Drug Development and Industrial Pharmacy, 2012; 38(3): 294–300

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI: 10.3109/03639045.2011.602407

informa
healthcare

RESEARCH ARTICLE

Development and evaluation of novel microemulsion based oral formulations of 5-fluorouracil using non-everted rat intestine sac model

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Abstract

Oral formulations of 5-fluorouracil (5-FU) with enhanced bioavailability were developed using microemulsion as a drug carrier system. The formulations were evaluated for drug content, physicochemical characteristics such as globule size, zeta potential, viscosity, stability and permeation characteristics. *Ex vivo* permeation studies were performed using *non-everted* rat intestinal sac technique. Results of the *ex vivo* permeation studies revealed that from aqueous solution only 25.08% drug was permeated, whereas, the optimized microemulsion formulation showed 97.5% drug permeation in 8 h, suggesting, approximately, four times enhancement in the drug permeability. Also a 7-fold increase in the flux of drug was observed from microemulsion formulation when compared with the aqueous solution. Further, *in vivo* pharmacodynamic studies were carried to check the therapeutic efficacy against benzo(a)pyrene [B(a)P]-induced stomach tumors in albino mice (Balb/C strain). The treatment of mice with 5-FU and microemulsion (5-FU II), after the last dose of B(a)P i.e. during the initiation period, resulted in 25% and 67% reduction in tumor incidence, respectively suggesting significant enhancement in the bioavailability and therapeutic efficacy of 5-FU when it was formulated as a microemulsion. These promising results suggest that microemulsion formulation of 5-FU may be used for the treatment of human cancers after pharmacokinetic and clinical evaluation.

Keywords: Microemulsion, 5-fluorouracil, solubility, permeability, benzo(a)pyrene, *non-everted* intestinal sac technique, anti-cancer, stability, bioavailability, metabolism

Introduction

5-Fluorouracil (5-FU) is one of the major anti-metabolites used in a variety of solid cancers, such as stomach, colon, lung and breast cancer[1,2]. It is a crystalline powder soluble in water (10 mg/mL) and is practically odorless. Owing to its hydrophilic nature and low lipophilicity (log *P*-0.83), it shows poor oral bioavailability[3]. Orally, its bioavailability is also impaired by high levels of the breakdown by enzyme dihydropyrimidine dehydrogenase present in gut mucosa. It has been reported that 80–85% of an administered dose of 5-FU is catabolized[4]. It is usually given intravenously, as the absorption from the gastrointestinal tract is erratic and unpredictable[5]. However, the intravenous route of administration is associated with severe systemic side effects due cytotoxic nature of 5-FU[6]. Numerous studies involving novel particulate carrier systems like pH-sensitive Eudragit microspheres[7], PEGylated dendritic nanoparticulate carrier[8], gold nanoparticles[9], poly(alkylcyanoacrylate) nanoparticles[10], PLGA nanoparticles[11] and chitosan-coated magnetic nanoparticles[12], have been reported. Acrylic acid-based molecularly imprinted hydrogels[13], pH-sensitive hydrogels[14], buccal bioadhesive tablets[15], transferrin-coupled liposomes[16], thermosensitive magnetoliposomes[17], gastro-retentive floating drug delivery systems[18,19] and niosomal delivery system[20] have also been reported for the effective and targeted delivery of 5-FU.

Microemulsion is defined as a dispersion consisting of oil, surfactants, cosurfactants and an aqueous phase, which is a single, optically isotropic and thermodynamically stable liquid solution, usually with a droplet diameter in submicron range[21]. They exhibit novel and

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(Received 20 October 2010; revised 09 May 2011; accepted 28 June 2011)

unique characteristic physical or biological behavior because of their small size. They possess higher saturation solubility and enhanced adhesion to biological surfaces thereby providing a rapid onset of therapeutic action and improved bioavailability[22,23]. Microemulsions also serve as protective media for the entrapped drugs. Stability of the drug is improved as the contact of drug with inactivating species such as enzymes present in the biological fluids is minimized[24]. It can also provide prolonged release of the drug and prevent irritation despite the toxicity of the drug[25,26].

In this study, we have developed microemulsion based oral delivery system of 5-FU, with an aim to enhance its oral bioavailability. Microemulsions were prepared employing Tween-20 (as a surfactant), Span-20 (as a cosurfactant), isopropyl myristate (IPM)/Captex 200 as an oil phase and triple distilled water (TDW) as an aqueous phase. *Ex vivo* permeation studies were performed using *non-everted* rat intestinal sac technique and finally the anti-cancer efficacy of the optimized formulation was evaluated against [B(a)P]-induced stomach tumors in mice.

Materials and methods

Materials

5-FU was a gift from Biochem Pharmaceutical Industries, Mumbai, India. Captex-200 (Propylene Glycol Dicaprylate/Dicaprate) was a gift from Abitec Corporation, Janesville, WI, USA. Labrasol (caprylocaproyl macrogol-8-glycerides) and Labrafac (medium chain triglyceride, C8-C10 fatty acids) were a gift from Colorcon Asia Ltd. Mumbai, India. IPM (isopropyl ester of myristic acid) Tween-20 and Span-20 were purchased from S.D. Fine Chemicals, Mumbai, India. All other reagents and chemicals used were of analytical reagent grade.

Ethical approval

Ethical approval to carry out *ex vivo* and *in vivo* studies in animals was obtained from Panjab University, Institutional Animal Ethics Committee, Chandigarh and their guidelines were followed for the studies (Registration No. 1334-50/CAH dated 3/9/2008). The male Wistar rats and albino female mice (Balb/C strain) required for study were obtained from Central Animal House, Panjab University, Chandigarh, India.

Methods

Selection of oil and surfactant

Solubility studies of 5-FU were carried out in various oils and surfactants so as to select the optimum components for microemulsion formulation, using conventional shake flask method[27]. An excess amount of drug was added to selected oil/surfactant in a vial and kept at 37 ± 1°C in a thermostatic water shaker bath for 48 h. Then, the contents were filtered through 0.22 μ filter and the filtrate was analyzed spectrophotometrically at λ_{max} 267 nm after appropriate dilution with the ethanol.

Construction of phase diagram

Pseudoternary phase diagrams were constructed to examine the formation of microemulsions using 4-component system consisting of an oil phase (Captex 200/IPM), a non-ionic surfactant Tween-20, a cosurfactant Span-20 and triple-distilled water (aqueous phase). The phase diagram was constructed by titration of homogenous liquid mixtures of water, surfactant, and cosurfactant, with oil phase, at ambient temperature[28]. A homogenous water-surfactant-cosurfactant blend was prepared, where contents of water and surfactant blend in the mixtures were varied from 9:1 to 1:9. Oil phase was added drop by drop to each mixture. During the titration, samples were stirred using a magnetic stirrer to allow equilibration. Following addition of aliquot of oil, the mixture was visually examined for transparency. Transparent, single-phase mixtures were designated as water-in-oil microemulsions. Again homogenous blends of the amphiphile and oil in the similar manner were prepared and titrated with water to obtain clear oil in water microemulsions.

Preparation of microemulsion formulation

After identification of water-in-oil microemulsion region in the phase diagram, the microemulsion formulations were prepared. 5-FU (0.2% w/w) was incorporated into the aqueous phase (10.0% w/w triple distilled water) in a beaker magnetically stirred at ambient temperature, then Tween-20 (20.0% w/w) and Span-20 (20.0% w/w) were added to the mixture and stirred. The oil phase IPM (formulation 5-FU-I)/Captex200 (formulation 5-FU-II) 49.8% w/w was added drop by drop into the above solution and clear water-in-oil microemulsions were obtained by stirring the mixtures for another 10 min[29] Similarly, blank microemulsions without drug were also prepared. The aqueous solution of 5-FU was prepared by dissolving 20 mg of drug in 10 mL of triple distilled water which would serve as control for *ex vivo* permeation studies against the microemulsion formulations.

Characterization of microemulsions

The microemulsion formulations were evaluated for percent drug content, content uniformity, pH, viscosity, particle size distribution, zeta potential, morphology and structure. Drug content was determined by taking 100 mg of accurately weighed formulation which was diluted to 10 mL with ethanol and analyzed spectrophotometrically after suitable dilution at 267 nm[30]. Viscosity studies were carried out using Brookfield DV-II+ pro viscometer with spindle no. 21 at 37.0 ± 1.0°C and 0–100 rpm spindle rotation speed. Particle size distribution, polydispersity index and zeta potential were determined by dynamic light scattering (DLS) method using Malvern Zetasizer 2000 HS. Surface morphology was characterized employing transmission electron microscopy (TEM).

Ex vivo intestinal permeation studies

Ex vivo permeation studies of microemulsion formulations were carried out using *non-everted* gut

sac technique in triplicate. Male Wistar rats (weighing 250–300 g) were used for the study and were sacrificed by spinal dislocation. The small intestine was removed by cutting across the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery. The small intestine was washed out carefully with cold normal oxygenated saline solution (0.9%, w/v, NaCl) using a syringe equipped with blunt end. The clean intestinal tract was cut into 8 ± 0.2 cm long sacs having a diameter of 3.0 ± 0.5 mm. After tying one end, each sac was filled with 0.5 mL of 5-FU formulation (aqueous solution/5-FU-I/5-FU-II; equivalent to 2 mg/mL of 5-FU) via a blunt needle, then other end of the sac was tied, keeping effective sac length 5 cm for permeation. Each sac was placed in a glass conical flask containing 50 mL of Krebs Ringer phosphate buffer saline (pH 7.4) [containing sodium chloride (0.67%, w/v), potassium chloride (0.034%, w/v), magnesium sulfate (0.059%, w/v), calcium chloride (0.011%, w/v), sodium dihydrogen phosphate (0.234%, w/v) and glucose (0.18%, w/v) in distilled water]. The entire system was maintained at 37 ± 1.0°C in a water shaker bath operating at 50 rpm and gassed with oxygen (10–15 bubble/min) using laboratory aerator. 2 mL samples were withdrawn from outside the sacs every 30 min for 8 h and replaced with 2 mL of fresh buffer. The samples were analyzed at λ_{max} 267 nm using a UV-visible spectrophotometer taking Krebs buffer as a blank. Similarly, permeation study was performed using blank microemulsions (without drug) and the absorbance values were subtracted from the test (5-FU-I and 5-FU-II) to account for the effect of excipients. The cumulative amount released per unit area of sac (μg/cm^2), flux (μg/h/cm^2) and percent drug permeation in the receptor compartment were calculated[31]. Surface area of the sac was calculated assuming it to be of cylindrical shape. The length 5 cm, the volume 0.5 mL and the inner diameter was 0.30 cm. The surface area for permeation was calculated to be 4.71 cm^2 per sac. Although, the method used took no account of the microvilli and villi, this was of little consequence for the results of the present study, since the surface area of the intestine between the experiments was standardized, by performing an identical surgical procedure and performing the permeation study in triplicate.

Anti-cancer studies

Albino female mice (Balb/C strain) 8–9 weeks old weighing 20–30 g were used for the study. The animals were kept under standard 12/12 light/dark cycle and were given food and water *ad libitum*. Animals in the treatment groups were administered two doses of 3 mg of B(a)P in 0.25 mL of corn oil per oral with a gap of two weeks. The B(a)P treated mice were divided into 3 groups ($n = 12$) i.e., only B(a)P treated, plain 5-FU treated and 5-FU-II microemulsion treated. The microemulsion without drug in an equal quantity was administered to the control group. The treatment groups were administered an oral dose of 20 mg/kg aqueous solution of 5-FU or its equivalent in case of microemulsion for five consecutive days followed by two drug free days. This dosage regimen was repeated until end of the experiment.

Tumor determination

Animals were sacrificed after 10 weeks of last dose of B(a)P by cervical dislocation. The forestomach was separated, sectioned longitudinally and fixed in 10% buffered formalin-phosphate. Stomach papillomas measuring 1.0 mm or larger were counted using magnifying glass[32,33]. The relative susceptibility to B(a)P induced tumors was expressed by the tumorigenic index as proposed by Shimkin[34].

Tumorigenic index = Percentage of mice with tumors X mean number of tumors per tumor bearing mouse

The statistical significance between was analyzed using one way ANOVA followed by Dunnett's test. P value < 0.05 was considered significant.

Histopathology studies

For histopathological evaluation the isolated and fixed forestomachs were carefully embedded in paraffin blocks. Then tissues sections were cut using a microtome and processed. Final staining was performed with hematoxylin and eosin. The tissue sections were mounted on microscopic slides and were observed under high magnification light microscope to examine the histopathological changes.

Stability studies

Three batches of each formulation were stored in sealed glass containers at 4, 25 and 40 °C for three months. The stability of the microemulsions was checked periodically with respect to transparency, phase separation, color change and drug content at three different temperature conditions.

Results and discussion

Solubility studies

Solubility studies were carried out in different media to select the appropriate oil phase and surfactant phase for developing water-in-oil microemulsion formulations of hydrophilic agent 5-FU. In Captex-200, the solubility of 5-FU was found to be highest (7.87 ± 0.46 mg/mL) followed by IPM (5.78 ± 0.70 mg/L. Among the surfactants, maximum solubility was observed in Tween-20 (22.40 ± 4.91 mg/mL). Accordingly, Captex-200 and IPM were selected as appropriate oil phase and Tween-20 was selected as the most appropriate surfactant for development of microemulsion (Table 1).

Phase diagram study

Phase studies were carried out to find the area of microemulsion existence and to investigate the effect of different surfactant/cosurfactant weight ratios on the extent of stable microemulsion region. The pseudoternary phase diagrams with two oils (Isopropyl myristate and Captex-200

are depicted in Figure 1A and B. Optimum microemulsion formula was selected using phase studies employing IPM and Captex-200 as oil phase, 1:1 mixture of Tween-20 and Span-20 as surfactant mixture and water as internal aqueous phase. A 1:1 mixture of Tween-20 and Span-20 was considered as optimum surfactant mixture with highest oil solubilization capacity and maximum microemulsion stability. Suitable formulas were selected from both the phase diagrams keeping internal phase as 10% w/w water.

Table 1. Solubility studies of 5-FU in different vehicles.

Ingredient	Solubility (mg/mL)
Captex 200	7.87 ± 0.46
IPM	5.78 ± 0.70
Labrafac	3.23 ± 0.46
Tween 20	22.40 ± 4.91
Labrasol	16.66 ± 1.46
Span 20	0.617 ± 0.07

Characterization of microemulsion

Results of our study indicate development of successful microemulsion formulations of 5-FU with optimum characteristics. The drug content was found to be 99.04 ± 0.04% and pH was found to be 5.68 ± 0.01. The viscosity of microemulsions ranged between 55.9–57.2 m Pas and exhibited Newtonian flow which may be attributed to the uniformity of nanostructures with respect to size and shape present in developed microemulsion, which does not allow change in the flow behavior on changing the shear rate and maintain constant viscosity of the emulsified system. The average diameter and polydispersity index was found to be 118 and 0.574 nm respectively (Figure 2). TEM revealed that the surface of water-in-oil globules of 5-FU II was spherical in nature (Figure 3). The zeta potential of the prepared microemulsion, measured using Malvern Zetasizer™, was found to be 0.143 mV.

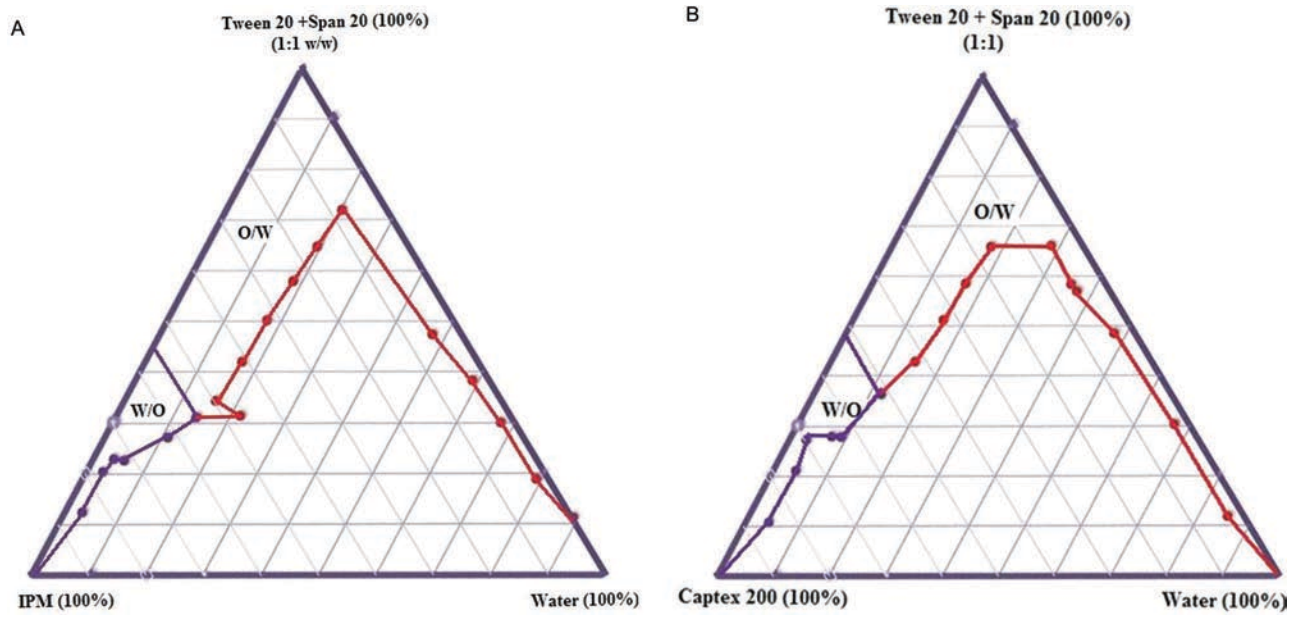

Figure 1. (A) Phase diagram of the system containing IPM (5-FU-I), tween-20, span-20 and water. (B) Phase diagram of the system containing Captex-200 (5-FU-II), Tween-20, Span-20 and water

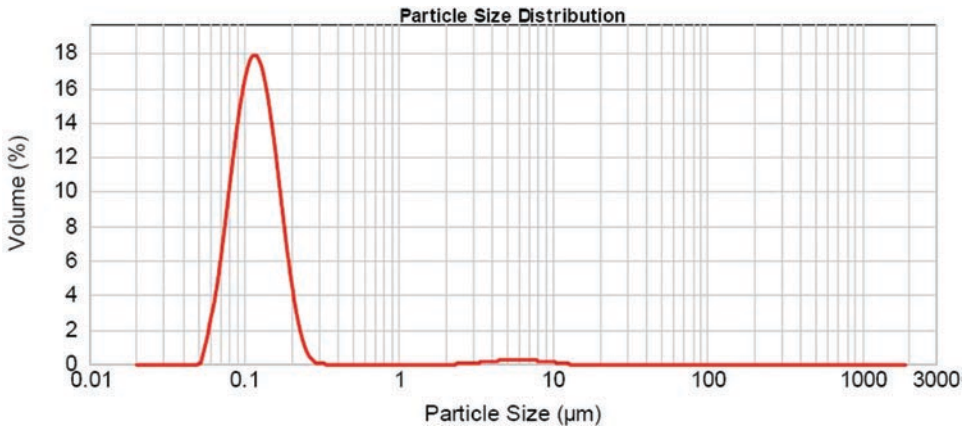

Figure 2. Particle size distribution graph for microemulsion formulation 5-FU-II.

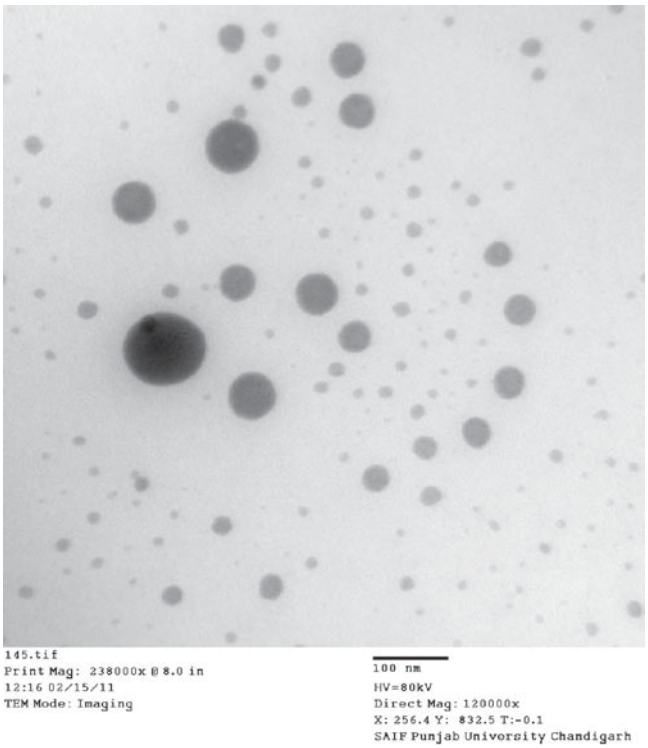

Figure 3. Visualization of globules of microemulsion 5-FU-II by TEM.

Ex vivo permeation studies

Figure 4 depicts the *ex vivo* permeation profiles of different formulations 5-FU from non-everted rodent gut sac. The cumulative amount of drug permeated from the control (aqueous solution of 5-FU) was found to be 66 ± 7 μg/cm^2 (25.08%) in 8 h. Whereas, a tremendous increase in permeability of 5-FU was observed upon its incorporation into microemulsions. The permeability of 5-FU from microemulsion 5-FU-I was found to be 185 ± 9 μg/cm^2 (70%) in 8 h which was significantly different and higher from the control at $P < 0.01$. Further, higher permeation was achieved with the formulation 5-FU-II containing Captex-200, as oil phase. In this case more than 80% (231 ± 9 μg/cm^2) permeation of the drug was obtained in 3 h. The cumulative permeability was found to be 258 ± 9 μg/cm^2 (97.5%) in 8 h which was significantly greater than the control and 5-FU-I respectively. The rate of permeation (flux) of 5-FU from control, 5-FU-I and 5-FU-II were observed to be 13, 55 and 85 μg/cm^2/h, respectively (Figure 5). A 7-fold increase in flux was achieved for 5-FU-II formulation when compared with control. Such a high degree of enhancement in permeability of the drug from the microemulsion system can be attributed to larger concentration gradient, created by increased solubility of drug in the microemulsion and favorable partitioning of the drug. These results were in accordance with previous report by Kim et al. suggesting that w/o microemulsions are very good candidates for solubilization and delivery of hydrophilic molecules[23]. Moreover, entrapment of drug molecules in the interior of w/o microemulsion system might have protected it from enzymatic degradation by

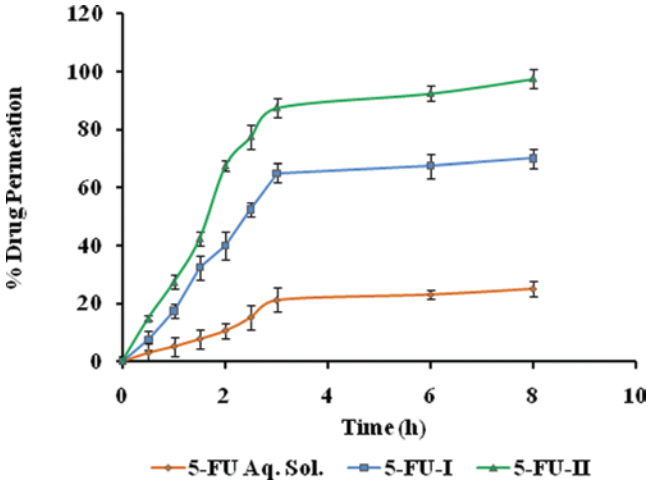

Figure 4. Comparison of permeation of 5-FU from different formulations using *non-everted* rat intestinal sac model.

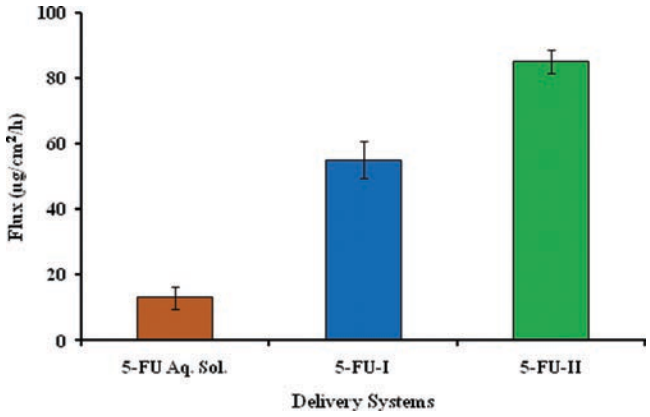

Figure 5. Flux values 5-FU from different formulations using *non-everted* rat intestinal sac model.

gut enzymes. Earlier investigations by Lyons et al., also suggest that w/o microemulsions might help in protection of the drugs from enzymatic degradation[24].

Anti-cancer studies

Based on the encouraging results of preliminary *ex vivo* permeation studies from rats intestine, further *in vivo* pharmacodynamic studies were performed to evaluate the therapeutic efficacy of developed 5-FU microemulsions against B(a)P induced forestomach neoplasia. It is a well-known animal model to examine the anti-cancer activities of therapeutic agents against GIT cancers. The studies indicated that the treatment of mice with B(a)P resulted in 100% incidence of fore stomach tumors after 10 weeks with an average of 1.75 tumors per mouse compared with corn oil–treated control animals. Treatment of mice with 5-FU and 5-FU-II after the last dose of B(a)P (i.e., during the initiation period) resulted in 25 and 67% reduction in tumor incidence (percentage of number of mice with tumors), respectively (Table 2). Number of tumors per tumor-bearing mouse was reduced to 76 and 57%, respectively. Similarly, the tumorigenic

Table 2. Effect of microemulsion 5-FU-II on B(a)P induced forestomach tumors in albino female mice.

Group No.	Treatment group	Mice with tumors (%) (tumor incidence)	No. of tumors per tumor bearing mice	Tumerigenic index[a]
1	Blank microemulsion	0.00	0.00	0.00
2	B(a)P	100.00	1.75	175.00 (100)[b]
3	B(a)P + 5-FU aqueous solution	75.00	1.33	99.75 (57)
4	B(a)P + 5-FU-II	33.33	1.00	33.33 (19)

[a]Tumorigenic index obtained by multiplying the percentage of mice with tumors times the mean number of tumors per tumor bearing mouse.
[b]Number in parentheses indicate percentage of the control tumorigenic index.

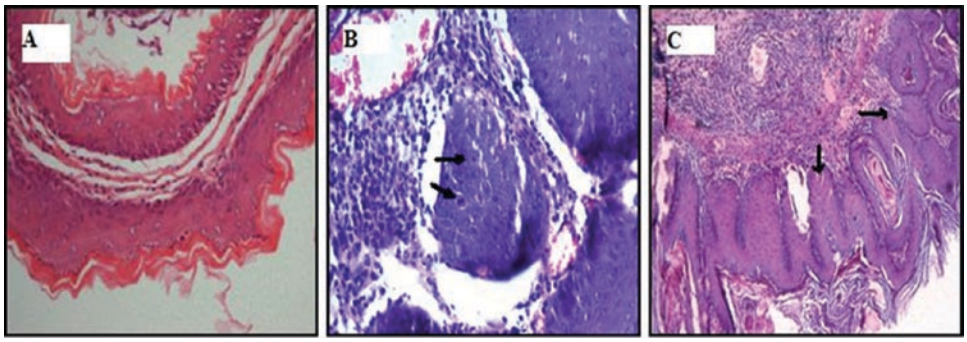

Figure 6. Histopathologic changes of forestomachs (A) untreated mice showing normal squamous epithelium; (B) mice treated with B(a)P (increase in number of cells in the squamous epithelium and hyperplasia representing tumor development of epithelial origin); (C) mice treated with B(a)P and 5-FU-II microemulsion (no hyperplasia and a decrease in number of cells in squamous epithelium). Arrows indicate changes in histopathology.

index (product of percentage of mice with tumors and number of tumor bearing mouse) was reduced to 99.75 and 33.33 with 5-FU solution and 5-FU-II, respectively. The statistically significant ($P < 0.05$) reduction in the number of mice with tumors was obtained with 5-FU-II as compared with control and plain 5-FU treatments suggesting the higher bioavailability and better therapeutic efficacy of 5-FU-II. These results can be correlated with *ex vivo* permeation studies of 5-FU where higher (more than 97% permeation in 8 h) was observed from 5-FU-II compared to 5-FU aqueous solution (only 25% permeation in 8 h). Enhanced solubility of the drug in the microemulsion system, partitioning and enhanced permeability due to submicron size and protection from intestinal degradation might have resulted in higher therapeutic levels to treat stomach tumors more effectively as compared to 5-FU aqueous solution.

Histopathological studies

Histopathological examination was carried out to check the effectiveness of developed formulation of 5-FU. Figure 6A depicts the normal esophageal squamous epithelial in untreated animal. An increase in number of cells in the squamous epithelium and hyperplasia of the squamous epithelial cells with mild atypia in the basal region, representing tumor development of epithelial origin was observed in the animals treated with B(a)P (Figure 6B). In contrast, in mice treated with 5-FU-II after B(a)P treatment, no hyperplasia was observed and a decrease in number of cells in squamous epithelium was seen as compared to B(a)P treated animals (Figure 6C).

Stability studies

Stability studies of microemulsions were performed at 4°C, 25°C and 40°C. It was observed that more than 99% of drug was present in all the formulations after three months at 4°C, whereas at 25°C, there was about 3% and at 40°C, there was about 4% decline in the drug content in the formulations after three months. Other characteristics like pH, transparency, viscosity, feel were also evaluated periodically and no significant change was observed (data not shown).

Conclusion

Efficient and stable microemulsion formulations were developed for oral delivery of 5-FU. Results of *ex vivo* permeation using *non-everted* gut sac technique showed drastic increase in the permeability of 5-FU upon formulation into microemulsion system. Further, the results of *in vivo* investigations using murine model of [B(a)P]-induced forestomach tumorigenesis confirms the better bioavailability and therapeutic efficacy of microemulsion formulation. These encouraging outcomes of preliminary investigations strongly warrant further pharmacokinetic and clinical investigations of these oral microemulsion based formulations of 5-FU so that they can be effectively used as an alternative to intravenous therapy for the treatment of cancer.

Declaration of interest

The authors gratefully acknowledge the financial assistance from the University Grants Commission, India; the gift sample of 5-FU supplied by Biochem Pharmaceutical Industries (Mumbai). The authors are also thankful to Abitec Corporation (Janesville, WI, USA) for providing the gift sample of Captex-200 and Colorcon (Asia Pvt. Ltd. Mumbai, India) for supplying the Labrasol and Labrafac.

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