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Research Paper

Design of Estradiol Loaded PLGA Nanoparticulate Formulations: A Potential Oral Delivery System for Hormone Therapy

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Abstract. Estradiol (E2), a highly lipophilic molecule with good oral absorption but poor oral bioavailability, was incorporated into poly(lactide-*co*-glycolide) (PLGA) nanoparticles to improve its oral bioavailability. Nanoparticles were prepared by using polyvinyl alcohol (PVA) or didodecyldimethylammonium bromide (DMAB) as stabilizer, leading to negatively (size 410.9 ± 39.4 nm) and positively (size 148.3 ± 10.7 nm) charged particles, respectively. Both preparations showed near zero order release *in vitro* with about 95% drug being released within 45 and 31 days for PVA and DMAB, respectively. *In situ* intestinal uptake studies in male Sprague–Dawley (SD) rats showed higher uptake of DMAB stabilized nanoparticles. Following oral administration to male SD rats, E2 could be detected in blood for 7 and 2 days from DMAB and PVA stabilized nanoparticles, respectively. Histopathological examination and blood counts indicated the absence of inflammatory response. These data suggest that DMAB stabilized PLGA nanoparticles have great potential as carriers for oral delivery of estradiol.

KEY WORDS: absorption; blood levels; DMAB; estradiol; nanoparticles; oral administration; PLGA; PVA.

INTRODUCTION

Estrogens are endogenous substances with a variety of effects on the human body, including growth and maintenance of the female reproductive system and female sex characteristics (1). The primary source of estrogen in normally cycling adult women is the ovarian follicle (2). Estradiol (E2) is the principal estrogen and is substantially more potent than estrone or estriol (its major metabolites) at the receptor and is prescribed for moderate to severe menopausal symptoms (3) and prevention of postmenopausal osteoporosis (4). Hormone replacement therapy (HRT) using E2 alone or in combination with progestins is prescribed to postmenopausal women for prevention of osteoporosis. It has been demonstrated that short-term treatment with pregnancy levels of E2 with or without progesterone is highly effective in decreasing the incidence of mammary cancer (5). On the other hand, estrogens have also been reported to increase the risk of endometrial cancer. This risk seems to depend on the dose and the length of time for which this drug is used. Hence, when long-term estrogen therapy is indicated, the lowest effective dose should be used (2).

Oral administration is the most convenient and commonly employed route for delivery of pharmaceuticals. However, many drugs are poorly bioavailable when administered by this route. Estradiol is one such compound that has good oral absorption but low bioavailability due to extensive intestinal and hepatic first pass metabolism. Hence the oral dose required exceeds many times those required extraintestinally leading to undesired side effects. An increased level of metabolite circulation in the blood is responsible for the side effects related to this drug. Besides, it is almost insoluble in water and only sparingly soluble in vegetable oils (Merck Index). Nonoral delivery of E2 has been successful because of the avoidance of first pass metabolism. Of the non-oral routes, transdermal route of administration of E2 is the most widely accepted. However, the physical size of transdermal patches and skin irritation lead to reduced patient compliance (6).

The inherent shortcomings of conventional drug delivery and the potential of nanoparticles as drug delivery systems have offered tremendous scope for researchers in this field and is moving from concept to reality. Nanoparticles may be used for oral administration of gut-labile drugs or those with low aqueous solubility (7). These colloidal carriers have the ability to cross the mucosal barrier as such. In addition to the potential for enhancing drug bioavailability via particle uptake mechanisms, nanoparticulate oral delivery systems also have slower transit times than larger dosage forms increasing the local concentration gradient across absorptive cells, thereby enhancing local and systemic delivery of both free and bound drugs across the gut. These colloidal carriers are expected to develop adhesive interactions within the

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mucosa and remain in the gastrointestinal tract, while protecting the entrapped drug from enzymatic degradation, until the release of the loaded drug or their absorption in an intact particulate form (8). Most evidence suggests that the favored site for uptake is the Peyer's patches (PP) lymphoepithelial M cell. It has been shown that microparticles remain in the Peyer's patches, whereas nanoparticles disseminate systemically. Nanoparticles bind to the apical membrane of the M cells, followed by rapid internalization and shuttling to lymphocytes wherein size and surface charge play a crucial role for their uptake (9), and these properties are themselves influenced by the stabilizer used.

Appropriate selection of the polymeric matrix is necessary in order to develop a successful nanoparticulate delivery system. Biodegradable polymers have received much attention in recent years (10). Poly(lactide-*co*-glycolide) (PLGA) has been most extensively used because of its biocompatibility and biodegradability with degradation products formed at a slow rate, thus not affecting the normal cell function. PLGA degrades *in vivo* to lactic and glycolic acids, which are subsequently eliminated as carbon dioxide and water via the Krebs cycle (11). The release of drug from the nanoparticles depends on polymer degradation, which is governed by the nature of copolymer composition and its molecular weight. For this study, we used PLGA 50:50, which is known to hydrolyze at a faster rate than those containing a higher proportion of polylactic acid (12). This article reports the design of biodegradable nanoparticles containing estradiol for oral delivery.

MATERIALS AND METHODS

Materials

Poly(lactide-*co*-glycolide) (PLGA) (Resomer RG 50:50 H; inherent viscosity 0.45 dL/g) and estradiol were gift samples from Boehringer Ingelheim (Ingelheim, Germany) and Orion Pharma (Espoo, Finland), respectively. Didodecyldimethylammonium bromide (DMAB) and polyvinyl alcohol (PVA; molecular weight = 30,000–70,000) were purchased from Aldrich (St. Louis, MO, USA) and Sigma (St. Louis, MO, USA), respectively. Ethyl acetate (AR grade) and acetonitrile (ACN; HPLC grade) were purchased from Rankem Fine Chemicals (New Delhi, India). Ultrapure water (SG Water Purification System, Barsbuttel, Germany) was used for all the experiments.

Method of Preparation of PLGA Nanoparticles

A modified emulsion–diffusion–evaporation method was used to make the nanoparticles. In brief, 50 mg of PLGA (50:50) was dissolved in 2.5 mL of ethyl acetate (EA) at room temperature for 2 h. The organic phase was then emulsified with 5 mL of an aqueous phase containing stabilizer. The resulting o/w emulsion was stirred at room temperature for 3 h before homogenizing at 15,000 rpm for 5 min with the use of a high-speed homogenizer (Polytron PT4000; Polytron Kinematica, Lucerne, Switzerland). To this emulsion, water was added with constant stirring on a water bath set at 40°C to remove the organic solvent, leading to nanoprecipitation (13). Drug loaded nanoparticles were prepared by dissolving the drug in a minimum volume of ethyl acetate and adding to polymeric solution following the same method.

Particle Size and Zeta Potential of Nanoparticles

The size of nanoparticles was determined by dynamic light scattering (Nano ZS, Malvern Instruments, Malvern, UK), taking the average of five measurements, whereas zeta potential was estimated on the basis of electrophoretic mobility under an electric field, as an average of 30 measurements.

Entrapment Efficiency

The percentage of drug incorporated into the nanoparticles was determined by centrifuging the drug-loaded nanoparticles and separating the supernatant. The pellet was washed twice with water and once with 15 mL of 5% methanol. The drug was analyzed using Shimadzu high-performance liquid chromatography (HPLC) system consisting of SPD-10 AVP Shimadzu Ultraviolet–Visible (UV–VS) spectroscopic detector and Merck LiChoCART® 100 RP-18 end-capped 5 μm column (Merck, Darmstadt, Germany). Acetonitrile/water (65:35) was used as the mobile phase with a flow rate of 0.5 mL/min. The injection volume was 15 μL and the retention time of E2 was 6.5 ± 0.5 min. The detection wavelength (λ_{max}) for E2 was 281 nm. E2 was dissolved in acetonitrile (HPLC grade) and diluted in appropriate concentrations as standard solutions (6). The peak areas correlated linearly with E2 concentrations in the range 0.5–10 μg/mL. the correlation coefficient varied in the range of 0.9997–0.9999 with an average of 0.9997.

Effect of Variables on Size and Entrapment Efficiency

Effect of Variation in Stabilizer Concentration on Particle Size and Entrapment Efficiency

DMAB, in four different concentrations (0.5, 1, 2, and 3% w/v of the aqueous phase) was used to optimize the stabilizer concentration required to obtain a stable particulate preparation. The effect of stabilizer concentration on the particle size, zeta potential, and drug encapsulation efficiency was evaluated.

Effect of Initial Drug Loading on Particle Size and Encapsulation Efficiency

Nanoparticles were prepared by varying the amount of initial drug loading. The initial amount of drug used for the formulations was 5, 10, 15, and 20% w/w of polymer to evaluate its effect on particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency.

In Vitro Drug Release Studies

The release of E2 from nanoparticles was determined by dialysis membrane method. PLGA nanoparticles (corresponding to 1 mg drug encapsulation) were redispersed in 1 mL of phosphate buffer in dialysis bags (Sigma) with a molecular mass cutoff of 12,000 Da. The bags were suspended in 5 mL of 10 mM phosphate buffer (pH 7.4) at

37°C and stirred using magnetic bead at 100 rpm. The release medium was completely replaced with fresh medium at 1, 6, and 12 h on the first day, followed by sampling at a 12-h interval daily. The amount of E2 released in the medium was evaluated using the HPLC method at λ_{max} = 281 nm.

In Situ Uptake Studies

In situ uptake studies were carried out on male Sprague–Dawley rats weighing between 300 and 360 g. All animal experiments were performed according to a protocol duly approved by the Institutional animal ethics committee (IAEC) of NIPER. The rats were fasted for 6 h and anesthetized by injecting Thiopental (50 mg/kg body weight). A midline incision was made and an intestinal segment of jejunum (length about 15 cm) was selected as the area for carrying out the study and separated from the rest of the intestine by ligating the distal end with thread. The drug suspension was made by dispersing the drug in 0.05% DMSO in water to obtain a concentration of 1 mg/mL. Drug loaded nanoparticles with DMAB and PVA as stabilizer and drug suspension, containing 1 mg drug each, were injected with the help of syringe, after which the proximal portion of intestine was ligated with thread. The segment of jejunum was excised after 2 h and the intestinal content was washed with methanol followed by washing with water. The intestinal contents were centrifuged and the supernatant was evaluated for drug content. The uptake was calculated by evaluating the amount of drug remaining to be absorbed.

IN VIVO DRUG RELEASE STUDIES

For *in vivo* studies, male Sprague–Dawley rats weighing 200–350 g were used. Nanoparticle suspension was administered orally over a period of 10 s by oral gavage needle. The dose administered was 3 mg/kg body weight of the animal. Blood samples were collected (500 μL) from the retroorbital plexus under mild ether anesthesia at 0, 0.5, 1, 2, 4, 6, 12, and every 24 h for 7 days in heparinized microcentrifuge tubes (50 units heparin/mL of blood). After each sampling, 1 mL of dextrose-normal saline and ringer lactate was administered to prevent changes in central compartment volume and electrolytes. Plasma was separated by centrifuging the blood samples at 10,000×*g* for 15 min at 4°C. To 200 μL of plasma, 800 μL of methanol was added to precipitate proteins and 60 μL of 10 μg/mL of internal standard Fluorescein sodium (Sigma) was added. The samples were vortexed and centrifuged at 15,000×*g* for 15 min. The supernatant was separated and vacuum-dried in Maxi Dry Plus (Heto Lab Equipment, Germany) for 6 h. The residue was reconstituted in 120 μL of ACN/water (50:50) and vortexed for 10 min, after which they were centrifuged at 20,000×*g* for 15 min. The clear supernatant was evaluated for drug concentration by HPLC using an injection volume of 100 μL utilizing a fluorescence detector (Multi λ Fluorescence Detector, 2475, Separation Module, 2695, Alliance; Waters, USA) with an excitation wavelength of 268 nm and an emission wavelength of 303 nm. The fraction of a sample remaining in the sample holder was diluted (500 times with water) and the fluorescence intensity of Fluorescein sodium was evaluated at emission λ_{max} = 519 nm. The peak area ratio of E2 to internal standard was determined. The regression line of peak area ratios vs. standard concentrations was calculated, and the concentrations in the unknown sample were estimated from the regression line. The peak areas correlated linearly with E2 concentrations in the range 2–500 ng/mL. The correlation coefficient had an average of 0.9866 with a percentage recovery of 98.45 ± 4.83%.

The efficiency of nanoparticle formulation was evaluated by oral administration of pure drug and measuring the blood levels at 15, 30, 45 min, 1, 2, 4, 6, and 8 h. The drug suspension was made by dispersing the drug in 0.05% DMSO in water to obtain a concentration of 1 mg/mL. The same dose of drug was administered by intravenous route. Because the drug has good solubility in alcohol, for intravenous administration E2 was dissolved in 40% ethanol (14) to obtain a concentration of 1 mg/mL and the blood levels were monitored over a time period of 15, 30, 45 min, 1, 2, 4, 6, and 8 h. In the case of intravenous administration of pure drug, the solution was administered by femoral vein cannulation and blood was withdrawn by femoral artery cannulation. The surgeries were carried out under anesthesia by intraperitoneal administration of Thiopental (50 mg/kg body weight).

After the oral administration of nanoparticles, the red and white blood cell (WBC) counts were monitored for a week.

Histopathological Studies

After 2 h, 24 h, and 7 days of administration of nanoparticles, animals were sacrificed and tissue sections collected. Segments from jejunum, ileum, and colon were isolated and fixed in 10% formal saline. Liver and spleen were also excised and fixed in 10% formal saline. The tissues were processed for light microscopy. All the tissues were first dehydrated by placing them in increasing concentration of absolute alcohol and xylene. The anhydrous tissue samples were then embedded in the paraffin blocks. Sections (5 μm thick) of the tissue were cut using a microtome and processed for hydration and final staining was performed with hematoxylin and eosin. The final section was mounted on the microscopic slide and coverslip was fixed with DPX solution. These sections were observed under high-magnification (100×/450×/630×) light microscope to check the histopathological changes.

Statistics

Statistical difference between treatment groups was evaluated via one-way analysis of variance (ANOVA) using SigmaStat 2.0 software (Jindal Scientific); a value of $p < 0.05$ was considered significant difference.

RESULTS AND DISCUSSION

Preparation of PLGA Nanoparticles

PLGA nanoparticles for gene/drug delivery are routinely prepared by emulsion–solvent–evaporation technique employing PVA as stabilizer (15). Recently, a new method, emulsion–diffusion–evaporation, a slight modification of an existing method, was reported using the PVA–chitosan blend

as the stabilizer for nanoparticles intended for gene delivery (13,16). This method was adopted with suitable modifications to design E2-loaded particles for oral delivery. DMAB is an infrequently used surfactant (17) that gives smaller particles than those obtained with PVA, and its concentration was optimized for the purpose. The dispersion of the solvent due to stirring results in irregular-sized globules in equilibrium with continuous phase. Homogenization resulted in reduction in size of globules and addition of water destabilizes the equilibrium to force the organic solvent to diffuse to the continuous phase. This led to nanoprecipitation resulting in the formation of nanoparticles. Stirring at 40°C for 3 h ensured complete evaporation of organic phase. Thus obtained particles had smooth surfaces and were spherical in shape as observed via atomic force microscopy (AFM). Drug loading did not change the surface appearance or shape of the nanoparticles (Fig. 1).

Effect of Variables on Size and Entrapment Efficiency

For drug carriers, the knowledge of the amount of drug associated to the system as well as the characterization of the drug release is of major importance. Drug content of nanoparticles is difficult to evaluate because of their small size. Drug entrapment can either be determined by centrifugation or by ultrafiltration/centrifugation (18).

Effect of Variation in Stabilizer Concentration on Particle Size and Drug Loading

Nanoparticles, because of their small size, have large surface energies, which drives the system toward aggregation. A stabilizer is required for nanoparticulate systems to prevent coalescence and formation of agglomerates during and after the emulsification process. The adsorption of stabilizers at the interface prevents this coalescence by lowering the interfacial tension and the energy of the system (19). The amount of stabilizer used also has an effect on the properties of nanoparticles. If the concentration of stabilizer is too low, aggregation of the polymer will take place, whereas, if too much stabilizer is used, drug incorporation could be reduced as a result of the interaction between the drug and stabilizer (20). The type of stabilizer affects bioadhesion, uptake, and drug release to a great extent (21,22). DMAB is a double-chain compound belonging to the class of cationic quaternary ammonium surfactants. However, DMAB did not show any concentration-dependent encapsulation efficiency and particle size variation. The formulations with 1 and 2% w/v DMAB (in aqueous phase) as stabilizer showed no statistically significant difference in terms of particle size and encapsulation efficiency within 95% confidence interval (Student's t test). The encapsulation efficiency of nanoparticles using 1 and 2% w/v DMAB was about 70% for initial drug loading of 10% w/w of polymer.

The zeta potential of formulation with 2% w/v DMAB was significantly higher compared with 1% DMAB (p = 0.025). The stabilizer concentration with DMAB was optimized at 1% w/v because, with lower concentration, reproducibility was compromised and a higher concentration did not give any significant advantage in terms of particle size and encapsulation efficiency (Table I); therefore, to maintain low stabilizer concentration without affecting the particle size or drug encapsulation, nanoparticles were subsequently made using 1% DMAB. The type of stabilizer was found to have a major role in determining the particle size and zeta potential of PLGA nanoparticles.

Effect of Initial Drug Loading on Particle Size and Encapsulation Efficiency

The effect of initial drug loading on particle size and encapsulation efficiency was investigated by varying the drug loading from 5 to 20% w/w of polymer. It was observed that with both stabilizers there was an increase in encapsulation efficiency with increase in drug loading. When DMAB was used as the stabilizer, the encapsulation efficiency was found to increase from 57.79% (±4.73) to 72.76% (±5.19) as the drug loading increased from 5 to 20% w/w of polymer. Similarly, for particles with PVA as the stabilizer, encapsulation efficiency was increased from 46.22% (±5.45) to 62.24% (±3.94) by increasing the initial drug loading from 5 to 20% w/w of the polymer (Table II). With the increase in encapsulation efficiency, an increase in size and PDI was also observed. However, no significant change in zeta potential was observed with variation in drug loading. The increase in size may be attributed to the entrapment of drug in the

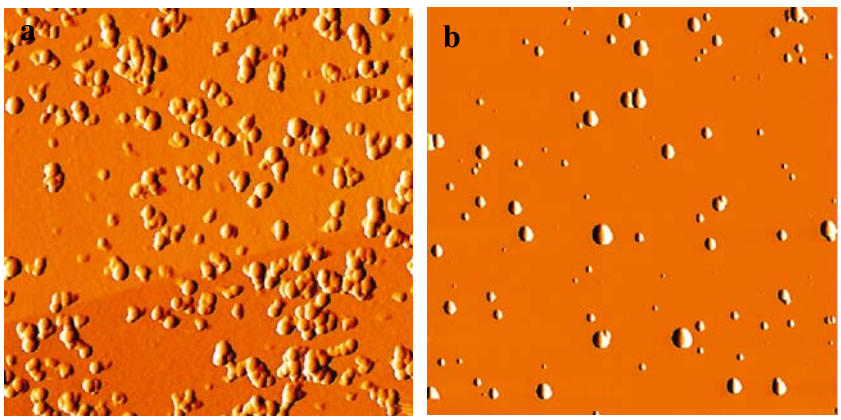

Fig. 1. AFM images of drug loaded particles stabilized using (a) didodecyldimethylammonium bromide (DMAB) (5 × 5 μm) and (b) polyvinyl alcohol (PVA) (10 × 10 μm) showing the spherical shape of particles.

Table I. Effect of Varying Concentration of DMAB on PLGA Nanoparticles ($n = 3$)

Blank				DL				
Conc. of DMAB (% w/v)	PS	PDI	ZP	Conc. of DMAB (% w/v)	PS	PDI	ZP	% EE
0.5	155.5 ± 7.5	0.13 ± 0.03	68.3 ± 4.3	0.5	175.7 ± 3.8	0.24 ± 0.07	70.2 ± 7.3	61.85 ± 5.75
1.0	100.8 ± 10.6	0.08 ± 0.02	84.4 ± 8.3	1.0	148.3 ± 10.6	0.12 ± 0.01	75.7 ± 3.1	66.77 ± 3.30
2.0	105.2 ± 3.2	0.22 ± 0.05	73.5 ± 4.6	2.0	108.3 ± 2.6	0.17 ± 0.03	92.9 ± 4.5	72.98 ± 2.93
3.0	123.7 ± 24.5	0.41 ± 0.05	80.3 ± 2.7	3.0	128.0 ± 9.5	0.19 ± 0.03	88.7 ± 3.2	72.16 ± 5.85

PS: particle size, PDI: polydispersity index, ZP: zeta potential, DL: drug loading, % EE: percent encapsulation efficiency.
DL indicates drug loaded nanoparticles (drug loading 10% w/w of polymer). The zeta potentials reported are in the pH range 4.11–4.71. Values reported are mean ± standard deviation.

nanoparticles (23). Considering the criteria of size and the PDI of the formulations, 10% w/w initial drug loading was finalized for further studies. The most significant effect of variation in initial drug loading was on reproducibility of the particle size, with an initial amount of 10% w/w of polymer providing highest reproducibility. The noticeable effect of initial drug loading is on particle size. The DMAB stabilized particles with 20% (w/w) drug resulted in 251.7 ± 44.6 nm showing the variability of size in the three experiments, whereas with 10% the particle size from three experiments was 148.3 ± 10.7 nm, showing the highest reproducibility. On the other hand, with PVA as stabilizer wide variations in the particle size were observed for the concentrations screened.

Particle Size and Zeta Potential of PLGA Nanoparticles

Particle size is an important parameter because it has a direct relevance to the stability of the formulation. Larger particles tend to aggregate to a greater extent compared to smaller particles, thereby resulting in sedimentation. In this study, two different stabilizers were used leading to positively and negatively charged nanoparticles. The blank particles with DMAB were 100.8 ± 10.7 nm in size, whereas the particles with PVA were 262.4 ± 15.1 nm. Drug-loaded nanoparticles were larger in size compared to blank ones with both stabilizers. The drug loaded (10% w/w of polymer) particles with 1% w/v DMAB as the stabilizer led to 148.3 ± 10.7 nm with a PDI of 0.12 ± 0.01, whereas with 1% w/v PVA, the particles were 410.9 ± 39.4 nm with a PDI of 0.43 ± 0.01 (Table II). The entrapment of drug slightly increased the size of nanoparticles but the volume distribution was narrow, as has been observed by Govender *et al.* (23). It was observed that nanoparticles prepared with DMAB as the stabilizer were more resistant against aggregation or sedimentation compared to particles prepared with PVA as the stabilizer. This could be attributed to the pronounced positive zeta potential with DMAB as compared to the slightly negative PVA stabilized nanoparticles (Table II). The process of centrifugation used in washing of the drug loaded nanoparticles did not have any significant effect on particle size and PDI. The % yield of particles made using DMAB and PVA was found to be 88.87 and 87.78, respectively.

The zeta potential value is an important particle characteristic because it can influence particle stability as well as mucoadhesivity. In theory, more pronounced zeta potential values, either positive or negative, tend to stabilize particle suspension to a greater extent. The electrostatic repulsion between particles with like charges prevents the aggregation of the particles. Mucoadhesion, on the other hand, can be promoted by positive zeta potential values because of the anionic nature of mucus.

Table II. Effect of Variation in Initial Drug Loading on PLGA Nanoparticles with DMAB and PVA ($n = 3$)

Drug loading (% w/w of PLGA)	Particle size (nm)	PDI	Zeta potential (mV)	% EE
		DMAB		
5%	124.1 (±4.4)	0.17 (±0.05)	70.34 (±11.28)	57.79 (±4.73)
10%	148.3 (±10.7)	0.12 (±0.01)	75.73 (±3.09)	66.77 (±3.31)
15%	150.8 (±9.1)	0.18 (±0.07)	85.24 (±2.46)	68.20 (±4.06)
20%	251.7 (±44.6)	0.29 (±0.05)	79.13 (±1.57)	72.76 (±5.19)
		PVA		
5%	348.0 (±45.4)	0.34 (±0.10)	−2.23 (±0.32)	46.22 (±5.45)
10%	410.9 (±39.4)	0.43 (±0.01)	−1.40 (±0.70)	55.12 (±3.44)
15%	458.5 (±80.4)	0.44 (±0.07)	−1.59 (±0.26)	58.60 (±1.87)
20%	654.0 (±103.5)	0.66 (±0.11)	−1.36 (±0.49)	62.24 (±3.94)

PDI: polydispersity index, % EE: percent encapsulation efficiency.
The zeta potential values reported are in the pH range 3.95–4.83 for particles with DMAB and 5.23–5.79 for particles with PVA as stabilizer. Values reported are mean ± standard deviation.

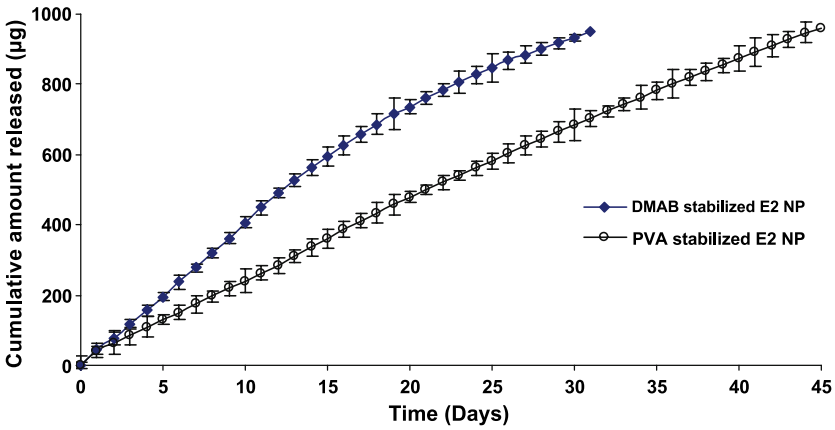

Fig. 2. *In vitro* release profiles of E2 (Estradiol) nanoparticles (NP) with DMAB and PVA as stabilizers in phosphate buffer. Both the formulations showed controlled release of estradiol.

In Vitro Drug Release Studies

Drug release from nanoparticles takes place by several mechanisms including surface and bulk erosion, disintegration, diffusion, and desorption (24). Release of drug from PLGA matrix has been found to occur by its diffusion from the polymer matrix, whereas during the later phases the release is mediated through both diffusion of therapeutic agent and degradation of polymer matrix itself. PLGA degradation occurs through a process of autocatalytic hydrolysis of ester bonds. The acidic monomers and the oligomers formed catalyze the further degradation of the polymer. Thus any factor that influences the formation or retention of the acidic monomers in the particles could affect the polymer degradation rate and *in vitro* release of the entrapped drug (25).

It is difficult to conduct an appropriate design of *in vitro* release study for nanoparticle formulations because of a number of technical problems associated with it. Sink conditions are difficult to achieve for a lipophilic drug during the whole course of the release experiment, because the sensitivity of the analytical assay usually does not allow for sufficient dilution in commonly used acceptor media. Dialysis bag diffusion, bulk-equilibrium reverse dialysis sac, ultrafiltration at low pressure, and centrifugation are some of the methods to evaluate drug release.

Most of the drug loaded nano- and microparticulate formulations show a biphasic release pattern wherein there is an initial burst release followed by a sustained release (26,27). E2 was released from PLGA nanoparticles for 1 month at a nearly constant rate. The cumulative release of E2 from nanoparticle formulations plotted against time is shown in Fig. 2. There was no initial burst effect observed with either of the formulations, which indicates a homogeneous encapsulation. The formulation with DMAB as the stabilizer was observed to release the drug at a constant rate for a period of 31 days at an average release rate of 15 µg/day with 50% of the drug releasing in 13 days. The cumulative release was fitted into different release models: zero order, first order, Higuchi's square root plot, and Hixson–Crowell cube root plot. The model giving a correlation coefficient close to unity was taken as the order of release. Zero order release pattern was observed with DMAB stabilized nanoparticles with R^2 of 0.975. The PVA stabilized nanoparticles also gave a zero order release ($R^2 = 0.9905$) for a period of 45 days. The average amount released was about 10 µg/day with 50% of the drug released from the formulation in 22 days. The formulation with PVA was found to give a slower release, which extended over 45 days as compared to formulation with DMAB where the release went below detectable levels after 31 days. The difference in initial release rates is probably attributable to the difference in encapsulation efficiencies. In the case of PVA stabilized nanoparticles, the encapsulation efficiency was less compared to the formulation with DMAB; thus, to obtain the same amount of drug more quantity of nanoparticles had to be used. For the formulation with DMAB, because of the comparatively higher encapsulation efficiency (66%), a smaller amount of the formulation had to be suspended to obtain the corresponding amount of drug for the release studies. The proportionate increase in polymer may have been reflected in controlling the release to a greater extent in the formulation with PVA as compared to DMAB formulation. The drug was found to be stable in 10 mM phosphate buffer throughout the duration of dissolution studies. Particle size is

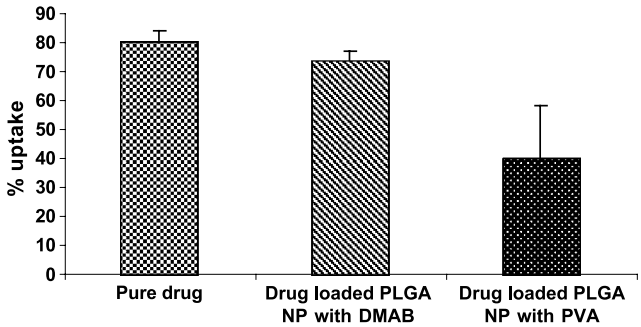

Fig. 3. The difference in uptake a dose equivalent to 1 mg of E2 (Estradiol) as pure drug, drug loaded nanoparticles with DMAB and PVA as the stabilizers. The drug loading was 10% w/w of polymer. The concentration of stabilizer was 1% w/v of aqueous phase. The results are given with standard deviation ($n = 3$–4).

an important parameter that could affect the degradation of the polymer matrix. With a decrease in particle size, surface area/volume ratio increases leading to increased buffer penetration and faster escape of drug. This may be another reason for the comparatively faster release of drug from DMAB stabilized nanoparticles as compared to PVA stabilized nanoparticles (25).

It is generally anticipated from a bulk eroding polymer such as 50:50 PLGA to give an initial burst release followed by a controlled release, in contrast to the release pattern observed. The surface eroding polymers generally give a zero order release. PLGA with a copolymer composition of 50:50 is known to have fastest degradation rate among the D,L-lactide/glycolide materials, degrading in about 50–60 days. In cases where there is an initial burst effect, the high initial release may be attributed to the presence of crystals of free and weakly bound drug on the surface of the particulate carriers. In this case, the release pattern can be explained by the dissolution of this poorly soluble drug from the polymer–drug matrix, which involves not only the erosion of the polymer entity but also the solubility of the drug into the surrounding media before its diffusion out of the dialysis membrane. The equilibrium solubility of the drug in phosphate buffer (pH 7.4) is reported to be 5.12 mg/L (about 25 μg/ 5 mL) (28). The volume of buffer media was optimized to 5 mL considering the sensitivity of the analytical method. At no sampling point was the release of drug found to be greater than 20 μg, indicating that the volume of the release media is not a factor governing the release of drug into the media.

In Situ Uptake Studies

In situ uptake studies were carried out to confirm the uptake of particles from the intestine, which is a prerequisite for orally administered colloidal delivery systems. It has been demonstrated that enhancement of electrostatic interaction between the mucosal surfaces and drugs has a significant effect on their uptake and overall bioavailability. The epithelial cells in various tissues including gastrointestinal tract carry a negative charge on their surface because of the presence of negatively charged residues of proteins in the outer membrane of the cell. Hence the epithelia are selective to positively charged solutes. It is anticipated that positively charged colloidal systems should strongly interact with cells and result in better permeability and overall bioavailability. A common conclusion drawn from several studies is that positively charged colloidal carriers increase the permeability and the potential uptake of slightly soluble drugs when compared with neutral or negatively charged ones. This behavior can also be attributed to the mucoadhesion elicited by positively charged particles with the negatively charged mucin on the mucosal surface (22). Hence cationized delivery systems have been used to overcome biological barriers and are suggested for drugs targeting such organs as the brain, eyes, nose, and inflamed intestinal epithelium (21).

Among the drug loaded nanoparticles, the absorption of those stabilized using cationic DMAB was found to be significantly higher ($p < 0.05$) in comparison to negatively charged PVA stabilized ones. The negative charge of mucin on intestinal epithelium results in higher absorption of the positively charged drug loaded nanoparticles. E2 itself has good oral absorption; however, it is poorly bioavailable because of its extensive gut wall and first pass metabolism. In our experiments, it was observed that the pure form of drug showed about 80% absorption. Estradiol nanoparticles prepared using DMAB as the stabilizer showed 70% uptake, whereas PVA stabilized nanoparticles showed 40% uptake, which is significantly less ($p < 0.05$) than the pure drug suspension and the DMAB stabilized nanoparticles (Fig. 3). The variation in the results obtained from PVA stabilized nanoparticles may be due to the PDI of the preparations. A size-dependent particle deposition in the gastrointestinal tract of healthy rats was reported (29). It has also been combined with bioadhesion depending upon the particle surface properties. The particles with small diameter were shown to have increased accumulation in the gut (30). The small size along with the positive charge could be correlated to the increased uptake of DMAB stabilized nanoparticles in comparison to the larger and negatively charged PVA stabilized nanoparticles.

In Vivo Drug Release Studies

The incorporation of E2 into PLGA nanoparticles was explored as a method to increase the oral bioavailability of this drug. The *in vivo* blood persistence properties of E2 entrapped in PLGA nanoparticles was evaluated in male Sprague–Dawley rats. Blood levels after intravenous and oral administration of pure drug were compared with those after oral administration of nanoparticle formulations using HPLC with fluorescence detector. The relevant pharmacokinetic parameters derived are listed in Table III. The mean plasma

Table III. Pharmacokinetic Parameters of E2 after Intravenous and Oral Administration of Pure Drug and Oral Administration of Nanoparticles

Administration	C_{max} (ng/mL)	T_{max} (h)	AUC (ng h/mL)
Estradiol, i.v.	–	–	1354.66 ± 347.72[a]
Estradiol only, oral	31.75 ± 7.87	1.40 ± 0.24	51.87 ± 9.13[a]
PVA NP, oral	61.33 ± 2.04*	12.80 ± 3.20*	1907.68 ± 63.37[b]
DMAB NP, oral	65.11 ± 5.35*	27.00 ± 7.55*	2541.94 ± 147.68[b]

Data are presented as mean ± SEM of three to five rats for each group.
NP: nanoparticles, C_{max}: maximum plasma concentration, T_{max}: time taken to attain C_{max}, AUC: area under the plasma concentration–time curve.
[a] $AUC_{0–6\ h}$.
[b] $AUC_{0–48\ h}$.
*$p<0.05$, in comparison to estradiol administered alone orally. Statistical significance was assessed by one-way ANOVA.

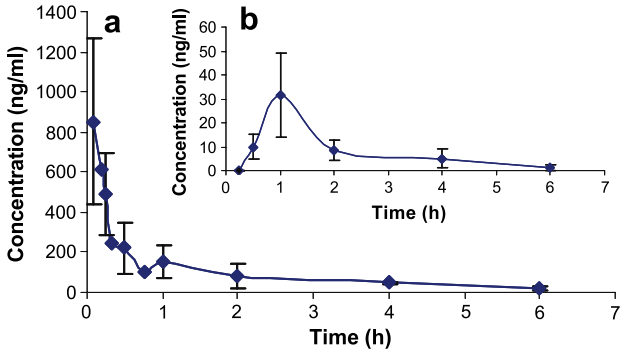

Fig. 4. Comparative *in vivo* plasma concentration vs. time profiles of E2 (3 mg/kg body weight of animal) pure drug on (a) intravenous and (b) oral administration (n = 4–5).

levels of E2 after oral and intravenous administration of pure drug solution and of orally administered nanoparticulate formulations are shown in Figs. 4 and 5, respectively.

The blood concentration–time curve showed a wide variability between the two formulations especially in the absorption phase. From the graphs obtained by plotting concentration vs. time for both formulations, it was observed that the formulation with DMAB as the stabilizer showed sustained release over a period of 7 days as against the formulation with PVA, which showed release for a period of 2 days beyond which the drug levels had fallen below the detectable limits. The results obtained revealed that i.v. pure drug solution showed initial high plasma concentrations, whereas oral pure drug suspension in 0.05% DMSO showed a C_{max} of 31.75 ± 7.87 ng/mL at T_{max} of 1.40 ± 0.24 h. In the case of nanoparticles with DMAB, the C_{max} of E2 was found to be 65.11 ± 5.35 ng/mL at T_{max} of 27.00 ± 7.55 h, whereas for nanoparticles with PVA the C_{max} was found to be 61.33 ± 2.04 ng/mL at 12.80 ± 3.20 h. There was a delay in reaching the peak concentration in the case of particles with DMAB, i.e., about 27 h, compared to the formulation with PVA where peak concentration was reached 14.2 h earlier. Blood levels in both formulations, when compared to the profile generated with orally administered pure drug, showed a significant delay in appearance of maximum concentration. This delay may be attributed to the uptake of nanoparticles by M cells into the lymphatic and systemic circulation, which involves the process of mucoadhesion and transcellular or paracellular transit through the intestinal membranes (9,31). A steep fall in blood levels was observed in oral and i.v. administration of pure drug within a period of a period of 2 h when compared to nanoparticulate formulations, indicating sustained release by the nanoparticulate formulations.

With PVA, the particles attain a negative charge. This anionic charge conferred upon the particles by PVA could have resulted in less absorption and lower blood levels. This result is also corroborated by the *in situ* uptake studies, where DMAB stabilized nanoparticles showed much higher uptake (70%), comparable to that of pure drug given orally (80%), than PVA, which showed a 40% uptake. El-Shabouri (22) reported an overall increase in relative bioavailability with Cyclosporine loaded positively charged chitosan nanoparticles by about 73% as compared to the marketed Cyclosporine formulation, Neoral®. The smaller particle size of nanoparticles with DMAB (148.3 ± 0.818 nm) compared to PVA (410.93 ± 39.42 nm) may also be largely responsible for prolonging the circulation time and sustained blood levels of E2. Larger particles are known to be readily taken up by the reticuloendothelial system (RES) cells (cells of liver and spleen) (32,29). *In situ* studies revealed good oral absorption of pure drug, but *in vivo* blood profiles indicated short circulation times and drastic fall in blood levels. With PLGA nanoparticulate formulations, the same dose of drug gave detectable blood levels for a period of 2–7 days. The positive charge of DMAB leading to the increased uptake, smaller size, and higher polymeric encapsulation of the drug

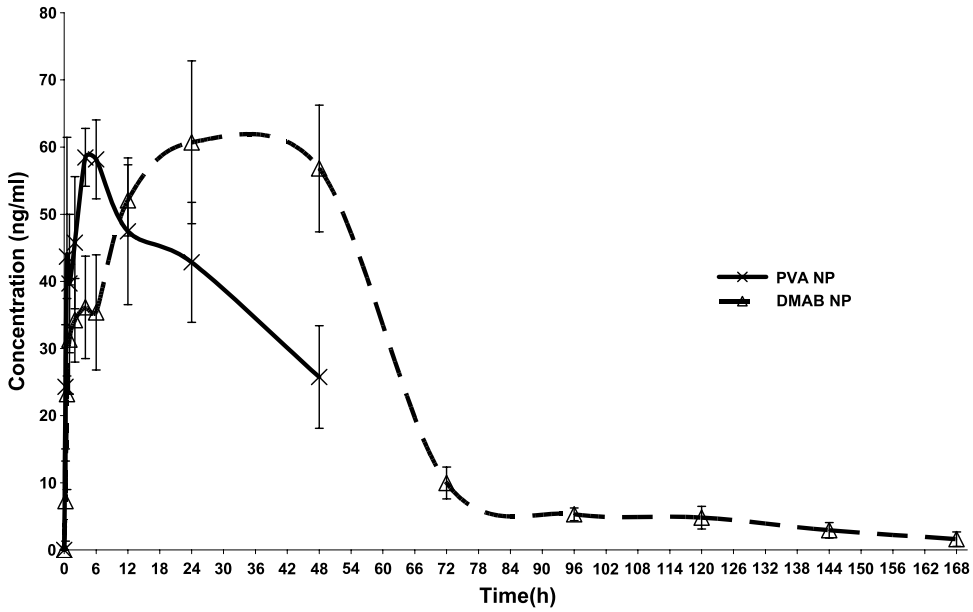

Fig. 5. Comparative *in vivo* profiles of PVA and DMAB stabilized E2 loaded nanoparticles (equivalent to 3 mg drug/kg body weight of animal) on oral administration. Blood levels of the drug was evaluated until concentrations were within the detectable limits of the analytical method (n = 4–5).

Table IV. Total Leukocyte and Erythrocyte Count of Control, DMAB Stabilized NP Treated, and PVA Stabilized NP Treated Animals (N = 1–3)

	Control		DMAB stabilized NP		PVA stabilized NP	
Samples	RBC[a] (million cells/mm^3)	WBC[b] (cells/mm^3)	RBC (million cells/mm^3)	WBC (cells/mm^3)	RBC (million cells/mm^3)	WBC (cells/mm^3)
2 h	10.36	8300	9.37 ± 0.16	6600 ± 1201.04	9.35 ± 0.66	8016.67 ± 2570.18
24 h	9.33	8350	8.18 ± 1.24	9000 ± 867.47	8.7 ± 0.28	9483.33 ± 629.15
7 days	–	–	8.30 ± 0.33	14,516.67 ± 880.81	7.36 ± 0.11	15,316.67 ± 2821.94

RBC: red BLOOD cells, WBC: white blood cells, NP: nanoparticles.
[a] Normal RBC count in healthy untreated rats 5–10 million cells /mm^3.
[b] Normal WBC count in healthy untreated rats 7000–13,000 cells/mm^3.

may be the primary factors for improving the overall bioavailability of E2. The results suggest that *in vitro* E2 release pattern from PLGA nanoparticles was significantly different from *in vivo* release, possibly because of the enzymatic reactions and biological effects in the animal body. Sandor *et al.* (33) reported differences in degradation patterns of PLGA implants between not only *in vitro* and *in vivo* conditions, but also amid different sites in the body and the observations were ascribed to enzymatic activity. Similarly for PLGA foam, the enzymatic degradation caused by trypsin (a proteolytic enzyme found in the gastrointestinal tract) was faster than hydrolytic degradation. Also, the pattern followed for porous and nonporous foams was exactly opposite to that observed with hydrolytic degradation (34). These studies indicate that the *in vitro* release rates can not be directly extrapolated to predict the behavior of these systems in a biological milieu, and differences exist for which explanations are still not very clear. Therefore, a more appropriate dissolution medium should be designed to establish a good *in vitro–in vivo* correlation. Various biorelevant dissolution media can be tried, incorporating enzymes in particular to study the *in vitro* release.

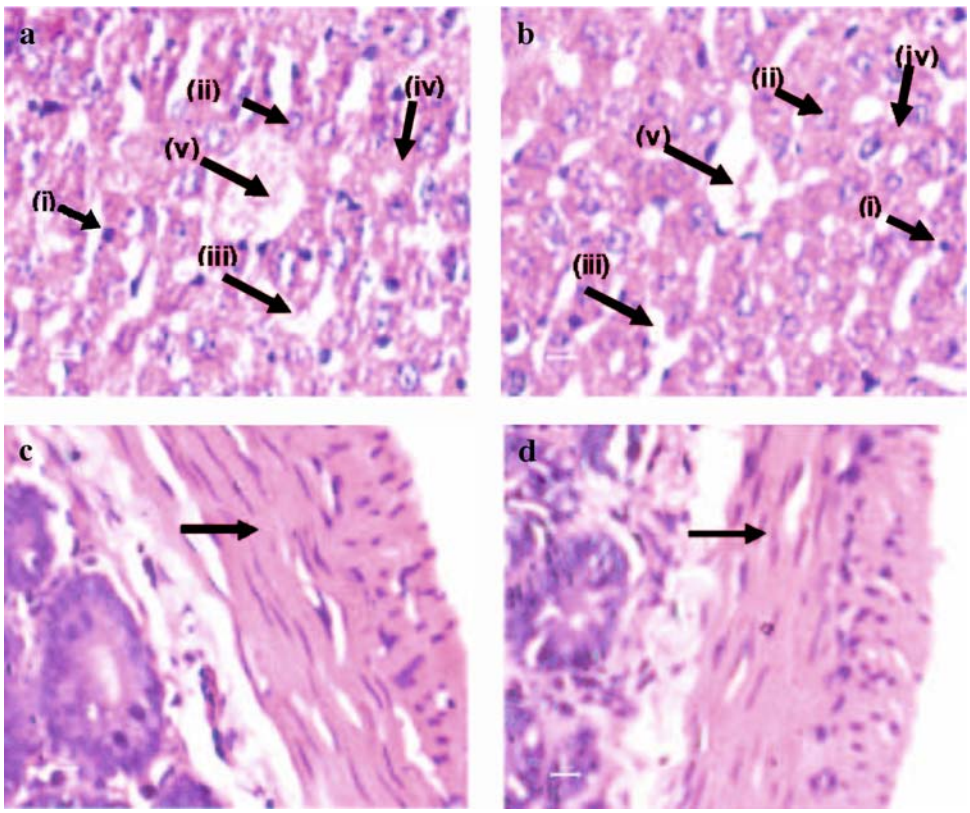

Fig. 6. Microscopic structure of sections of liver of (a) control and (b) treated animal 24 h after dosing, showing (i) Kupffer's cells, (ii) binucleate hepatocytes, (iii) sinusoids, (iv) hepatic plates, (v) central vein, and sections of jejunum of (c) control and (d) treated animal 24 h after dosing, showing the absence of inflammatory cells in intestinal muscle layer in control and treated animal, respectively. For assessing induction of inflammatory response, these histological structures were observed closely as the most likely loci of visible physical changes. The arrows highlight the absence of any markers— inflammatory cells and damage therein.

Histopathological Studies

Particulate matter is generally captured by macrophages or the mononuclear phagocytes in the body. Studies have made it evident that particle distribution in the body may influence the toxicity of the polymer drug entity. In some cases, alteration in the drug distribution profile by incorporation into nanoparticles would considerably reduce the toxicity of the drug by decreasing their accumulation in the organs where the most acute toxicity effects are exerted. For example, doxorubicin encapsulated in polyalkylcyanoacrylate (PACA) nanoparticles was found to be less cardiotoxic than free doxorubicin. In contrast, bone marrow toxicity was found to increase after linkage to PACA nanoparticles. Temporary depletion of blood opsonins was observed with PACA nanoparticles (35). Hence hemocompatibility also needs to be evaluated for hemolysis due to erythrocyte damage by nanoparticles or their degradation products, in case of intravenous administration. Histocompatibility is essential in oral, transmucosal, and regional administration. Evaluation of cytotoxicity caused by nanoparticles is important because these are phagocytosed by cells. The cytotoxicity of nanoparticles may be caused by the degradation products, membrane adhesion, stimulation of cells, and release of inflammatory mediators (36). The morphology of cells and the total leukocyte and total erythrocyte counts give insight into the inflammatory status after administration of polymeric colloidal carriers. Hence from the toxicological point of view, it was noteworthy to study the interaction between blood, tissues, and nanoparticles.

A systemic inflammation results in an alteration of the blood cell count. An increase in total leukocyte (WBCs) count is an inherent defense mechanism of the body to combat infection and xenobiotics. A comparison of the blood cell counts of control and treated animals is shown in Table IV. No significant increase in WBC count was observed in treated animals as compared to the control group. A slight decrease in red blood cell (RBC) count from 9.37 to 8.30 million cells/mm^3 with DMAB stabilized nanoparticles, and from 9.35 to 7.36 million cells/mm^3 with PVA stabilized nanoparticles over a period of 2 h to 7 days were observed. This may be attributable to the withdrawal of blood during the study period. A nominal increase in WBC counts on the seventh day may be attributable to the inflammation caused by the process of retroorbital bleeding for drawing blood samples. The overall change in the blood cell count, supported by histological examinations, indicates the absence of any inflammatory response as a result of the administration of a formulation.

Histopathological examination of the primary metabolizing organ (liver), the lymphoid tissue (spleen), and the intestinal segments (jejunum, ileum, and colon) was carried out to study the presence of any inflammation after the administration of E2 nanoparticles. Compared to the normal tissue, the liver in treated animals showed no difference in number, arrangement, or appearance of Kupffer cells, hepatocytes, hepatic plates, and sinusoids. No inflammatory cells were seen in portal triads, which are normally the first place where lymphocytes appear in case of inflammation (Fig. 6a, b). Inflammation of the spleen is rare. The spleen

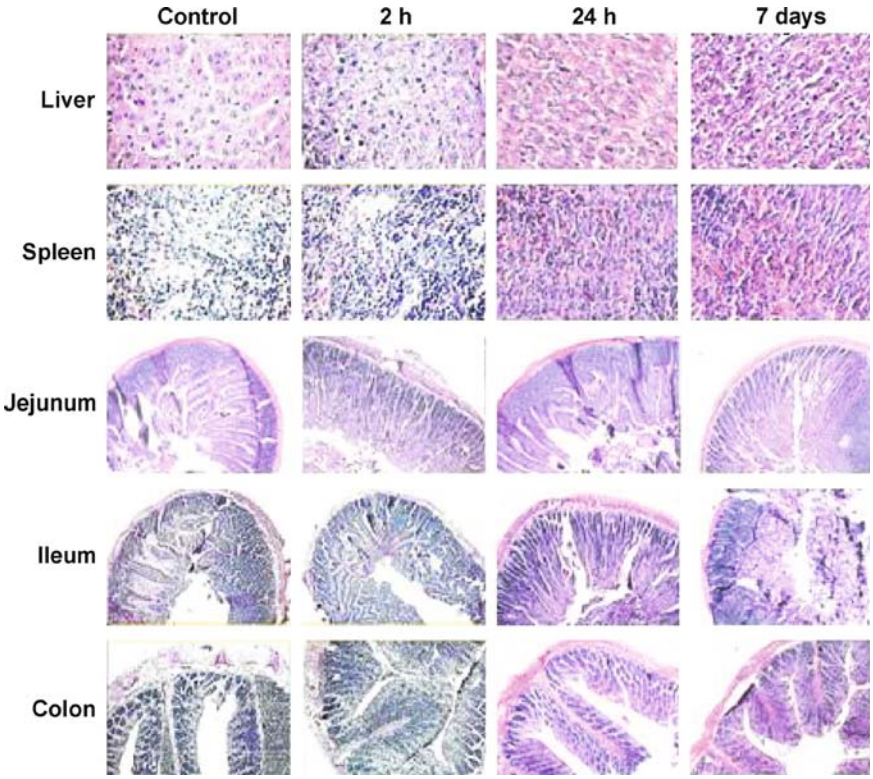

Fig. 7. Microscopic images of sections of tissues in control (untreated) and animals treated with DMAB stabilized nanoparticles 2 h, 24 h, and 7 days after dosing.

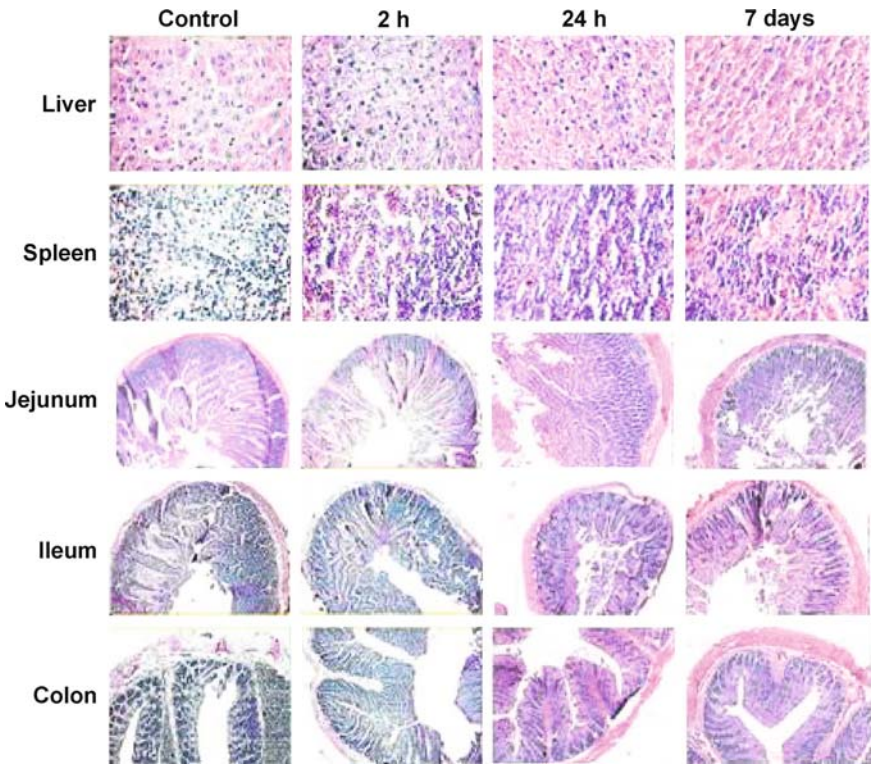

Fig. 8. Microscopic images of sections of tissues of control (untreated) and animals treated with PVA stabilized nanoparticles 2 h, 24 h, and 7 days after dosing.

histology of treated animals was no different from normal, untreated ones in terms of the lymphoid tissue mass, the sinusoids, and the number and appearance of RBCs.

In the normal intestinal segments of jejunum, ileum, and colon, the inflammatory cells (lymphocytes) are inherently present in significant numbers because the gastrointestinal tract is constantly challenged by the ingested xenobiotics, food, and microorganisms. The treated animals did not show any increase in number or distribution of these cells with respect to control. Inflammation in the intestine is normally evidenced by the appearance of inflammatory cells in the muscle layer immediately encircling the mucosa. None of the intestinal segments showed any inflammatory cells in the muscle layer (Fig. 6c, d). The mucosa was also intact and showed no signs of degeneration, suggesting that the nanoparticles did not induce damage or initiate an inflammatory response in these tissues. A comparison of healthy tissue from untreated animals with that of treated animals at different sampling points (2 h, 24 h, and 7 days) shows the absence of inflammation (Figs. 7 and 8).

CONCLUSIONS

The results presented in this work indicate that PLGA nanoparticles can be designed as potential carriers for oral delivery of E2, showing promising uptake and sustaining the release of E2 *in vivo*. *In situ* uptake studies showed higher uptake with DMAB stabilized nanoparticles than those stabilized with PVA. *In vivo* blood levels showed that nanoparticles with DMAB sustained the release of E2 for about 7 days. Histopathological examination and blood counts suggest good compatibility of this drug delivery system in animals. Together, these results indicate that DMAB stabilized PLGA nanoparticles have great potential as oral delivery systems for estradiol intended for hormone therapy.

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