

PREPARATION, CHARACTERIZATION AND EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF NNDMAC BIODEGRADABLE PARENTERAL SUSTAINED RELEASE MICROSPHERES

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ABSTRACT

The objective of this work was to prepare biodegradable sustained release parenteral microspheres of NNDMA-curcumin (NNDMAC), a novel curcumin analogue, characterize the formulation and evaluate the hepatoprotective activity of the microsphere formulation. Microspheres were prepared using solvent evaporation technique using polycaprolactone and were further characterized for various parameters. *In vivo* animal studies for 10 days were carried out for the determination of hepatoprotective activity of the formulation in a CCl₄ model. Particles of 12 µm size with 75.41% yield with no drug-polymer interaction were obtained. The drug release was sustained for 10 days. Significant hepatoprotection which was better than repeated I.V. administration was offered by the microsphere formulation in this 10-day study. Prepared NNDMAC microspheres have potential use in liver toxicity/cirrhosis.

Keywords: NNDMAC, Microspheres, *In vitro* release, Biodegradable polymer, Hepatoprotection

INTRODUCTION

Liver disease is defined as any change in the anatomy or function of the liver. Liver is one of the major organs of the body which has tremendous capacity to detoxify toxic principle and synthesize useful principles. Thus, damage to liver can affect our body tremendously. Various types of liver disorders such as cirrhosis, jaundice, tumors, metabolic and degenerative lesions, liver cell necrosis and hepatitis etc exist¹. Liver disorders can result from viruses, xenobiotics, excessive drug therapy, environmental pollution and alcohol intoxication. The management of liver diseases is still a challenge to the modern medicine. Few treatments exist in modern allopathy. Thus, drug discovery for these diseases is the need of the hour. Curcumin, an ayurvedic natural product previously demonstrated hepatoprotective activity². Similarly, another curcumin analog BDMCA also demonstrated significant hepatoprotective activity which was higher than compared to that of curcumin³. In the continuation of quest for new drugs in liver disorders, we synthesized a series of curcumin analogs. These analogs demonstrated better hepatoprotective activity as well as antidepressant activity when compared to that of curcumin (4). This can be attributed to better pharmacokinetics, stability and low metabolism. In this study, we investigated a delivery approach to one of these curcumin analogs, NNdimehyaminocurcumin (NNDMAC). The structure of NNDMAC is shown in Figure 1. Sustained release parenteral formulations offer better pharmacokinetics to the drug compared to the conventional formulation thereby improves the activity. In this study, we compared the hepatoprotective activity of NNDMAC using a sustained release formulation developed in this

study with an equivalent parenteral I.V. formulation. Advantages with such parenteral sustained release delivery systems were previously reported^{5, 6}. The results of hepatoprotective activity of the molecule will be better clarified using a delivery system approach when compared to the administration in a solution form. Thus, the objective of this study was to prepare a sustained release parenteral depot for NNDMAC, characterize the formulation and evaluate its hepatoprotective activity.

MATERIALS AND METHODS

The required aromatic aldehyde p N N dimethylamino benzaldehyde was obtained from Merck. All the solvents and accessories used in the synthesis of the compound were procured from standard sources. Polycaprolactone was purchased from Sigma-aldrich, Germany. Ethanol LR, ethyl acetate, tween 80 and dichloromethane were procured from Finar reagents. To conduct in vitro drug release studies, magnetic stirrer and cyclo mixer from Remi Equipments Pvt. Limited were used. A SL 164 Elico Double Beam UV-Vis Spectrophotometer was used to analyze the samples. HPLC from Cyberlabs was used for analysis of synthesized compound. Male Wister rats (100 to 150 gms, 5 to 6 weeks old) purchased from animal center of Mahaveera enterprises, Hyderabad were used in this study.

Synthesis of NNDMAC

A mixture of acetyl acetone (0.01 mole), p N N dimethyl benzaldehyde (0.02 moles), boric acid (0.01 mole), dimethyl formamide (10 ml), few drops of diethanolamine and acetic acid mixture were refluxed in a mantel for 16 hours at 150⁰C temperature in a round bottom flask. The reaction was monitored by TLC for the confirmation of the formation product. After refluxing the reaction mixture was poured into a 10% acetic acid solution and stirred for one hour to get a solid mass which was filtered and washed with water to get crude drug. This crude drug was purified and separated by column chromatography using 60-120 mesh TLC grade silica gel. Purification of the compound was done by using column chromatography.

The column was filled with silica gel of mesh size 60 to 120 and wet packing method was followed. The reaction product mixed with silica gel was loaded on top of the column and column was run with a mixture of n hexane and ethyl acetate (75:25) of 500 ml volume. The pure product was subsequently eluted by running the column with a mixture of methanol and benzene (50:50). The elutant was allowed to air dry. It was recrystallized by subsequent solubilization in benzene followed by methanol to get pure product. The purity of the compound obtained was determined using a HPLC. A HPLC procedure employing a C-18, 100 X 4.6 column, SPD-10A UV-Vis detector, LC-10 AD pump and C-R7A Plus integrator was used. HPLC grade methanol and water in the ratio of 70:30 was taken as the mobile phase.

Fabrication of NNDMAC Microspheres

Microspheres of NNDMAC using biodegradable polycaprolactone as the polymer were prepared by emulsion-solvent evaporation method. Dichloromethane was taken as organic phase in which polymer (400mg) and drug (200mg) in a ratio of 2:1 were dissolved (20ml). This organic phase was added to the aqueous phase containing tween 80 as surface active agent (1% w/v solution) drop by drop while the aqueous phase was kept for stirring on a magnetic stirrer. Stirring was continued till complete evaporation of dichloromethane occurred. As the organic phase evaporates precipitation of the polymer and drug occurs due to which drug gets entrapped in the polymer and stirring results in size reduction as well as spherical particle formation.

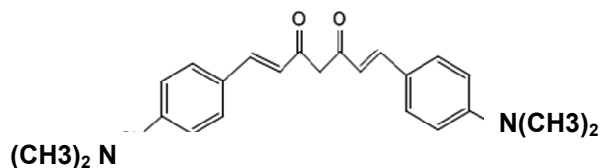


Figure 1. Chemical Structure of NNDMAC

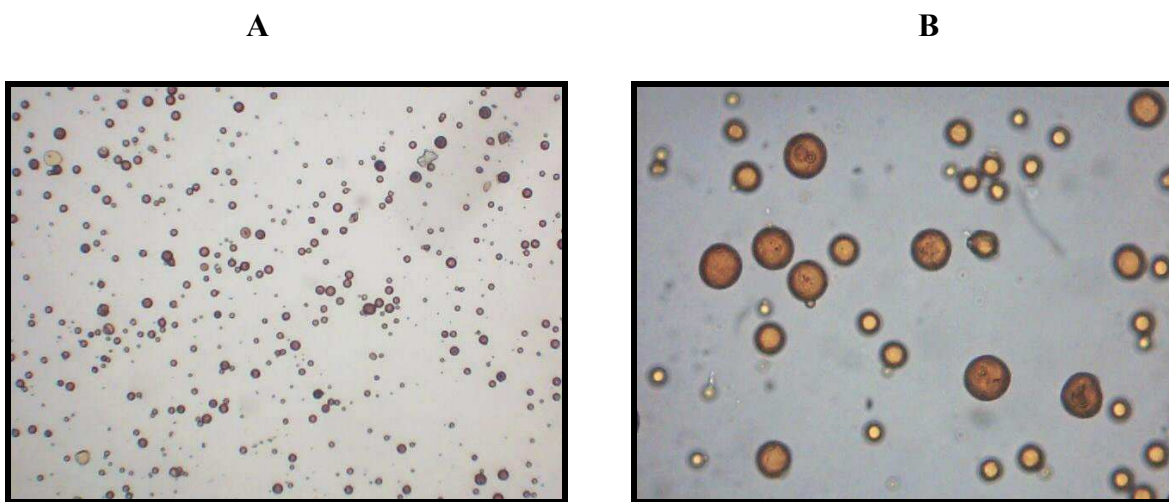


Figure 1: Microscopic view of NNDMAC microspheres

A) NNDMAC Microspheres under 10x

B) NNDMAC Microspheres under 40X

Characterization of Microspheres

Percentage Yield:

It is the ratio of the practical yield to the theoretical yield and was calculated using the formula

$$\text{Percentage Yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

Particle Size Analysis:

Particle size was determined using optical microscope. Size of 50 particles was taken and the average was reported.

Entrapment Efficiency:

High performance liquid chromatographic analysis was used for estimation of entrapment efficiency. HPLC method was as previously mentioned. Briefly, 10 mg of the microspheres were dissolved in 1 ml of chloroform and 20 μ l of the solution was injected into the HPLC and the presence of the drug was determined.

***In vitro* Drug Release Studies:**

A dialysis membrane was used for the release study. The dissolution medium, PBS (7 ml) was taken into the receiver compartment. The donor compartment was immersed into the receiver compartment so that the edge just touches the receiver compartment. A 100mg of the micro particles were dispersed in 2 ml of distilled water and placed in the donor compartment. The rpm of the system was maintained by using magnetic stirrer and bead. Samples (1 ml) were removed from the receptor compartment and replaced with fresh medium immediately. The samples were analyzed using a UV-visible spectrophotometer at 425 nm wavelength.

Drug-Excipients Compatibility study:

This was carried out by FTIR analysis of pure drug (NNDMAC), pure polymer (Polycaprolactone), drug loaded microparticles and placebo microparticles (polycaprolactone microspheres).

Evaluation of Hepatoprotective Activity of NNDMAC Microspheres

The study was carried out on male Wister rats weighing 100-150gms. The animals were housed in clean cages and maintained in controlled temperature. They were fed with standard diet and ad libitum. Animals were divided into 3 groups each group containing 4 rats.

Group I: Served as normal control and received normal saline solution orally.

Group II: Served as toxic control and was given 0.7 ml/kg of carbon tetrachloride (25 ml in 75 ml olive oil) intraperitoneally every third day.

Group III: Administered NNDMAC micro particular suspension at a dose of 100mg/ml to each rat on day 1 of the study.

For pharmacodynamics group II and group III were give carbon tetrachloride (25% v/v in olive oil) after every 3 days. On day 10 the rats were sacrificed and blood was collected. Serum was separated by centrifugation and used for the estimation of biochemical parameters such as SGOT and SGPT. Then liver was carefully isolated and washed. This fresh liver was fixed in 10%

formalin solution and stored for histopathological studies. After embedding in paraffin wax, thin sections of 5 μm thickness of liver tissue were cut and stained with haematoxylin-eosin stain.

Processing of liver tissue:

Liver tissues were taken out from fixing solution and dehydrated for 30 minutes each in 30, 50, 70, 90 and 100% alcohol successively to remove the alcohol from the dehydrated tissue, they were kept for 30 minutes each in alcohol: xylene (1:1) followed by pure xylene. The tissues were then kept in xylene: paraffin wax mixture (1:1) for 1 hour and then in molten paraffin wax at 62°C, after which they were trimmed and mounted on wooden blocks for thin sectioning. Hand microtome (Yorco precision rotary microtome model no. YS1114) was used to cut sections of the liver tissues of 5 μm thickness. These thin sections of the liver were then stained with eosin hematology stain. The slides were observed under microscope at a magnification of 40X.

RESULTS AND DISCUSSION

NNDMAC was prepared by condensation of p N N dimethyl amino benzaldehyde with acetyl acetone in presence of boric acid. Final product obtained after purification by column chromatography and recrystallization with benzene and methanol was fine brownish-yellow coloured powder. Total yield was found to be 83.68 \pm 6.33%. Melting point of the pure compound was 134 \pm 3°C. Percentage purity estimated by HPLC in water: methanol as the mobile phase with retention time of 14.23 minutes was 99.845%. The NNDMAC microspheres prepared by emulsion solvent evaporation method were spherical, brown colored and free flowing with an

average particle size of $20 \pm 5 \mu\text{m}$ (Figure 2). The percentage yield of the microspheres was $65.88 \pm 9.55\%$. Entrapment efficiency estimated by HPLC analysis was found to be $22 \pm 0.84\%$.

NNDMAC was found to be compatible with the polymer in the microparticulate formulation. This compatibility was concluded by comparing the FTIR profiles of pure polymer, pure drug, placebo biodegradable microparticles and NNDMAC biodegradable polycaprolactone microspheres. From the graphs (Figure 3) it was clear that there is no interaction between the drug NNDMAC and polymer polycaprolactone at the end of the fabrication of microspheres. *In vitro* drug release from the NNDMAC microspheres was carried out by dialysis membrane technique. The data (Figure 4) indicated that the release of the drug was prolonged for 10 days. For estimation of hepatoprotective activity at the end of 10 days, the administration of CCl_4 to the animals resulted in a marked increase in SGPT and SGOT activities (Table 1).

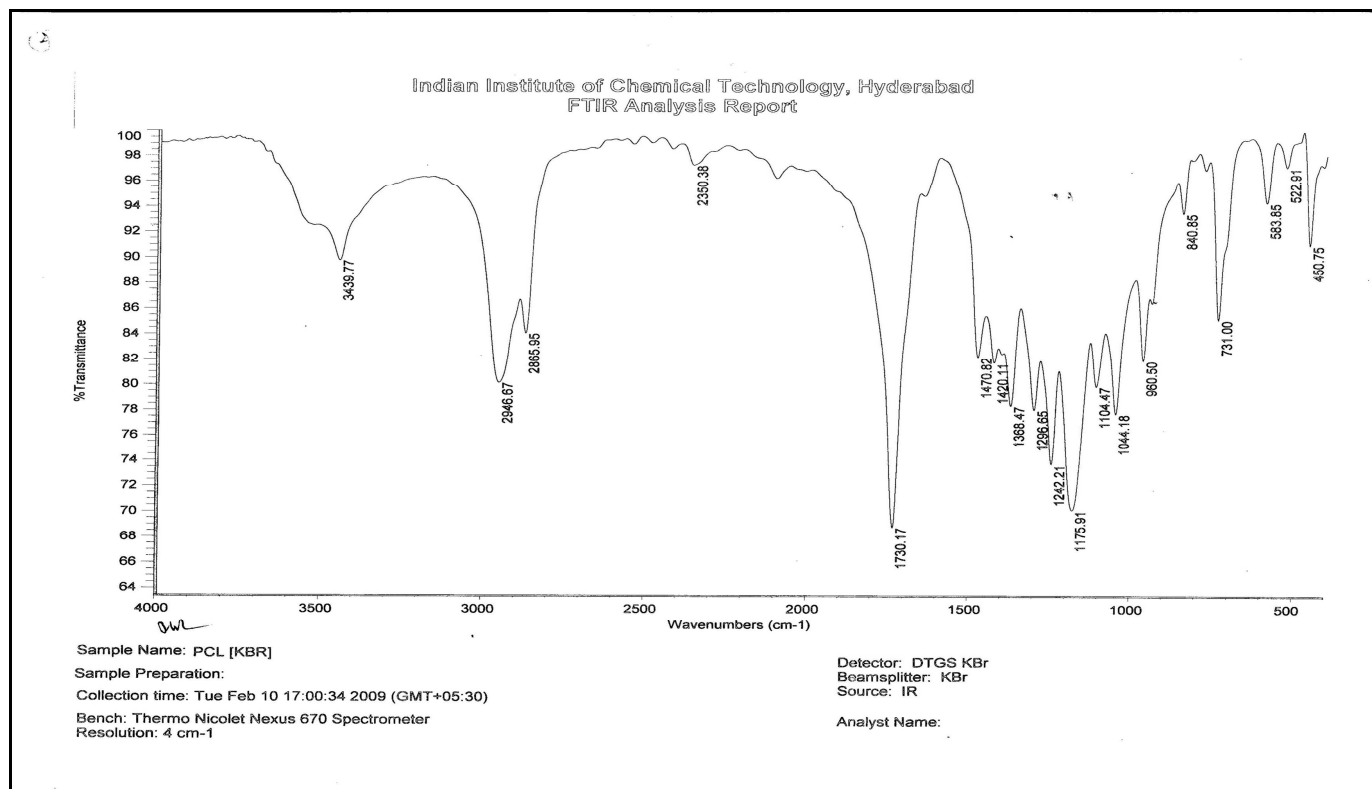


Figure 2A: FTIR profile of Pure Polycaprolactone

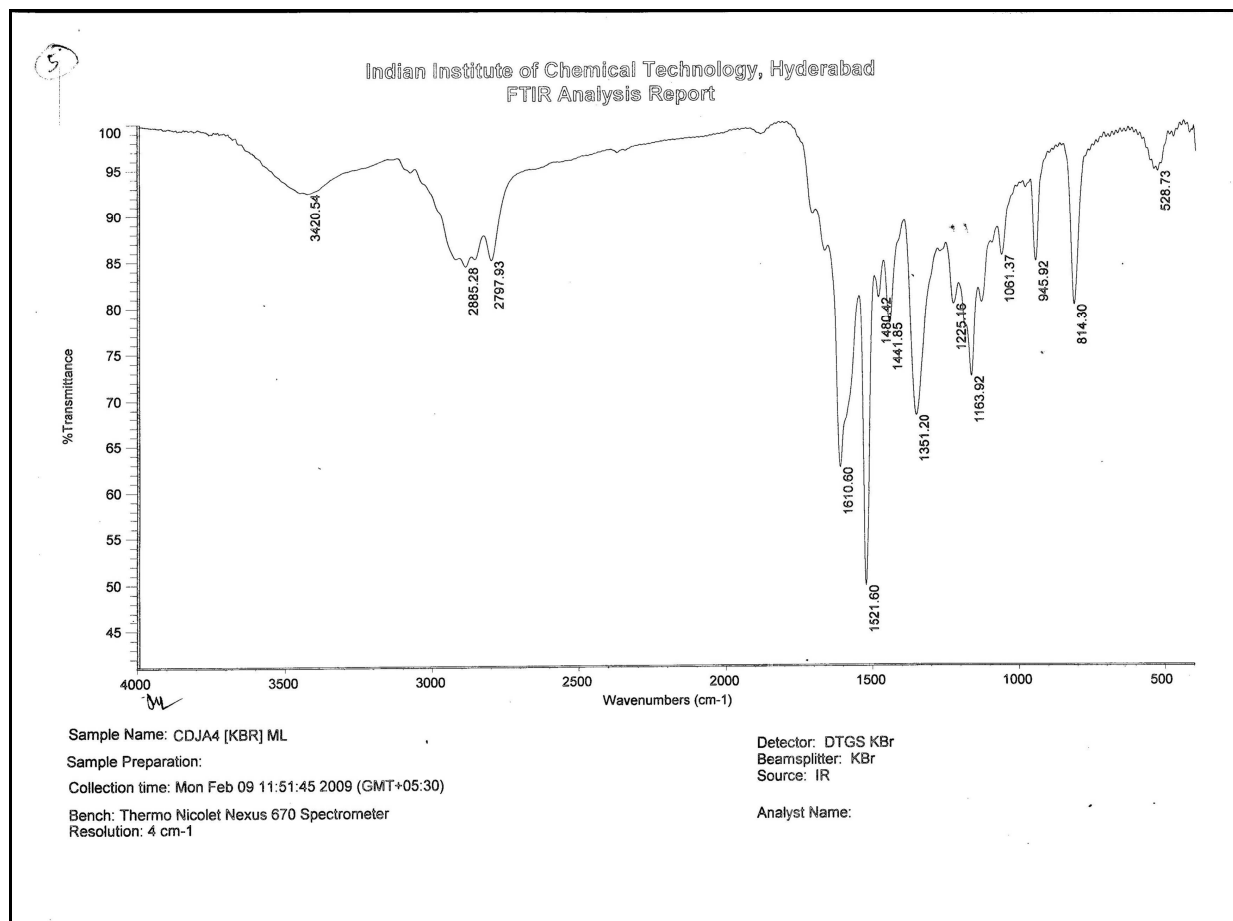


Figure 2B. FTIR profile of Pure NNDMAC

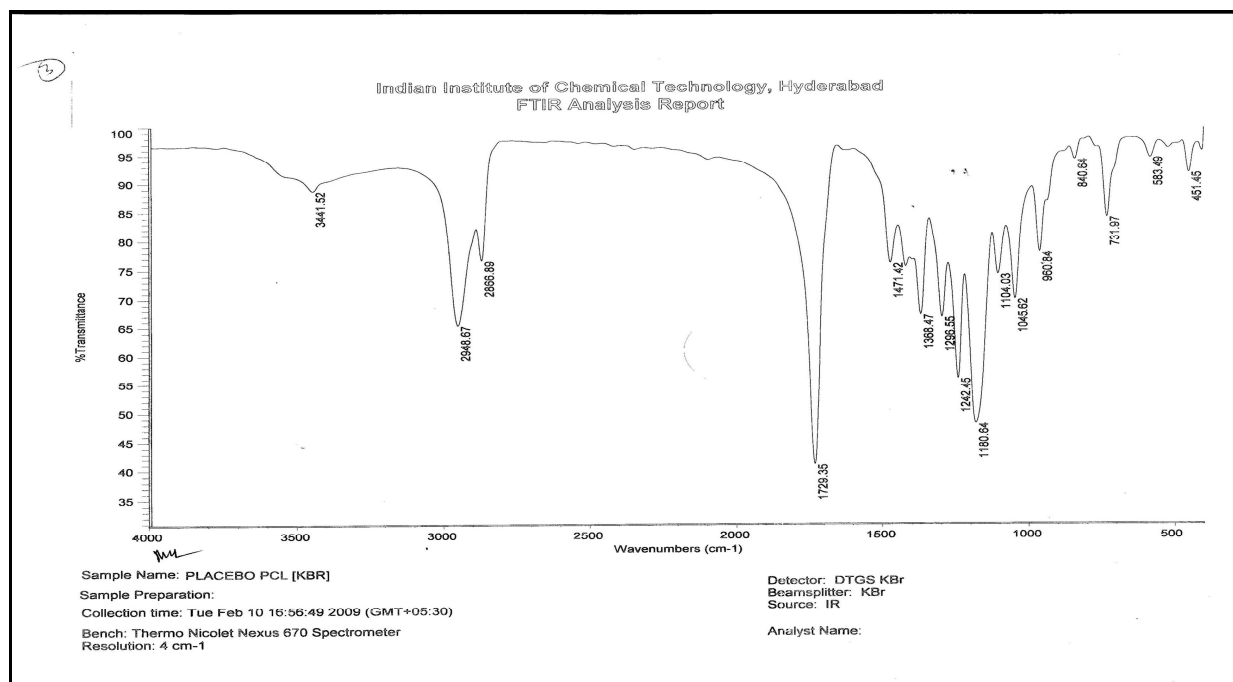


Figure 2C. FTIR profile of Placebo microspheres

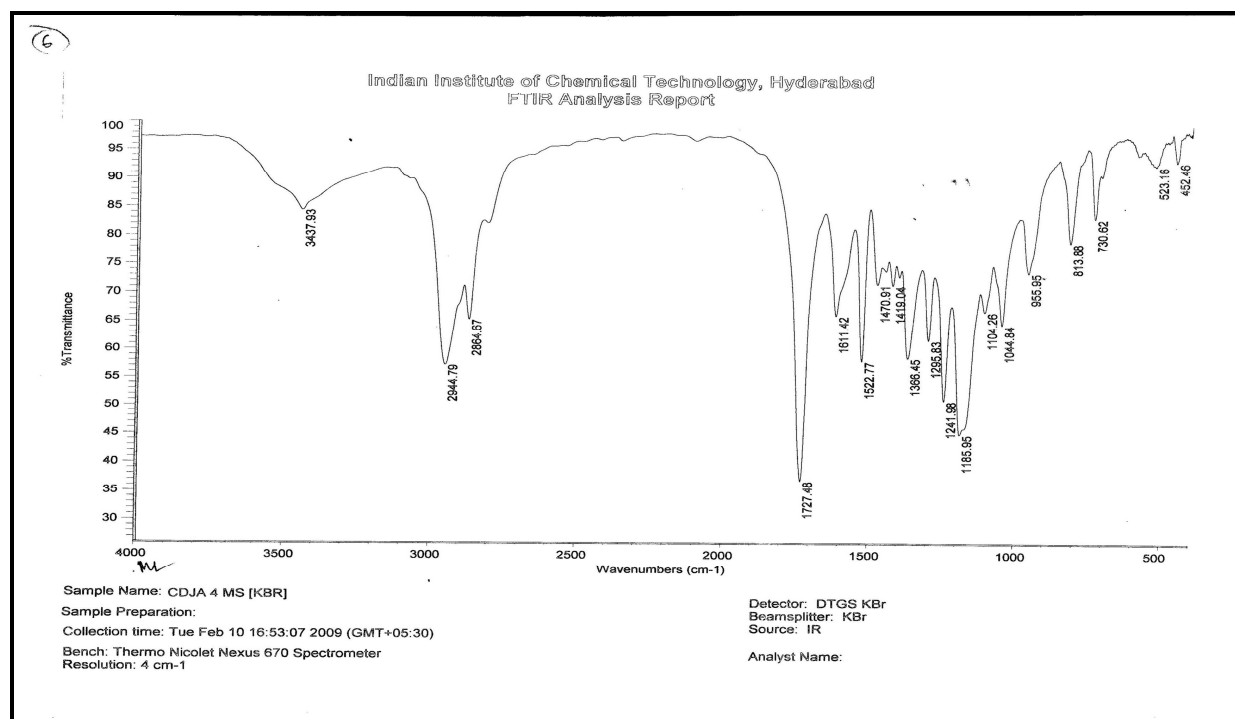


Figure 2 D. FTIR profiles of NNDMAC microspheres

Treatment with 100 mg dose of microspheres containing 20mg of NNDMAC intraperitoneally reversed the elevation of the liver enzymes found in CCl_4 treated mice. Histological profile of the control animals showed normal hepatic architecture with distinct hepatic cells, well presented cytoplasm sinusoidal spaces and central vein (Figure 5A). Disarrangement of normal cells with intense centrilobular necrosis was observed in CCl_4 intoxication liver (Figure 5C). The 20 mg of intraperitoneal NNDMAC microspheres exhibited a significant liver protection against CCl_4 induced liver damage, as evidenced by the presence of normal hepatic cords and well defined cytoplasm and absence of necrosis (Figure 5B).

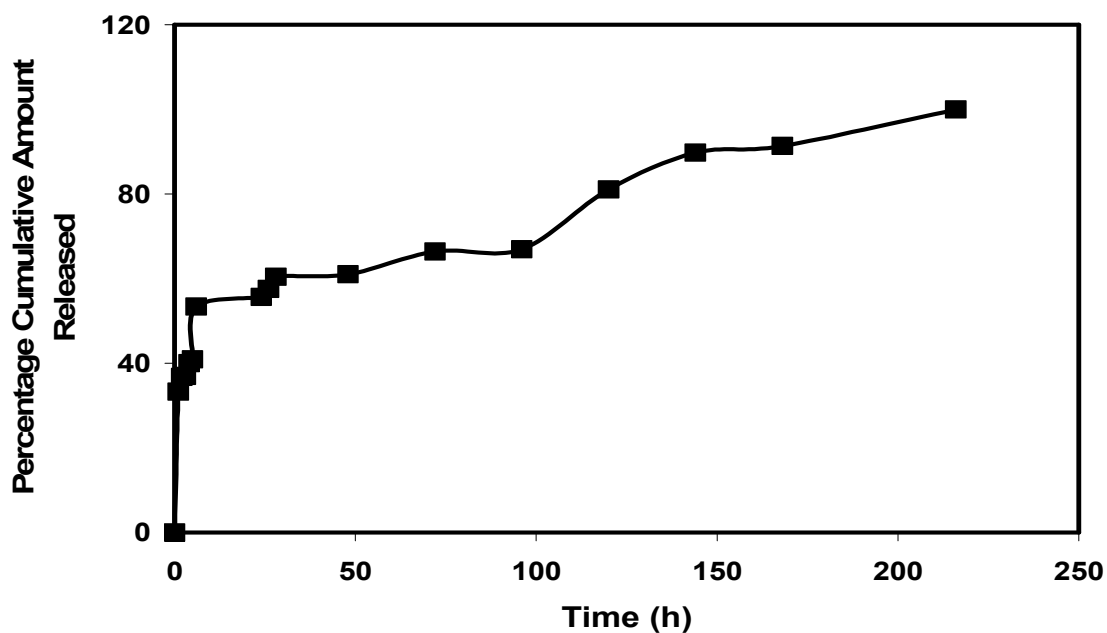
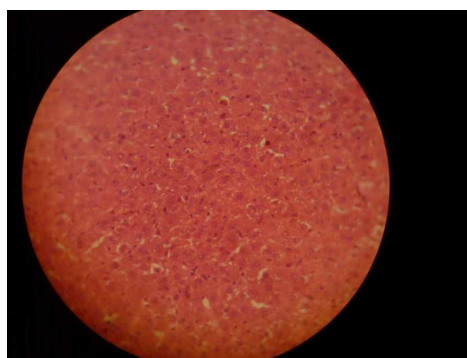


Figure 3: *In vitro* release profile of NNDMAC microspheres



A) Saline Control



B) NNDMAC Microparticulate System



C) CCl₄ Toxic Control

Figure 4: Histology of rat livers

Table 1: SGOT and SGPT levels for Hepatoprotective activity

Group Name	Drug and Dose	ROA	SGOT U/L	SGPT U/L
Saline Control	Normal Saline, 1ml/rat	Oral	18.66±0.62	8.85±0.88
IP MS	NNDMAC microspheres in normal saline, equivalent to 100mg/kg of NNDMAC + CCl ₄ , 0.7ml/kg, 25%v/v in olive oil	IP	19.99±0.92	9.52±0.93
CCl ₄ toxic	CCl ₄ , 0.7ml/kg, 25%v/v in olive oil	IP	53.6±2.14	37.128±1.89

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