European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 01, 2020

Development Of Rifampicin Loaded Hyaluronic Acid Coated Chitosan Nanoparticles.

Suchita Prabhakar Dhamane[1], Swati Changdeo Jagdale[2]

[1]MAEER's Maharashtra Institute of Pharmacy, Maharashtra, India.
[2]Professor, Dept of Pharmaceutics, School of Pharmacy, Dr. Vishwanath Karad MIT World Peace University, MIT Campus, Kothrud, Pune, Maharashtra, India.

Email: - [1]spd.jscopr@gmail.com

ABSTRACT: There is a promising potential for nanoparticles in developing controlled and guided drug delivery systems. The most effective formulations are currently considered to be chitosan based nanoparticles; biocompatible, biodegradable, less toxic and simple to use in preparations. Chitosan is a natural biopolymer and has many advantages that can easily be used to achieve the ideal drug delivery mechanism. It is licensed and classified in General Safe (GRAS) by U.S. Food and Drug Administration (USFDA). In the current study, chitosan nanoparticles loaded with Rifampicin were prepared and published in-house. Hyaluronic acid (HA) was coated and further characterized using effective in-vitro methods. The research also included the determination of pharmacokinetic parameters. The findings were confirmatory and can be used to increase the accumulation of medicines.

KEYWORDS: Rifampicin, Hyaluronic Acid, Chitosan, Nanoparticles.

1. INTRODUCTION

Tuberculosis is Mycobacterium tuberculosis' most widespread lethal infectious disease. In 2018, 10 million people globally suffered from TB; 1.6 million tuberculosis-related deaths and 26, 90,000 people had TB in India and maximum patients had drug-resistant TB. Mycobacterium tuberculosis triggers the lung infection that has primordiality and can spread to different parts of the body such as kidney, lymphatic system, central nervous system (meningitis). Tuberculosis is treated with many medications sensitive to the organism and avoids mycobacterium mutation. According to the WHO recommendations, Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA) and ethambutol are treated regularly for two months followed by INH and RIF for a further four months. The major issues with TB care include long-term treatment and repeated multiple dosing of medications that result in a patient having poor or non-compliance with the current therapy. Treatment non-compliance is one reason why MDR-TB and XDR-TB.1, 2,3 Intra-venous or oral administration of anti-TB medicines distributes to the body target and non-target organ through systemic circulation and very less of drug molecules meet its target and adverse effects on the non-target site4.
Ramkhenda G. G. et al (2019) stated in their analysis that the unfavorable outcome of TB in India is due to the concentration of anti-TB drugs (First line agent) in subtherapy. They concluded that pulmonary TB patients treated as an independent risk factor for unfavorable TC treatment in India have a low rifampicin concentration two hours after dosage. Owing to a

low serum concentration of anti-TB medications, the subsubtherapy concentration of medications enters the cavitarian pulmonary and extrapulmonary tissue contributing to inability to treat, recurrence, and acquired drug tolerance. The results of the anti-TB treatment outcome are bacterial load, strain type, virulence, MIC in relation to the concentration of the drugs at the site of action, drug concentration at the place of action, infections duration, disease severity, and immunity, and nutrition status. Increased medication dose aggravates many side effects related to earlier-line chemotherapy, including hepatotoxicity / nephrotoxicity / ocular toxicity / ototoxicity. While evidence suggests an increased dose of the anti-TB drug to minimize therapy and retain the therapeutic concentration of an anti-TB medication, the dosage that could be used for a favorable tuberculosis result is highly toxic to anti-TB drugs. The therapeutic index of these anti-TB agent can be supplemented by delivering a high drug concentration to the action site i.e. macrophage: a cell which engulfs and gets infected with mycobacterium tuberculosis. This macrophage represents various receptors of the cell surface due to infection with mycobacterium. Surface receptors are intended to identify mycobacterium. Following mycobacterium recognition, receptors like mannose, dectin-1, complement, surfactant protein, scavengers, CD14 and CD44 receipts join macrophage. The destiny of mycobacterium is determined of internalization of receptor type. The presence of muramyl dipeptide on the mycobacterium cell wall induces the expression of CD44 receptors, which also mediates Mycobacterium tuberculosis internalization. CD44 receptors are characterized by an internalisation of the non-sulphated, natural non-protein glycosaminoglycan (GAG) ligand hyaluronic acid with distinct physicochemical properties and are bound to the CD44 cell surface receptor. Chitosan, partially deacetylated chitin, consisting of polycationic N-acetyglucosamine, biocompatible, low immunogenic, and low-cost polymer9. Hyaluronic acid conjugated chitosan nanoparticles of anti-TB may be a promising approach to active macrophage delivery by internalization through CD44 receptors 7 , 8. For the preparation of chitosan nanoparticles, multiple interlinkers such as glutaraldehyde, aldehyde, glyoxal, sodium tri polyphosphates-be used. These crosslinkers interact with the chitosan functional amino and hydroxyl group. Cross-linkers such as glutaraldehyde, aldehydes and glyoxal have a less residual cytotoxicity and mechanical power of sodium tripolyphosphate or sodium sulphates prepared chitosan nanoparticles, which results in the explosion of drug 10,11. Therefore, in this report, an attempt has been made to formulate and evaluate chitosan rifampicin nanoparticles, with vanillin as a linker and nanoparticles obtained covered with hyaluronic acid.

2. MATERIALS AND METHODS

MATERIALS

Chitosan was procured from Analab Fine Chemicals, Mumbai. Rifampicin was obtained as kind gift from Lupin laboratories Ltd. Pune. Vanillin was purchased from Loba Chem, Mumbai. All other chemicals were of analytical grades.

METHODS

Development of Rifampicin loaded chitosan nanoparticles

Chitosan nanoparticles (FR5) were prepared by the procedure reported by Dhamane S. P. et. al. Chitosan was dissolved in aqueous acetic acid solution. Vanillin and RIF was dissolved in ethanol. Vanillin and RIF solution was added drop wise in chitosan solution and the obtained solution was stirred for 5hrs to obtain chitosan nanoparticles. Obtained RIF nanoparticle suspension was stored for further characterization[12].

Coating of optimized rifampicin nanoparticles with Hyaluronic acid

Optimized nanoparticle was coated by hyaluronic acid (HA1FR-0.5mg/ml, HA2FR- 1mg/ml, and HA2FR-1.5mg/ml).

1. Optimized nanoparticle suspension was centrifuged at 1300RPM. Obtained nanoparticles were washed with ethanol and redispersed in 10ml of 1% acetic acid solution.
2. Required quantity of hyaluronic acid was dissolved in 10ml of 1% acetic acid.
3. Hyaluronic acid solution and redispersed nanoparticle suspension was added simultaneously in a beaker and stirred vigorously for 1hrs.
4. Hyaluronic acid coated nanoparticle suspension was centrifuged and obtained nanoparticles were washed with distilled water, freeze dried and stored for further characterization.

Characterization of RIF loaded Hyaluronic acid coated chitosan nanoparticles suspension:
Prepared RIF loaded Hyaluronic acid coated chitosan nanoparticle suspensions were evaluated for the following parameters.

1. Physical examination:
The prepared nanoparticle suspensions were visually inspected for colour and appearance.

2. Entrapment efficiency (% EE):
EE (%) was determined by measuring the concentration of unentrapped drug after separation. To separate the nanoparticle, nanoparticle suspension was centrifugated at 12000rpm for 30 min. The supernatant was diluted with ethanol and analyzed by UV spectroscopy. Then the percent entrapment was determined using the following equation 1:

$$\%\, EE = \frac{Weight\ of\ drug\ used\ in\ formulation - Weight\ of\ unbound\ drug\ in\ supernatant}{Weight\ of\ drug\ used\ in\ formulation} \times 100 \text{---------} \quad (1)$$

PS, Zeta potential and Polydispersity index (PDI)

*2. **Dynamic Light Scattering***
(MAL1098084, Zetasizer 7.12, Malvern Instruments, UK) was used to measure the PS and zeta potential (ZP) of all the nanoparticles. All samples were diluted with distilled water to make up a suitable concentration. The Z-average PS, polydispersity index (PI), and zeta potential (ZP) were determined.

3. Transmission Electron Microscopy
The morphology of optimized nanoparticles in suspensions was observed by using transmission electron microscopy (TEM, H-7650, Hitachi, Japan). Nanoparticle suspension was dropped onto film-coated copper grids and stained with 2% phosphotungstic acid solution for 3 min. The excess of solution was removed using a filter paper and the copper grids were dried for observation.

4. In-vitro drug release study of rifampicin
For *In Vitro* drug release study, 2ml of optimized nanoparticle suspension was placed in dialysis bag with molecular weight cut off 1000. The dialysis bag was then immersed into 60ml of 7.4 phosphate buffer solution and shaken on rotary shaker at 60 rpm at 37°C. A sample was withdrawn at different time intervals of 0, 1, 3, 5, 10, 16, 24hrs and analyzed simultaneously by UV spectrophotometry at 332.2 nm[10].

5. FTIR study

FTIR spectrum of optimized rifampicin nanoparticles were determined by FTIR spectrophotometer (FTIR) technique wherein 1-2 mg of rifampicin nanoparticles was used. The sample was placed in contact with the surface of an IR transmitting crystal. The scanning range was 450 to 4,000 cm^{-1} and the resolution was 1 cm^{-1}.

6. Differential scanning calorimetry (DSC):

Differential scanning calorimetry (DSC) thermograms were recorded for rifampicin, Chitosan, vanillin and the optimized rifampicin nanoparticles. Samples were placed in an aluminium pan and pan was sealed with lid. Thermograms were obtained using a differential scanning calorimeter (DSC STARe, Mettler Toledo, USA). The samples were heated from 40°C to 300°C under a nitrogen atmosphere at a heating rate of 10°C/minute.

7. X-ray diffraction studies

X-ray powder diffraction patterns of rifampcin, chitosan, vanillin and optimized dried rifampicin nanoparticles (Batch FR5) were recorded on X-ray diffractometer (Bruker D8 Advance) at a scan rate of 5°/min in the 2θ range from 2.5° to 80°

8. Macrophage uptake study of rifampicin loaded hyaluronic acid coated chitosan nanoparticles:

Cell lines and culture medium

RAW264.7 cells line was procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cells were dissociated using cell scraper at 80% confluence. The viability of the cells was checked and centrifuged. Further, the cells were counted and seeded at appropriate density.

Procedure

The cells were aspirated from the culture flask and centrifuged at 1000rpm for 5mins. The cell pellet was then resuspended in 1mL of DMEM (Dulbecco's Modified Eagle's Medium) complete media and cell count was adjusted using hemocytometer at a density of $1x10^6$ cells/ml. To each P35 dish, 1ml of cell suspension containing $1X10^6$ cells was added. After 24 h, to determine the effect of samples on phagocytic activity, RAW264.7 cells were treated with various concentrations of test samples at 37ºC in 5% CO_2 atmosphere. Post incubation, the cells were inoculated with heat inactivated yeast cells at a density of 10^8 cells/dish and the cells were further incubated for another 4hrs to assess the phagocytic activity. Post incubation, test solutions were removed and the cell monolayer was gently washed with 1X PBS. The cells were fixed with 3.7% formaldehyde for 2 mins and phagocytic cells were scored by observing the cells under inverted light microscope[13,14,15].

9. Pharmacokinetic study

In vivo studies were performed to compare the plasma profile of pure drug, chitosan nanoparticles and hyaluronic acid coated chitosan nanoparticles. Adult male wistar rats (150-200 g) were used in the study. The animals were kept under standard environmental conditions of room temperature (22 ±2°C), relative humidity (50% ± 5%) and 12 h light and dark cycle. The animals were housed in the colony cages (either three rats or six mice per cage) and provided feed (commercial pellets contain a balanced diet) and water ad libitum. All the animals were acclimatized to the laboratory environment 5 days prior to experiment. The animals were fasted overnight just prior to the experiment but was allowed free access to drinking water. All the experiments were carried out in accordance with the guidelines of Institutional Animal Ethics Committee CPCSEA/IAEC/PT-14/01-2K19.

Group I: Pure drug Rifampicin

Group II: Rifampicin nanoparticle
Group III: HA coated Rifampicin nanoparticle
For rifampicin dose calculated was 62mg/kg. Pure drug was dissolved in 1ml of gum acacia solution (2%w/v). Formulations were administered orally by feeding needle.

Collection of blood samples
Blood samples (1 ml) were collected in EDTA coated bottles through retro orbital route during a dosing interval at the following times: 0 (prior to drug administration), 0.5, 1, 2, 3, 5, 8, 12, 18, and 24 h post dose. Samples were centrifuged for 15 min at 1300 rpm to collect plasma and then frozen at −20° C until analysis.
Pharmacokinetic analysis
Plasma samples were analyzed for Rifampicin concentrations by H.P.L.C. with U.V. detection under conditions mentioned in "Preparation of spiked plasma samples". Pharmacokinetic parameters like Cmax, tmax, AUC (0,24 Hr), AUC (infinity), etc were observed for reference standard and its formulations.

3. RESULTS AND DISCUSSION

Macrophage possesses various receptors such as Fc, mannose, complement and scavenger. As this activated macrophage express CD44 and therefore can internalize hyaluronic acid which is a natural selective CD44 targeting moiety. For targeted delivery of anti-tuberculosis drug to the macrophage hyaluronic acid coated chitosan nanoparticles were prepared by the method reported by Wang T. et. al. Formation of HA coated chitosan nanoparticles is due to the strong electrostatic interaction between the cationic amino group of CS and the anionic carboxyl group of HA, HA was conjugated at the surface of the CS NPs by charge adsorption to obtain Rifampicin-loaded HA-coated CS NPs[16].

Physical examination:
Formulated HA coated chitosan nanoparticles were found to be red in colour.

Table 1. Particle size and zeta potential of formulated batches.

Formulation Code	**Colour**	**Particle size**	**Zeta Potential**	**Entrapment efficiency**
H1FR	Reddish orange	211.23nm	16.5	82.56±1.26
H2FR	Reddish orange	221.44nm	12.5	86.23±1.08
H3FR	Reddish orange	301.67nm	9.11	87.21±0.98

As the concentration of hyaluronic acid increases there is increase in particle size and entrapment efficiency. As hyaluronic acid is anionic due to the presence of carboxyl group which strongly interacts with the cationic amino group present on the surface of chitosan nanoparticles and decreases the zeta potential. Similar results were reported in literature by Wang T. et al [16]. Increase in entrapment efficiency may be due to drug which is present on the surface of a particle get entrapped within nanoparticle.

Transmission electron microscopy:

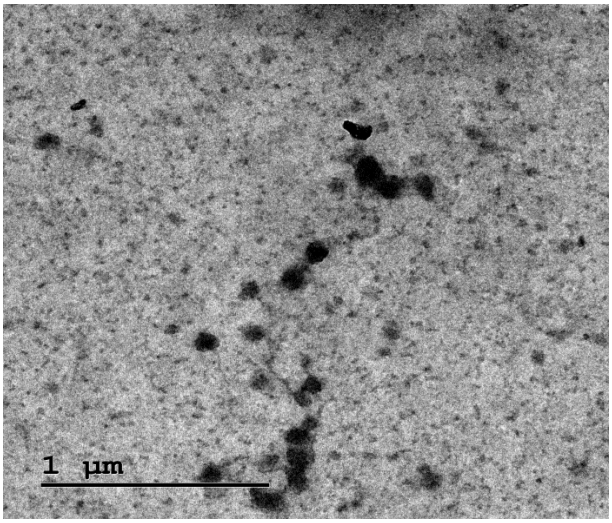
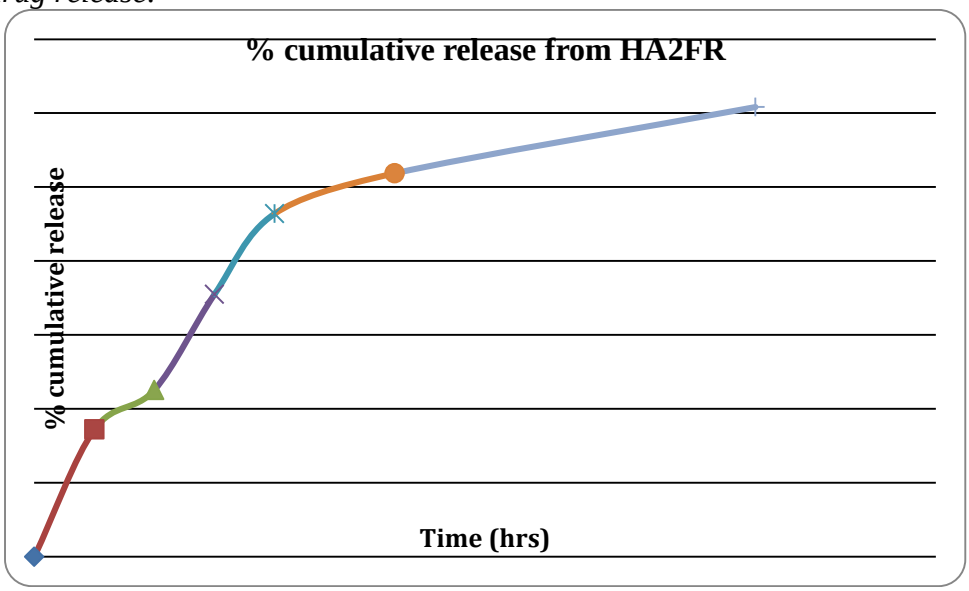


Fig. 1. Transmission electron microscopy of HA2FR

Transmission electron microscopy of HA2FR shows that HA coated nanoparticles are spherical.

In vitro drug release:



Graph 1. % cumulative release from HA2FR

Drug release from HA2FR was found to be sustained release. At the end of 24 hrs 60.84% of rifampicin was released from the formulation.

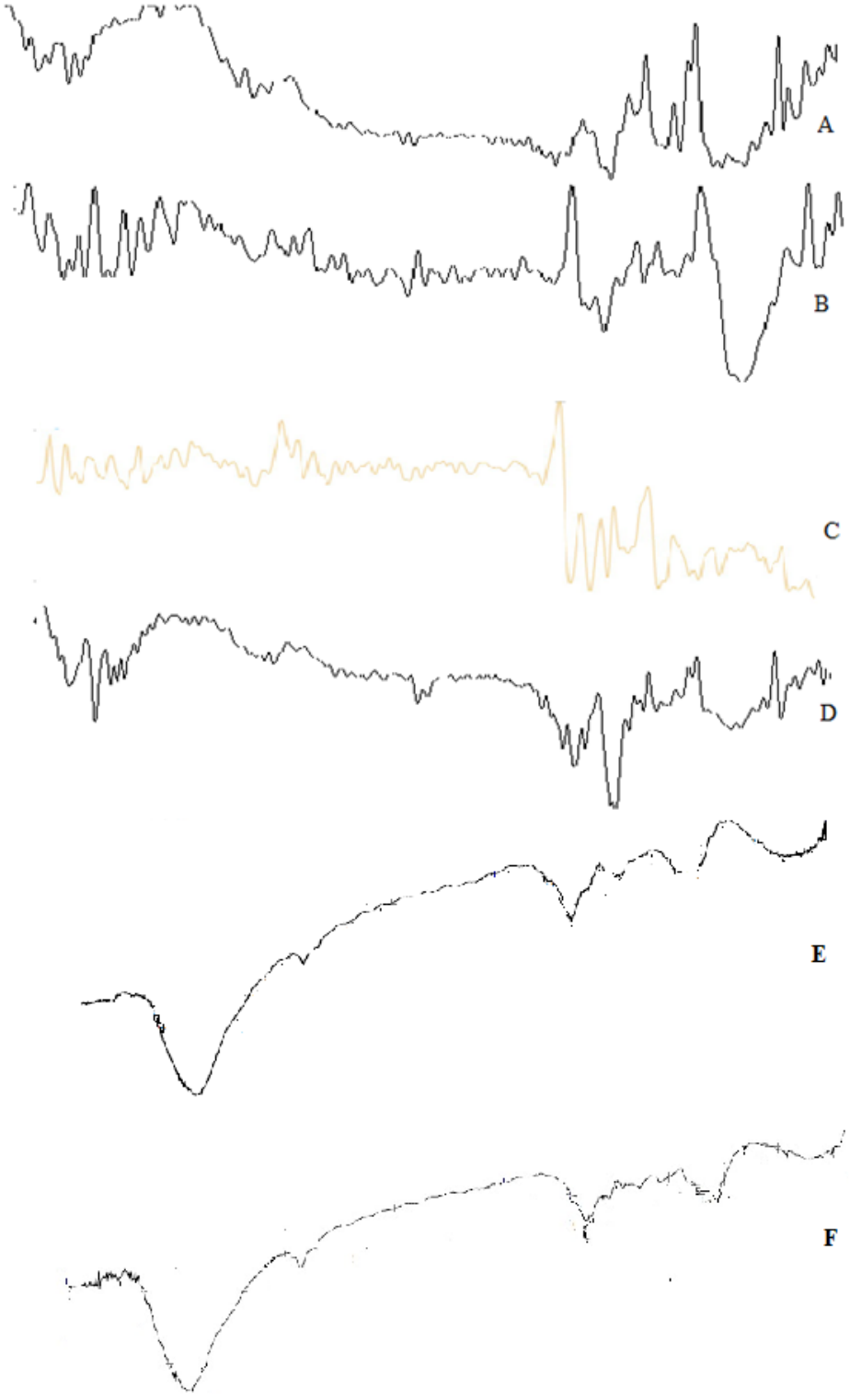


Fig 2: FTIR spectrum of A) Rifampicin B) Chitosan C) Vanillin D) Rifampicin loaded chitosan nanoparticle E) Hyaluronic acid F) Rifampicin loaded hyaluronic acid coated chitosan nanoparticles.

In FTIR spectrum of pure RIF (Fig.2A) absorption band at 3447 cm^{-1} was due to presence of –OH and –NH group, peak at 1726 cm^{-1} corresponds to C=O acetyl stretching, peak at

1620cm^{-1} indicates presence of C=N stretching, 1620cm^{-1} indicates C-O-C stretching and peak at 811cm^{-1} is due to C-H stretching. In FTIR spectrum of pure chitosan (Fig.2B), stretching vibrations at 3300-3500cm-1 indicates presence of –OH and –NH group. Stretching vibrations at 1589 cm-1 is due to presence of amino group. Peak at 1028cm-1 indicates stretch vibrations of C-O. Peak at 1317cm-1 indicates stretching vibrations of C-N bond. In FTIR spectrum of pure vanillin (Fig.2C) peak at 3150cm-1 is due to stretching vibrations of –OH, peak at 1675 cm-1 corresponds to stretching vibrations of C=O of aldehyde group, peak at 1590, 1514 and 812 cm-1 is due to presence of benzene ring. In FTIR spectrum of RIF loaded chitosan nanoparticles (Fig.2D) peak at 1697 cm-1 corresponds to characteristics vibrations of C=N which indicates formation nanoparticles due to interaction between the aldehyde group of vanillin and amino group of chitosan. In FTIR spectrum of hyaluronic acid (Fig 2E) broad peak at 3443cm^{-1} is due to presence of amine group, peak at 1629cm^{-1} indicates presence of C=O. In FTIR spectrum of hyaluronic acid coated chitosan nanoparticles shifting of peaks were observed, new peaks were not observed which indicates that coating of chitosan nanoparticles with hyaluronic acid is due to electrostatic attraction.

Characteristics of freeze dried HA coated chitosan nanoparticles of Rifampicin

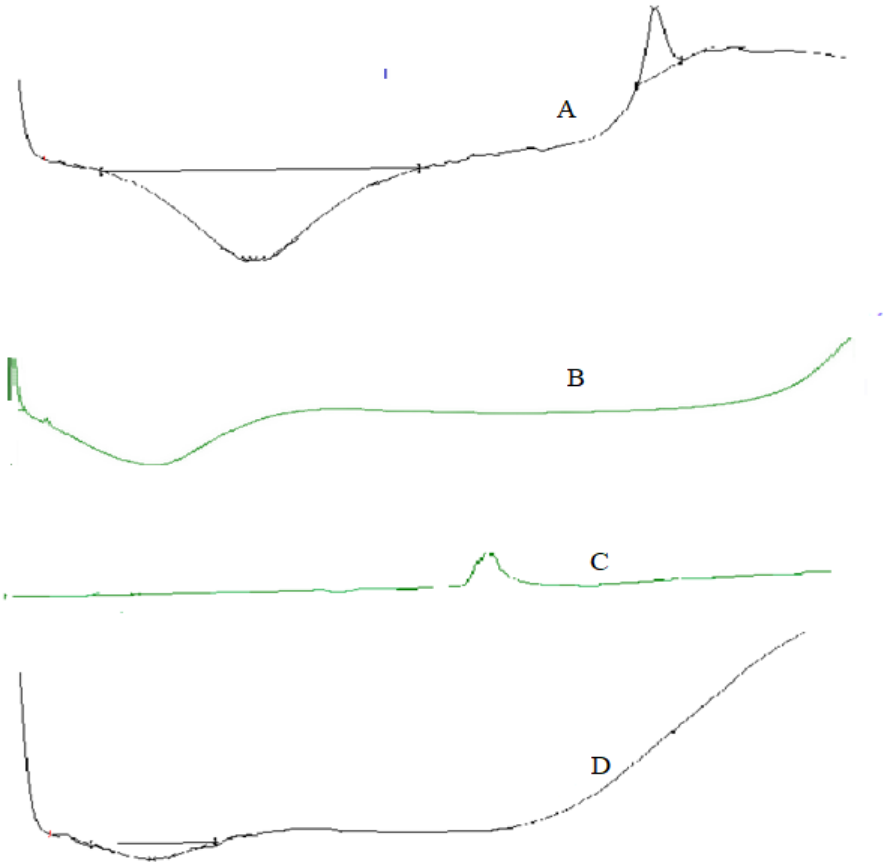


Fig. 3. DSC thermogram of A. Hyaluronic acid, B. Chitosan C.Rifampicin, D. Hyaluronic acid coated chitosan nanoparticles of rifampicin

The DSC thermograms of rifampicin exhibit an endothermic peak showed melting around 196.23 °C. HA exhibited one exothermic peak presenting crystallization around 237 °C and

broad endothermic peak of chitosan at 84.0°C. Characteristic peaks of rifampicin and HA were not exist in the thermograms of HA coated chitosan naoparticles of rifampicin. These findings confirm the development of HA coating of chitosan nanoparticles of rifampicin.
XRPD.

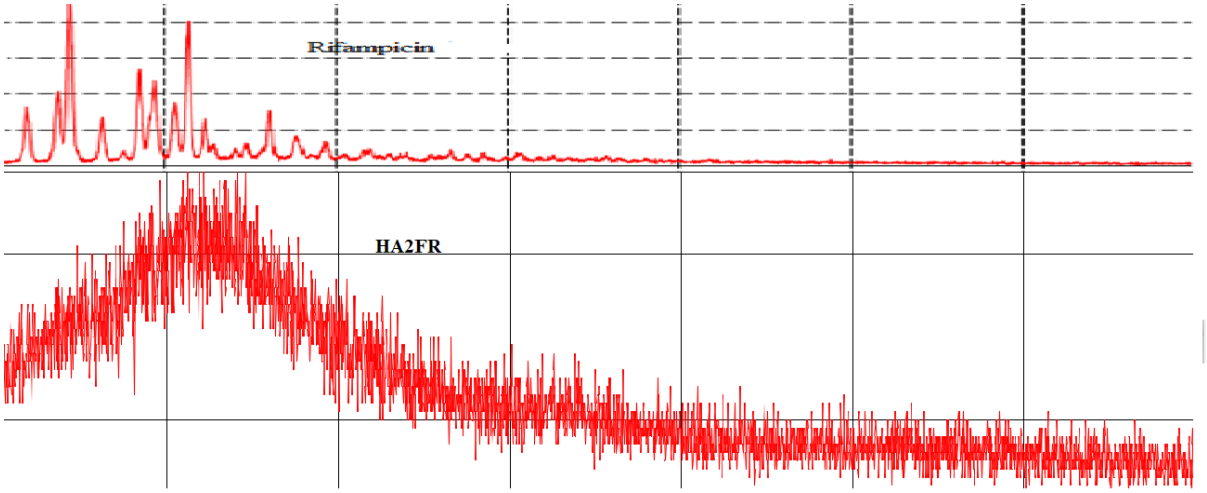


Fig. 4: XRPD of rifampicin and rifampicin loaded hyaluronic acid coated chitosan nanoparticles.

X-ray diffractogram of RIF indicates crystalline nature of drug and X-ray diffractogram of RIF loaded hyaluronic acid coated chitosan nanoparticles confirms formation of nanoparticles as crystalline peaks of rifampicin are disappeared indicating rifampicin is converted into amorphous form.

Macrophage uptake study
Macrophage uptake analysis will perform targeting efficiency of nanoparticles. To test the effect of hyaluronic acid coating on macrophage uptake, the study used raw 264.7 cell lines. Cell lines were incubated with pure drug rifampicin, rifampicin-loaded chitosan nanoparticles (FR), HA-coated rifampicin chitosan nanoparticles (HA2FR) and the degree of cell uptake was determined by examining cells under inverted light microscope. Macrophage uptake analysis findings are shown in fig. Results suggest the macrophage absorption of HA-coated chitosan nanoparticles compared to chitosan nanopaticles and pure drug rifampicin.
HA2FR5>FR> Pure drug rifampicin

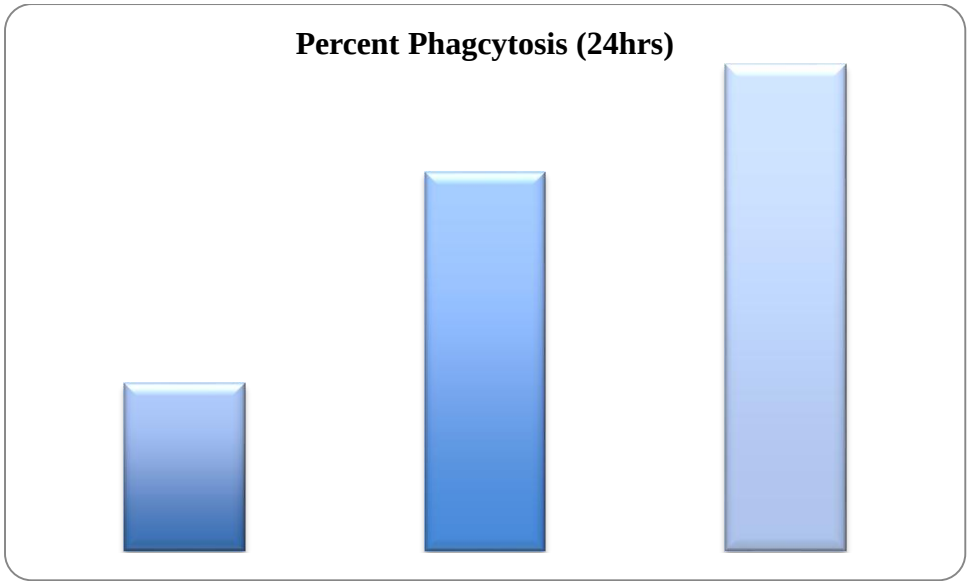


Chart 1. Percent phagocytosis of nanoparticles.

Pharmacokinetic studies:

Rifampicin loaded chitosan nanoparticles (FR), hyaluronic acid coated chitosan nanoaprticles (HA2FR) were given orally to rats to study pharmacokinetic parameters. Rifampicin's plasma concentration profile in rat plasma.

Rifampicin in hyaluronic acid-coated chitosan nanoparticles increased rifampicin AUC and Cmax relative to chitosan nanoparticles & pure drug. Coating chitosan nanoparticles with hyaluronic acid provides stealth layer targeting CD44 receptor. Hyaluronic acid also inhibits protein adsorption and nanoparticles opsonization.

Table 2. Pharmacokinetic parameters of optimized batches.

Formulation Code	**Cmax (µg/ml)**	**Tmax (hrs)**	**AUC (µg.hr/ml)**
Rifampicin (Pure drug)	4.165	2.17	98.894
FR	7.506	2.33	179.332
HA2FR	8.554	2.83	209.0.89

As the Cmax of HAFR is higher as compared to pure drug and FR it may reduce the dose of a drug.

4.CONCLUSION

Rifampicin loaded hyaluronic acid coated chitosan nanoparticles were prepared by electrostatic attraction coating of hyaluronic acid on the surface of rifampicin loaded chitosan nanoparticles. Surface modification of nanoparticles increased particle size, trap strength, and decreased chitosan nanoparticles' zeta potential. The analysis FTIR, DSC, and XRPD showed the formation of loaded hyaluronic acid coated chitosan nanoparticles. Macrophage absorption of loaded hyaluronic acid-coated chitosan nanoparticles was higher. Hyaluronic acid nanoparticles also strengthened rifampicin AUC and Cmax. Study results indicate that established nanoparticles could effectively target macrophage, a residual site of mycobacterium tuberculosis.

Acknowledgement
Authors are thankful to MAEER's Maharashtra Institute of Pharmacy, Pune for providing technical assistance during research work.

Conflicts of the interest
All authors declare that they have no conflict of interest.

5. REFERENCES

[1]. WHO (2018) Global Tuberculosis Control. Geneva, Switzerland.
[2]. WHO (2010) Treatment of tuberculosis guidelines. 4th ed. Switzerland
[3]. Kempker R, Kipiani M, Mirtskhulava V, Tukvadze N, Magee M, Blumberg H. Acquired Drug Resistance in Mycobacterium tuberculosis and Poor Outcomes among Patients with Multidrug-Resistant Tuberculosis. Emerging Infectious Diseases. 2015; 21(6), 992-1000.
[4]. Nasiruddin M, Neyaz K, Das S. Nanotechnology-Based Approach in Tuberculosis Treatment. Tuberculosis Research and Treatment. 2017.1-12.
[5]. Ramchandran G, Kumar H, Chandrasekaran V, Kannan T, Vijaylashmi R, Dusthakeer A, Ramesh K, Lavanya J, Swaminathan S. Factors influencing tuberculosis treatment outcome in adult patients treated with thrice- weekly regimens in India. Antimicrob. Agent Chemother. Feb.2017. DOI: 10.1128/AAC.02464-16.
[6]. Ruslami R, Nijland H., Alisjahbana B, and Parwati I, Crevel R, Aarnoutse R. Pharmacokinetics and Tolerability of a Higher Rifampin Dose versus the Standard Dose in Pulmonary Tuberculosis Patients. Antimicrobial Agents and Chemotherapy Jun 2007, 51 (7) 2546-2551; DOI: 10.1128/AAC.01550-06
[7]. Schafer G, Jacobs M, Wilkinson R, Brown G. Non-opsonic recognition of Mycobacterium Tuberculosis by Phagocytosis. 2009; 1: 231-243.
[8]. Vachon E et al. CD44 is a phagocytic receptor. Blood, 2006; 107(10), 4149-4158.
[9]. DOI: 10.1182/blood-2005-09-3808
[10]. Ahmed TA, Aljaeid BM. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. Drug Design, Development and Therapy. 2016;10: 483–507
[11]. Li PW , Wang G ,Yang ZM ,Duan W , Peng Z , Kong LS, Wang QH Development of drug-loaded chitosan–vanillin nanoparticles and its cytotoxicity against HT-29 cells. Drug Delivery. 2014; 1–6.
[12]. Xiong H, Peng H, Li J, Xie M, Liu Y, Bai C, Chen L.Vanillin cross-linked chitosan microspheres for controlled release of resveratrol. Food chem. 2010;121(1):23-28.
[13]. Dhamane S., Jagdale S Development of Rifampicin loaded Chitosan nanoparticles by 3^2 full Factorial design. Research J. Pharm. and Tech. 13(6): June 2020
[14]. http://www.protocol-online.org/prot/Protocols/In-vitro-Phagocytosis-Assay-of-Macrophages-3460.html
[15]. Marcil A, Harcus D, Thomas D, Whiteway M. Candida albicans killing by RAW 264.7 mouse macrophage cells: effects of Candida genotype, infection ratios, and gamma interferon treatment. Infection and immunity; 2002. 70(11), 6319-6329.

[16]. Lewis, L. E., Bain, J. M., Okai, B., Gow, N. A., & Erwig, L. P. (2013). Live-cell video microscopy of fungal pathogen phagocytosis. Journal of visualized experiments: JoVE, (71).

[17]. Wang, T., Hou, J., Su, C. et al. Hyaluronic acid-coated chitosan nanoparticles induce ROS-mediated tumor cell apoptosis and enhance antitumor efficiency by targeted drug delivery via CD44. J Nanobiotechnol 15, 7 (2017). https://doi.org/10.1186/s12951-016-0245-2