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Research Paper

Design of Biodegradable Nanoparticles for Oral Delivery of Doxorubicin: *In vivo* Pharmacokinetics and Toxicity Studies in Rats

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Purpose. Doxorubicin, a potent anticancer drug associated with cardiotoxicity and low oral bioavailability, was loaded into nanoparticles with a view to improve its performance.
Methods. Doxorubicin loaded PLGA nanoparticles were prepared by a double emulsion method. The pH dependent stability of nanoparticles in simulated fluids was evaluated. DSC and XRD studies were carried out in order to ascertain the nature of doxorubicin in formulations in conjunction with accelerated stability studies. The *in vitro* release was investigated in phosphate buffer. The pharmacokinetic and toxicity studies were conducted in rats.
Results. Nanoparticles had an average size of 185 nm, with 49% entrapment at 10% w/w of polymer. The particles displayed good pH dependent stability in the pH range 1.1–7.4. DSC and XRD studies revealed the amorphous nature of doxorubicin in nanoparticles and the accelerated stability studies revealed the integrity of formulations. Initial biphasic release (20%) followed by a sustained release (80%) for 24 days was observed under *in vitro* conditions. The doxorubicin loaded nanoparticles demonstrated superior performance *in vivo* as evident by enhanced bioavailability and lower toxicity.
Conclusions. Together, the data indicates the potential of doxorubicin loaded nanoparticles for oral chemotherapy. Further, these formulations could be explored for new indications like leishmaniasis.

KEY WORDS: bioavailability; cardiotoxicity; oral delivery; oxidative stress.

INTRODUCTION

The efficacy of cancer chemotherapy is limited by the incidence of toxicity to healthy tissues, attributed by the lack of specificity exhibited by anticancer agents for cancerous cells and in part to due poor biopharmaceutical properties of the drug which together dictates the success of chemotherapy. In addition to this, most of the anticancer agents are administered as intravenous drug injections/infusions which lead to an initial rapid increase and subsequent decay of drug concentrations, below therapeutic levels in blood. It is generally believed that long-term exposure to drug at modest concentrations would be more beneficial than a pulsed supply of the drug at higher concentrations (1). Toxicity associated with these anticancer agents and their poor biopharmaceutical properties has lead to numerous attempts in developing more rational formulations for chemotherapy (2) and the most successful has been DOXIL®. Oral chemotherapy is a great challenge and its success is expected to revolutionize cancer chemotherapy. Classical anti-neoplastic drugs have been available as oral medications for some time and many of these compounds are analogues of their parenteral counterparts (3). Oral chemotherapy can maintain an optimum concentration of drug in circulation which can provide prolonged exposure to cancerous cells, which will in turn improve the efficacy and decrease the adverse effects (4,5). Advantages associated with oral chemotherapy include better patient compliance and acceptance and significant cost savings, both in terms of treatment costs and lost wages incurred by patients and family during physician visits (6). Recently, there has been a surge in the development of oral chemotherapeutic agents and many molecules are undergoing clinical trails or already approved for their oral efficacy (7).

Doxorubicin, an anthracycline antibiotic and one of the most widely used anticancer agents, shows high antitumor activity (8,9). However, its therapeutic effects are limited due to its dose dependent cardiotoxicity and myelosuppression (10,11). Indeed, nearly 2000 analogs were synthesized and evaluated; yet only few of them have reached the stage of clinical development and approval. Second generation analogs like mitoxantrone, epirubicin or idarubicin exhibited lower cardiotoxicity; but had lower efficacy compared to the parent molecule. Hence, doxorubicin becomes indispensable when it comes to cancer chemotherapy (12,13). Oral bioavail-

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ABBREVIATIONS: AUC, Area under the curve; BA, Bioavailability; Dox, Doxorubicin; EE, Entrapment Efficiency; IAEC, Institutional Animal Ethics Committee; i.v., intravenous; mV, milli volts; nm, Nanometers; NPs, Nanoparticles; PDI, Polydispersity Index; SGF, Simulated gastric fluid; SIF, Simulated intestinal fluid.

ability of doxorubicin is less because it is eliminated by the first-pass extraction of the cytochrome P450-dependent metabolic process and the over expression of the multidrug efflux pump transporter P-glycoprotein (P-gp), which is rich in the intestine, liver and kidney, thus making it difficult to administer doxorubicin *via* oral route (14,15) along with poor permeability. The general idea is to apply P-gp/P450 inhibitors such as cyclosporine A to suppress the elimination process. But these inhibitors suppress body's immune system and cause medical complications. Moreover, molecules like cyclosporine have their own side effects thus making it more difficult to incorporate them into drug delivery system along with anticancer agents (16). Advanced drug delivery strategies can offer alternatives which can circumvent the issues associated with drug's toxicity and on the other hand can lead to enhanced therapeutic performance by increasing the bioavailability of the drug. Nanoparticles prepared from biodegradable polymers seem to be a promising approach that can provide drug delivery system for oral chemotherapy with high therapeutic efficacy and better profile in terms of adverse effects (17). Moreover, the loaded drug can be released from the nanoparticles in a controlled fashion at a desired rate over a sufficiently long duration (18,19) thereby increasing the overall pay load of drug to cancerous cells. Further, the poor success rate of the drug discovery program results in exploring molecules like doxorubicin for new indications where there is no therapy, for example leishmaniasis (20), and oral formulations would certainly benefit such causes.

Therefore, we report doxorubicin loaded biodegradable poly (D,L-lactide-*co*-glycolide) (PLGA) nanoparticles for oral chemotherapy. The nanoparticles were characterized in terms of their particle size/polydispersity index (PDI), morphology, Entrapment Efficiency (EE) and zeta potential. Physical state of drug in nanoparticles was investigated using differential scanning calorimetry (DSC) and X-ray diffraction (XRD). Formulations were evaluated *in vitro* for release behavior and *in vivo* for their toxicity profiling and pharmacokinetic behavior.

MATERIALS & METHODS

Materials

Doxorubicin (purity~98.5%) was purchased from RPG Life Sciences limited Ankleshwar, India and PLGA 50:50 (intrinsic viscosity 0.35 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly-vinyl alcohol (PVA; MW 125000 Da) was purchased from S.D. Fine Chemicals India, Pluronic F-68 and Didodecyldimethylammonium bromide (DMAB) from Sigma Aldrich, US. Thiobarbituric acid (GR grade), sodium lauryl sulphate (LR grade) and acetic acid (LR grade) were purchased from Loba Chemie, India. Superoxide Dismutase and Catalase assay kits were purchased from Cayman Chemicals, USA and Fluka, USA respectively. Ultra pure water (SG Water Purification Systems, Barsbuttel, Germany) was used for all experiments. All other solvents used were of HPLC grade. All chemicals were used as received.

Nanoparticle Preparation

Due to high aqueous solubility of doxorubicin, double emulsion method was adopted for particles preparation with appropriate modifications (21). In brief, doxorubicin was dissolved in water to form the aqueous phase, which was then added to a solution of 50 mg PLGA in 2.5 ml ethyl acetate to give a w/o emulsion which was then sonicated and added dropwise under stirring to aqueous solution containing 3% PVA to form the secondary emulsion. The secondary emulsion was again sonicated to reduce the particle size and then was diluted with sufficient water to aid solvent diffusion and precipitation of the polymer resulting into formation of NPs.

Particle Size and Zeta Potential of NPs

Particle size was measured by dynamic light scattering (Nano ZS, Malvern, UK) and zeta potential was estimated on the basis of electrophoretic mobility under an electric field.

Entrapment Efficiency

Efficiency of NPs in entrapping Doxorubicin was determined by centrifugation method which involved separation of NPs from their aqueous suspension followed by drug analysis in the pellet. The freshly prepared nanoparticles were centrifuged at 30,000 rpm for 20 min and supernatant was separated from the pellet. The obtained pellet was dissolved in acetonitrile and analyzed by reversed-phase (RP) HPLC using Shimadzu HPLC system with LC software coupled to RF-10AXL fluorescence detector. The separation was achieved on the C18 column, 4.6× 250 mm, 5 μm analytical column (Merck, Germany) maintained at 30°C. Doxorubicin was isolated isocratically at flow rate of 1.2 ml/min using mobile phase consisting of methanol, acetonitrile and 10 mM acetate buffer; 45:30:25 v/v) at pH 3.0. The absorbance of the eluent was optimized for fluorimetric measurement at 470 nm (excitation) and 550 (emission).

Effect of Type/Concentration of Stabilizer and Drug Loading on Particle Properties

NPs were prepared with different stabilizers viz. PVA, DMAB and PF-68 and their effect on the particle size and entrapment efficiency was evaluated. Initial drug loading was varied (2.5, 5 and 10% w/w with respect to polymer) in the internal aqueous phase to investigate its effect on nanoparticles characteristics.

Nature of Doxorubicin in Nanoparticles using DSC and XRD

The physical state of doxorubicin loaded in PLGA NPs was investigated using DSC (DSC 821e Mettler Toledo, Switzerland) and XRD (Brooker D8, Germany). Freeze dried NPs and freshly prepared NPs were subjected to DSC and XRD studies with native Doxorubicin as control. For DSC a heating rate of 10°C/min was used whereas for X-ray diffraction the diffraction angle 2θ was recorded from 3°C to 40°C with a scanning speed of 3°C/min and copper was used as source of x-ray radiation at 40 Kv with 40 mA.

pH Dependent Stability of Nanoparticles in Simulated Fluids

When NPs are given orally, they pass through different gastrointestinal regions where they are exposed to different pH and different enzymatic conditions which can influence

their physicochemical properties and drug release behavior and can alter their stability characteristics. To test this hypothesis, doxorubicin loaded NPs were subjected to different pH media where they encountered different ionic strengths and enzymatic conditions and the change in their properties was elucidated by counter checking their particle size and polydispersity. pH dependent stability studies were carried out in following media:

1. pH 1.1: 12 ml HCl (32%)with 1188 ml H_2O
2. pH 3.5: 150 ml solution (10.5 g citric acid+100 ml NaOH (1 M)+395.5 ml H_2O) with 100 ml HCl
3. pH 7.4: 68 g NaH_2PO_4 with 15.6 g NaOH with 10 L H_2O
4. Simulated Gastric Fluid (SGF): 0.2% NaCl, Pepsin 0.7% HCl with pH 1.2
5. Simulated Intestinal Fluid (SIF): 0.685 Monobasic Potassium Phosphate, 1% NaOH and 1% Pancreatin with pH 7.4

Nine milliliters of simulated fluids were added to 1 ml of Polymeric NPs. The samples were investigated at the interval of 2 h for the pH 1.1, 3.5 and SGF while for pH 7.4 and SIF the samples were assessed after 6 h (22). The above time intervals were selected for the study based on expected formulation residence time in stomach and intestine. Particle size, PDI and Zeta potential were determined on the preset time periods.

Accelerated Stability Studies

Accelerated stability studies were carried out using freeze dried NPs as per ICH Q1AR2 guidelines meant for refrigerated product over a period of 90 days. NPs were transferred in 5 ml glass vials sealed with plastic caps and were kept in stability chamber with temperature of (25±2)°C/(60%±5%) relative humidity. Trehalose (5%) was used as cryoprotectant during the freeze drying process. The sampling time points were 30, 60 and 90 days. The formulations were monitored for changes in particle size and morphology, PDI, zeta potential, and entrapment efficiency. The physical appearance, ease of reconstitution and volume used for reconstitution were also recorded.

In vitro Drug Release from Doxorubicin Loaded PLGA Nanoparticles

The release of doxorubicin from the nanoparticles was determined by dialysis membrane method. Drug loaded NPs (corresponding to 2 mg loaded drug) were redispersed in 2 ml of phosphate buffer (pH 7.4) in dialysis bags of 12 kDa molecular weight cut-off. The bags were suspended in 5 ml of phosphate buffer (pH 7.4) at 37°C in shaking water bath at 50 rpm to simulate the peristaltic conditions *in vivo*. At predetermined intervals, aliquots of 100 µl of sample were withdrawn and estimated by HPLC to calculate the amount of drug released.

The Ability of Nanoparticles in Lowering Drug Induced Toxicity

Healthy Sprague–Dawley (SD) female rats of uniform body weight (150–170 g) with no prior drug treatment were used. The animal protocol was duly approved by the Institutional Animal Ethics Committee of NIPER, Mohali, India. All the animals were acclimatized for a week and were fed with standard rat diet, water *ad libitum* and were maintained at 12 h dark and 12 h light periods. The animals were randomly divided into six groups having 6 rats in each group. Group-I was kept as control without any treatment; Group-II &III received blank nanoparticles *via* oral and i.v. routes respectively; Group-IV & V received drug loaded nanoparticles *via* oral and drug solution *via* i.v. respectively, and Group VI received Doxorubicin-loaded nanoparticles *via* i.v. route. The treatment regimen had two doses of 4 mg/kg, one on the 1st day and other on the 15th day. At the end of 28th day animals were euthanatized and heart tissues were collected for further examination and were stored at −20°C till analysis. The body and heart weight of all the animals were recorded. Heart tissue were homogenized in phosphate buffer at pH 7.4 (five times the volume of heart weight) using tissue homogenizer at 20,000 rpm for 2 min. Homogenized heart tissue were used for estimation of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA). SOD and CAT were estimated using kits from Fluka and Cayman chemicals respectively. The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances assay (TBARS) (23).

Bioavailability Studies

The blood persistence properties of drug loaded NPs was evaluated in female SD rats weighing between 220–250 g with 3 animals per group. The animal protocol was duly approved by the Institutional Animal Ethics Committee of NIPER, Mohali, India. Doxorubicin nanoparticles (equivalent to a dose of 10 mg/kg of Doxorubicin) were redispersed in 1 ml of water and were administered orally using oral gavage needle. The relative oral bioavailability of Doxorubicin was assessed by administering the nanoparticulate formulations against Doxorubicin solution orally. The blood samples were collected (200 µl) from the retro-orbital plexus under mild ether anesthesia at 0.5, 1, 2, 6, 12 and every 12 h for 5 days in the heparinized micro-centrifuge tubes (20 units heparin/ml of blood). Blood samples were centrifuged at 10,000 rpm for separation of plasma. Briefly, methyl paraben, (dissolved in methanol), as an internal standard, and 0.2 ml of acetonitrile were added to 0.2 ml of the plasma sample. The resulting mixture was then vortex-mixed vigorously for 2 min and centrifuged at 13,000 rpm for 10 min and 0.05 ml of the supernatant was injected into the HPLC system. The detector was operated at an excitation wavelength of 254 nm with an emission cut-off filter of 360 nm for first 6 min (for methyl paraben) and then excitation wavelength of 470 nm with an emission cut-off filter of 550 nm for next 6 min (for Doxorubicin). A C18 column (4.6 mm×150 mm, 5 um,) was used at a temperature of 30°C set by HPLC column temperature controller. Mobile phase consisted of 50% acetate buffer (pH 5.0), 30% methanol and 20% acetonitrile. The flow rate was maintained at 0.8 ml/min.

RESULTS AND DISCUSSION

Nanoparticle Preparation

In emulsification–diffusion method, the stabilization of emulsion droplets and 'proto-nanoparticles' after diffusion

process are important to avoid coalescence and inhibit formation of agglomerates. When the interface is formed, the role of the stabilizer at interface is to lower the energy of the system and to hinder the coalescence of particles. Table I shows the influence of stabilizer type and concentration on the nanoparticles characteristics. The results clearly indicate that PVA was the choice among the stabilizers as it provided higher entrapment efficiency coupled with optimum particle size and polydispersity. On the contrary smaller particle size and lower entrapment efficiency were obtained with DMAB. This may be attributed to the solubility enhancement of doxorubicin in presence of DMAB which provides sink conditions due to which more number of drug molecules migrated to aqueous phase rather getting loaded in hydrophobic polymeric matrix. PF-68 on the other hand gave particles with larger diameter and polydispersity though encapsulation efficiency was appreciable.

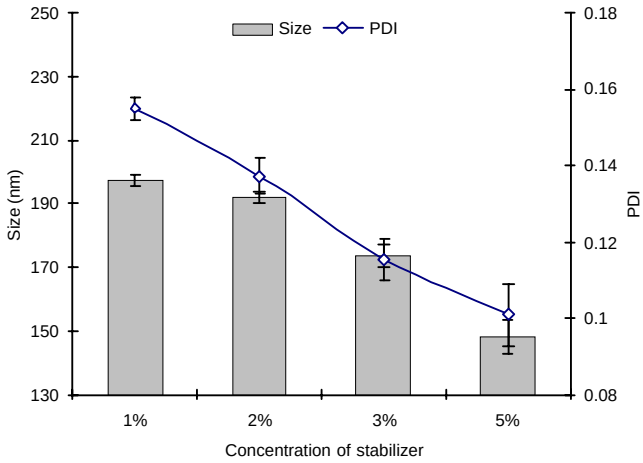

Fig. 1. Effect of PVA concentration on size and PDI of doxorubicin-loaded PLGA nanoparticles. Data represents Mean ± S.D. (n=3).

Influence of Stabilizer Concentration and Drug Loading on Nanoparticles

The effect of different concentration of PVA on particle size and PDI was also investigated. As shown in Fig. 1, an increase in PVA concentration in external aqueous phase corresponded to decrease in particle size and PDI. These results were consistent with already available literature reports (24,25). For a PVA concentration below 2.5%, the solution contains PVA single molecules and therefore the double emulsion was unstable and formed particles showing higher PDI. At a concentration higher than 2.5%, the solution showed aggregation behavior which allows droplet stabilization leading to decrease in size and PDI (25). The prepared nanoparticles were freeze dried with trehalose as the cryoprotectant and were imaged using atomic force microscopy (Fig. 2).

Initial drug loading in the internal phase was varied to investigate its effect on nanoparticles characteristics including size, PDI and entrapment efficiency. Three concentrations tested were 2.5, 5 and 10% w/w. As shown in the Fig. 3 drug loading had minimum effect on particle size and polydispersity. A minor increase in particle size can be attributed to higher drug loading. Drug loaded particles were very similar to blank particles. Entrapment efficiency of drug in nanoparticles was also found to be consistent (Fig. 4). Hence loading of 10% provided particles with optimum size, PDI and entrapment.

DSC and XRD Studies

The state of drug in nanoparticles is crucial for the stability of formulation as controlling crystallization is one of the key issues in formulating successful stable nanoparticulate formulations. Increasing the amorphous fraction of the drug increases its saturation solubility much above its crystalline form and this prevents the phenomenon of Ostwald ripening in such systems leading to their higher shelf life and long term stability as aqueous suspensions. The melting endotherm of pure doxorubicin appeared at 218°C. However, no melting peak was detected for drug-loaded PLGA nanoparticles (Fig. 5) even at higher temperatures of 290°C. It can thus be concluded that doxorubicin in nanoparticles was in amorphous or disordered crystalline phase which inhibited crystal growth thereby leading to enhanced stability of formulation. The information obtained from XR diffractograms complied with the results obtained from DSC analysis for the developed formulations (Fig. 6). The disappearance of characteristic peak of doxorubicin in NPs along with presence of halo pattern spectrum suggested that doxorubicin in the NPs was in amorphous state.

Table I. Influence of Surfactant type and Concentration on Size, PDI and EE of Doxorubicin Loaded PLGA Nanoparticles

Surfactant	Size (nm)	PDI	ZP (mv)	EE
3% PVA	176.3±1.5	0.119±0.006	2.99±0.46	49.00±2.00
3% PF-68	211.0±5.2	0.168±0.011	3.05±1.52	40.33±2.08
1% DMAB	95.4±1.2	0.126±0.021	71.21±4.23	5.12±1.02

Values reported are mean ± S.D. (n=3)

Accelerated Stability Studies

After three months of storage at accelerated conditions, nanoparticles freeze-dried with 5% trehalose were stable without any collapse or shrinkage of the dried cake (Table II). The measurement of particles size demonstrated the conservation of nanoparticle properties during the stress testing. There was also no change in the physical appearance and encapsulation efficiency. The physical separation of dry nanoparticles by bulky cryoprotectant glass could be an important factor for preventing nanoparticles aggregation in the glassy state. The glassy state is characterized by a high viscosity which lowers the mobility of molecules and thus prevents the aggregation (26,27). XRD analysis of freeze dried product along with nanoparticles after stability studies is shown in Fig. 7. It can be clearly seen that there was no recrystallization of Doxorubicin or trehalose after accelerated stability studies in presence of 60% relative humidity. Nanoparticles subjected to stability studies showed the same amorphous character as exhibited after freeze drying. This demonstrated the stable nature of nanoparticles in presence of trehalose after accelerated stability studies.

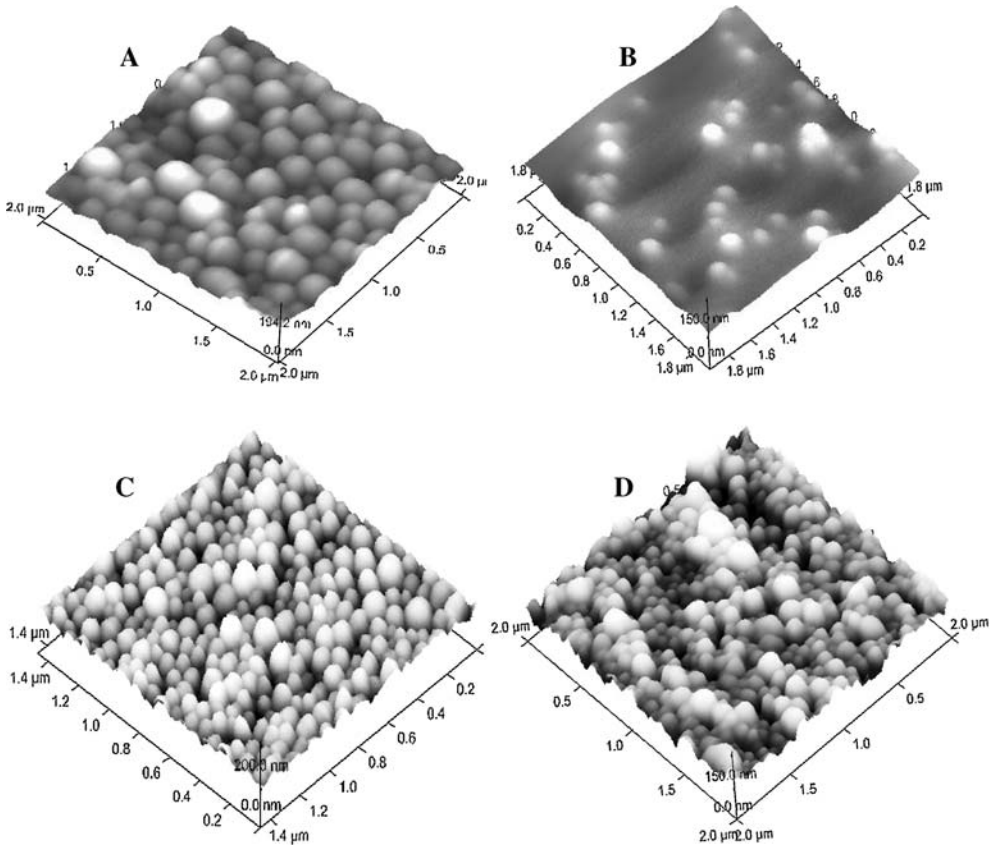

Fig. 2. Atomic force micrographs of NPs before and after freeze drying with 5% trehalose as cryoprotectant. **A** & **B** shows blank nanoparticles (with 3% PVA as surfactant) before and after freeze drying and **C** & **D** shows doxorubicin loaded nanoparticles (with 3% PVA as surfactant) before and after freeze drying. The particles display a smooth spherical topography with homogeneous size distribution and low PDI without formation of major aggregates. All images were acquired using contact mode at room temperature with 2×2 μm as scan area using Atomic force Microscope (Veeco).

pH Dependent Stability of Nanoparticles in Simulated Fluids

The GI stability of the particles was investigated by subjecting the particles to simulated GI fluids and found to be quite stable (Table III) under the studies conditions and duration. This formed an important exercise, as stable particles would show better uptake and subsequent bioavailability as aggregation would be undesirable and may compromise the uptake as well as activity.

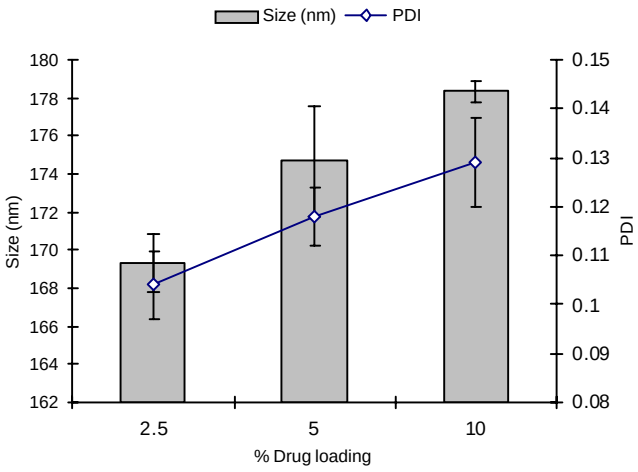

Fig. 3. Effect of drug loading on particle size and polydispersity of doxorubicin loaded PLGA nanoparticles. Data represents mean ± S.D. (n=3).

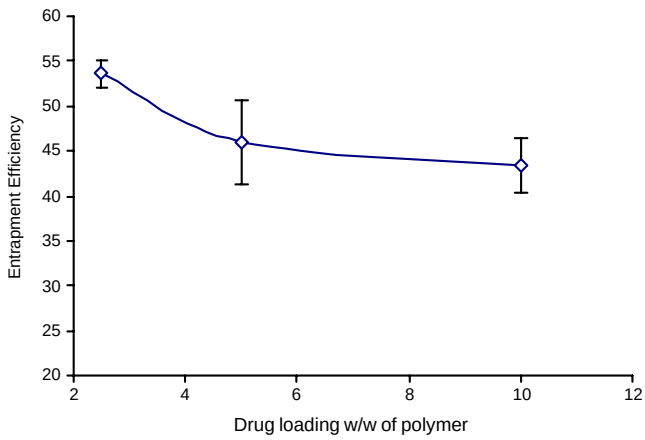

Fig. 4. Entrapment Efficiency of doxorubicin *vs.* drug loading *w*/*w* of polymer. Data represents mean ± S.D. (n=3).

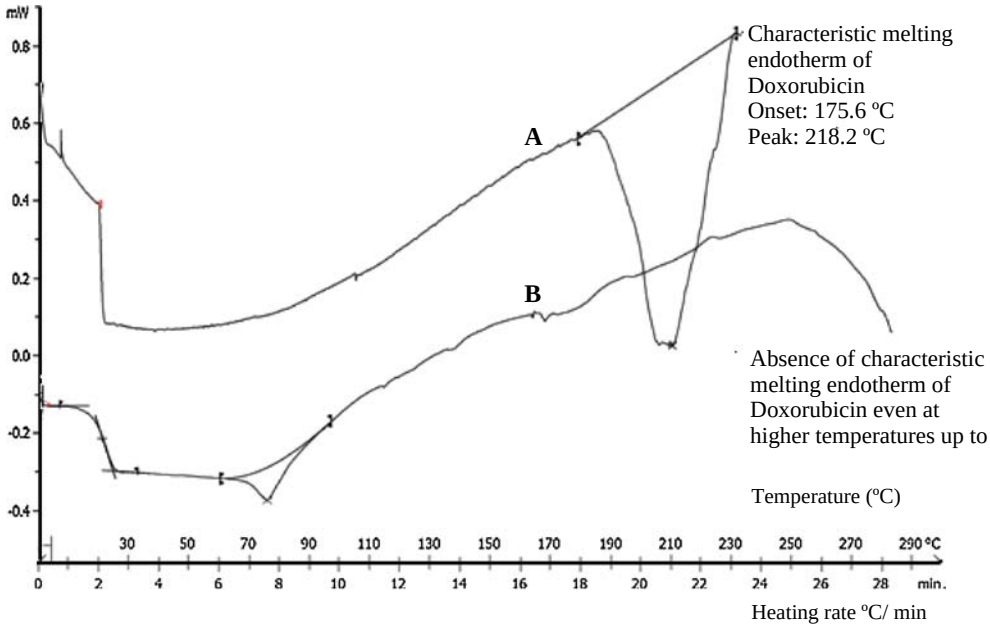

Fig. 5. DSC thermograms of freeze dried doxorubicin-loaded PLGA NPs and native doxorubicin at heating rate of 10°C/min. The melting point of doxorubicin is 218°C and absence of characteristic melting endotherm around this temperature shows that phase transformation has already occurred and doxorubicin exists in amorphous form in PLGA nanoparticles. **A** Native doxorubicin. **B** Doxorubicin loaded freeze dried nanoparticles. It can be clearly seen that even heating up to 290°C, there is no melting of doxorubicin in freeze dried nanoparticles confirming amorphous nature of drug in nanoparticles.

In Vitro Drug Release Studies

Nanoparticulate drug delivery systems are crucial where the sustained release of drug is desired for a longer time period and chronic illness like cancer forms no exception to this. One of the desired attributes of oral chemotherapy is reduced dosing frequency and accumulation of the dosed drug in the tumor tissues by enhanced permeation retention effect (EPR) that can be attained using nanoparticles. Figure 8 shows an initial rapid release, with about 20% release in first

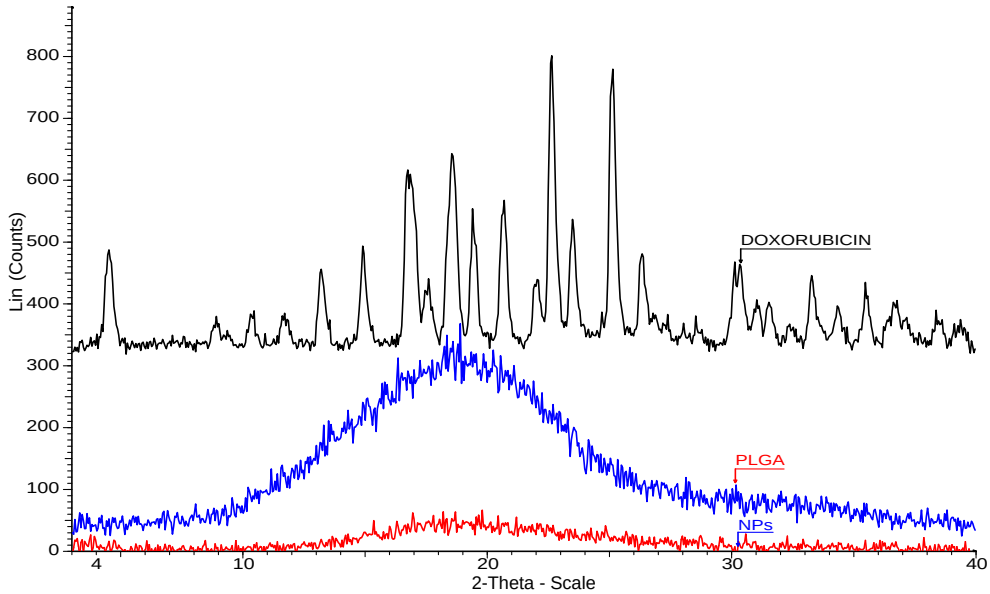

Fig. 6. XRD spectrum of doxorubicin loaded NPs with PVA as stabilizer, doxorubicin in native form and PLGA in native form. For doxorubicin clear peaks are visible in the diffractogram indicating the presence of crystalline phase in the native form whereas PLGA shows a typical amorphous pattern. Absence of peaks in the diffractograms of doxorubicin loaded PLGA nanoparticles indicates the phase transformation of crystalline doxorubicin to amorphous doxorubicin.

Table II. Characterization of Formulation after 90 Days of Accelerated Stability Studies

Parameters	Initial	Final
Particle size (nm)	205.2±4.3	207.0±5.0
PDI	0.134±0.025	0.139±0.041
Zeta-potential	−5.18±4.00	−8.77±5.06
Entrapment efficiency	46.0±2.5%	45.7±3.8%
Physical appearance	Intact cake	Intact cake
Volume for reconstitution	2 ml	2 ml
Ease of reconstitution	Simple Inversion	Simple Inversion

Values reported are mean ± S.D. (n=3)

Table III. Initial and Final Particle Size/PDI of Nanoparticles after Exposure to Simulated GIT Media

Medium	Initial size (nm)	Final size (nm)	Initial PDI	Final PDI
pH 1.1	187.0±5.0	190.7±2.8	0.126±0.050	0.171±0.051
pH 3.5	187.0±5.0	189.0±5.3	0.126±0.050	0.178±0.030
pH 7.4	187.0±5.0	192.7±3.6	0.126±0.050	0.188±0.049
SGF	187.0±5.0	193.0±2.7	0.126±0.050	0.179±0.025
SIF	187.0±5.0	191.4±4.2	0.126±0.050	0.163±0.043

Values reported are mean ± S.D. (n=3)

day followed by a slower sustained release for over 24 days. The faster release of drug was due to the faster migration of the exterior drug molecules within the particle, while the sustained release was attributed to the diffusion/ erosion of the polymeric matrix. Thus, the release mechanism was diffusion for the initial period followed by diffusion/degradation in later part.

The Ability of Nanoparticles in Lowering Drug Induced Toxicity

Body Weight

Literature reports suggest reduction in body weight as a result of doxorubicin induced toxicity, which forms the basis of this study. Table IV shows the changes in body weight and heart weight on different doxorubicin treatment. Body weights were significantly lower in all treatment groups compared to that of control, whereas in groups administered blank NP (oral and i.v.) body weights were comparable to control suggesting the effect was due to doxorubicin treatment. Moreover, the body weights displayed statistically significant differences among different treatment groups, which were conclusive in showing efficacy of nanoparticulate formulation in reducing toxicity associated with doxorubicin. It was also observed that there was reduction in heart size in treatment groups compared to control, although the differences were not statistically different and the results pertaining to them were inconclusive (Table IV). The dox NPs (oral) evidence a statistically different protective of doxorubicin toxicity, when compared to the doxorubicin solution which was quite evident from the changes in the body weights of animals, however, could not completely eradicate this phenomenon.

Biochemical Parameters

It is widely accepted that oxidative stress and the production of free radicals are involved in doxorubicin action, both in terms of antitumor effects and cardiotoxicity (28). It has been reported that doxorubicin results in direct oxidative injure to DNA and generates lipid peroxidation products. In

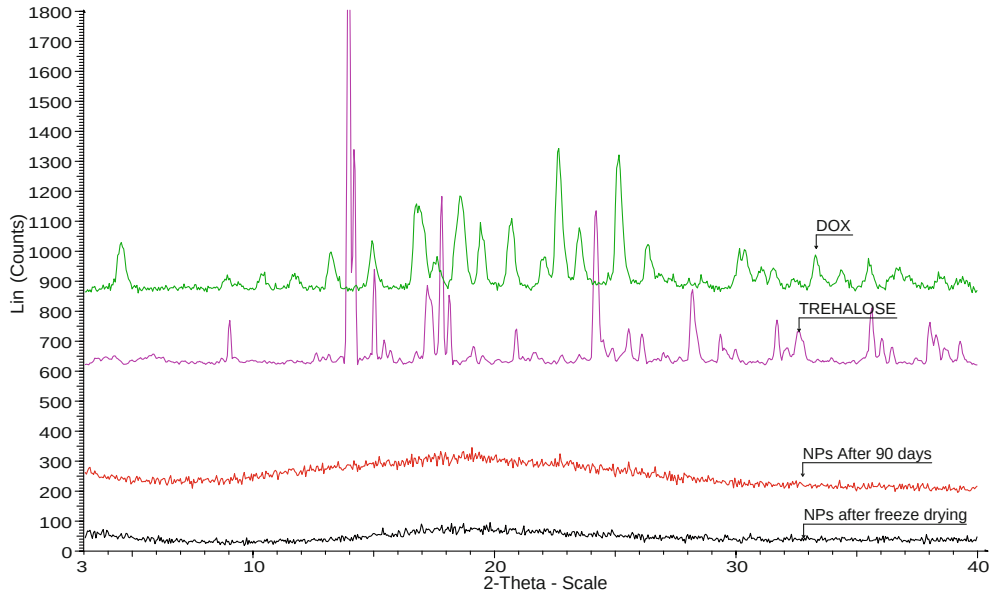

Fig. 7. XRD spectrums of doxorubicin, trehalose, doxorubicin-loaded NPs after freeze drying and after 90 days of accelerated stability study with PVA as stabilizer. The diffractograms after 90 days of accelerated stability testing clearly shows the absence of crystalline structures confirming the amorphous nature of doxorubicin in nanoparticles.

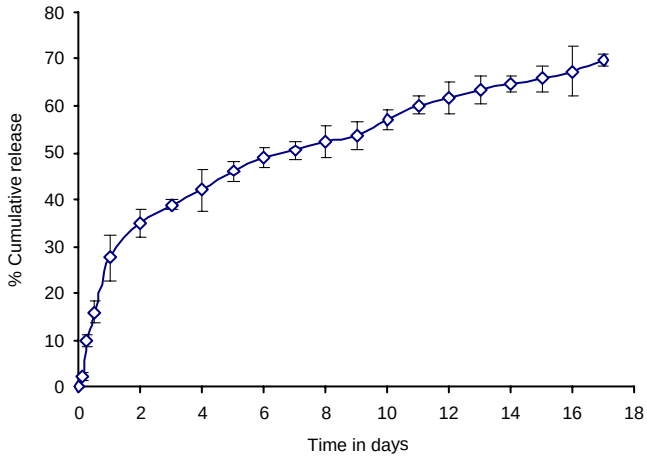

Fig. 8. *In vitro* release of doxorubicin-loaded nanoparticles (10% drug loading) in phosphate buffer pH 7.4. Data represents mean ± S. D. (n=6).

relation to doxorubicin cardiotoxicity, it is of value to remember that heart tissues are very sensitive to free radical damage, among other reasons, because of its highly oxidative metabolism and the lower amount of antioxidant defense in this organ compared with others like liver. Additionally, it has been reported that Doxorubicin has a very high affinity for cardiolipin phospholipids species which are mainly present in mitochondrial membranes of heart and are known to cause selective accumulation of doxorubicin inside cardiac tissues. Hence, estimation of oxidative stress markers in heart tissue was carried out to investigate the protective effects of nanoparticulate formulation compared to plain doxorubicin solution. Parameters like MDA, CAT and SOD were assessed for predicting the potential of nanoparticles in lowering cardiotoxicity.

A significant increase in MDA levels (*p<0.05) as shown in Fig. 9 was observed in rats treated with i.v. doxorubicin solution in comparison to control group, indicating incidence of cardiotoxicity in that group. An increase in the MDA levels treated with NP doxorubicin both i.v. and oral was observed, however the levels were significantly lower that the i.v. doxorubicin solution suggesting the protective effect of the NPs. Where as blank nanoparticles did not showed any appreciable increase in the MDA levels suggesting that particles per se do not alter the lipid peroxidation under the studied conditions.

Table IV. Body Weight Gain and Weight Indices of the Rat Internal Organ (heart) on Day 28 Post-treatment Using Doxorubicin Formulations

Group	Heart weight (g)	% Increase in body weight
Control	0.80±0.11	42.21±6.22
Blank NPs	0.81±0.08	45.23±4.15
Blank NPs i.v.	0.82±0.04	40.50±3.63
Dox-NPs oral	0.75±0.05	27.91±3.62
Dox solution i.v.	0.66±0.02	15.19±1.23
Dox NPs i.v.	0.73±0.07	20.21±4.35

Values reported are mean ± S.D. (n=6)

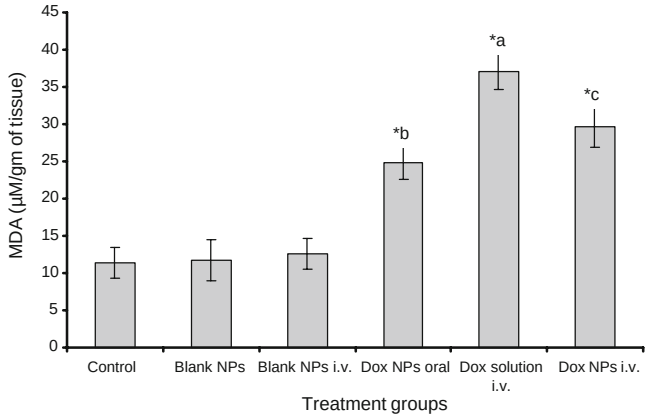

Fig. 9. MDA levels in homogenized rat heart tissue after 28th day. *Control* Without treatment; *Blank NPs* Blank NPs administered orally; *Blank NPs i.v.* Blank NPs administered intravenously; *Dox NPs oral* Doxorubicin loaded NPs administered orally; *Dox solution i.v.* Doxorubicin solution administered intravenously; *Dox NPs i.v.* Doxorubicin loaded NPs administered intravenously. p<0.05, *a vs.* Control; p<0.05, *b vs.* Dox solution i.v. and p<0.05, *c vs.* Dox solution i.v.; Data represents mean ± S.D. (n=6). Difference in means between the groups was calculated using one way ANOVA.

Different endogenous antioxidant enzymes were examined in heart tissue in all the groups and data are shown in Figs. 10 and 11. Endogenous antioxidant enzyme levels in blank NPs (oral and *via* i.v.) showed no difference with the control group. Intravenously administered Doxorubicin solution demonstrated lower levels of SOD and CAT in comparison to control group indicating higher level of oxidative stress due to doxorubicin. Nanoparticles by oral as well as i.v. route also showed lower levels of SOD and CAT compared to control but had higher levels in comparison to i.v. doxorubicin solution. The reduced toxicity observed was attributed to the slow release of the incorporated Doxorubicin from the NPs

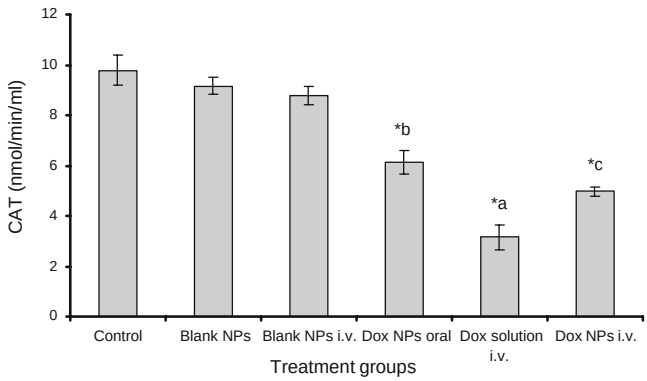

Fig. 10. CAT levels in homogenized rat heart tissue after 28th day. *Control* without treatment; *Blank NPs* Blank NPs administered orally; *Blank NPs i.v.* Blank NPs administered intravenously; *Dox NPs oral* Doxorubicin loaded NPs administered orally; *Dox solution i.v.* Doxorubicin solution administered intravenously; *Dox NPs i.v.* Doxorubicin loaded NPs administered intravenously. p<0.05, *a vs.* Control; p<0.05, *b vs.* Dox solution i.v. and p<0.05, *c vs.* Dox solution i.v.; Data represents mean ± S.D. (n=6). Difference in means between the groups was calculated using one way ANOVA.

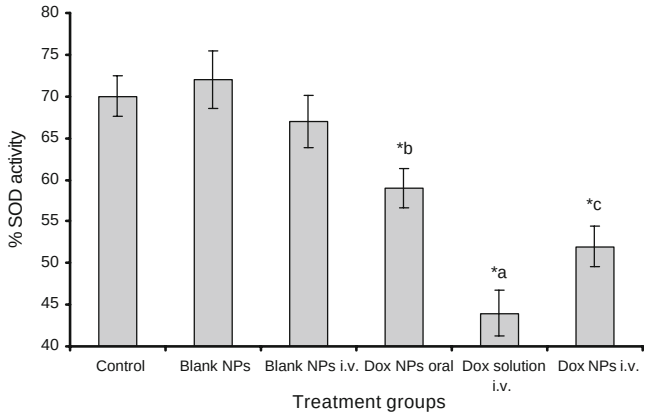

Fig. 11. SOD in homogenized rat heart tissue after 28th day. *Control* Without treatment; *Blank NPs* blank NPs administered orally; *Blank NPs i.v.* Blank NPs administered intravenously; *Dox NPs oral* Doxorubicin loaded NPs administered orally; *Dox solution i.v.* Doxorubicin solution administered intravenously; *Dox NPs i.v.* Doxorubicin loaded NPs administered intravenously. $p<0.05$, **a vs.* Control; $p<0.05$, **b vs.* Dox solution i.v. and $p<0.05$, **c vs.* Dox solution i.v.; Data represents mean ± S.D. ($n=6$). Difference in means between the groups was calculated using one way ANOVA.

formulation unlike the i.v. administration where the systemic circulation was exposed to a high pay load of doxorubicin resulting in higher circulating levels of the drug.

Bioavailability Studies

Doxorubicin loaded PLGA nanoparticles were designed to improve the oral bioavailability of the drug which was other wise poorly absorbed due to permeability issues. Blood levels after oral administration of nanoparticulate formulation were compared with doxorubicin solution using validated HPLC method. The mean concentrations in the plasma after oral administration of doxorubicin NPs and solution (as a reference) at 10 mg/kg single dose in rats are illustrated in Fig. 12. The relevant pharmacokinetic parameters including C_{max}, T_{max} and AUC_{0-inf} are listed in Table V.

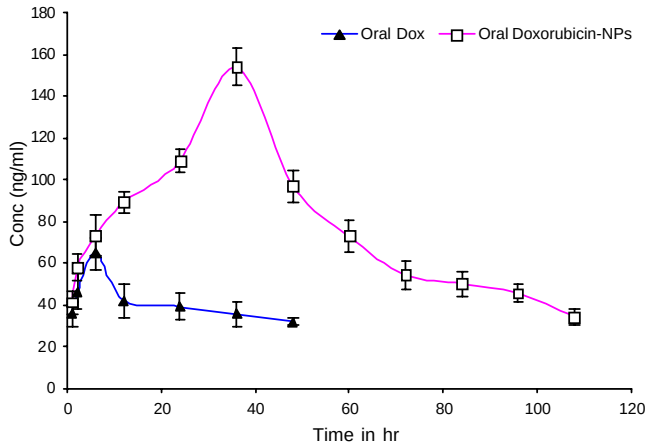

Fig. 12. Comparative *in vivo* plasma concentration *vs.* time profile of doxorubicin (10 mg/kg of animal as body weight as single dose) administered orally as doxorubicin solution and doxorubicin NPs orally. Data represents mean ± S.D. ($n=3$).

Table V. Pharmacokinetic Parameters of Two Formulations Upon Oral Administration. Comparison of Orally Administered Doxorubicin Loaded PLGA Nanoparticles and Doxorubicin Solution

Formulation	C_{max}(ng/ml)	T_{max} (h)	$AUC_{0-\infty}$ (ng-/ml-h)	Relative BA
Dox solution oral	64.68±7.50	6	1452±1017	–
Dox NPs oral	154.08±8.63	36	5282±1089	363%

Values reported are mean ± S.D. ($n=3$)

Concentration (ng/ml) of doxorubicin in plasma was plotted against time to generate the pharmacokinetic behavior of test formulations *in vivo*. From the graph obtained by plotting concentration (ng/ml) *vs.* time profile for both formulations, it was observed that the nanoparticulate formulation showed sustained release over a period of 5 days against the solution, which showed release for a period of only 2 days beyond which the drug levels had fallen below the detectable limits. Doxorubicin upon oral administration in solution form lead to very low plasma concentration with C_{max} of 64.68±7.50 ng/mL and T_{max} of 6 h which was followed by rapid decline in the plasma concentration. Whereas, relatively slow increase and sustained plasma concentrations of doxorubicin were observed with nanoparticle form having a C_{max} of 154.08±8.63 ng/ml, with significantly delayed T_{max} at 36 h.

There was a marked difference in the oral bioavailability of doxorubicin, from solution and the nanoparticulate formulation. The doxorubicin loaded nanoparticle formulations (oral administration) showed lower toxicity than the corresponding i.v. doxorubicin (dox NPs and dox solution). The reduced toxicity observed could be due to the gradual release of the incorporated doxorubicin from the NP formulation or as a result of reduced exposure as the doxorubicin loaded nanoparticles are sequestrated in tissues. Further, the doxorubicin loaded nanoparticles administered as oral and i.v. differed in toxicity, where i.v. dosing showed increased toxicity which could be due differences in the plasma/tissue exposure.

CONCLUSION

In conclusion, nanoparticles showed promise in improving oral bioavailability of doxorubicin and reduced cardiotoxicity, though tissue distribution of these particles remained to be investigated. The particle characteristics appear to be ideal for targeting tumors by EPR effect. Further, such formulations should allow treatment of new diseases where there is no proper treatment, for example leishmaniasis.

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