

FORMULATION OF NANOPARTICLES OF TELMISARTAN INCORPORATED IN CARBOXYMETHYL CHITOSAN FOR THE BETTER DRUG DELIVERY AND ENHANCED BIOAVAILABILITY

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ABSTRACT

Objective: In this study, we have made an attempt to the developed formulation of nanoparticles (NPs) of telmisartan (TLM) incorporated in carboxymethyl chitosan (CMCS) for the better drug delivery and enhanced bioavailability.

Materials and Methods: The NPs size and morphology were investigated by high-resolution transmission electron microscopy and field emission scanning electron microscopy, respectively. The crystal structures and surface functional groups were analyzed using X-ray diffraction pattern, and Fourier transform infrared spectroscopy, respectively.

Results: To increase the solubility of TLM by targeted delivery of the drug through polymeric NPs is an alternative efficient, option for increasing the solubility. TLM nanosuspension powders were successfully formulated for dissolution and bioavailability enhancement of the drug. We focused on evaluating the influence of particle size and crystalline state on the *in vitro* and *in vivo* performance of TLM.

Conclusion: In summary, we have developed a new approach toward the delivery of poorly water-soluble drug TLM by CMCS NPs. The particles having a good drug loading content and drug encapsulation efficiency. The cytotoxicity of the synthesized NPs is also very less.

Keywords: Telmisartan, Nanoparticle, Carboxymethyl chitosan, Transmission electron microscopy, Scanning electron microscopy.

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INTRODUCTION

The administration of drug telmisartan (TLM) by the oral route is desirable but difficult due to the variable pH of the gastrointestinal tract. The presence of enzymes such as pepsin and the highly acidic pH in the stomach can cause protein degradation. There is a substantial loss of drug activity due to pancreatic enzymes secreted by the lumen of the intestine and also by membrane-bound brush-border enzymes. Finally, the physical barrier of the intestinal cells must be crossed before a drug can reach the circulation. To reduce all the above problems, nanoparticles (NPs), a new drug delivery system has been developed [1-3]. For medical purposes NPs are an attractive material which depends on their important and unique properties, such as their surface to mass ratio is much larger than that of other particles, their quantum properties and their ability to absorb and carry other compounds [4-6]. NPs of polymer not only protect the bioactive substance but also facilitate the control release of the material in delivering system [7-10]. In comparison to other polymer, chitosan is used extensively in drug delivery applications [11-16]. Chitosan can be produced in a different form such as fiber, film, paste, powder due to its reactive nature [17,18]. The solubility of chitosan depends on the free distribution of amino and *N*-acetyl group [19]. Chitosan slowly metabolizes into less harmful metabolite (amino sugars) which are completely absorbed by human body [20]. Carboxymethyl chitosan (CMCS) can be obtained by direct alkylation of chitosan. The CMCS achieved can be in various types: *N*, *O*-CMCS and *N,N*-CMCS, *N*-CMCS, *O*-CMCS. Different degree of substitution and different reaction conditions will result in the *N* versus *O* selectivity. CMCS is soluble in water at neutral pH. It possesses large hydrodynamic volume, high viscosity, film and gel forming capabilities and the other useful properties, such as biodegradation, biological activity, and low toxicity, biocompatibility all the above properties makes it an attractive

option in relation with its use in drug delivery, food, and cosmetics. To synthesize *O*-CMCS NP we have used CaCl_2 as crosslinking agent. To achieve targeting property, CaCl_2 is incorporated into the amphiphilic polymers through coupling reaction between the -COOH group of CMCS through 2, 2'-(ethylenedioxy)-bis-(ethylamine) to obtain the final product. At neutral pH is expected to constitute a hydrophobic core of self-assembled NPs which can serve as a depot for hydrophobic drugs. At acidic pH, the free amines group of chitosan is to be protonated, causing disassembles of the NPs and eventual release of drugs [21-23].

Chitosan, a copolymer of *N*-acetyl-D-glucosamine and glucosamine linked by glycosidic bonds, is the second plentiful biomass produced by deacetylation of chitin. It can absorb toxic metals such as mercury, cadmium, and lead. It also has following properties such as antimicrobial, immuno-stimulation, coagulation ability, good adhesion, bio-degradability, biocompatibility, hemostatic, and fungistatic. In view of the above properties, chitosan has been used as a biomaterial for drug delivery, gene delivery, and other biomedical application [24-28]. For the preparation of mucoadhesive formulation, chitosan has been used [29-32]. It improves the rate of dissolution of poorly soluble drug for the targeted drug delivery and enhances the absorption of the peptide. Cell adhesion and potential cell uptake of NPs of chitosan should be efficient due to its positive charge which is attracted by negatively charged cell membranes. Since it is poorly soluble in water and inorganic solvents which limit its wide applications. Chitosan has positive charges ($-\text{NH}_3^+$) thus at relatively low pH (<6.0) are soluble in dilute aqueous solutions, but as the pH increases they lose their charge and precipitate from the solution due to de-protonation of the amino group [33-37].

For the prevention and treatment of hypertension TLM an, angiotensin II receptor antagonist is used. TLM is less soluble and highly permeable

because it belongs to Class II drug. The low solubility of the drug in biological fluids which results into poor bioavailability after oral administration is one of the big problems [38-41]. The solubility of TLM in water is very low, i.e., 0.078 mg/ml in water. Poor bioavailability after oral administration of the TLM is due to its bioavailability that is 42-58%, and biological half-life is only 24 hrs. Thus, increasing dissolution and aqueous solubility of TLM is of therapeutic importance [42-49]. Various approaches for enhancing solubility and oral efficacy of TLM used were solid dispersion and inclusion complexation [50-52]. None of the above approaches offered an adequate improvement in therapeutic potential due to the dosage form limitations itself. Thus, we used the CMCS polymers to load TLM, which have not been used to best of our knowledge and there is no report about the use of this based drug delivery. In light of these, the aim of the present investigation was done to design and develop novel nanoparticulate solid oral dosage forms of TLM by incorporating it into the CMCS.

In this research, the solubility and bioavailability of TLM in conjugation with various carriers using various methods were studied. Besides this solubility and dissolution study, the various evaluation methods were carried out to determine the physicochemical properties of solid dispersions and physical mixture in comparison to pure drug. To increase the solubility and for targeted drug delivery of TLM use of polymeric NPs is an alternative efficient, option. In this study, TLM nanosuspension powders were successfully formulated for dissolution and bioavailability enhancement of the drug. We focused on evaluating the influence of crystalline state and particle size on the *in vitro* and *in vivo* performance of TLM.

MATERIALS AND METHODS

Materials

Drugs and chemicals

Purchases were as follows: Chitosan, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TLM, and monochloroacetic acid were purchased from Sigma-Aldrich (USA). Calcium chloride, isopropanol were obtained from Merck India. Drug solutions were freshly prepared in millipore water. Organic solvents such as methanol and ethanol were purchased from Qualigens Fine Chemicals (Mumbai). Dimethyl sulfoxide (DMSO) and *N,N*-dimethyl formamide were obtained from Merck India. Fetal bovine serum and minimum essential medium were procured from Hyclone (USA) and Himedia (India), respectively. All chemicals were used without further purification.

Methods

General procedure for preparation of CMCS

CMCS were synthesized using chitosan (1.0 g) was swelled in 50% (w/v) sodium hydroxide solution (40 ml) at 0°C for overnight. Further chitosan was washed with isopropanol and dissolve in isopropanol (30 ml). A reaction mixture of monochloroacetic acid (3.0 g) and isopropanol (5 ml) was added dropwise into the chitosan mixture and reacted for 12 hrs at 40°C. The reaction was stopped by adding 70% ethyl alcohol (25 ml). The solid was filtered and washed with ethyl alcohol and vacuum dried at room temperature. The resulted product was sodium salt of CMCS [53].

Preparation of CMCS NPs

CMCS NPs were synthesized by ionotropic gelation of CMCS solution with calcium chloride solution. About 0.5% CMCS solution was prepared in distilled water. The solution (5 ml) was added in 1.5 % CaCl₂ (1 ml) solution under continuous stirring. The resulting NPs were purified by centrifugation for 10 min at 15,000 rpm and lyophilized. A diagrammatical representation is given in Fig. 1 for better insight into the development of nanoparticles (NPs).

Drug loading into NPs

TLM (1 mg/mL) in the poorly water soluble drug was dissolved in glacial acetic acid solution (10 ml). CMCS NPs (10 mg) were

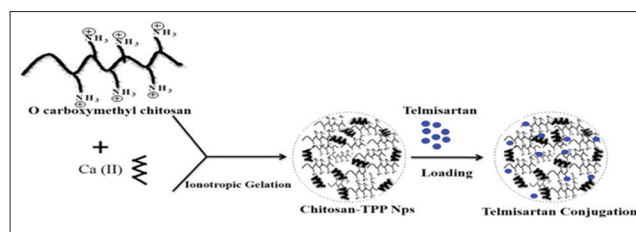


Fig. 1: Schematic representation of synthetic procedure of carboxymethyl chitosan-telmisartan

introduced into the previously prepared drug solutions. The solution was shaken in an orbital shaker for 24 hrs. The suspension was then centrifuged at 15,000 rpm for 20 minutes to separate the NPs from the solutions. The procedure was repeated 3 times to eliminate any unloaded TLM from the suspension. The amount of loaded TLM was measured spectrophotometrically at 295 nm with a ultraviolet (UV)-1700 spectrophotometer (Shimadzu). The drug loading content and entrapment efficiency were calculated according to the following equations.

$$\text{Drug loading contents (\%)} = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles taken}} \times 100$$

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles taken}} \times 100$$

Characterization of CMCS nanoparticles

Particle size and zeta potential measurement

Mean particle size its distribution were determined using dynamic light scattering (DLS) technique. Zeta potential was evaluated by measuring electrophoretic mobility of the particle using a laser-based multiple angle electrophoresis analyzer. TLM NPs were sufficiently diluted with deionized water to reduce the count rate up to 100-250 kcps and analyzed by employing Malvern Zetasizer (Nano ZS, Malvern Instruments, UK). Observations were recorded in triplicate for each sample at 25±1°C.

Particle size measurement by transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

Particle's morphological characterization of TLM NPs was executed by SEM and TEM. Samples for SEM studies were prepared by clinging the NPs on a double adhesive tape stuck to an aluminium stub and were coated with gold and palladium under an argon atmosphere utilizing a high-vacuum evaporator (Polaron SEM coating system). Samples were scanned through an electron microscope (EVO-50, ZEISS; UK) and photomicrographs were captured. For TEM, a drop of optimized NP suspension in aqueous solution was placed on a carbon film coated on a copper grid and freeze dried. Then, NPs were observed at 80 kV using Tem TECNAI G² 20 S-TWIN (FEI Netherlands) instrument, from Indian Institute of Toxicology Research (IITR), Lucknow.

Aggregation state of TLM nanoparticles

The physical and chemical aggregation state of TLM was evaluated using UV and Fourier transform infrared (FTIR) spectroscopy. The FTIR spectrums of TLM and drug loaded chitosan NPs (freeze dried) were obtained using spectrometer (Perkin Elmer). A total of 2 mg of sample was mixed with 100 mg of dry potassium bromide (KBr) and the mixture was ground into fine powder using a mortar before compressing into (KBr) disk under a hydraulic press at 10,000 psi. Each KBr disk was then scanned at 4 mm/s at a resolution of 2/cm over a wave number region of 400-4000/cm using IR solution (software ver. 1.10). The characteristics peaks of functional groups in the drug samples were determined and compared with pharmacopoeial standards, and the

possible interactions between drug and excipients were analyzed by obtained data. The electronic configuration of TLM NPs was elucidated by a UV spectrophotometer (UV 1700, Shimadzu, Japan). Lyophilized DTX-NCs were dispersed in triple-distilled water (3.0 mL) and scanned in the range from 200 to 400 nm. The UV spectrum of pure TLM was taken after solubilizing it in methanol.

Evaluation studies

Dissolution studies

In-vitro drug release study for optimized formulations of TLM loaded polymeric NPs was conducted in triplicate by equilibrium dialysis membrane method. The amount of drug release from the formulation was measured using spectroscopic method. The experiment was performed by taking specified volume of formulation in hermetically sealed dialysis membrane. The dialysis bag was suspended in pH 7.4 phosphate buffer saline (PBS) at 37°C being stirred at 240 rpm. Samples were collected at predetermined time points, and subsequently, media was replenished with pH 7.4 PBS after each sampling to maintain sink conditions.

In vivo studies

The experiments were carried out using adult male animals spontaneously hypertensive rat (SHRs) (225-250 g, 8-9 weeks old) procured from the laboratory animal services division of Central drug research Institute, Lucknow, India. Experiments were performed according to internationally followed ethical standards and approved by the Institutional animal ethics committee. They were kept in a polyacrylic cage (22.5 cm × 37.5 cm) and were maintained under standard housing conditions (24-27°C and humidity 60-65%) with a 12 hrs light and dark cycle. They were fed a standard chow diet and water were available but were withdrawn 1 hr before administration of drugs till completion of the trial. After the 1-week acclimatization period, animals were used of experiments, and all efforts were made to minimize animal suffering.

The animals were divided into the following three groups (TLM dose, 5 mg/kg):

- Group I: Treatment TLM (control group)
- Group II: Blank formulation (CMC-TLM)
- Group III: Formulation (CMC-TPP-TLM).

Measurement of blood pressure (BP) and heart rate (HR) using the telemetry system SHRs and maintained at 37°C on a servo controlled, heated rodent operating table. SHRs had a BP catheter attached to a telemeter inserted into their abdominal aorta. Hemodynamic data were measured over a 10 seconds interval every 5 minutes.

Dose 1 was used for vehicle-treated control. BP (mmHg) and HR (beats/min) were measured. Each treatment by single injection (n=6) was performed with an interval of several days between each injection for washout and return to higher systolic BP (SBP) (200 mmHg). Mean arterial pressure and HR were recorded continuously in telemetered rats and data were collected over 24 hrs after injection.

Cytotoxicity study

MTT assay

Non-cytotoxic dose of chitosan, formulation, and combination of chitosan + formulation was identified in HaCaT, MCF-7, A549, and HepG2 human cell lines. Cytotoxicity assessment was done using standard endpoint, i.e., tetrazolium bromide MTT assay. In brief, cells (1×10^4 cells/well) were seeded in 96-well tissue culture plates and incubated in the CO₂ incubator for 24 hrs at 37°C. Then, the medium was aspirated and cells were exposed to medium containing either of chitosan, formulation, and combination of chitosan + formulation (0.001-100 µg/ml) for 24-96 hrs at 37°C in 5% CO₂-95% atmosphere under high humid conditions. Tetrazolium salt (10 µl/well; 5 mg/ml of stock in PBS) was added 4 hrs before completion of respective incubation periods. At the completion of incubation period, the reaction mixture

was carefully taken out and 200 µl of culture grade DMSO was added to each well. The content was mixed well by pipetting up and down several times until dissolved completely. Plates were then incubated for 10 minutes at room temperature and color was read at 550 nm using multiwell microplate reader (Synergy HT, Bio-Tek, USA). The unexposed sets were also run parallel under identical conditions that served as a basal control.

Neutral red uptake (NRU) assay

The assay was carried out following the protocol described earlier by Siddiqui *et al.* [54]. Cells were exposed to compound in identical experimental setup as to MTT assay. On the completion of incubation period, medium was aspirated and NRU salt (50 µM/mL in medium) was added 100 µl per well plate and incubated for 3 hrs. Then, the reaction mixture was carefully taken out and plates were washed with washing solution (100 µl/well) containing 1% CaCl₂ (w/v) and 0.5% Formaldehyde for HCHO (v/v) to remove unincorporated dye. Washing solution was removed and mixture of 200 µl; 1% acetic acid and 50% ethanol was added. The plates were kept on rocker shaker for 10 min at room temperature and then analyzed at 540 nm using multi-well microplate reader (Synergy HT, Bio-Tek, USA). Unexposed sets were also run under identical condition and served as control.

Lactate dehydrogenase (LDH) release assay

LDH release assay is a method to measure the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay was carried out using readymade commercially available LDH assay kit for *in vitro* cytotoxicity evaluation (TOX-7, Sigma St. Louis, MO., USA). The assay was based on the reduction of nicotinamide adenine dinucleotide (NAD) by the action of LDH. The resulting reduced NAD (NADH⁺) was utilized in the stoichiometric conversion of a tetrazolium dye. The resulting colored compound was measured using multiwell plate reader at wavelengths 490 and 690 nm. In brief, the cells were exposed with (0.001-100 µg/ml) for different time periods after the completion of respective time periods the cells were processed for LDH release assay similar to MTT assay. Culture plates were removed from CO₂ incubator as per the experimental schedule and centrifuged at 250 × g for 4 minutes. Then, supernatant of each well was transferred to a fresh flat bottom 96 well culture plate and processed further for enzymatic analysis as per the manufacturer's instructions.

RESULTS AND DISCUSSIONS

Characterization studies

FTIR analysis

FTIR study was conducted to characterize any possible interaction between drug and CMCS NPs. The characteristic peaks at 3420/cm (O-H stretching and N-H stretching vibrations), 1583/cm (corresponding to the amino groups), 1645, 1548/cm (amide I and amide II of amino group), and 1076/cm (C-O-C stretching vibration) confirms the formation of CMCS, as shown in Fig. 2.

DLS

The hydrodynamic diameter of CMCS NPs are measured by DLS is 150±10 nm, whereas that of TLM loaded NPs is 160±10 nm as shown in Fig. 3. Results representing that there is no big change between the two types of NPs, which demonstrates that the size distribution are not significantly affected during the encapsulation of TLM. In physiological conditions, the NPs are very stable and the stability was maintained for 12 days. In the stability study, on aggregation or precipitation of NPs was observed during the course of storage. Moreover, changes in particle size of the NPs of the study were very negligible. Zeta potential (ζ) is an electric potential of the shear plane attached to the moving particles in the medium and it is generally utilized for the prediction of stability of nanosuspensions. The decrease in zeta potential value with decrease in concentration of stabilizer may be attributed to the inability of stabilizer to prevent particle agglomeration at lower concentrations.

The results of the optimized batches of TLM loaded nanosuspension depicted the zeta potential values of more than 30 mV, which suggested physical stable formulation developed [9].

TEM

The characterization of morphology and size of the nanoparticles are performed using the TEM technique. TEM micrographs were obtained

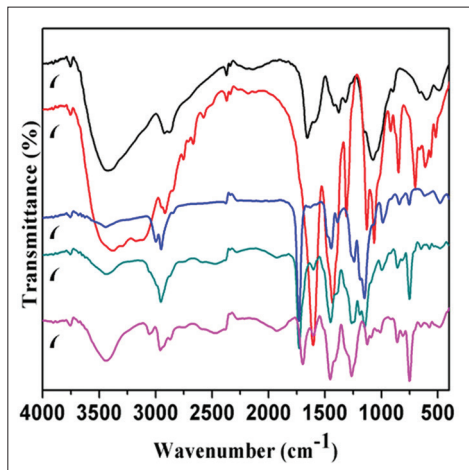


Fig. 2: Fourier transform infrared spectra of carboxymethyl chitosan (CMCS), CaCl₂, telmisartan (TLM) and CMCS-TLM

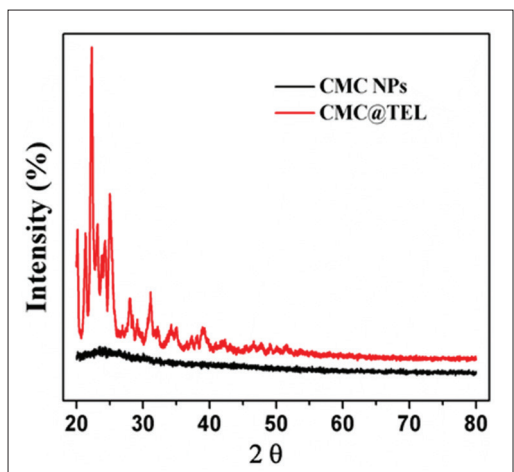


Fig. 3: Hydrodynamic diameter of telmisartan loaded nanoparticles measured by dynamic light scattering

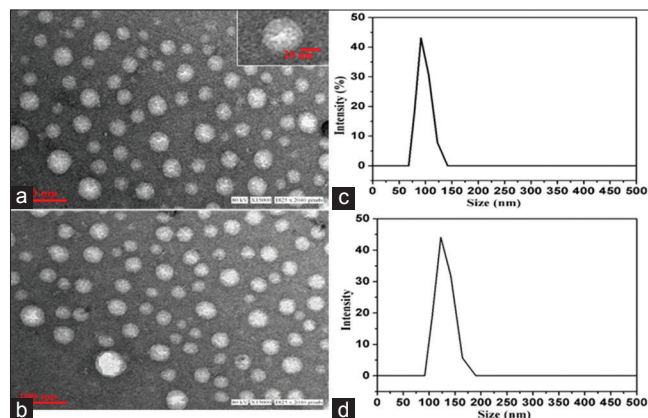


Fig. 4: (a-d) Transmission electron microscopy photograph of optimized batch of telmisartan loaded nanosuspension

with an acceleration voltage of 200 kV. A drop of sample solution was placed onto a copper grid with carbon, taped with a filter paper to remove surface solvent, and air-dried for 2 minutes. The study results are shown in Fig. 4.

SEM

SEM was used to determine the particle size and surface morphology of the NPs. The study reveals that the surface of the NPs was smooth and

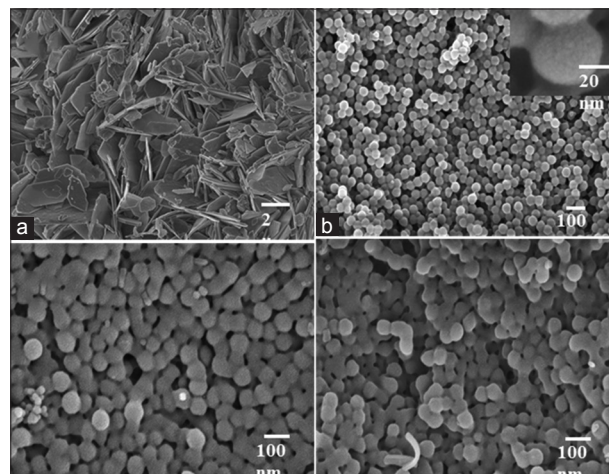


Fig. 5: (a-b) Scanning electron microscopy microphotographs of telmisartan nanoparticles

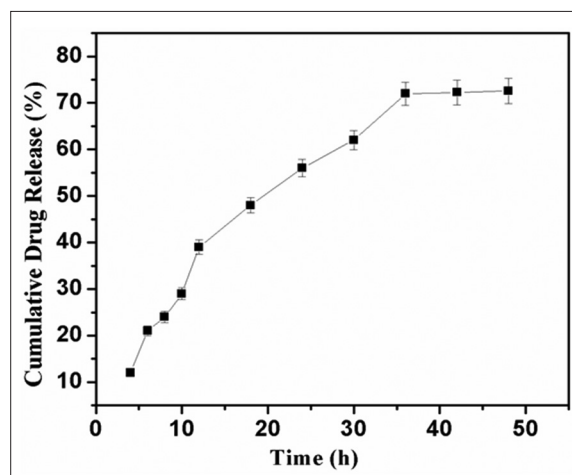


Fig. 6: In-vitro release studies of telmisartan nanoparticles

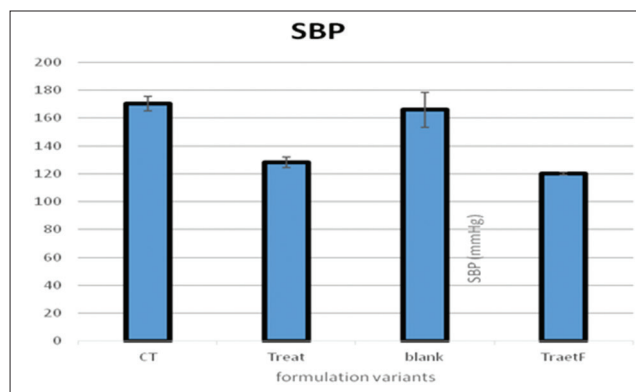


Fig. 7: The effect of telmisartan treatment on blood pressure in spontaneously hypertensive rats

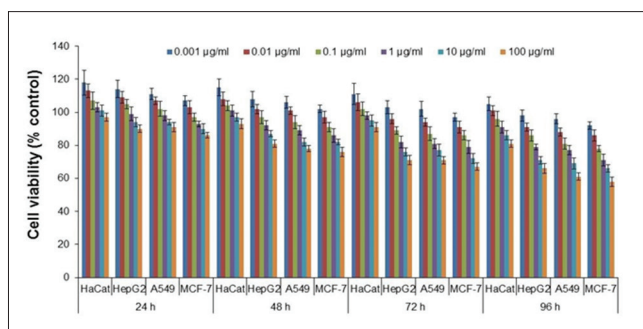


Fig. 8: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of chitosan + formulation

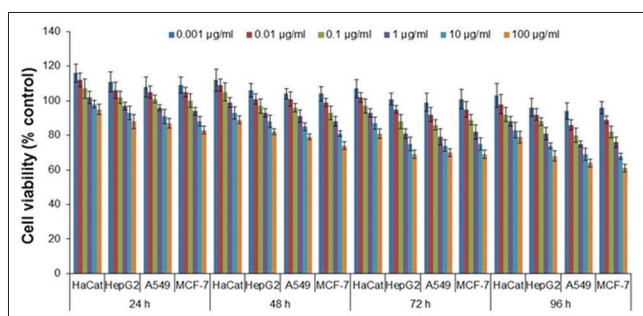


Fig. 9: Neutral red uptake assay of chitosan+ formulation

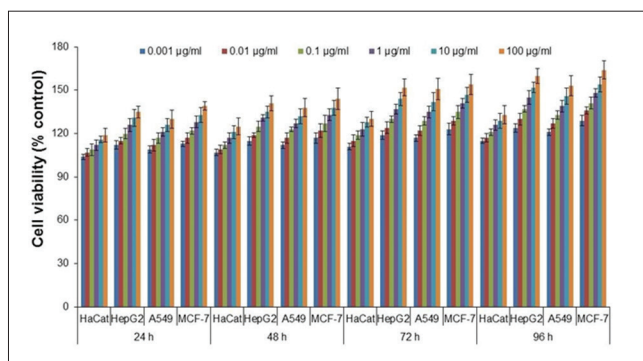


Fig. 10: Lactate dehydrogenase assay of chitosan + formulation

no aggregate were found from the sample used for SEM analysis. The particle size of the NPs was found to be 100 nm. The study results are shown in Fig. 5.

Evaluation studies

In-vitro drug release studies

The *In-vitro* release studies were carried out using dialysis membrane. The cumulative percentage drug release after 48 hrs was found to be. The significantly lower values of TLM loaded nanosuspension suggested market improvement in dissolution rate of by the developed nanosized formulation in dissolution velocity of TLM NPs could be attributed to the reduction in particle size, in surface area, and decrease in diffusion distance, whereas the incomplete drug release of drug particles could be attributed to the large crystal size Fig. 6.

In vivo studies

To determine the effect of TLM treatment on BP in SHR rats, we measured the SBP of the group rats every week. The baseline SBP in SHRs was 170 ± 2 mm Hg which was much higher than in WKY (121 ± 1 mmHg, $p < 0.001$). Administration of nano TLM in the dose of 5 mg/kg concentration showed a significant decrease in SBP than the

administration of 5 mg/kg TLM showed a substantial decrease in SBP at the end of week, showed significant reduction when compared with the vehicle control SHR rats Fig. 7.

Cytotoxicity study

MTT assay

The highlights of the data of cytotoxicity activity of test compounds on independent exposure as well as in combination are summarized in Figs. 8-10. A time and dose-dependent decrease in percent cell viability was observed in all the cells used in the study. However, none of the concentration could induce loss of all viability upto statistically significant level in any cell type. HaCat, a normal human cell line was used as positive control against the human cancer cell lines of different origin viz., HepG2 (hepatoma cells), A549 (lung epithelium carcinoma cells) and MCF-7 (human breast adenocarcinoma cells). However, no cell specific changes could be recorded and there was a non-significant loss of cell viability with more or less equal magnitude in all the cell types. Hence, the effect of chemicals could be speculated due to cytostatic responses, not due to anti-cancer activity. Although the combination of both the test compounds increases the magnitude of percent cell viability loss, could not attain the significant levels. In the present investigations, both the test compounds were found to be non-cytotoxic/non anti-carcinogenic upto 100 µg/ml concentration for a long term exposure, i.e., till 96 hrs in human cancer cells of different origin. Even the combination of these two test compounds were also not able to brought the effect to the significant level. In cytotoxic investigations, both the test compounds (CMCS, CMCS-TLM NP) were found to be non-cytotoxic/non-anti-carcinogenic upto 100 µg/ml concentration for a long term exposure, i.e., till 96 hrs in human cancer cells of different origin. Even, the combination of these two test compounds were also not able to brought the effect to the significant level.

CONCLUSION

In summary, we have developed a new approach toward the delivery of poorly water soluble drug TLM by CMCS NPs. The particles having a good drug loading content and drug encapsulation efficiency. The cytotoxicity of the synthesized NPs are also very less.

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