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Optimizing Polymeric Nanoparticles for Enhanced Protein Delivery: Strategies for Improving Loading Efficiency, Stability, and Targeted Release Through Surface Modification and Polymer Design

Ms. Arti Bhagat

Assistant Professor, Pharmacy, Sant Gahira Guru University sarguja , Ambikapur Chhttisgarh

Abstract

This study investigates the development and evaluation of catalase-loaded chitosan nanoparticles (CNPs) as a sustained drug delivery system for anti-arthritic therapy. Chitosan nanoparticles were prepared using ionotropic gelation, achieving an optimal particle size of approximately 190 nm and a drug entrapment efficiency of 67-68%. The nanoparticles exhibited a zeta potential of 40.26 ± 1.4 mV, indicating good stability, and were characterized for their spherical morphology via transmission electron microscopy. In vitro release studies demonstrated a controlled release profile, with 58.42% of catalase released over 24 hours in phosphate buffer saline (pH 7.4). Stability studies revealed minimal changes in particle size and drug content at 4°C, though aggregation was observed at 27°C. In vivo pharmacodynamic studies in a carrageenan-induced arthritis model in albino rats showed that CNPs significantly enhanced anti-inflammatory effects, achieving up to 67.38% inhibition of arthritis compared to 41.26% for free catalase. Biodistribution analysis indicated higher drug retention in the arthritic joint and prolonged blood levels with CNPs compared to free catalase, suggesting improved targeting and sustained release. These findings highlight the potential of chitosan nanoparticles as an effective delivery system for catalase in the treatment of rheumatoid arthritis, offering enhanced biodistribution and therapeutic efficacy.

INTRODUCTION

Nanoparticles are defined as particulate dispersions or solid particles with a size in range of 10-1000 nm. Nanoparticles, first developed around 1970, they were initially devised as carriers for vaccines and anticancer drugs. Polymeric nanoparticles with biodegradable and biocompatible polymers are good candidates as particulate carrier for peptide drug delivery (Bilati *et al*., 2005) and there has been considerable interest in the use of nanoparticles (NPs) as potential protein delivery systems.

Numerous investigations have shown that nanoparticles can not only improve the stability of therapeutic agents against enzymatic degradation, but by modulating polymer characteristics, they can also achieve desired therapeutic levels in target tissues for the required duration for optimal therapeutic efficacy (Bravo-Osuna *et al*., 2006).

Depending on their composition and intended use, they can be administered orally, parenterally, or locally (Campos De, *et al.,* 2000). Different NP manufacturing methods were described allowing modification of physicochemical characteristics such as size, structure, morphology, surface texture and composition to meet different requirements

The main issues in this field are the loading efficiency, stability of bioactive agent during preparation and release, release profiles and surface modification. Particles size and surface property (surface charge and hydrophobic or hydrophilic property) are primary factors for the in-vivo fate of NPs. Surface modification of NPs has been achieved mainly by two methods:

- Surface coating with hydrophilic polymers/surfactants
- Development of biodegradable copolymers with charged functional group or hydrophilic segments.

Nanoparticles in Drug Delivery

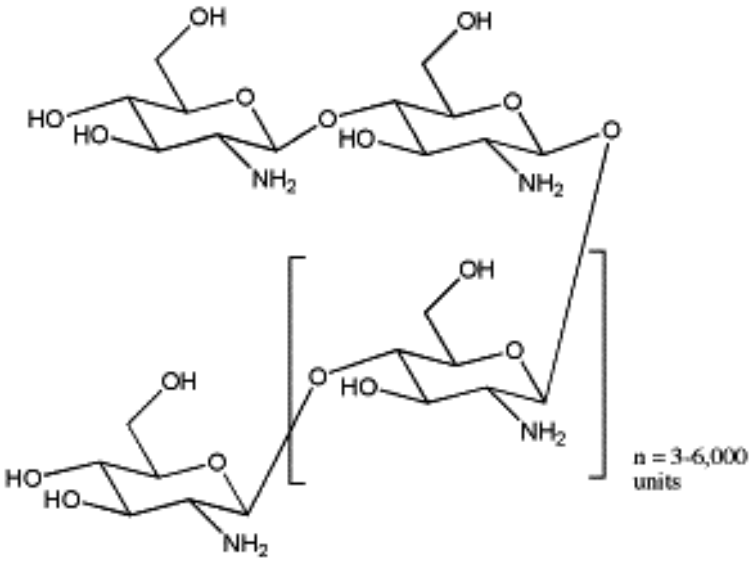
The interest in using nanoparticles for drug delivery has increased at an exponential rate in the past few years. Nanoparticles can offer significant advantages over the traditional delivery mechanisms in terms of high stability, high specificity, high drug carrying capacity, ability for controlled release, possibility to use in different types of drug administration and the capability to transport both hydrophilic and hydrophobic molecules. The drugs may be enclosed inside the sphere of the nanoparticle or linked to the surface. Once they are at the target site, the drug payload may be released from the nanoparticle by diffusion, swelling, erosion or degradation. Active systems are also possible, e.g. drug release in response to the input of external energy such as targeted ultrasound, light or magnetic field.

Protein nanoparticles

- Albumin nanoparticles
- Chitosan and Lectin nanoparticles
- Gold nanoparticles
- Magnetic nanoparticles
- Nanoshells
- Aptamer-nanoparticle conjugates

Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. Chitosan is also found in some microorganisms, yeast and fungi (Illum *et al*., 1998). This is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi.



Chemical formula of chitosan

The amino group in chitosan has a pKa value of ~6.5, thus, chitosan is positively charged and soluble in acidic to neutral solution with a charge density dependent on pH and the % Deacetylation value. This makes chitosan a bioadhesive which readily binds to negatively charged surfaces such as mucosal membranes. Chitosan enhances the transport of polar drugs across epithelial surfaces and is biocompatible and biodegradable. Purified qualities of chitosans are available for biomedical applications.

Preparation method

Over the past 30 years, chitosan NP preparation technique has been developed based on chitosan microparticles technology. There are at least four methods available:

- Ionotropic gelation
- Micro emulsion
- Emulsification solvent diffusion
- Polyelectrolyte complex.

The most widely developed methods are **ionotropic gelation** and self assemble **polyelectrolytes**.

Criteria for ideal polymeric carriers for nanoparticles & nanoparticle delivery systems

- Polymeric carriers
- Easy to synthesize and characterize
- Inexpensive
- Biocompatible
- Biodegradable
- Non-immunogenic
- Non-toxic
- Water soluble

Advantages of nanoparticles delivery systems

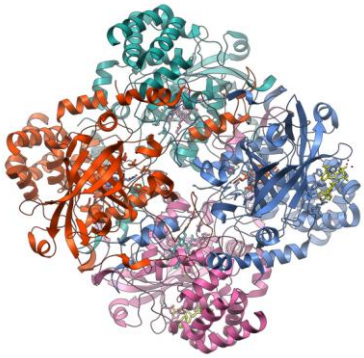
- Simple and inexpensive to manufacture and scale-up
- No heat, high shear forces or organic solvents involved in their preparation process

- Reproducible and stable
- Applicable to a broad category of drugs; small molecules, proteins and polynucleotides
- Ability to lyophilize
- Stable after administration
- Non-toxic

ENZYME PROFILE

Name: Catalase (I.U.B.:1.11.1.6)

Catalase was first noticed as a substance in 1811. when Louis Jacques Thenard, who discovered H_2O_2 (hydrogen peroxide), suggested that its breakdown is caused by a substance. In 1900, Oscar Loew was the first to who named it catalase and found its presence in many plants and animals (Loew, 1900). In 1969 the amino acid sequence of bovine catalase was worked out. Then in 1981, the 3D structure of the protein was revealed.



Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al*., 2004). Catalase has one of the highest turnover numbers of all enzymes; one molecule of catalase can convert 40 million molecules of hydrogen peroxide to water and oxygen each second.

The reaction of catalase in the decomposition of hydrogen peroxide is:

$$\underset{\text{(substrate)}}{2H_2O_2} \xrightarrow[\text{(enzyme)}]{\text{Catalase}} \underset{\text{(products)}}{2H_2O + O_2\,(gas)}$$

Catalase is an antioxidant enzyme uniquitously present in aerobic cells. It catalyses the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Hydrogen peroxide is formed in cells by controlled pathways. H_2O_2 elicits a broad spectrum of cellular response ranging from mitogenic growth stimulation to apoptosis to necrosis at different concentration levels. Hydrogen peroxide at high concentration is deleterious to cells and its accumulation causes oxidation of cellular targets such as DNA, proteins and lipids leading to mutagenesis and cell death. Removal of the H_2O_2 from the cell by catalase provides protection against oxidative damage to the cell.

The enzyme is composed of 4 subunits of the same size, each of which contains a heme active site to accelerate decomposition of hydrogen peroxide. Catalase exhibits an unusual kinetic behaviour, i.e., it is not possible to saturate the enzyme with substrate H_2O_2 up to 5 M concentration but there is a rapid inactivation of enzymes above 0.1 M H_2O_2. Therefore, its activity assay is typically carried out at 10- 50 mM H_2O_2, because substantially lower concentration then saturated substrate is used, the enzyme activity is dependent on precise concentration of H_2O_2.

The most common definition of one catalase units is the amount of catalase decomposing 1.0 mM of hydrogen peroxide per minute at pH 7.0 at 25°C, with initial H_2O_2 concentration of 10.3 mM.

Characteristics of catalase from Bovine Liver:-

Molecular weight	250,000 Dalton
Description	Brownish green color powder
Solubility	Soluble in aqueous buffer and water
Protein	95%
Potency	5000 unit/mg
Half life	20 min
Storage	2-8 ^{0}C
Stroke's radius	5.12 nm[4]
Extinction Coefficient	E1% = 36.5 (276 nm)[3]
Optimum pH	Approximately 7.0
Isoelectric point	pH 5.4

Composition: Catalase from bovine liver is a tetramer consisting of 4 equal subunits with a molecular weight of 60 kD. Each subunit contains iron bound to aprotoheme IX group. The enzyme also strongly binds NADP, of which the NADP and heme group are within 13.7 Å of each other.
Inhibitors: inhibition by ascorbate alone as well as with Cu^{2+} has been shown. Freezing and lyophilization cause inactivation and Anderson indicate catalases to be inactivated by sunlight under aerobic conditions. Catalase inactivation by peroxide has been reported also.
Preparation Instructions: Enzyme is soluble in 50 mM potassium phosphate buffer, pH 7.0 (1 mg/ml).
Activators: No activators or cofactoctors are necessary.
Stability: All preparations are stable for 6-12 months when stored at 5 °C. Do not freeze liquid preparations.
Approved uses: Food industry, skin disorders, contact lens cleaning
Market preparation: Lensan-B, Citrizan, Biocatalase, Oxycept, Ultracare

IDENTIFICATION

1 Colorimetric Reactions of Protein Identification

Biuret reaction: Proteins give a violet colour (λmax = 540 - 560 nm) with alkaline copper sulphate solution containing sodium potassium tartarate because the CO-NH groups of protein form complex with cupric ions (Jayaraman, 1996).
Ninhydrin test: Proteins give violet or purple colour (Rheumann's purple complex) when treated with ninhydrin reagent due to the condensation of two molecules of ninhydrin with a molecule of ammonia from amino acid (Brewer *et al.*, 1995; Kunkel and Ward, 1950; Sadasivam and Manikam, 1996).
Xanthoproteic reaction: Upon boiling with HNO_3 proteins give yellow colour due to formation of nitro derivatives of the aromatic amino acids (Sadasivam and Manikam, 1996).
Modified Millon's test: When protein is boiled with 10 % w/v mercuric sulphate in 10 %w/v H_2SO_4 yellow precipitate appears because mercury combines with the tyrosine of protein (Sadasivam and Manikam, 1996).

2 Spectrophotometric Methods for Protein Identification

Bradford method: Bradford assay is a rapid and accurate method. The assay is based on the observation that the absorbance maxima for an acidic solution of Coomassie Brillient Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change, within the linear range of assay (5-50 mcg/ml), if more protein present, it binds with more coomassie (Bradford, 1976).

Lowery's method: The Lowery procedure is one of the most widely used protein assays. Under alkaline conditions, copper complexes with protein. When folin phenol reagent (phospho-molybdic-phosphotungstic reagent) is added, the Folin- phenol reagent binds to the protein. Bound reagent is slowly reduced and changes colour from yellow to blue (Lowery *et al.*, 1951).

UV Spectrophotometric method: This is the simplest technique, used for identification. Scanning of the aqueous solution of catalase enzyme in phosphate buffer saline (pH 7.4) shows the absorption maxima at 240 nm (Beers and Sizer, 1952).

Enzyme estimation by BCA method: Protein assay based on BCA is the most sensitive and detergent compatible method for colorimetric detection and quantitation of total protein.

U.V. SPECTROSCOPIC METHOD

1 Determination of λ_{max}

Accurately weighed 10 mg of catalase was dissolved in 100 ml of phosphate buffer solution. From the stock solution 1 ml was pipetted out and volume was made up to the 10 ml with PBS. The resulting solution was then scanned between 200-400 nm using Shimadzu-1700 UV/Visible spectrophotometer. The λ_{max} was found to be 240 nm.

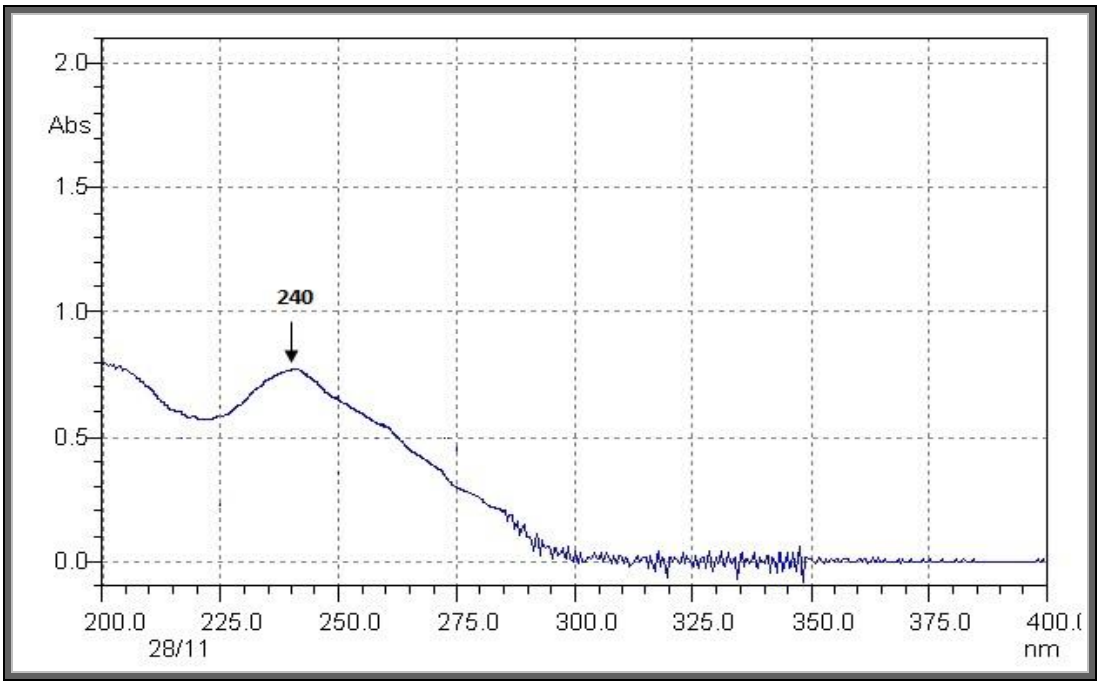


Fig. UV absorption spectra of catalase enzyme

Enzymatic Assay of Catalase Enzyme (Beers and Sizer, 1952; Stern, 1937)

Condition: T = 25 ^{0}C, pH = 7.0, A_{240nm}, Light Path = 1 cm

Method: Continuous Spectrophotometric Rate determination

Reagents:

A. 50 mM Potassium phosphate buffer pH 7.0 at 25 ^{0}C (Prepare 200 ml in deionized water using anhydrous monobasic potassium phosphate, Adjust to pH 7.0 at 25 ^{0}C using 1 M KOH.

B. 0.036% Hydrogen peroxide solution (H2O2) (Substrate Solution) (Prepare in reagent A using Hydrogen peroxide 30%, Determine the λ_{max} 240 nm of this solution using reagent A as a blank. The λ_{max} 240 nm should be between 0.550 and 0.520 absorbance units. Added hydrogen peroxide to increase the absorbance and reagent A to decrease the absorbance.)

C. Catalase solution (Immediately before use prepared a solution containing 10 - 80 units per ml in cold reagent A.)

Unit defination

The amount of enzyme which catalyzes the decomposition of one micromole of H_2O_2 per minute at 25°C and pH 7.00. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at λ_{max} 240 nm.

Procedure

1. The spectrophotometer was set at λ_{max} 240 nm.
2. In quartz cuvette, taken diluted H_2O_2 solution (substrate), incubated in spectrophotometer at 25°C for 5 min. temperature equilibration and the absorbance was determined at λ_{max} 240 nm (blank)
3. Initiate reaction 0.1 ml diluted enzyme (catalase) solution to the cuvette. Record decrease in absorbance was recorded at λ_{max} 240 nm for 2-3 minutes

The absorbance of these solutions were determined at 240 λ_{max} nm using Shimadzu UV-1700. Standard curve was constructed and shown (Table 3.1 & Fig. 3.2). Standard curve was linearly regressed and statistical parameters related to it were derived.

Table 2.1: Linearly regressed curve of catalase at λ_{max} 240 nm

S. No.	Conc. (unit/ml)	Absorbance	Regressed value	Statistical parameter
1	0.0	1.258	1.2526	y = -0.0128x +1.2526 R^2 = 0.9994
2	10.0	1.106	1.1246	
3	20.0	1.004	0.9966	
4	30.0	0.873	0.8686	
5	40.0	0.741	0.7406	
6	50.0	0.609	0.6126	
7	60.0	0.489	0.4846	
8	70.0	0.344	0.3566	
9	80.0	0.227	0.2286	

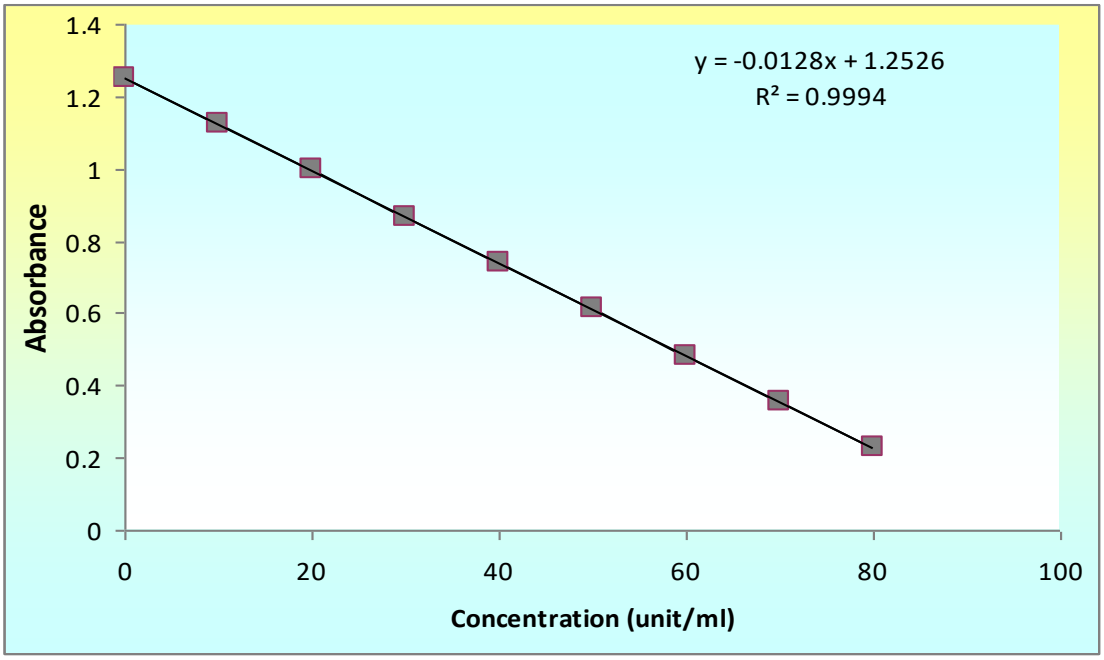


Fig 2.2: Linearly regressed curve of catalase in PBS (pH 7.4)

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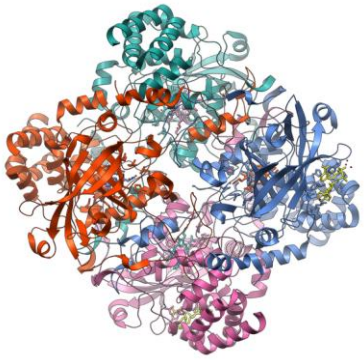

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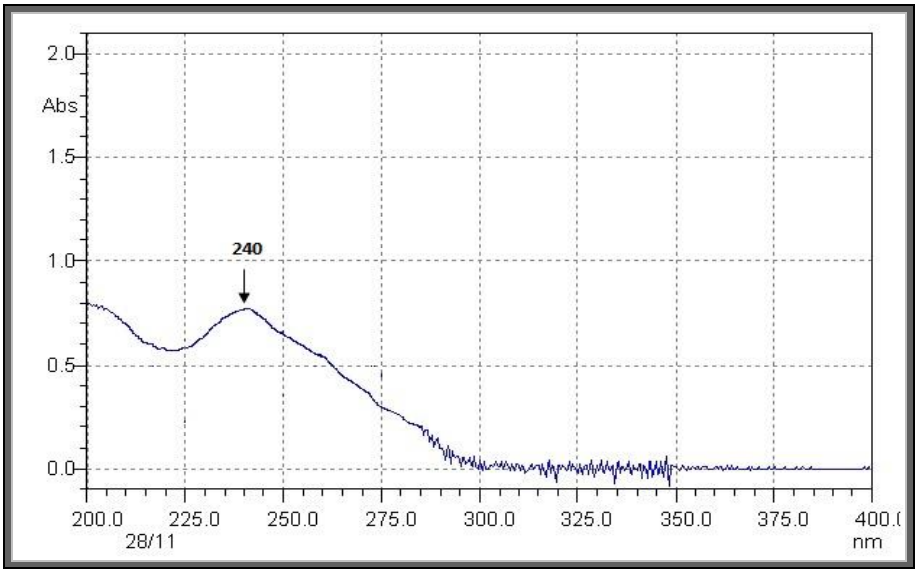

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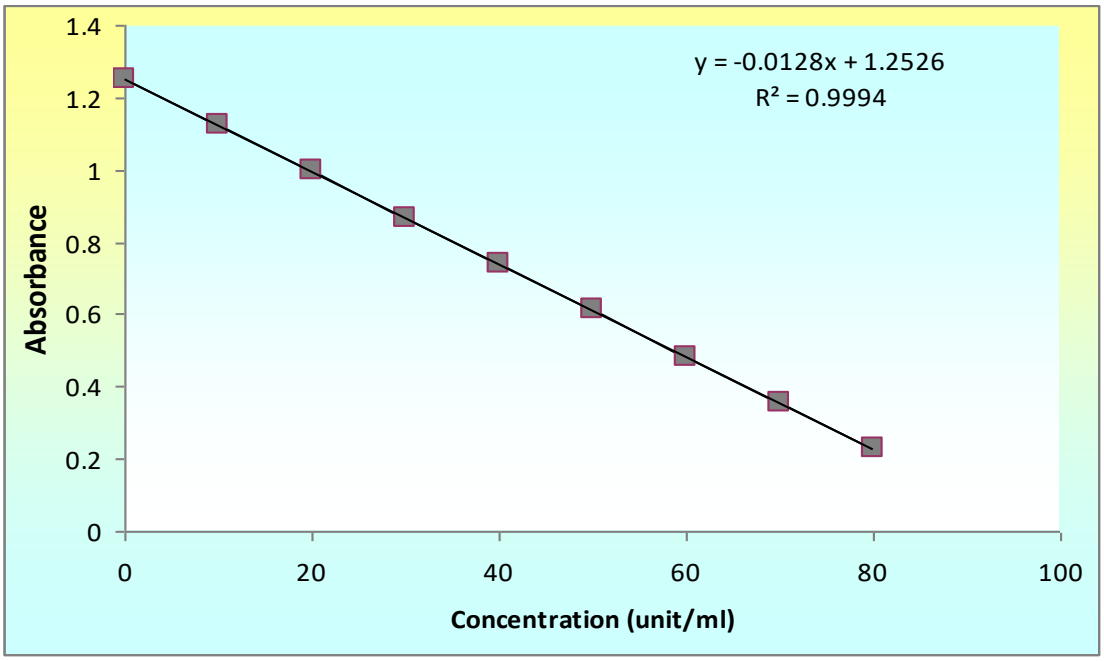


Linearly regressed curve of catalase in PBS (pH 7.4)

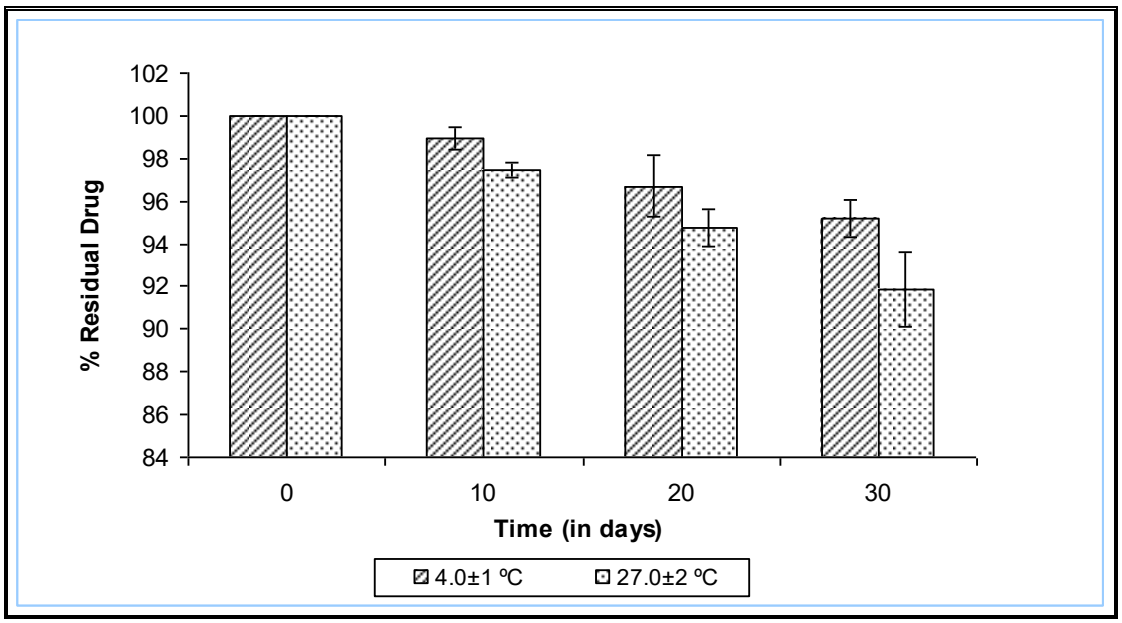
EFFECT OF STORAGE ON DRUG CONTENT

Stability of CNPs formulations on storage is of great concern as it is the major restraint in their development as marketed preparation. The prepared formulations were tested for stability at 4.0±1 ºC & 27±2 ºC temperatures formulation were stored in amber colored glass vials and then they were evaluated after 10, 20 & 30 days for change in residual drug content. Samples were taken periodically & residual

drug was estimated spectrophotometrically. The initial catalase content was considered as 100%. Results are given in Table 5.1 and shown in
Fig. 5.1.

Effect of storage on drug content of chitosan nanoparticles at different temperatures

Days	% Residual drug content on storage	
	4.0±1 ºC	**27.0±2 ºC**
Initial	100	100
10	98.92±0.54	97.43±0.35
20	96.69±1.43	94.75±0.89
30	95.18±0.84	91.87±1.77



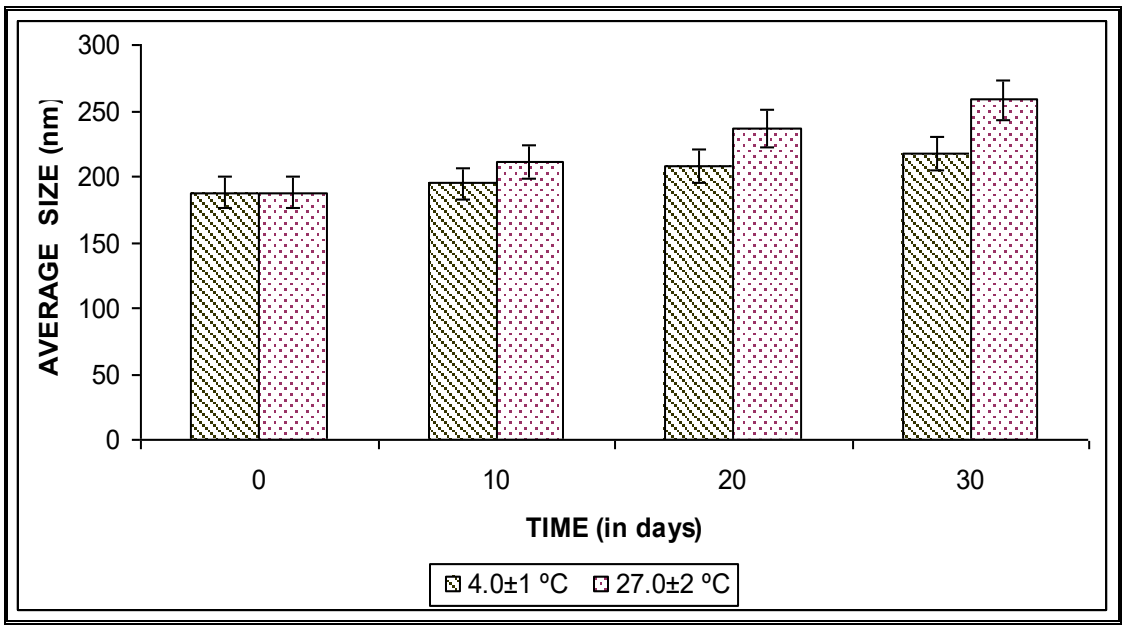
Effect of storage on drug content

EFFECT OF STORAGE ON SIZE OF NANOPARTICLES

The optimized formulation was stored in amber colored glass vials at 4.0±1 ºC & 27±2 ºC for period of 30 days. At specified time intervals i.e. 10, 20 & 30 days the particles size of the formulation was determined. Results are given in Table 5.2 and shown in Fig 5.2.

Effect of storage temperature on particles size of drug loaded nanoparticles

Days	Average Particles Size (nm)	
	4.0±1 ºC	**27.0±2 ºC**
Initial	188±1.48	188±1.48
10	195±2.16	201±2.45
20	208±2.24	236±3.28
30	217±2.81	258±2.16



Effect of storage on size of nanoparticles

***In-vivo* Evaluation**

It is quite essential to evaluate the delivery system *in-vivo* because many factors like pH of different biological fluids, enzymes system and variable affinity of the carrier system for the various biological fluids including the tissue are expected to influence its performance. These factors affect the *in-vivo* biological distribution and drug release profile from a novel carrier system, *In-vivo* studies are important to evaluate the bioavailability of drug from the designed formulations. Eight weeks old Albino rats (Sprague Dawley, 100-150 gm) of either sex were used for the present *in-vivo* studies. The experimental protocol was duly approved by the Institutional Animal Ethical Committee of Dr. H. S. Gour Vishwavidyalaya, Sagar, M.P. India (CPCSEA).

***In-vivo* Studies of Catalase loaded Chitosan nanoparticles**

The Pharmacodynamic study of catalase loaded chitosan nanoparticles includes carrageenan induced Arthritis includes blood level, tissue distribution and pharmacodynamic studies as compared to free drug.

Screening for Anti-inflammatory Activity

The screening methods for evaluation of anti-inflammatory activity have been classified as follows:-

A. Non Immunological methods

1. Evaluation of acute inflammation

- Carrageenan induced arthritis **(Hartmann *et al*., 2009)**
- Histamine induced hind paw method **(Hassan *et al*, 1974)**
- Carrageenan granuloma pouch technique **(Winter, 1965)**

2. Evaluation of chronic inflammation

- Formaldehyde induced arthritis **(Swingle, 1974)**

B. Immunological methods

- Adjuvant arthritis **(Satyavati *et al*., 1969)**
  - Complete Freund's adjuvant induced arthritis **(Newbould, 1963)**

- Collagen-induced arthritis **(Nakae *et al.*, 2003)**
- *Borrelia burgdorferi* induce arthritis **(Chateau *et al.*, 1996)**

C. Miscellaneous

- UV erythema (Schimacher and Phelps, 1974)

Materials and Methods

The experiments were performed on male Albino rats (average weight 200±20 g). The animals were housed in plastic cages in a thermoneutral environment and were supplied with standard rat chow and water ad libitum.

Induction of Arthritis

Carrageenan induced arthritis method was selected for evaluation of acute inflammation (Hartmann *et al*., 2009). This assay was based on the single intra articular injection of 2% w/v carrageenan and 4% w/v kaolin. In the present study 0.1 ml mixture of 2% w/v carrageenan and 4% w/v kaolin was taken as phlogistic agent. Joint inflammation was determined by measuring the change in the volume of inflamed knee joint, produced by injection of mixture of carrageenan and kaolin after 24 hrs. The paw volumes were measured using plethysmometer (UGO, BASILE, Italy).

Pharmacodynamic study

The carrageenan arthritis test was performed for Catalase loaded chitosan nanoparticles. Albino male rats selected for the present study were weighed, numbered and marks were made on the right knee joint on each animal, so that every time paw was dipped in the plethysmometer up to the fixed mark to ensure constant paw volume. The day time was chosen for the study to avoid any significant changes in the circadian rhythms. Animals were divided into 3 groups including one controlled group, each group comprised of three animals. Test formulation of catalase loaded chitosan nanoparticles (0.2% w/v) and plain enzyme (catalase) suspended in normal saline in the dosage of 10 mg/kg body weight was administered through *i.v.* route in albino rats of respective group excluding control group. The controlled group was injected with normal saline (PBS, 7.4 pH).

After administration of the test formulation of catalase loaded chitosan nanoparticles, the paw volume was measured every hour till 24th hour and subsequently readings were taken at ½, 1, 2, 3, 4, 5, 6, 8, 12, 18 & 24 hours. The percentage inhibition of arthritis induced by mixture of carrageenan and kaolin was calculated for each group. The results are reported in Table 5.1. A graph was plotted between % inhibitions of arthritis vs. time (hrs) (Figure 5.1).

Percentage inhibition was calculated using the following equation:

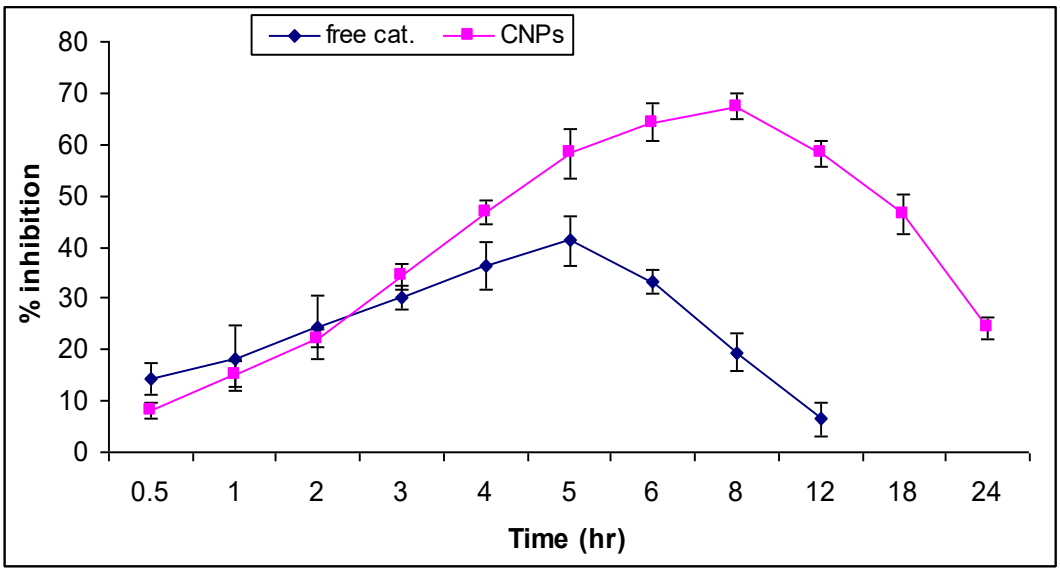
$$\% \text{ Inhibition of arthritis} = \left[\frac{V_{control} - V_{treated}}{V_{control}} \right] \times$$

Where, $V_{control}$ = mean edema volume of rats in control group, $V_{treated}$ = mean volume of each rat in test group

Screening data for anti-arthritic activity of Catalase loaded Chitosan Nanoparticles and free Catalase Enzyme

Time (hr)	Percent inhibition of arthritis	
	Free catalase	CNPs
½	14.34±3.2	8.14±1.6
1	18.26±6.4	15.26±2.4
2	24.38±6.1	22.11±1.8
3	30.16±2.4	34.27±2.6
4	36.18±4.6	46.8±2.2
5	41.26±4.8	58.25±4.8
6	33.14±2.3	64.29±3.6
8	19.46±3.8	67.38±2.4
12	6.41±3.2	58.26±2.6
18	ND	46.26±3.8
24	ND	24.26±2.2

*n=3, ±SD; Dose= 10 mg/kg body weight; ND= not detectable



: Percentage inhibition of arthritis with plain Catalase and catalase loaded chitosan nanoparticles

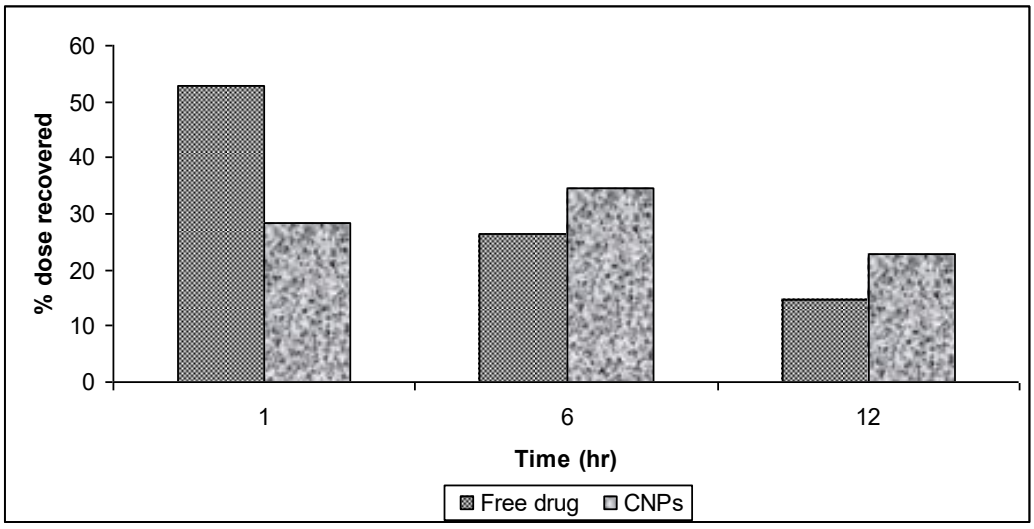
Estimation of Catalase in Serums

Blood was collected from cardiac puncture in a centrifuge tube containing heparin sodium (anticoagulant) and centrifuged at 5000 rpm for 10 minutes. Supernatant was collected and acetonitrile (1 mg/ml) was added to precipitate the proteins. The precipitated proteins were settled by centrifugation at 5000 rpm for 10 minutes and supernatant was collected. One ml of collected supernatant was filtered

through 0.45-µm membrane filter and concentration was determined by HPLC method. Results are given in Table 5.2 and shown in Fig. 5.2.

Percentage of administered dose present in serum as free drug at different time intervals after i.v. administration of CNPs

S. No.	Formulation	% dose recovered after (hrs)		
		1	6	12
1.	Free Drug	52.8	26.4	14.8
2.	CNPs	28.4	34.6	22.9



Percentage dose recovered in serum after administration of free drug & CNPs at different time intervals

Biodistribution Study

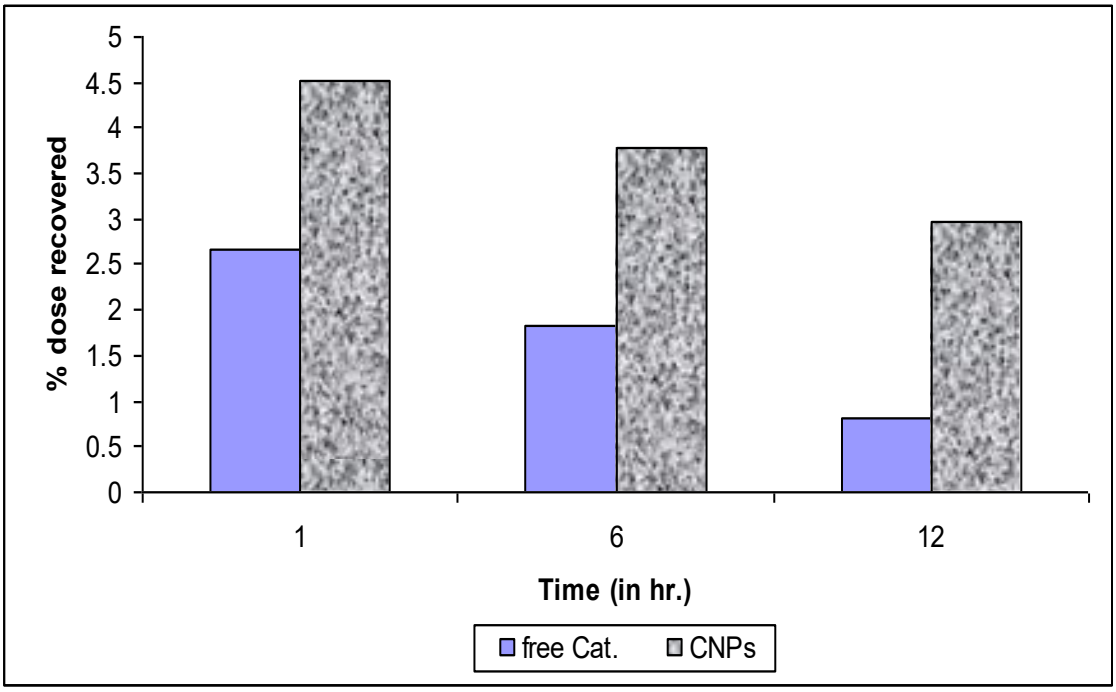
The rats were divided in 3 groups the each group comprising of three rats, marked properly. After the induction of arthritis the test formulation of catalase loaded chitosan nanoparticles and plain catalase suspended in phosphate buffer saline (pH 7.4) in a dose of 10 mg/kg body weight were administered through *i.v.* route in albino rats. After 1, 6, 12 hours following administration of formulations, rats were sacrificed and various organs such as liver, kidney, spleen lungs and arthritic knee joint were removed. The organs were weighed and stored in freezer until further required. These organs were homogenized well after adding methanol using tissue homogenizer (York Scientific Instrument, Delhi). After proper homogenization, the homogenates were centrifuged at 5000 rpm for 15 min and the supernatant was collected and assayed for Catalase content by HPLC method (Table 5.3 & 5.4; Fig. 5.3-5.5).

: Organ distribution of Catalase after i.v. administration of free Catalase

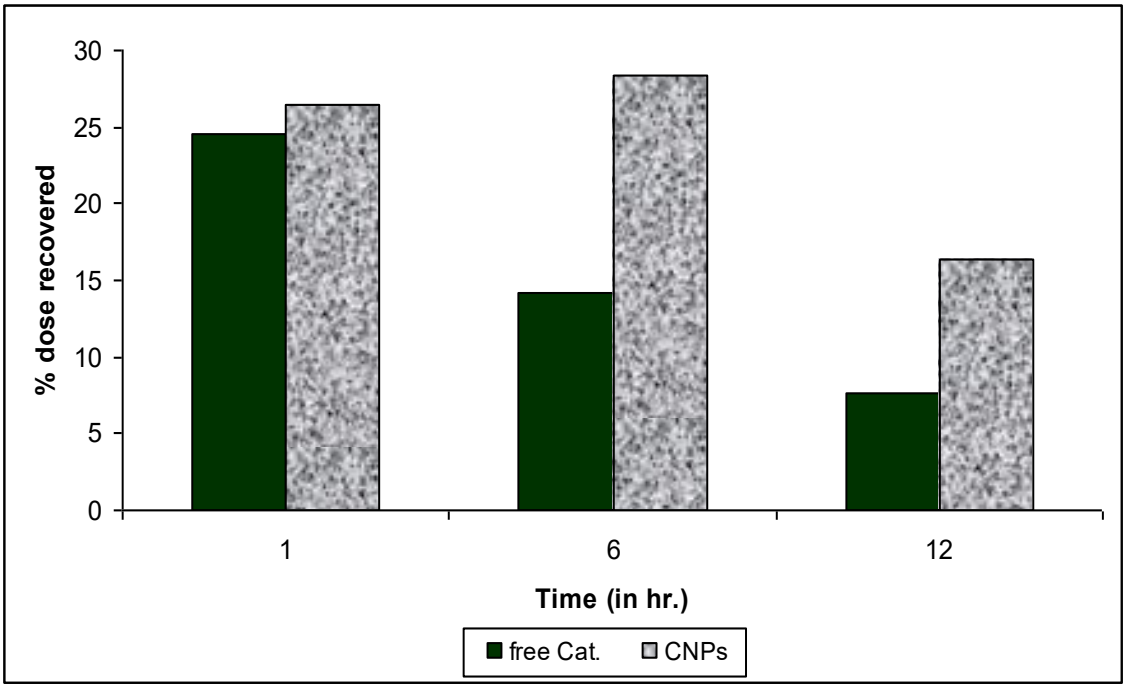
S. No.	Time (hrs.)	% dose recovered				
		Liver	Kidney	Spleen	Lungs	Joints
1	1.0	24.6	6.11	4.54	5.11	2.67
2	6.0	14.2	3.74	2.87	2.38	1.82
3	12.0	7.6	1.86	1.21	0.39	0.81

Organ distribution of Catalase after IV administration of Catalase loaded Chitosan Nanoparticles

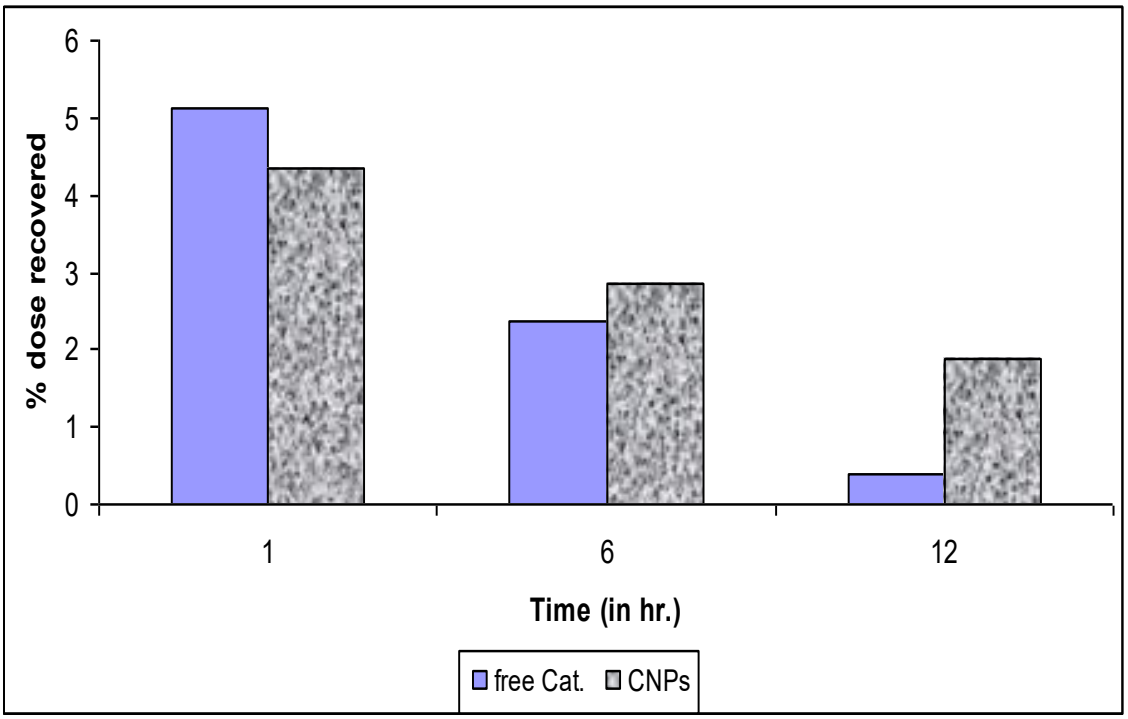
S. No	Time (hr.)	% dose recovered				
		Liver	Kidney	Spleen	Lungs	Joints
1	1.0	26.5	5.6	4.3	4.34	4.52
2	6.0	28.3	4.2	2.6	2.87	3.78
3	12.0	16.4	2.18	1.1	1.89	2.98



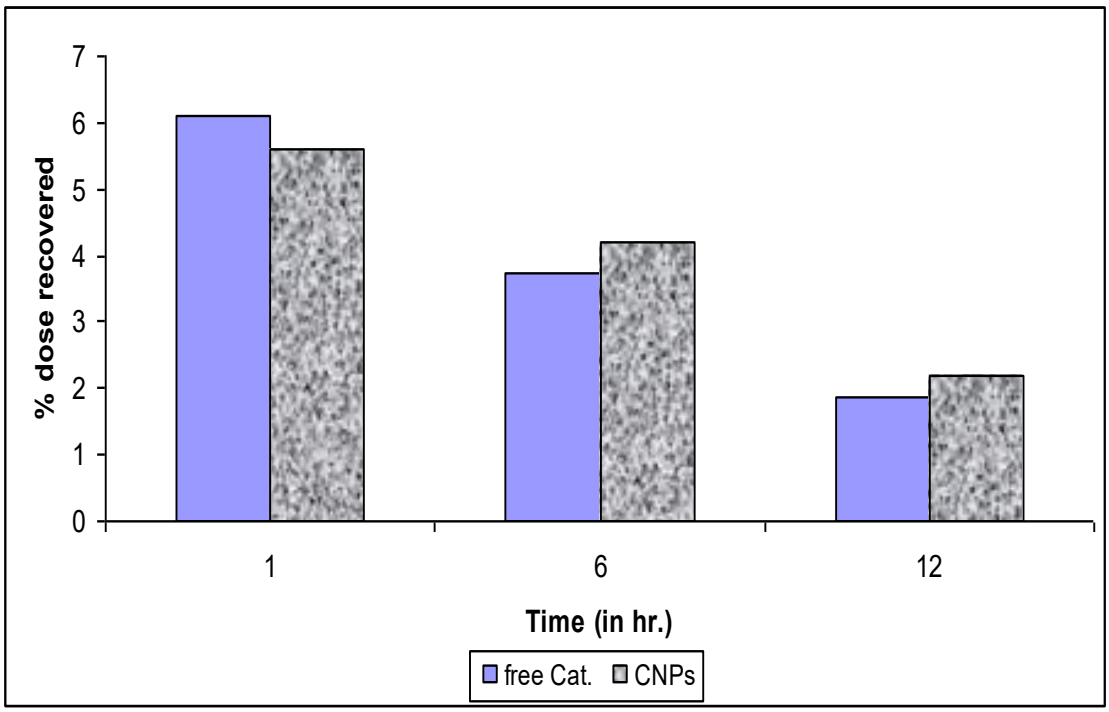
Percent dose recovered in Joints



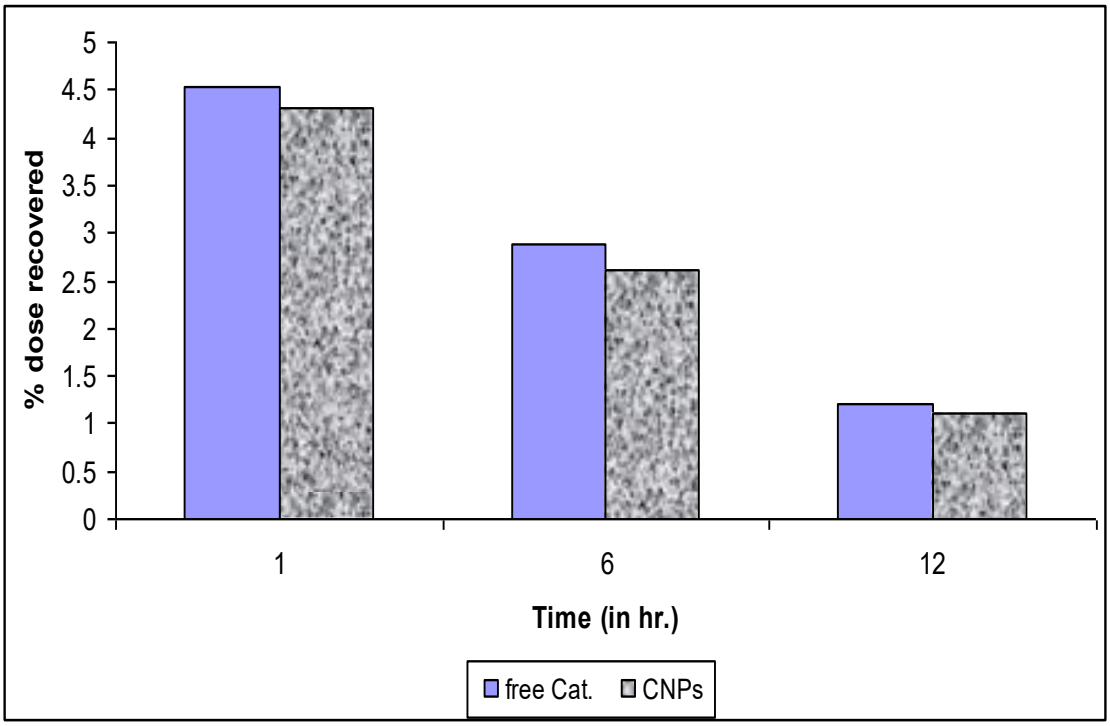
Percent dose recovered in Liver



Percent dose recovered in Lungs



Percent dose recovered in Kidney



: Percent dose recovered in Spleen

Results and Discussion

The objective of the present investigation was to evaluate performance of Catalase loaded Chitosan Nanoparticles *via i.v.* route with a view to enhance the controlled release efficacy of drug. The estimation of Catalase concentrations in plasma and tissues was important in order to understand the altered disposition caused due to variability in formulation. The plasma and tissue concentrations of Catalase were determined by HPLC method (Bohmer *et al.*, 2010). The maximum plasma concentration was observed for *i.v.* administration of plain Catalase as compared to Catalase loaded Chitosan Nanoparticles. This might be due to direct availability of free drug in case of plain Catalase in the blood.

The total percentage of administered dose present in serum as free drug at different time intervals was calculated. The results are given in Table 5.2 and shown graphically in Fig 5.2.

In case of free drug, maximum dose 52.8% was recovered in serum after 1 hour but in case of CNPs 28.4% after 1 hour was recovered in serum. After 12 hours 14.8% in case of free drug and 22.9% drug in case of CNPs recovered respectively. These results indicate the drastic reduction in serum concentration of drug entrapped in CNPs. The significant reduction in the serum concentration of free drug in CNPs formulations can be accounted for the fact that the most of the drug present in blood was entrapped in NPs.

The estimation of amount of drug present in liver, kidney, lungs, and spleen at different time interval after *i.v.* administration of free catalase revealed that maximum accumulation of the drug in these organs was achieved with in few hours. However, the maximum amount of drug accumulated in liver, lungs, spleen & kidney was considerably higher in case of free drug than CNPs.

As shown in Table 5.2 free drug was cleared quickly from the blood. In contrast, blood level in case of CNPs remained high for a longer period. The estimation of drug present in liver, kidney, spleen, lungs, at different time intervals after *i.v.* administration of free drug revealed that maximum accumulation of the drug in these organs was achieved within 1–6 hours. The accumulation in different organs was

5.11% in lungs, 24.6% in liver, 4.54% in spleen, 2.67% in joints and 6.11% in kidney after 1 hour in case of free drug and 4.34% in lungs, 26.5% in liver, 4.3% in spleen, 4.52% in joints and 5.6% in kidney after 1 hour in the case of the CNPs.

The percentage inhibition of arthritis was found to be significantly higher ($p<0.005$) from CNPs as compared to plain drug. The CNPs significantly increased the concentration of drug at the site of inflammation (knee joint) as well as increased the biological half life as compared to free drug (Table 5.1, Fig. 5.1).

These findings strongly suggest that chitosan nanoparticles have potential to improve the biodistribution properties of the anti-arthritic drug and can deliver the drug to arthritic region. The results of these studies suggest the ability of drug loaded CNPs may as sustained drug delivery system.

The aim of the present investigation was to develop catalase loaded chitosan nanoparticles for delivery to arthritic region. The drug characterization study was designed with at least two objectives in mind. Firstly, comparison of certain experimentally determined properties of the drug with those reported in literature would authenticate the drug Purchased from Himedia. Secondly these preliminary studies of drug properties would help further during more detailed experimental workup.

The enzyme was found to be brownish green in color in powdered form confirmed standards of the enzyme, The absorption maxima was found to be 240 nm which assured the identification of enzyme. The linearity was found to be 0.99. It shows the clear identification and purity of enzyme.

The CNPs were prepared using different concentration of chitosan and the particle sizes of CNPs as well as their polydispersity index were determined. It was found that on increasing the ratio of chitosan (2-6 %) with constant concentration (1%) of TPP, the size of CNPs increased from 162 to 236 nm. (Table 3.1 & Fig. 3.2). The 5: 1 of chitosan: TPP ratio found to be optimum, stirring speed & stirring time also affect the in average particle size & drug entrapment efficiency. On increasing the stirring speed from 200 to 800 rpm & stirring time from 10 to 40 min., the size of CNPs gradually decreased which may be due to high shearing to the media & might have caused particle reduction in size of nanoparticle. At an optimum 600 rpm stirring speed & 30 min. stirring time, average particle size of 190±2.14 nm & 186±2.11 nm with drug entrapment efficiency 67.34±1.66 & 68.14±1.98, respectively were obtained.

The TEM (Fig. 3.5) photomicrograph shows that NPs are spherical in shape but surface of chitosan NPs is less smooth as compared to other NPs. The zeta potential of chitosan NPs was found to 40.26±1.4 mV.

The *In-vitro* release study shows that around 32% of the loaded catalase enzyme was immediately released into PBS. The burst might be ascribed to protein molecules that were loosely bound to chitosan nanoparticles surface. The release of catalase enzyme from the chitosan nanoparticles in the PBS pH 7.4 was found to be 58.42% in 24 hrs of total catalase enzyme loaded in the formulation. The percentage release obtained in PBS shows that the encapsulated enzyme in nanoparticles was delivered into the systemic circulation.

Stability studies were carried out with selected formulations which were stored for a period of 10, 20 & 30 days at 4.0±1 ºC & 27±2 ºC. The particle size of the nanoparticles was found to slightly increase at 4.0±1 ºC & more at 27±2 ºC (Table 5.2 & Fig. 5.2) which could be due to aggregation of CNPs at elevated temperature. Thus, from these studies it can be concluded that for better stability, the formulation should be stored only at refrigerated conditions.

The *in-vivo* estimation of catalase concentration in plasma and tissues is important in order to understand the altered disposition caused due to variability in formulation. The plasma and tissue concentrations of

catalase were determined by HPLC method Bohmer *et al*, 2010). The maximum plasma concentration was observed for *i.v.* administration of plain catalase as compared to catalase loaded chitosan nanoparticles. This might be due to direct availability of free drug in case of plain catalase in the blood.

The total percentage of administered dose present in serum as free drug at different time intervals was calculated. The calculated data is given in Table 5.2 and shown graphically in Fig. 5.2.

In case of free drug, maximum dose 52.8% was recovered in serum after 1 hour but in case of CNPs 28.4% was recovered after 1 hour, 12 hours 14.8% and 22.9% drug were recovered in case of free drug and CNPs respectively. These results indicate the drastic reduction in serum concentration of drug entrapped in CNPs. The significant decrease in the serum concentration of free drug in CNPs formulations can be accounted for the fact that most of the drug present in blood was entrapped in NPs.

The estimation of amount of drug present in liver, kidney, lung, and spleen at different time interval after *i.v.* administration of free catalase revealed that maximum accumulation of the drug in these organs was achieved with in few hours. However, the amount of drug accumulated in liver, lungs, spleen & kidney were considerably higher in case of free drug than CNPs.

As shown in Table 5.2 the free drug was cleared quickly from the blood. But the blood level in case of CNPs remained high for a longer period. The estimation of drug present in liver, kidney, spleen, lungs, at different time intervals after intravenous administration of free drug revealed that maximum accumulation of the drug in these organs was achieved within 1–6 hours. The accumulation in different organs were 5.11% in lungs, 24.6% in liver, 4.54% in spleen, 2.67% in joints and 6.11% in kidney after 1 hour in case of free drug and 4.34% in lungs, 26.5% in liver, 4.3% in spleen, 4.52% in joints and 5.6% in kidney after 1 hour in the case of the CNPs.

The percentage inhibition of arthritis was found to be significantly higher ($p<0.005$) from CNPs as compared to plain drug. In case of CNPs the concentration of drug and its biological half life was more as compared to free drug (Table 5.1, Fig. 5.1).

These findings strongly confirmed that chitosan nanoparticles have potential to improve the biodistribution properties of the anti-arthritic drug and can deliver the drug to arthritic region. The results of these studies confirm that the drug loaded CNPs may passively be used as sustained drug delivery system.

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