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Formulation and characterization of Paclitaxel, 5-FU and Paclitaxel + 5-FU microspheres

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Abstract

The purpose of this study was to compare the combination (Paclitaxel + 5-FU microspheres) with a single drug chemotherapy (Paclitaxel and 5-FU microspheres) against metastatic breast cancer cell line (MDA-MB 435 S). The physicochemical characteristics of the microspheres (i.e. encapsulation efficiency, particle size distribution, in vitro release, thermal characteristics) were studied. The results demonstrated that the encapsulation efficiency of Paclitaxel was high (90%) when the drug was encapsulated in poly(lactic-co-glycolic acid) (PLGA) microparticles with or without 5-fluorouracil (5-FU). However, the encapsulation efficiency of 5-FU was low (19%) and increased to 30% when the drug was encapsulated with Paclitaxel. The mean particle size of microspheres was 2.5 μm and were spherical in shape. The in vitro release of both 5-FU and Paclitaxel from the microspheres was relatively fast initially followed by a slower and more controlled release. The cytotoxic activity of Paclitaxel microspheres was far greater compared to either the microspheres containing 5-FU + Paclitaxel or 5-FU alone. Overall results demonstrated that incorporation of Paclitaxel or 5-FU in microspheres enhances the cytotoxicity in more controlled manner compared to that of free drugs and also that careful consideration should be made when combining drugs acting in different phases of cell cycle.

Keywords: Microspheres; Poly(lactide-co-glycolide); Paclitaxel; 5-Flourouracil

1. Introduction

Paclitaxel and 5-fluorouracil (5-FU) are two of the most widely used chemotherapeutic agents for the treatment of metastatic breast cancer. Clinical protocols frequently combine chemotherapeutic agents that exhibit their cytotoxic action at different phases of cell cycle (Sartorelli, 1969; Johnson et al., 1999; Fan et al., 1998). Paclitaxel (Taxol®) is one of the best anti-neoplastic drugs to be found in the past decades (Rideout and Chou, 1991). It has been clinically used in the treatment of various cancers especially breast and ovarian cancers. Paclitaxel has a unique mechanism of action. It promotes the polymerization of tubulin unlike other microtubule agents like vinca alkaloids, which induce the disassembly of microtubules. The microtubules formed in the presence of Paclitaxel are extraordinarily stable and dysfunctional, thereby causing the death of the cell by disrupting the normal tubule dynamics required for the mitotic cell division (Singla et al., 2002). It is well known that the clinical success of taxol is limited due to its

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low solubility in water and most of the other pharmaceutical solvents compatible for intravenous (i.v.) administration (Panchagnula, 1998; Fonseca et al., 2002). Paclitaxel (Taxol®) is currently available as a solution in a vehicle composed of Cremophor® EL and dehydrated alcohol.

5-Fluorouracil is one of the oldest chemotherapeutic drugs, and has been around and in use, for decades. It is commonly used against many cancers namely colon, stomach, breast and pancreatic cancers (Grem, 1996; Grem et al., 1999). It is a fluorinated analog of pyrimidine base uracil, which is metabolized intracellulary to its active form, fluorodeoxyuridine monophophate (FdUMP). The active form inhibits DNA synthesis by inhibiting the normal production of thymidine. Fluorouracil is also cell cycle specific (S-phase).

5-FU is sparingly soluble in water and slightly soluble in alcohol (McEnvoy et al., 1998). Due to apparent physicochemical characteristics of Paclitaxel and 5-FU, an oral formulation for the two drugs cannot be a possible alternative.

Polymeric delivery systems have been used over the years in the delivery of both water soluble and insoluble drugs. The delivery of chemotherapeutic agents using polymeric microspheres has become one of the most popular areas of research because of the possibilities of reducing toxicities, enhancing controlled release activity and also localizing the drug delivery (Liggins et al., 2000). Poly(lactic-co-glycolic acid) (PLGA), biodegradable and biocompatible polymer that has developed tremendous interest involving the development of microparticulate formulations (Jain, 2000) was used in this study to prepare the Paclitaxel, 5-FU and the Paclitaxel + 5-FU microspheres. Although there have been many earlier attempts to develop PLGA microspheres loaded with Paclitaxel (Wang et al., 1996; Sato et al., 1996; Mu and Feng, 2001) and 5-FU (Ciftci et al., 1996; Chiang et al., 2001), none of them have attempted to compare their cytotoxic activity.

The purpose of the present work was to develop Paclitaxel, 5-FU and their combination (Paclitaxel + 5-FU) loaded microspheres and to determine the physicochemical characteristics (i.e. encapsulation efficiency, in vitro release, thermal profile, size distribution) of the developed microspheres. In addition, the cytotoxic effects of these microspheres in metastatic breast cancer cells were investigated.

2. Materials and methods

2.1. Materials

PLGA (L/G = 50/50); MW, 40,000–75,000; inherent viscosity, 0.82 dl/g) was purchased from Birmingham Polymers (Birmingham, AL), Polyvinyl alcohol (PVA, 88°mol% hydrolyzed, MW 25,000) was purchased from Polyscience Inc. (Warrington, PA). Paclitaxel and 5-FU were purchased from Sigma Aldrich Inc. (St. Louis, MO) methylene chloride (dichloromethane, DCM analytical grade) was purchased from Fischer Scientific (Pittsburgh, PA). The in vitro release was carried out at pH 7.4 and at 37 °C in phosphate buffer saline (PBS), which was purchased from Sigma Aldrich. The metastatic breast cancer cell lines MDA-MB 435 S was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The Cell Titre-Glo™ Luminescent cell viability assay kit was purchased from Promega Inc. (Madison, WI). All other chemicals used were of reagent grade.

2.2. Methods

2.2.1. Preparation of the drug loaded PLGA-microspheres

The PLGA particles containing drugs were prepared by modified solvent evaporation method (Ciftci et al., 1996). For single agent microspheres, 5-FU was dispersed and Paclitaxel was dissolved in methylene chloride containing 1–2% of PLGA (50/50, inherent viscosity, 0.82 dl/g) using a probe sonicator. The resulting organic phase was then poured into aqueous phase containing 0.2–1% (w/v) of PVA. For the combination microspheres, 5-FU was dispersed in a solution of PLGA and Paclitaxel in methylene chloride. The mixture was then emulsified in the aqueous phase containing 0.2–1% (w/v) of PVA to form a o/w emulsion (Perez et al., 2000).

The final emulsion was then placed on the magnetic stirrer plate and continuously stirred at 1600 rpm at room temperature to evaporate methylene chloride. The microspheres were collected by centrifugation and washed two times with distilled water. The microspheres were then lyophilized and stored at −20 °C before further analysis. The percentage yield was calculated based on the amount of lyophilized

microspheres of each formulation obtained to the amount of solid material used in the dispersed phase. The encapsulation efficiency is defined as the ratio of amount of drug encapsulated to that of the drug used in microshere preparation. To determine the drug content in the microspheres, a specific amount of lyophilized microspheres was dissolved in 1 ml of dichloromethane (DCM) and mixed with 5 ml of the respective mobile phase for the two drugs. The mixture was then vortexed vigorously for 5 min and DCM was evaporated under a nitrogen stream until a clear solution was obtained. The final solution was diluted with more mobile phase, filtered with 0.22 μm membrane filter and used for the analysis of Paclitaxel and 5-FU concentration with the HPLC assay method described below.

2.2.2. Determination of Paclitaxel and 5-FU content in the microspheres

The Paclitaxel or 5-FU content in the PLGA-microspheres was analyzed by an HPLC (Agilent 1100 series, Agilent Technologies) assay. Paclitaxel and 5-FU concentrations were determined by modifying methods proposed by Lee et al. (1999) and Micoli et al. (2001), respectively. The HPLC assay for both drugs was performed on a reverse phase Zorbax® C18 column (25 cm × 0.45 cm i.d pore size 5 μm, Hewlett Packard Inc., Palo Alto, CA). The column temperature was maintained at 20° C. The mobile phase was a mixture of acetonitrile: 0.1% phosphoric acid in deionized water (50:50, (v/v)) for Paclitaxel delivered at a flow rate of 1.3 ml/min. The mobile phase for 5-FU consisted of 0.02 M phosphoric acid and methanol (98:2, (v/v)) delivered at a flow rate of 0.8 ml/min. Samples (50 μl aliquots) were filtered and sonicated before injection. Paclitaxel was detected at 227 nm and 5-FU at 265 nm with a variable wavelength detector (VWD). The calibration curve for the quantification for both Paclitaxel and 5-FU was linear over the range of standard concentration between 5 and 50,000 ng/ml with a correlation coefficient of $R^2 = 0.9996$ for Paclitaxel and 0.9983 for 5-FU. The solvent for calibration was same as the mobile phase used above.

2.2.3. Size distribution and morphology (SEM)

The particle size distribution of the prepared microspheres was measured by the laser light scattering technique (Accusizer 770 model, Particle Sizing Systems Inc., Santa Barbara, CA). Weighed microsphere samples were suspended in deionized water (1 ml) and vortexed before measurement. The obtained homogenous suspension was examined to determine the particle size distribution. For the morphology studies, freeze-dried particles were visualized using scanning electron microscope (30 kV). Samples were dusted on a double-sided adhesive tape applied previously to an aluminum stub. Excess samples were removed and stub sputter coated with 30-nm layer of gold. The coated samples were viewed under a scanning electron microscope (JSM-5510 LV JEOL, Peabody, MA).

2.2.4. In vitro release

The release rate of Paclitaxel or 5-FU from microspheres was measured in phosphate buffer saline (PBS) medium (pH 7.4) by an HPLC assay in triplicate. Paclitaxel, 5-FU or Paclitaxel + 5−FU loaded microspheres (0.1% w/v) were suspended in PBS containing 0.01% Tween 80 in screw capped tubes and placed in an orbital shaker (C 24 incubator shaker, New Brunswick Scientific, New Jersey) maintained at 37 °C and shaken at 50 rpm. At predetermined time intervals, the tubes were taken out of the shaker and centrifuged at 2000°rpm for 2 min. The supernatant was taken for analysis. The precipitated microsphere pellets were resuspended in 10 ml of fresh buffer and placed back in the shaker.

2.2.5. Differential scanning calorimetry (DSC)

Thermal characterization of microspheres was performed with a Perkin–Elmer DSC 7 (Perkin–Elmer, Wellesley, MA). Samples were weighed (2.00 ± 0.5 mg) and placed in sealed aluminum pans. The equipment was calibrated with indium. The samples were scanned at 20 °C/min from 25 to 300 °C. All the determination was performed in triplicate.

2.2.6. In vitro anti-tumoral activity

MDA-MB 435 S (ATCC, Rockville, MD) cells (6×10^3 cells/well) were seeded in 100 μl of Levobitz-15 (ATCC, Rockville, MD) medium in a 96 well plate. Cells were then incubated with a low (0.01 μM) or high (1 μM) doses of Paclitaxel, low (1 μM) or high (2 μM) doses of 5-FU and Paclitaxel or 5-FU loaded microspheres and the combination (Paclitaxel + 5−FU) loaded microspheres.

Cytotoxicity of the treatment was determined at 15, 30 min, 1, 4, 24, 48 h, respectively.

The effect of the doses of free Paclitaxel, 5-FU and the prepared microspheres was assessed using Cell Titre-Glo™ luminescent cell viability assay (Promega Corp., Madison, WI). This assay is a homogenous method of determining the number of viable cells in culture based on the quantitation of ATP present, which signals the presence of metabolically active cells.

After the treatment the cells were incubated with 100 μl of Cell Titre-Glo™ reagent and contents were allowed to mix on an orbital shaker in accordance with the assay protocols, this resulted in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is proportional to the number of cells present in culture. The luminescence signal was recorded with a single tube luminometer (TD 20/20, Turner Biosystems Inc., Sunnyvale, CA).

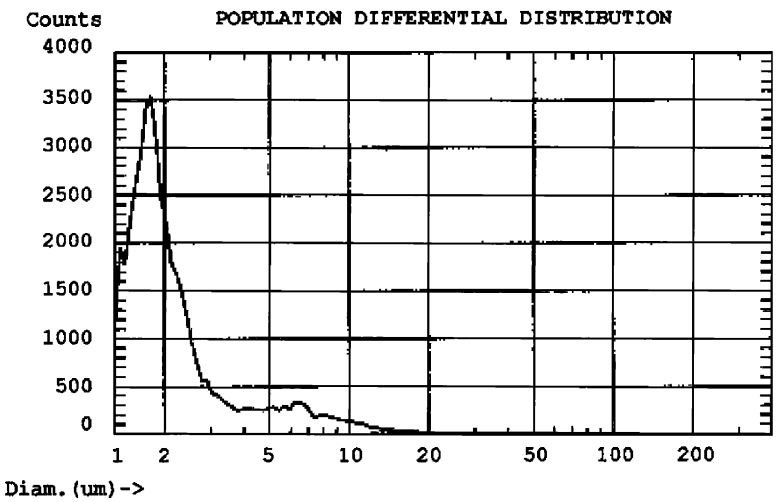
3. Results

3.1. Yield and encapsulation efficiency

All microspheres were prepared by using the modified solvent evaporation method. The initial loading for both drugs was 1.5% (w/w). The yield for Paclitaxel, 5-FU and Paclitaxel + 5−FU microspheres were 45, 59 and 50%, respectively. The encapsulation efficiency for Paclitaxel was very high (90%) in both Paclitaxel alone and in the combination microspheres. Previous studies have reported similar high drug loading with Paclitaxel microspheres prepared with various polymers, Paclitaxel loaded PCL (Dordunoo et al., 1995), PLGA (Wang et al., 1996), PLLA (Liggins et al., 2000). The high encapsulation efficiency of a hydrophobic drug like Paclitaxel is due to its high partition coefficient, and therefore, its retention in the organic phase as the microspheres solidify (Liggins et al., 2000). The encapsulation efficiency of 5-FU in the combination microspheres was ∼30% which was higher than that achieved for 5-FU microspheres alone (∼19%).

3.2. Particle size distribution and morphology

The size distribution for Paclitaxel, 5-FU and Paclitaxel + 5−FU microspheres is shown in Figs. 1–3. The mean particle size for the prepared microspheres was 2.5 μm. Scanning electron microscopy showed that the microspheres were spherical in shape and had relatively smooth surface as shown in Figs. 4–6 for Paclitaxel, 5-FU and Paclitaxel + 5−FU, respectively. The size range of the microspheres was found to be consistent with that deduced from the particle size experiments.



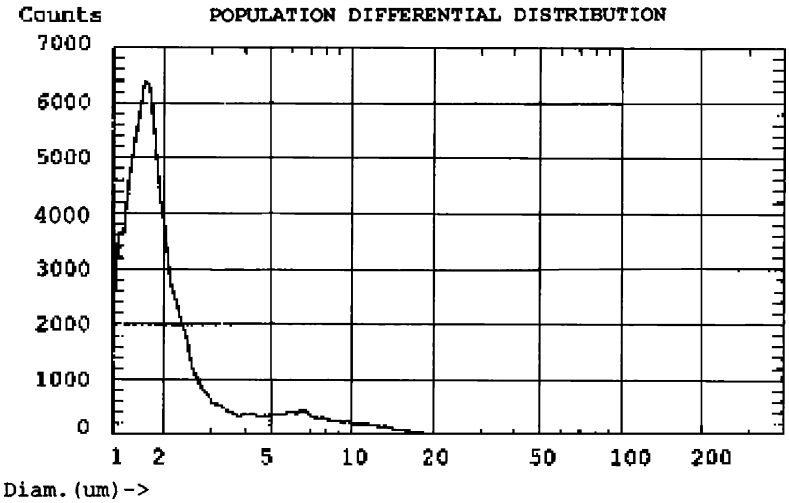


Fig. 2. Particle size distribution of 5-FU microspheres.

3.3. In vitro release

The in vitro release profiles for all the formulations are shown in Fig. 7. There was an initial release of approximately 12 and 18% for Paclitaxel and 5-FU, respectively, from the microspheres containing the combined drugs. Whereas the initial release from the single drug microspheres was about 10 and 15% for Paclitaxel and 5-FU, respectively. This initial release of drugs could be explained by the release of some drugs loosely bound on the surface of the microspheres (Bodmeier and Chen, 1989). This loosely bound drug would be released by a mechanism of diffusion through the aqueous pores on the surface of the microspheres created by the water uptake by the PLGA-microspheres immediately after being exposed to water (Mu and Feng, 2001; Kissel et al., 1991). This initial release was later followed by more controlled release for microsphere formulations for the 3-week study period, releasing a cumulative 45% of Paclitaxel and 64% of 5-FU from microspheres containing drugs alone, and it was 60 and 37% for 5-FU and Paclitaxel from the microspheres containing combined drugs, respectively.

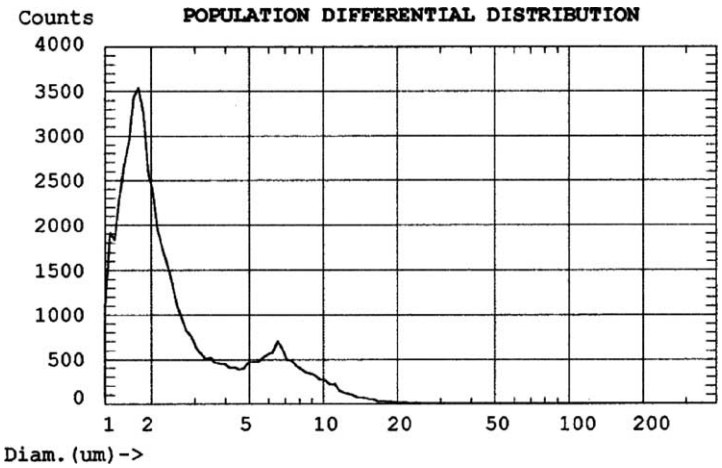


Fig. 3. Particle size distribution of Paclitaxel + 5−FU microspheres.

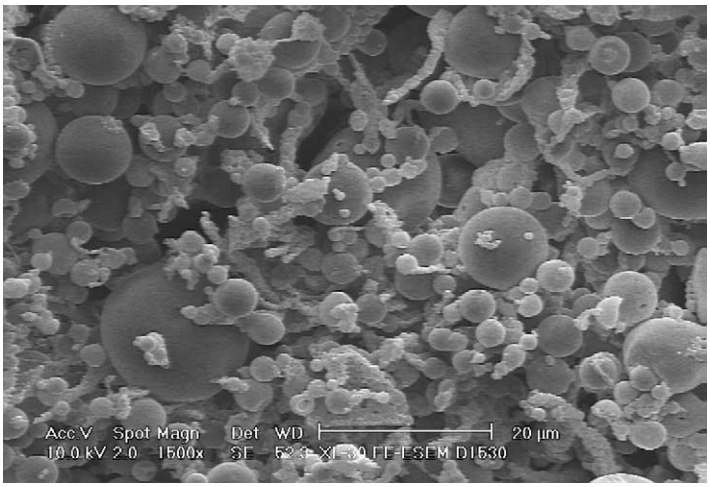


Fig. 4. SEM photograph of Paclitaxel microspheres.

3.4. Thermal characterization of microspheres

DSC is a very useful tool in the investigation of thermal properties of microspheres and can provide both qualitative and quantative information about the physicochemical state of the drug inside the microspheres (Mu and Feng, 2001; Dubernet, 1995). There is no detectable endotherm if the drug is present in a molecular dispersion or a solid solution state in the polymeric microspheres loaded with enough amount of drug (Mu and Feng, 2001). This amorphous nature of the drug may have pronounced pharmaceutical significance as it could lead to increased solubility and finally to an improved biological activity. In the present study, DSC thermograms of pure Paclitaxel, pure 5-FU, empty PLGA-microspheres, Paclitaxel, 5-FU and Paclitaxel + 5−FU loaded PLGA microspheres were obtained as explained in Section 2.2. As seen in Fig. 8 under the experimental conditions previously described, melting endotherm of pure

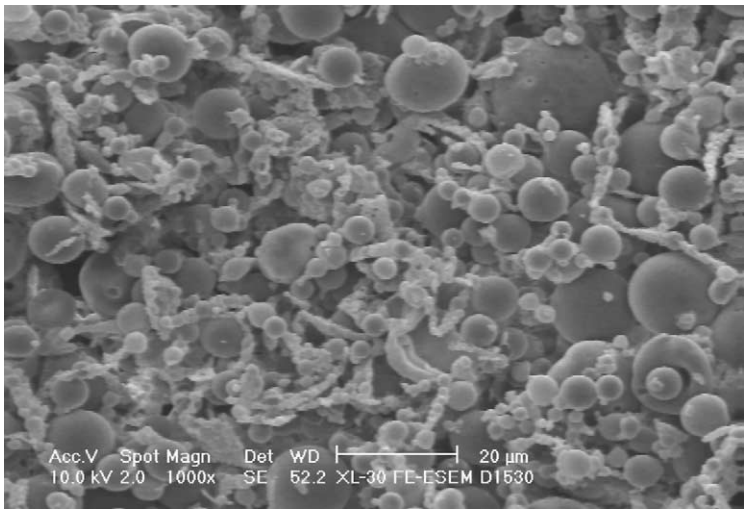


Fig. 5. SEM photograph of 5-FU microspheres.

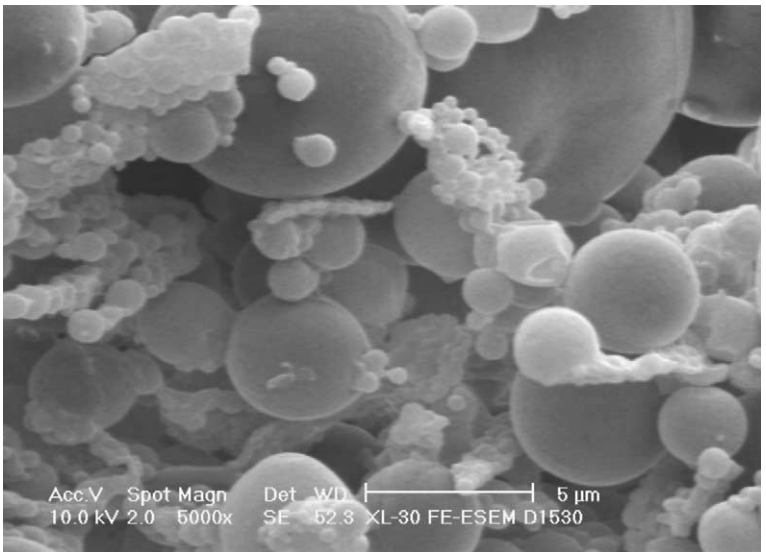


Fig. 6. SEM photograph of Paclitaxel + 5−FU microspheres.

Paclitaxel was found to be 211 °C (Mu and Feng, 2001) and 276 °C for 5-FU. We did not observe any peak in the temperature ranges of 150–250 °C and 100–300 °C for blank microspheres and the Paclitaxel or Paclitaxel + 5−FU loaded microspheres, respectively. This result suggests that Paclitaxel and/or 5-FU formulated in the PLGA-microspheres existed in amorphous or disordered-crystalline form of a molecular dispersion or solid solution state in the polymer matrix after the fabrication (Dubernet, 1995). On the contrary, 5-FU alone microspheres showed an endothermic peak around 276 °C that suggested the presence of at least some crystalline 5-FU after the microsphere fabrication.

3.5. Cell cytotoxicity study

The cytotoxic activity of free drugs and microspheres was assessed as described earlier with Cell Titre-Glo™ assay kit using metastatic breast cancer cell line MDA-MB435 S. The concentration range of free 5-FU and paclitaxel was selected based on our previous studies and the data reported in the literature (Liebmann et al., 1993).

As seen in Fig. 9, marked reduction in cell viability was observed when MDA-MB 435 S cells were incubated with 1 μM free Paclitaxel. At this concentration cell growth was reduced to ∼56% after 24 h and to 41% at the end of 48 h. It is also seen in Fig. 9 that lesser reduction in cell viability was observed with lower (0.01 μM) concentration of Paclitaxel where cell viability was reduced to ∼69% after 24 h and to ∼55% at the end of 48 h. While incubation with high concentration of free 5-FU, the viability was reduced to ∼70% after 24 h and to ∼58% within 48 h, the lower concentration of 5-FU reduced the viability to ∼80% and to ∼70% at the end of 48 h. There was no significant reduction in cell viability with the control microspheres (empty PLGA microspheres) within 48 h experiment period. Also as seen in Fig. 9, Paclitaxel microspheres incubation with MDA-MB 435 S resulted in a decline in cell viability which was less than that observed with both the lower and the higher concentrations of free Paclitaxel tested and resulted in cell viability to be reduced to ∼72% after 24 h and finally to ∼66% after 48 h incubation. 5-FU microspheres reduced cell viability to ∼81% in 24 h and ∼73% after 48 h. The combination microspheres resulted in a ∼86 and ∼80% reduction in cell viability of 24 and 48 h after treatment, respectively.

Moreover, encapsulation of Paclitaxel or 5-FU in microspheres prolonged the cytotoxic effect of the drugs compared to the free drugs 48 h after treatment.

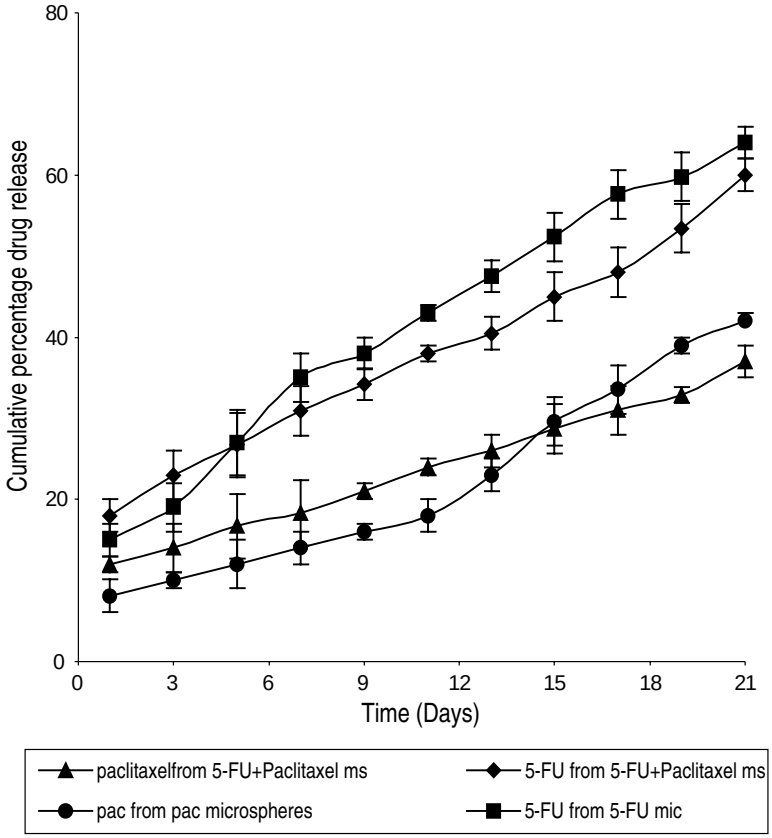


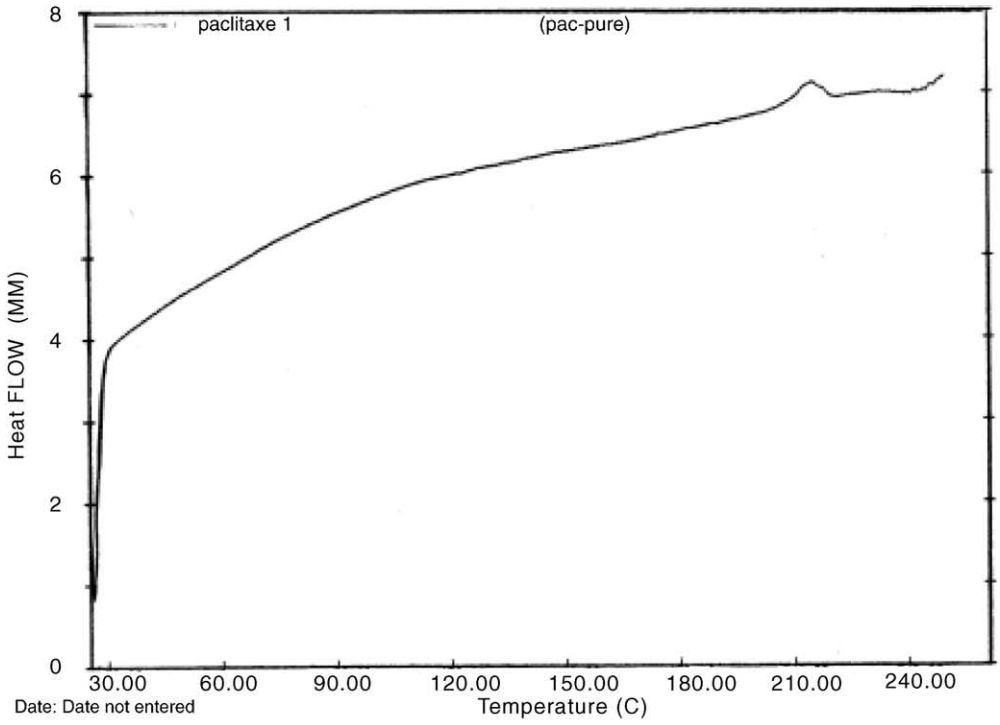
Fig. 7. In vitro release of Paclitaxel and 5-FU from microspheres. Dissotuion studies were conducted in PBS buffer (pH 7.4) as explained in Section 2.2 ($n = 3$).

This could be due to controlled release of the drugs from the formulations. In contrast, the combination microspheres of these drugs proved to be the least cytotoxic in comparison to either the free drugs or Paclitaxel or 5-FU microspheres, possibly as a result of antagonistic effect.

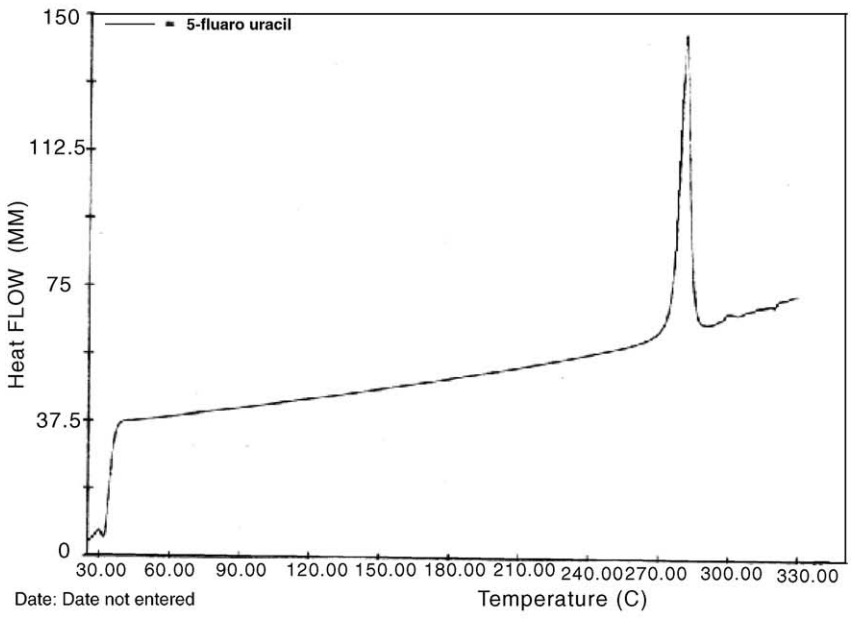
4. Discussions

Paclitaxel is one of the most promising drugs currently available for the treatment of metastatic breast cancer. But its effectiveness is restricted due to the problems associated with the current formulation. Thus, it could be clearly deduced that the development of an alternative formulation would be of prime importance. The purpose of this study in part was also to prepare microspheres formulation for Paclitaxel that could be administered systemically which would lead to enhancement of the therapeutic index of the drug and to reduce the side effects associated with the current formulation. Also Paclitaxel has been recently shown to possess potent anti-angiogenic activity against CAM model, and therefore, its targeted delivery to a tumor via Paclitaxel loaded microspheres may have the potential of inhibiting blood vessel growth into tumors, preventing them from increasing in size and also to provide cytotoxic effect to the tumor cells themselves (Wang et al., 1997).

The encapsulation efficiency achieved for Paclitaxel with the modified method was approximately 90%, which was consistent with earlier reports of Paclitaxel formulated in a polymeric delivery system. This high encapsulation efficiency is attributed to the lipophilic

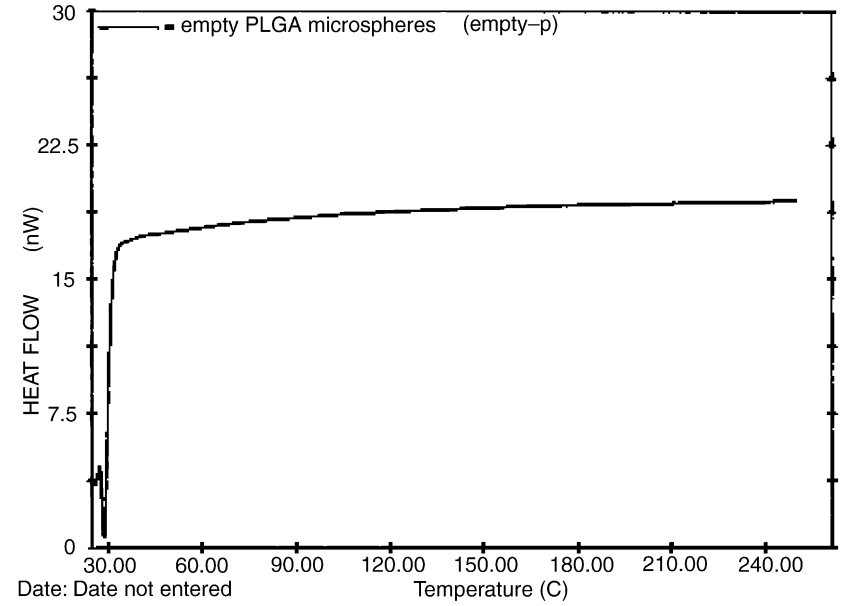


(a)



(b)

Fig. 8. DSC thermographs of (a) pure Paclitaxel, (b) pure 5-FU, (c) empty PLGA, (d) Paclitaxel, (e) Paclitaxel + 5−FU microspheres, (f) 5-FU microspheres.



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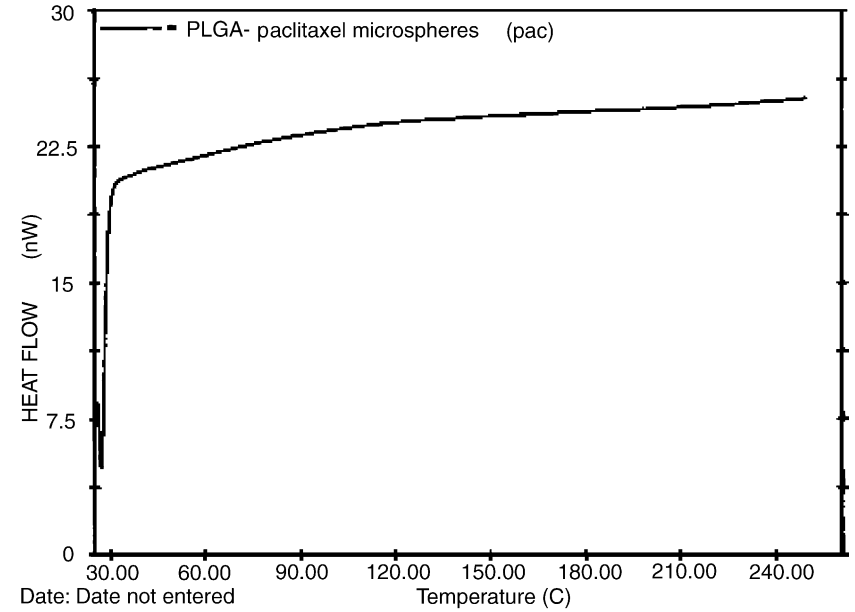
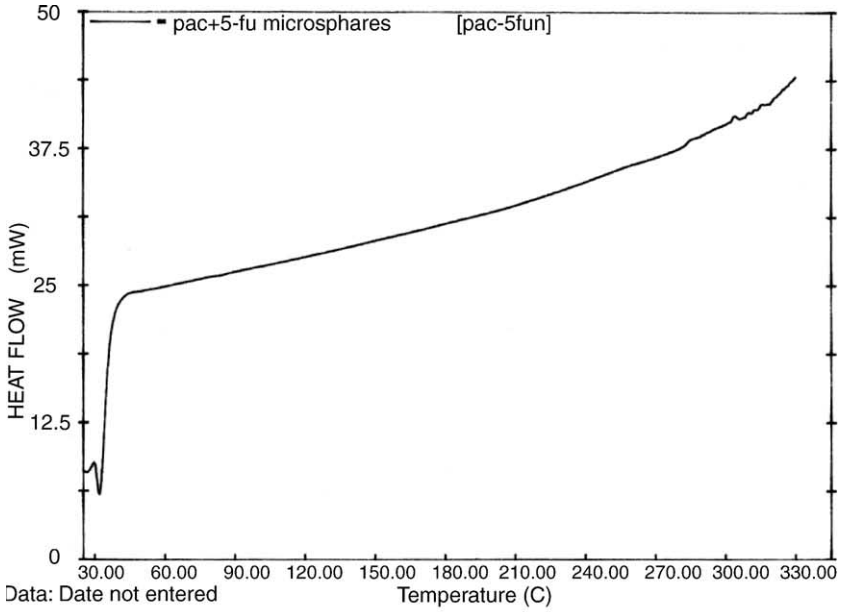
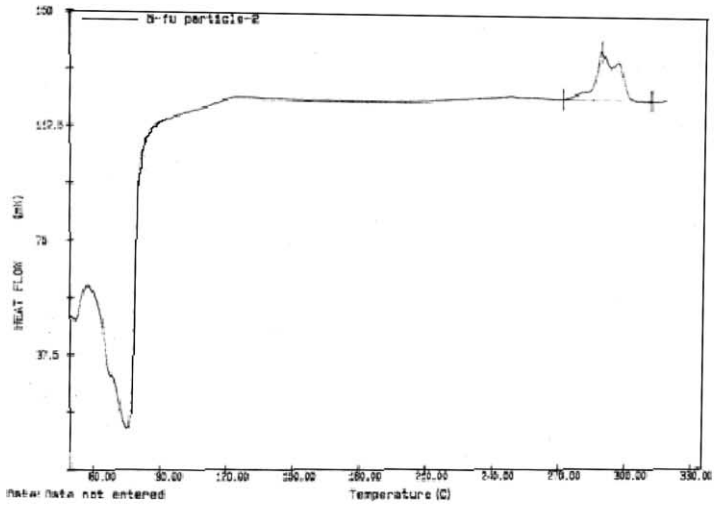


(d)

Fig. 8. (*Continued*)



(e)



(f)

Fig. 8. (*Continued*).

nature of Paclitaxel that allows it to be retained in the organic phase as the microspheres solidify (Liggins et al., 2000). Also 30% encapsulation efficiency was achieved for 5-FU, which was greater than that in our previous studies with 5-FU, alone (19%). This relatively high 5-FU loading could be in part due to the formation of a physical blend with the highly lipophilic drug, Paclitaxel. The microspheres prepared with the present modified method had a mean diameter of 2.5 μm with narrow particle size distribution

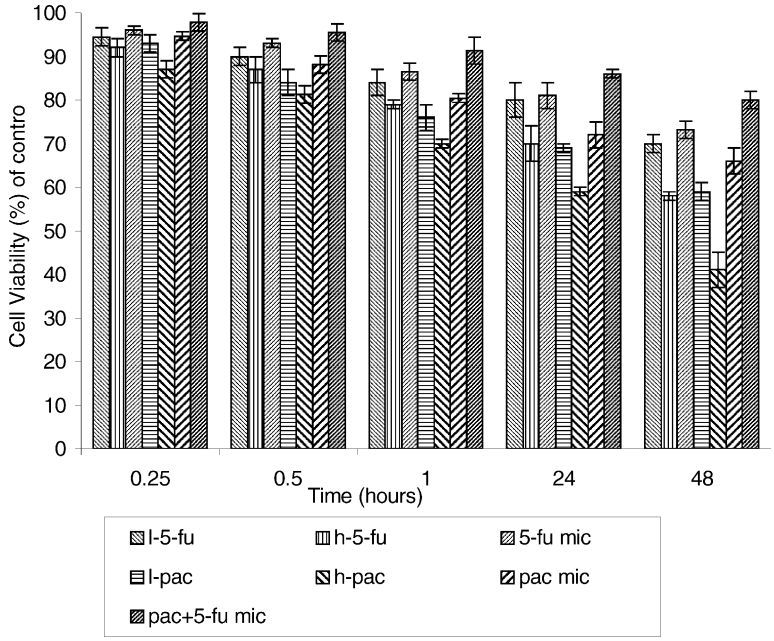


Fig. 9. Cytotoxicity of free Paclitaxel, 5-FU and Paclitaxel or Paclitaxel + 5FU loaded microspheres in MDA-MB 435 S. Cytotoxicity studies were conducted using the Cell Titre-Glo^{TM} luminescent cell viability assay as explained in Section 2.2 ($n = 3$).

and had spherical shape with smooth surface. There was an initial release of 10% from the Paclitaxel microspheres for the first 2 days, which was later followed by a constant release of the drug from the polymer matrix for 13 days. Similar release patterns have been previously reported relating to the release of Paclitaxel from other PLGA polymeric systems (Wang et al., 1997; Feng and Huang, 2001). For the combination (Paclitaxel + 5−FU) microspheres the initial release of Paclitaxel and 5-FU was 12 and 18%, respectively, which could be explained with the release of loosely bound drug on the microsphere surface and the solubility of 5-FU in the aqueous release media. Also as described by Fonseca et al. (2002) the initial release of the drug from microspheres may be due to the dissolution of the drug that was poorly entrapped in the polymer matrix, while slower and continuous release is generally attributed to the diffusion of the drug localized in the PLGA core of the microspheres. For the cytotoxicity assay, rapid reduction of cell viability of MDA-MB 435 S cells was observed when incubated with a high concentration of free Paclitaxel and free 5-FU, the cell growth being reduced to ∼41 and ∼58% after 48 h incubation, respectively. On the other hand a lower Paclitaxel and 5-FU concentration caused a lesser reduction in cell viability of ∼55 and ∼70% for 48 h, respectively. This result also goes on to prove the well known higher cytotoxicity of Paclitaxel over 5-FU at varied concentration ranges.

On the other hand, Paclitaxel or 5-FU alone microspheres resulted in the reduction of cell viability that was less than both the previously described lower and higher concentrations of free drugs. While the combination microspheres resulted in the least reduction in cell viability compared to the free drugs, the Paclitaxel or 5-FU microspheres, which suggested that the combination of Paclitaxel and 5-FU leads to reduction in cytotoxic activity as compared to the single drug formulations. The results demonstrated that careful consideration or experimental evaluation is required when combining anti-neoplastic drugs that exert their cytotoxic action at different phases of cell cycle. This antagonistic relationship between Paclitaxel and 5-FU arises due to the G1-S arresting agents such as 5-FU prevents the majority of cells from progressing to the G2-M phase of cell cycle, where the anti-mitotic agents such as Paclitaxel are known to exert their greatest cytotoxic effect (Johnson et al., 1999).

Thus, incorporation of Paclitaxel in microspheres resulted in a sustained release of drug. Microspheres would protect the drug from hydrolysis and epimerization during its passage to the tumor site. There are several ways to administer microspheres including intravascular, oral as well as subcutaneous injections. We think the main challenge for administering Paclitaxel or 5-FU microparticles into the vascular compartment is the targeting of these drugs to the distant tumor sites, allowing a site selective action of these drugs. As with all foreign colloidal particles, microspheres are quickly taken up by cells of the mononuclear phagocytic system (MPS), chiefly macrophages in liver and spleen and quickly removed from blood circulation. Several strategies have been developed to achieve the targeting to sites other than the MPS such as specific type of coating on the surface of microspheres can result in prolonged circulation time without being trapped by the MPS organs or attaching anti-bodies to the microparticle surface can also facilitate their target to specific sites.

Based on these results it can also be concluded that microsphere formulation could be considered useful alternative formulation for the systemic Paclitaxel delivery in the treatment of various cancers including metastatic breast cancer. At best the results obtained from this study were encouraging for continued research into development of microspheres as carriers of Paclitaxel.

Acknowledgements

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