Ghosh and Murthy, 2006). For oral administration the viscosity of the formulation should be minimum so as to facilitate its ease of intake. So, the viscosity of all the final test formulations was determined. The formulation F5 (150 ± 0.2 cps) showed minimum viscosity followed by F6 (210 ± 0.2 cps). The viscosity of other formulations were significantly higher ($p < 0.01$), except that of F5 which is lower than that of F6 but F6 was found to be promising than F5 with respect to other parameters. The refractive index of formulation ensures its homogeneous character thus, the nanoemulsion formulation could be easily administered as a uniform dose. Also the percentage transmittance reflects the clarity and visual appearance which increases the acceptability of formulation. The refractive index of formulation F3 (1.461 ± 0.002) was maximum followed by F6 (1.399 ± 0.004) and the percentage transmittance through formulation F6 (99.2 ± 0.42) was observed to be maximum.

6.6.3. Surface morphology

Surface morphology of nanoemulsion formulation F6 by TEM analysis is shown in Fig. 3. Nanoemulsion globules appeared as

Table 2
Mean (±S.D., $n = 3$) value of globule size, PDI, viscosity, drug content, refractive index, percentage transmittance, and zeta potential.

Formulation	Average globule size ± S.D. (nm)	Polydispersity index ± S.D.	Viscosity ± S.D. (cps)	Drug content ± S.D. (%)	R.I. ± S.D.[a]	%Transmittance ± S.D.	Zeta potential ± S.D. (mV)
F1	140.50 ± 6.24	0.419 ± 0.012	261 ± 0.8	95.00 ± 1.24	1.391 ± 0.004	97.6 ± 0.09	−10.17 ± 1.16
F2	89.31 ± 7.46	0.413 ± 0.032	300 ± 0.9	97.50 ± 2.06	1.397 ± 0.003	98.8 ± 0.05	−17.50 ± 2.24
F3	48.64 ± 12.24	0.399 ± 0.022	360 ± 0.0	93.00 ± 2.68	1.461 ± 0.002	98.2 ± 0.10	−15.20 ± 2.86
F4	195.20 ± 16.28	0.531 ± 0.014	240 ± 1.1	94.00 ± 1.84	1.383 ± 0.003	88.2 ± 0.38	−10.80 ± 3.22
F5	137.90 ± 22.46	0.274 ± 0.011	150 ± 0.2	95.05 ± 2.36	1.390 ± 0.005	96.7 ± 0.04	−12.70 ± 1.80
F6	97.69 ± 10.24	0.263 ± 0.020	210 ± 0.2	98.00 ± 1.38	1.399 ± 0.004	99.2 ± 0.42	−23.60 ± 2.46

[a] R.I. is refractive index.

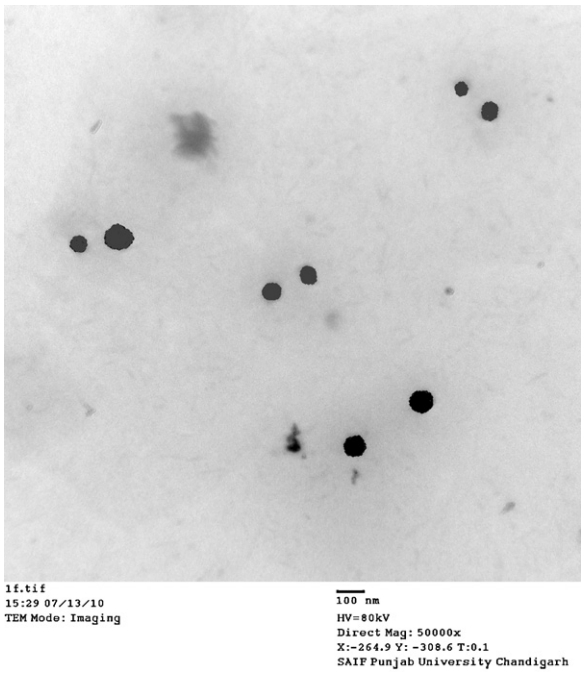

Fig. 3. Transmission electron microscopic positive image of melphalan nanoemulsion consisting of Capmul MCM, Tween 80 and Transcutol.

dark spots against a light background and were spherical in shape. Droplet sizes measured using TEM was found to be in conformity with the results obtained using zeta sizer.

6.7. In vitro drug release

The *in vitro* release behavior of melphalan from nanoemulsion formulations (F1–F6) and pure drug suspension (F7) is shown in Fig. 4. It was observed that the release of melphalan showed a controlled release pattern in all the formulations. Formulation F6 showed the maximum drug release (98.8%) followed by F2 showing release of 82% in 12 h as compared to formulation F7 (pure drug suspension) showing a release of 45.94%. The reason describing this may be, the composition of both formulations was similar except the amount of Tween 80 was higher in F2. Since, Tween 80 is highly viscous than other components used in the current study for the preparation of nanoemulsion, increased amount of Tween 80 in F2 might be responsible for higher viscosity of nanoemulsion F2 in comparison to F6. Higher viscosity of F2 might be one of the contributing factors toward slower rate of drug release from nanoemulsion F2 than that from nanoemulsion F6. Furthermore, polydispersity index of F6 was found to be lower than that of F2

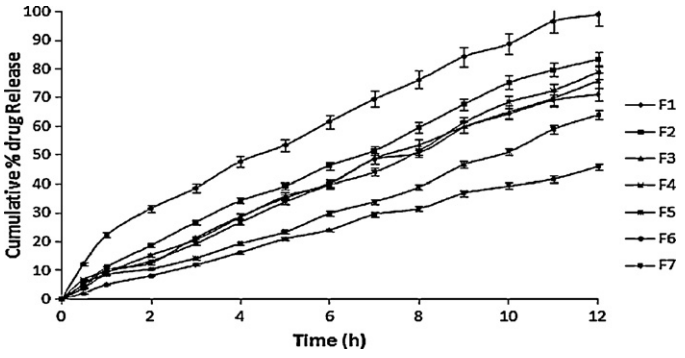

Fig. 4. Dissolution profile of melphalan (Mean percent release ± S.D., $n = 3$) from nanoemulsion formulations F1–F6 and pure drug suspension (F7).

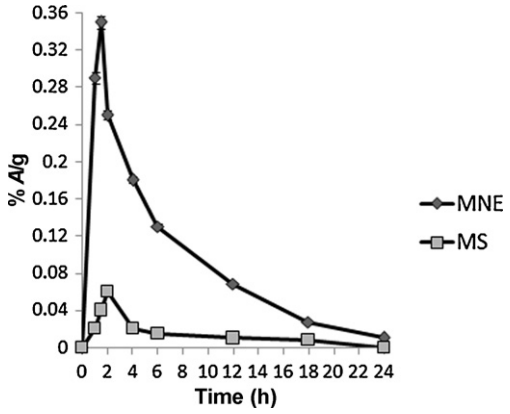

Fig. 5. % *A*/g of blood *versus* time profile of melphalan nanoemulsion (F6-MNE) and melphalan suspension (MS).

ensuring uniformity in globule sizes and hence, surface area for drug release in F6 was more as compared to that of nanoemulsion F2. On applying one-way ANOVA followed by Dunnett's test it was found that significant difference was observed in the release profile of F2 ($p < 0.05$), F6 ($p < 0.01$) as compared to F7 (pure drug suspension).

6.8. Pharmacokinetic study

The blood % *A*/g–time plot, obtained after oral administration of melphalan nanoemulsion and suspension are shown in Fig. 5. When the logarithm of % *A*/g values (from peak values onwards) was plotted against time (plot not shown here), a biphasic curve was obtained. This suggested that melphalan follows two compartment open model which was also in agreement with the literature reports stating that melphalan follows two compartment open model. So, two compartment open model was used to analyze various pharmacokinetic parameters obtained after administration of melphalan formulation (Mou et al., 2008; Woodhouse et al., 2007). The C_{max} of melphalan nanoemulsion (MNE) was found to be 0.35% *A*/g where as C_{max} of melphalan suspension (MS) was found to be 0.06% *A*/g (Table 3). C_{max} of MNE was found to be significantly increased ($p < 0.01$) in comparison to MS when analyzed by Student's '*t*' (two tailed-unpaired) test. In comparison to melphalan suspension, the nanoemulsion formulation displayed significantly ($p < 0.001$) reduced T_{max} indicating rapid absorption of drug from nanoemulsion. This is significant considering the fact that it would enable rapid onset of action and thus, the therapeutic level of drug in blood would be reached earlier. The reduction in T_{max} in case of nanoemulsion can be explained on the basis of the fact that the drug in nanoemulsion is present in the solubilized form. Hence, when it reaches to the gastrointestinal tract, the drug dissolution which is the major rate-limiting step in the absorption of a poorly water-soluble drug is absent. While, in case of suspension the drug is suspended in the form of fine particles and is yet to undergo dissolution in GI fluids to get absorbed. Moreover, in case of nanoemulsion, the nano sized globules demonstrate an enormously large surface area for the dissolution of the drug leading to rapid release of drug and subsequent absorption. Thus, the requirement of the drug to undergo dissolution in order to get absorbed from the dosage form and the surface area available for the release of drug from the dosage form could be the contributing factors toward the observed pattern of T_{max}. Volume of distribution of MS was found to be 14.7127 L which was significantly ($p < 0.0005$) reduced to 0.0084 L in MNE. The reduction in volume of distribution could be attributed to the nanometric size range of the developed MNE, leading to further weakened extravasation in non-target tissues. This would in

turn lead to reduction of toxic side effects (Tamilvanan, 2009). Thus, reduction in volume of distribution of drug through nanoemulsion would ensure better localization of the drug in the systemic circulation leading to attainment of higher plasma concentration with the same administered dose in comparison to a suspension. This, in turn, would help to reduce the dose of the drug required to achieve the therapeutic effect besides reduction in dose-associated side effects of the drug. The rate of transfer of melphalan from central compartment to peripheral compartment, K_{12} in case of nanoemulsion (0.0015 h^{-1}) was found to be lower as compared to that in case of suspension (0.0082 h^{-1}). On the contrary, the rate of transfer of drug from peripheral compartment to central compartment, K_{21} in case of nanoemulsion was found to be higher (0.3397 h^{-1}) than in case of suspension (0.0775 h^{-1}). This could be ascribed due to high affinity of melphalan toward the plasma proteins. It is reported in literature that melphalan is 60–90% plasma protein bound. The elimination half-life of drug from nanoemulsion was found to be 15.2316 h which was significantly ($p < 0.0005$) higher than that of 0.0419 h from suspension indicates that nanoemulsion is present in the body for much longer time than the melphalan suspension. The reason describing this could be the presence of polyoxyethylene surfactant (Tween 80) in the melphalan nanoemulsion. Tween

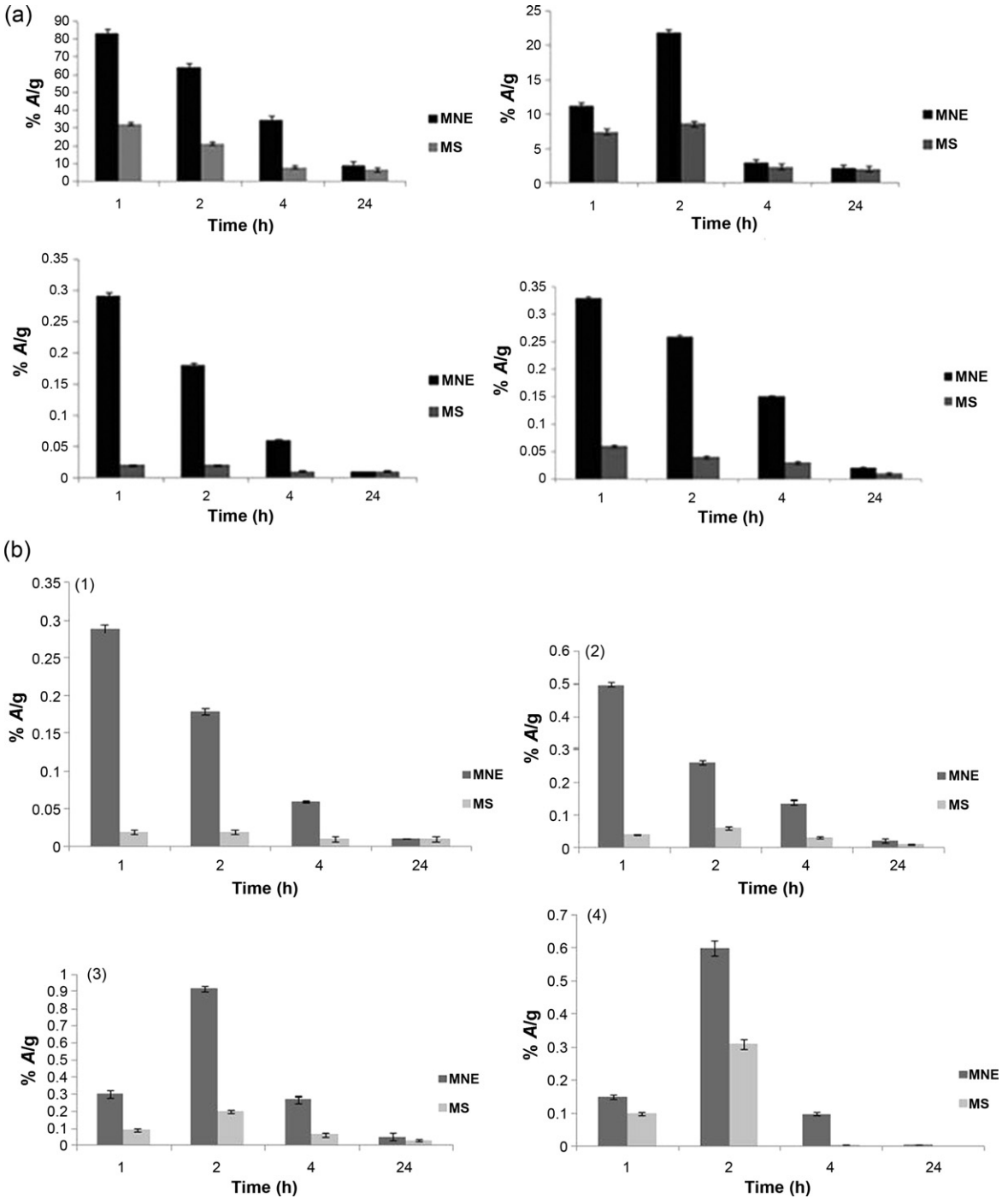

Fig. 6. (a) Biodistribution pattern of radiolabeled nanoemulsion formulation (F6-MNE) as compared to pure drug suspension (MS) in (1) stomach, (2) intestine, (3) heart, (4) lungs. (b). Biodistribution pattern of radiolabeled nanoemulsion formulation (F6-MNE) as compared to pure drug suspension (MS) in (1) liver, (2) spleen, (3) kidneys, (4) ovaries.

80 in the MNE might be responsible for surface modification of the nanoemulsion globules making them long circulating in the systemic circulation (Tamilvanan, 2009). The elimination rate constant of nanoemulsion (0.0455 h^{-1}) was observed to be lesser than that of suspension (16.5455 h^{-1}). This further supports longer residence time of nanoemulsion in the body than the drug suspension. The total clearance of MNE (0.1274 L/h) indicates that drug is cleared from the body at slower rate than that in case of MS (0.6162 L/h), this also supports the longer circulation time of MNE in the body as compared to MS. The area under the curve ($AUC_{(0\rightarrow\infty)}$) of nanoemulsion (3.13% *A* h/g) is much higher than that of suspension (0.64% *A* h/g) indicates 4.83 times enhancement in the bioavailability of the melphalan from nanoemulsion as compared to that from suspension. The relative bioavailability was found to be 483 as compared to that of suspension. The reduction in particle size, increase in solubility, surfactant induced permeability changes, prevention of P-glycoprotein efflux (Ghosh and Murthy, 2006) could be the reason for increase in absorption of drug and thus, bioavailability.

6.9. Biodistribution study

Biodistribution of ^{99m}Tc-labeled formulations (melphalan nanoemulsion and melphalan suspension) in heart, lungs, liver, spleen, kidneys, stomach, intestine and ovaries was studied. Fig. 6(a) and (b) shows the biodistribution pattern of melphalan in various organs. Uptake of melphalan from suspension and nanoemulsion was found to be different in different organs/tissues. It was found that free melphalan eliminated from the body much faster than the melphalan encapsulated in nanoemulsion formulation. The distribution of drug was observed to be significantly ($p < 0.01$) higher in stomach [Fig. 6(a)(1)] within 1 h of administration from both the formulations. This could be due to the reason that stomach is the first major organ of contact after oral administration. In the subsequent hours, the radioactivity in the stomach was found to decrease. This might be due to the short residence time of the suspension and nanoemulsion formulation in the stomach owing to the liquid nature of these formulations. After 24 h of administration, negligible amount of drug was found to be present in the stomach as indicated by the % *A*/g values. In case of intestine [Fig. 6(a)(2)], maximum activity was seen only after 2 h of administration of the formulations. This may be attributed to the time taken for transit of the formulation from stomach to intestine. However, % *A*/g values in intestine was found to be significantly higher ($p < 0.0005$) from nanoemulsion than that from the stomach indicating superior absorption of drug from nanoemulsion than that from drug suspension. This could be due to reduction in particle size, increase in solubility, surfactant induced permeability changes and prevention of P-glycoprotein efflux of drug in the form on nanoemulsion. Comparatively higher uptake of melphalan from nanoemulsion formulation was observed in heart [Fig. 6(a)(3)], lungs [Fig. 6(a)(4)], liver [Fig. 6(b)(1)], and spleen [Fig. 6(b)(2)] than that from suspension. This could be because of the high perfusion rate of these organs (Kang et al., 2004). No stealth effect can be provided with Tween 80 to achieve prolonged circulation in case of nanoemulsion because physical status of the nano-ranged emulsion globule itself is in question when administered in to blood. It may improve the bioavailability and consequently its deposition in the said tissues. The distribution pattern of melphalan formulations was found to be different in kidneys [Fig. 6(b)(3)] in comparison to other organs. The uptake was found to be maximum after 2 h. This could be attributed to the time taken by melphalan to undergo process of absorption, distribution and metabolism before eventually reaching kidneys for excretion. In case of ovaries [Fig. 6(b)(4)], activity/uptake per gram from nanoemulsion was observed to be significantly higher ($p < 0.0001$) than that from the suspension. Especially, at 2 h, the uptake per gram from nanoemulsion was almost 2 folds higher than that from melphalan suspension. Furthermore, after 4 h the uptake per gram was found to be much higher from nanoemulsion than suspension. This indicates that retention of melphalan from nanoemulsion in ovaries is for longer duration than that from suspension. Even after 24 h, some activity was still detectable in case of MNE but it was undetectable in case of drug suspension. Decrease in the ovarian uptake in subsequent hours was less from nanoemulsion in comparison to drug suspension which again indicated increased retention of the drug in the ovaries. A comparative analysis of uptake of melphalan from nanoemulsion in various organs at 2 h [Supplementary Fig.] reveals that with the exception of kidneys, % *A*/g value was found to be maximum (0.65% *A*/g) in case of ovaries. This observation can be explained on the basis of physiological nature of kidneys *i.e.* major organs of elimination. The biodistribution study of melphalan nanoemulsion in mice substantiates the observations made during pharmacokinetic study. Thus, formulation of melphalan into nanoemulsion leads to higher bioavailability and ovarian uptake of melphalan in comparison to the drug suspension.

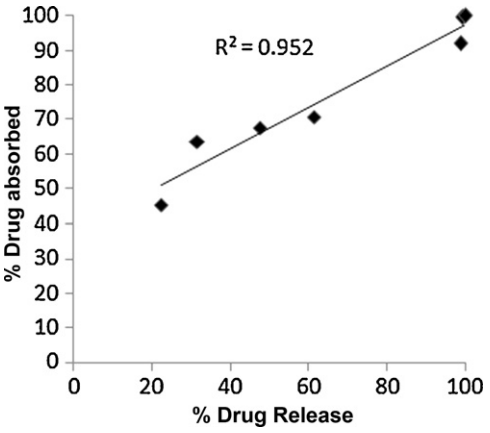

Fig. 7. *In vitro–in vivo* correlation of melphalan nanoemulsion (F6).

Supplementary material related to this article found, in the online version, at doi:10.1016/j.ijpharm.2012.01.027.

6.10. In vitro–in vivo correlation

The result of IVIVC study is shown in Fig. 7. The value of regression coefficient (r^2) was found to be 0.9526. There are four levels of correlation established by the USP Biopharmaceutics subcommittee for setting up the correlation between *in-vitro* dissolution and *in-vivo* absorption.

Table 3
Pharmacokinetic parameters obtained by two compartment open model analysis of the data obtained from pharmacokinetic study.

Parameters	Melphalan suspension	Melphalan nanoemulsion
C_{max} (% *A*/g × h)[a]	0.06	0.35
T_{max} (h)[b]	2	1.5
Volume of distribution (L)	14.7127	0.0084
Rate transfer constant (K_{12}) (h^{-1})	0.0082	0.0015
Rate transfer constant (K_{21}) (h^{-1})	0.0775	0.3397
Elimination half-life (h)	0.0419	15.2316
Elimination rate constant (K_E) (h^{-1})	16.5455	00.0455
Total clearance (L/h)	0.6162	0.1274
$AUC_{(0\rightarrow\infty)}$ (% *A*/g × h)	0.6491	3.1393
Relative bioavailability (%)	100	483

[a] C_{max} is the maximum concentration of drug in blood.
[b] T_{max} is the time at which the maximum concentration of drug is present in blood.

Table 4
Mean (±S.D., $n = 3$) value of refractive index, viscosity, droplet size and percentage drug remaining in nanoemulsion F6 stored at 40 ± 2 °C and 75 ± 5% RH.

S. No.	Time (days)	Mean refractive index ± S.D.	Mean viscosity ± S.D. (cps)	Mean droplet size ± S.D. (nm)	% Drug remaining
1	0	1.399 ± 0.003	210 ± 0.20	97.69 ± 10.24	100
2	30	1.401 ± 0.004	210 ± 0.30	97.82 ± 10.32	99.34
3	60	1.404 ± 0.004	211 ± 0.50	98.14 ± 10.46	98.92
4	90	1.410 ± 0.003	214 ± 0.40	98.38 ± 10.58	98.74

1. Level A: The *in-vitro* dissolution curve match with the *in-vivo* absorption curve in a point to point (1:1) manner and the two curves nearly or completely overlaps each other.
2. Level B: The *in-vitro* mean dissolution time (MDT), calculated by moment analysis, shows correlation with the *in-vivo* mean residence time (MRT).
3. Level C: Among $t_{50\%}$, $t_{90\%}$, etc. one of the dissolution parameters shows correlation with one of the pharmacokinetic parameters like AUC, C_{max}, T_{max}, etc.
4. Level D: The disintegration correlates qualitatively with the *in-vivo* behavior or the *in-vitro* data correlates qualitatively with the *in-vivo* data.

Level A correlation is point-to-point correlation and thus is the highest degree of correlation which is applicable to modified release systems than any other level of correlation. The *in-vitro* dissolution data was determined and compared with the *in-vivo* drug absorbed (Khoo et al., 1998). A value of $r^2 = 0.9526$ indicated linear relationship between the percent drug release and percent drug absorbed establishing a level A correlation between the two data, as shown in Fig. 7, where

$$\%\ \text{drug absorbed from melphalan nanoemulsion (F6)} = 0.9526 \times \%\ \text{drug dissolved.} \quad (4)$$

This signifies that *in-vivo* absorption of the drug from nanoemulsion can be predicted from *in vitro* dissolution data.

6.11. In vitro cytotoxicity study

The *in vitro* cytotoxicity study was done to compare the cytotoxic potential of developed formulation with respect to blank nanoemulsion and pure melphalan solution. The cytotoxic concentration (CTC_{50}) value corresponds to the concentration of drug at which 50% of cells are inhibited or killed. CTC_{50} value for the blank nanoemulsion was not measurable whereas the CTC_{50} value for melphalan loaded-nanoemulsion formulation was found to be 280 μg/mL, which was highly significant ($p < 0.0001$) as compared to the CTC_{50} value of 340 μg/mL for pure drug solution. Enhanced cytotoxic potential of the drug from the nanoemulsion formulation in comparison to pure drug solution indicates better cellular uptake of drug in the form of nanoemulsion against true solution. This could be due to presence of surfactant which is known to be a potent inhibitor of P-glycoprotein efflux as well as because of surfactant induced permeability changes in the cell membrane (Bali et al., 2011; Ghosh and Murthy, 2006).

6.12. Stability studies

No significant ($p > 0.05$) change was found in the values of refractive index, viscosity, droplet size, and drug content when estimated at the end of 0, 1, 2 and 3 months (Table 4). The percentage declination in the content of melphalan at the end of 3rd month was found to be 1.26%. The shelf life (Fig. 8) of the formulation was calculated as 1.30 years.

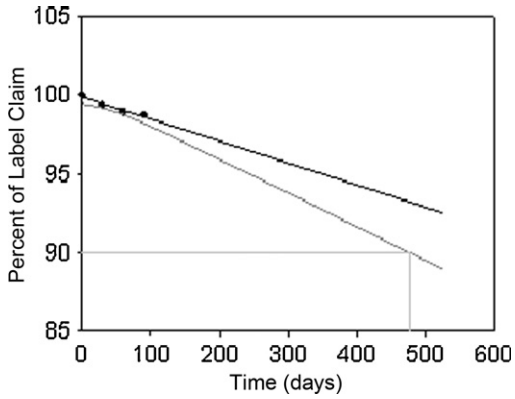

Fig. 8. Determination of shelf life of nanoemulsion (F6). The thin line shows the curve obtained by plotting the percent drug remaining *versus* time whereas the bold line shows the plot of 95% one-sided confidence limit for the percent drug remaining values *versus* time.

7. Conclusion

A nanoemulsion comprising of Capmul MCM (10%, v/v), Tween 80 (26.25%, v/v), Transcutol P® (8.75%, v/v) and double distilled water (55%, v/v) was successfully developed which exhibited superior bioavailability and enhanced ovarian uptake as compared to the pure drug suspension. Zeta potential of the formulation confirmed stability of the formulation. Bioavailability studies in mice demonstrated 4.83 folds increase in bioavailability of the drug in comparison to drug suspension. Biodistribution studies revealed an increase in the duration as well as the extent of ovarian uptake of the drug. Level A correlation on the basis of IVIVC studies established usefulness of the *in-vitro* drug release studies in precise prediction of the *in-vivo* behavior of the drug. Cytotoxicity studies divulged superior anticancer potential of the nanoemulsion formulation in comparison to drug suspension. The shelf life of the formulation was found to be 1.30 years. The present study corroborated surface modification of nanoemulsion to be a promising approach so as to facilitate augmented passive uptake, reduce dose, dose associated side effects and eventually improve clinical application of the drug.

Acknowledgments

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References

Bali, V., Ali, M., Ali, J., 2010. Novel nanoemulsion for minimizing variations in bioavailability of ezetimibe. J. Drug Target. 18, 506–519.
Bali, V., Ali, M., Ali, J., 2011. Nanocarrier for the enhanced bioavailability of a cardiovascular agent: *in vitro*, pharmacodynamic, pharmacokinetic and stability assessment. Int. J. Pharm. 403, 46–56.
Brightman, K., Finlay, G., Jarvis, I., Knowlton, T., Manktelow, C.T., 1999. A stability-indicating method for the determination of melphalan and related impurity content by gradient HPLC. J. Pharm. Biomed. Anal. 20, 439–447.

Cornaire, G., Woodley, J., Hermann, P., Cloarec, A., Arellano, C., Houin, G., 2004. Impact of excipients on the absorption of P-glycoprotein substrates in vitro and in vivo. Int. J. Pharm. 278, 119–131.
Ghosh, P.K., Murthy, R.S.R., 2006. Microemulsions: a potential drug delivery system. Curr. Drug Deliv. 3, 167–180.
Hauss, D.J., 2007. Oral lipid based formulations. Adv. Drug Deliv. Rev. 59, 667–676.
Hoeller, S., Sperger, A., Valenta, C., 2009. Lecithin based nanoemulsions: a comparative study of the influence of non-ionic surfactants and the cationic phytosphingosine on physicochemical behavior and skin permeation. Int. J. Pharm. 370, 181–186.
Humberstone, A.J., Charman, W.N., 1997. Lipid based vehicles for the oral delivery of poorly water soluble drugs. Adv. Drug Deliv. Rev. 25, 103–128.
Kang, B.K., Lee, J.S., Chon, S.K., Jeong, S.Y., Yuk, S.H., Khang, G., Lee, H.B., Cho, S.H., 2004. Development of self-microemulsifying drug delivery (SMEDDS) for oral bioavailability enhancement of Simvastatin in beagle dogs. Int. J. Pharm. 274, 65–73.
Karsulu, H.Y., Karabulut, B., Goker, E., Guneri, T., Gabor, F., 2007. Controlled release of methotrexate from W/O microemulsion and its in vitro anti-tumor activity. Drug Deliv. 14, 225–233.
Khoo, S.M., Humberstone, A.J., Porter, C.J.H., Edwards, G.A., Charman, W.N., 1998. Formulaion design and bioavailability assessment of lipidic self-emulsifying formulation of halofantrine. Int. J. Pharm. 167, 155–164.
Kommuru, T.R., Gurley, B., Khan, M.A., Reddy, I.K., 2001. Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q_{10}: formulation development and bioavailability assessment. Int. J. Pharm. 212, 233–246.
Kumar, M., Misra, A., Babbar, A.K., Mishra, A.K., Mishra, P., Pathak, K., 2008. Intranasal nanoemulsion based brain targeting drug delivery system of risperidone. Int. J. Pharm. 24, 285–291.
Lawrence, M.J., Rees, G.D., 2000. Microemulsion-based media as novel drug delivery systems. Adv. Drug Deliv. Rev. 45, 89–121.
Mou, D., Chen, H., Du, D., Mao, C., Wan, J., Xu, H., Yang, X., 2008. Hydrogel-thickened nanoemulsion system for topical delivery of lipophilic drugs. Int. J. Pharm. 353, 270–276.
Philip, A.K., Pathak, K., 2008. Wet process-induced phase-transited drug delivery system: a means for achieving osmotic, controlled, and level A IVIVC for poorly water soluble drug. Drug. Dev. Ind. Pharm. 34, 735–743.
Ping, L., Ghosh, A., Wagner, R.F., Krill, S., Joshi, Y.M., Serajuddin, A.T.M., 2005. Effect of combined use of nonionic surfactant on formation of oil-in-water microemulsions. Int. J. Pharm. 288, 27–34.
Pinguet, F., Joulia, J.M., Martel, P., Grosse, P.Y., Astre, C., Bressolle, F., 1996. High performance liquid chromatographic assay for melphalan in human plasma: application to pharmacokinetic studies. J. Chromatogr. B 686, 43–49.
Pouton, C.W., 1997. Formulation of self-emulsifying drug delivery systems. Adv. Drug Deliv. Rev. 25, 47–58.
Shafiq, S., Shakeel, F., Talegaonkar, S., Ahmad, F.J., Khar, R.K., Ali, M., 2007. Development and bioavailability assessment of Ramipril nanoemulsion formulation. Eur. J. Pharm. Biopharm. 66, 227–243.
Tadros, T.F., Vandamme, A., Levecke, B., Booten, K., Stevens, C.V., 2004. Stabilization of emulsions using polymers surfactants based on inulin. Adv. Colloid Interface Sci. 108/109, 207–226.
Tamilvanan, S., 2009. Formulation of multifunctional oil-in-water nanosized emulsions for active and passive targeting of drugs to otherwise inaccessible internal organs of the human body. Int. J. Pharm. 381, 62–76.
Tripathi, K.D., 2003. Essentials of Medical Pharmacology, 5th ed. Medical Publishers, New Delhi, India, pp. 772–773.
USP-27 NF-22, 2004. Canada, pp. 2513–2518.
Vyas, S.P., Khar, R.K., 2005. Targeted and Controlled Drug Delivery Systems. CBS Publishers & Distributors, New Delhi, pp. 331–386.
Wandel, C., Kim, R.B., Stein, M., 2003. "Inactive" excipients such as Cremophor can affect in vivo drug disposition. Clin. Pharmacol. Ther. 73, 394–396.
Wang, L., Xi, L., Zhang, G., Dong, J., Eastoe, J., 2007. Oil-in-water nanoemulsions for pesticide formulations. J. Colloid Interface Sci. 314, 230–235.
Woodhouse, K.W., Hamilton, P., Lennard, A., Rawlins, M.D., 2007. The pharmacokinetics of melphalan in patients with multiple myeloma: an intravenous/oral study using a conventional dose regimen. Eur. J. Clin. Phamacol. 24, 283–285.