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"TO DEVELOP AN ALTERNATE EFFICIENT MICROPARTICULATE SYSTEM(S) FOR ANTITUBERCULAR DRUG(S) BY ENGINEERING THE SURFACE OF PARTICLES TO REACH MYCOBACTERIUM INTRACELLULARLY"

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ABSTRACT:

Due to its status as the biggest infectious disease killer globally, along with HIV infection, tuberculosis (TB) continues to be a serious global health concern (HIV). An important opportunistic illness among people with a high incidence of AIDS is tuberculosis (TB). Mycobacterium tuberculosis (MTB), which causes TB, is a lethal infectious illness that primarily affects the respiratory system. Meningitis, circulatory tuberculosis, lymphatic tuberculosis, genitourinary tuberculosis, bones, and joints are other major public health concerns caused by MTB. Pharmacokinetic event showed that an experimental carrier's drug plasma profile is significantly influenced by the physico-chemical properties of the polymer. Tmax for RIF (Free) was found to be 2.890.31hr, whereas Tmax for the chitosan ascorbate formulation was found to be 13.250.01hr and 16.410.81hr for C3S3P4T2R and C3S3P4T2I, respectively. The results make it abundantly apparent that MMAD did not significantly change when the formulations were kept in a refrigerator. Although the MMAD significantly varied when the formulations were held at room temperature, it nevertheless stayed within the range of respirable particles that alveolar macrophages can effectively absorb. The amount of drug that has been incorporated into the formulation determines its therapeutic impact; as a result, the residual drug content was tracked and contrasted with the results from the stability testing phase. The results suggested that the conjugation with ligand greatly decreased the leaching of medication from formulations. This finding may be related to the bulky group on the surface of microspheres, which may have prevented some drug leakage.

KEYWORDS: Rifampicin, Chitosan, antitubercular drug, In-vivo study.

INTRODUCTION:

Tuberculosis (TB) is still a major global health concern, being the leading cause of death worldwide among infectious diseases, alongside the infection by the human immunodeficiency virus (HIV). Tuberculosis (TB) has become a significant opportunistic disease among populations with a high incidence of AIDS. TB, a pervasive and deadly infectious disease primarily affect respiratory system, is caused by *Mycobacterium tuberculosis* (MTB) the main challenges in public health can also affect central nervous system (meningitis), lymphatic system, circulatory system (Miliary tuberculosis), genitourinary system, bones and joints . Among the various forms of tuberculosis, pulmonary tuberculosis is most commonly characterized by the involvement of alveolar macrophages harboring a large number of tubercle bacilli. The bacilli secrete molecules that prevent phagosome lysosome fusion. Moreover, due to their very hydrophobic waxy cell wall, bacilli are resistant to digestion by lysosomal enzymes and hence resist the killing effects of macrophages.

Inside the macrophages the bacteria will be either destroyed or begin replicating, or remain latent indefinitely. If replication is not prevented, the bacilli multiply and may eventually cause the macrophage to break. Problems created by bacterial infection are linked to their ability to survive and multiply inside the body, especially in the lungs, and to the natural immune response of the infected host. Treatment of TB remains a challenge for clinicians, because of the oral uptake of high systemic doses of single or combined antibiotics, which causes many side effects due to high systemic exposure. The prognosis for patient with TB improved dramatically with the discovery and introduction of antitubercular drugs, starting with streptomycin in 1946 and incidence continued to plummet in industrialized countries throughout the 20th century. All the while, TB continued to take its toll on poor and vulnerable populations in low-income countries, as well as marginalized populations in high-income countries.

EPIDEMIOLOGY:

MTB, the bacteria that cause tuberculosis (TB) was discovered by Dr. Koch on March 21, 1882 and the M. Tuberculosis genome was sequenced in 1999. MTB divides every 15 to 20 hours, extremely slowly compared to other bacteria, which tend to have division times measured in minutes. It is a small, rod-like bacillus that can withstand weak disinfectants and can survive in a dry state for weeks but can grow only within a host organism. TB is spread through inhalation of airborne M. tuberculosis cells, which multiply in macrophages, and within the large cystic tubercles, they form liquefied tissue surrounded by infected macrophages. After the inhalation of TB bacteria, it establishes with primary infection and it may cure if human have a strong immune system. But if the immune system response is weaker than bacteria will spread and the infection become Latent TB, which may responsible for Tuberculosis disease. Despite global TB eradication efforts, it is still a global public health concern, especially in low and middle-income countries. As the human race began to form denser

population centers, culminating in urbanization, MTB spread more easily, and it became one of the leading causes of death by the beginning of the twentieth century.

An important consideration in the treatment of tuberculosis is the fact that the etiological agent, MTB, has the ability to persist intra-cellular in the host macrophage for long periods of time. This becomes even more important when one considers the ability of MTB to persist in a dormant state, thus giving rise to a large group of infected individuals who carry the organism in a subclinical state without having active disease.

Role of World Health Organization (WHO) in TB control:

WHO has been a pioneer and major player in the global war against TB? It guides and coordinates the research aimed at developing new anti-TB drugs, monitoring their trials, developing suitable dosage regimens, selecting appropriate drug delivery systems and also monitoring the patient compliance at the grass root level.

Current Treatment Strategies Against Tuberculosis

Since the control measures for TB such as Bacillus Calmette-Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, the main avenue for its control is case finding and treatment. The goals of treatment are to ensure cure without relapse, to prevent death, to impede transmission, and to prevent the emergence of drug resistance. Long-term treatment with a combination of drugs is required. Various drugs which are used in the treatment of TB are tabulated in Table 1.2. The current recommended TB chemotherapy, also known as DOTS (directly observed treatment, short-course), consists of a 6-month therapy of 4 co-administered drugs. As suggested by WHO, treatment of TB and drug resistant cases requires multi-drug therapy, comprising:

- An initial intensive phase of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ), and ethambutol (ETB) daily for 2 months.
- A continuation phase of RIF and INH for a further 4 months, either daily or 3 times per week.

Even though the availability of powerful anti-tubercular drugs makes TB, curable disease, the main reason being that multiple anti-tubercular dugs need to be administered for 6-9 months. Patients often find it troublesome to begin their day with a mouthful of pills. Further, as clinical symptoms improve, they may not consider the need to continue ATD and may actually forget to take the drugs. All these factors result in non-compliance and eventually lead to therapeutic failure.

Need for novel and Effective delivery systems:

TB has been a leading killer disease globally. Even though an efficient treatment regimen against TB is existing, multiple ATDs (first-line drugs) must be administered frequently for at least 6 months, resulting often in patient non-compliance and treatment-related side effects. A common and dangerous side effect of these ATDs is hepatotoxicity, and the effect is dose related. Further to this, INH causes neurotoxicity, EMB causes ocular toxicity and STR (though not hepatotoxic) causes nephrotoxicity and irreversible ototoxicity. Over the

past two decades, pharmaceutical text has conversed itself with diverse facets of drug delivery and problems linked therein. The aspect is of utmost importance in pharmaceutical dosage forms, since the presence of drug in systemic circulation and reaching sufficient concentration at the target site to exercise therapeutic response is essential. However, presence of greater than the optimal drug concentration in plasma can result in toxic side effects.

Factors responsible for the failure of chemotherapy of TB

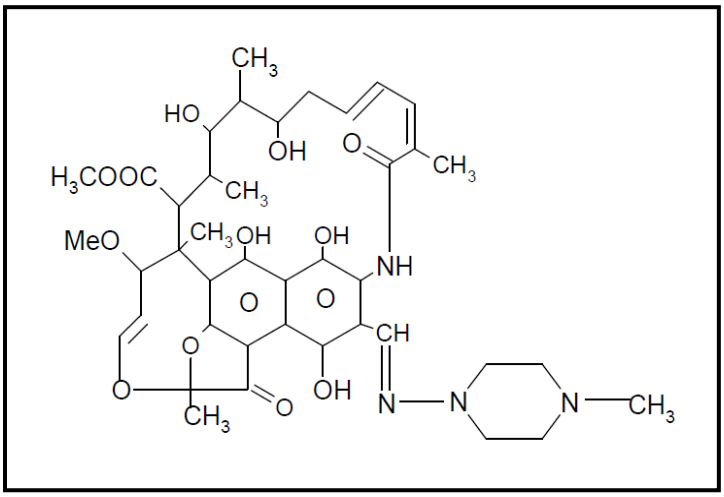
- M. tuberculosis finds its victims in developing countries with degraded social and health conditions where access to medicines is limited.
- Therapeutic regimen of long duration, patients usually do not take the prescribed medications with sufficient regularity and duration to achieve a cure.
- Treatment with a multi-drug regimen. Combined therapies are more effective than single ones, but their fulfillment becomes more difficult for patients and this leads to a poor patient compliance.
- Patients have to consume a large number of tablets (up to eight at one time),
- Which is a common cause for non-compliance.
- Concomitant presence of conditions compromising the immune system functionality such as HIV infection.

Novel drug delivery systems has been a boon to current pharmacological and biopharmaceutical enhancement of drug performance. Nowadays it is feasible to design drug delivery systems capable of targeting phagocytic cells which are infected by intracellular pathogens, such as mycobacteria. Delivery systems based on novel drug delivery systems offer wide opportunities for improving the therapy for a range of diseases including TB.

Keeping in mind the previously discussed facts in terms of increased frequency of multidrug resistant strains as an outcome of erratic and poorly maintained dosage regimen, toxicity, and side effects, it seems appropriate and worthwhile to develop novel ways of improved bioavailability and site specific targeting of these antimycobacterial therapeutics with lesser toxicity due to reduced concentrations in the plasma at any given time, assuming that the carrier system with entrapped drug acts as a reservoir of drug in plasma.

Rifampicin

Rifampicin (RIF) is a first line bactericidal antibiotic drug of the rifamycin group. It is a semisynthetic compound derived from *Amycolatopsis rifamycinica* (formerly known as *Amycolatopsis* mediterranei and Streptomyces mediterranei. It is red-orange, odorless, crystalline powder**.** RFP has two pKa values pKa_1 (1.7) and pKa_2.



Mechanism of action:

RFP inactivates bacterial RNA polymerase and it has activity against a wide range of microorganisms such as mycobacteria including Mycobacterium tuberculosis and M. leprae. Rifampicin binds to RNA polymerase at a site adjacent to the RNA polymerase active center and blocks RNA synthesis by physically preventing extension of RNA products beyond a length of 2-3 nucleotides ("steric-occlusion" mechanism).

Uses: Rifampicin is typically used to treat *Mycobacterium* infections, including tuberculosis and Hansen's disease. It can be used to treat BCG-oma, which follows as an uncommon complication of BCG vaccination for tuberculosis. With multidrug therapy used as the standard treatment of Hansen's disease, rifampicin is always used in combination with dapsone and clofazimine to avoid eliciting drug resistance. Rifampicin is used in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) in combination with fusidic acid.

Adverse effects:

1. **Nervous system reactions:** headache, drowsiness, fatigue, ataxia, dizziness, inability to concentrate, mental confusion, visual disturbances, muscular weakness, pain in extremities and generalized numbness.
2. **Gastrointestinal disturbances:** in some patients heartburn, epigastria distress, anorexia, nausea, vomiting, gas, cramps, and diarrhea.
3. **Hepatic reactions:** transient abnormalities in liver function tests e.g., elevations in serum bilirubin, BSP, alkaline phosphatase, serum transaminases have been observed. Rarely, hepatitis or a shock like syndrome with hepatic involvement and abnormal liver function tests.
4. **Renal reactions:** elevations in BUN and serum uric acid have been reported. Rarely, haemolysis, haemoglobinuria, hematuria, interstitial nephritis, renal insufficiency, and acute renal failure have been noted. These are generally considered to be hypersensitivity reactions. They usually occur during intermittent therapy or when treatment is resumed following intentional or accidental

interruption of a daily dosage regimen, and are reversible when RIF is discontinued and appropriate therapy instituted.

Study Design:

In-vivo studies were performed on healthy young albino rats, 3-4 months old (weight between 200-280g) with no prior drug treatment were used. The animals were kept with free access to water and foods.

Pharmacokinetic and Biodistribution Studies:

The drug distribution to various organs/tissues after intratracheal administration of microparticulate formulations and free drug solutions was investigated. Aerosolization was performed using a MicroSprayerTM aerosoliser (IA-1C; Penn-Century, Philadelphia, PA, USA) suitable for rat, attached to a high-pressure syringe (FMJ- 250; Penn-Century). This device is an aerosol generator consisting of a sub-miniaturized atomizer located in the tip of a 1.25' stainless steel tube, which is attached to a hand operated, high-pressure syringe. The rats were divided in 9 groups with 6 rats in each group. Throughout the study, the drugs were used at therapeutic dosage (RIF 10mg/kg body weight, INH, 5mg/kg body weight) for each of the treatment groups. Formulations were dispersed in 500µl PBS (pH 7.4) through vortex mixing for 30s. It was then administered as given below:

Table 1: Treatment groups for pharmacokinetics and Biodistribution study

Group	Formulations
Group 1	Control
Group 2	Free INH drug solution
Group 3	Free RIF drug solution
Group 4	C3S3P4T2

(n=6 in each grouFollowing the administration of plain drug and formulations, blood was collected from each group of animals after 1, 2, 4, 8, 12 and 24 hr through retro-orbital plexuses. Collected blood samples (0.5 ml) were centrifuged at 1000 rpm for 20 min; plasma was separated and filtered through membrane filter (0.45 µm). Drug estimation in plasma was done using a developed HPLC method. The plasma concentration v/s time data, obtained after intra-tracheal instillation of free drugs and different formulations were analyzed. The various pharmacokinetic parameters such as Cmax, Tmax and area under plasma drug concentration over time curve were calculated on Sigma Plot software (version 8.0). At the same time,

selected organs (lungs, liver, spleen and kidney) were excised, isolated, dried, weighed and stored at -70º in deep freezer.

In bio-distribution study, the stored isolated organs were recovered from deep freezer and kept out-side for some time at room temperature. Each tissue weighed separately, minced into pieces, and homogenized by tissue homogenizer in PBS solution. The homogenized tissues were deproteinized with Acetonitrile, vortexed for 5 min, centrifuged at 5000rpm for 10 min at 4-8°C, kept in dark for 30 min and filtered. Supernatant was filtered through a membrane filter and used for the analysis of RIF upon suitable dilution with mobile phase by HPLC technique and compared with developed calibration graphs to obtain the tissue drug concentration with respect to time **(Tian *et al.*, 2013)**.

Statistical analysis:

Result were expressed as Mean±SD and n=3. Statistical analysis was performed using Graph Pad InStat version 3.1, Graph pad software, San Diago California.

Table 2: Pharmacokinetic data of all formulations

Formulations	Cmax µg/mL	Tmax (hr)	AUC $_{0\text{-}24h}$ (µg hr/mL)
Free drug RIF	6.65±1.18	2.89±0.31	45.51±3.71
C3S3P4T2R	4.98±0.91	13.25±0.01	130.91±4.10
C3S3P4T2I	4.76±0.14	16.41±0.81	309.1±7.10
m-C3S3P4T2R	5.21±0.01	18.22±0.14	145.41±4.75
m-C3S3P4T2I	5.30±1.74	19.48±0.44	331.24±1.1

(n=3), All values are expressed as Mean± SD

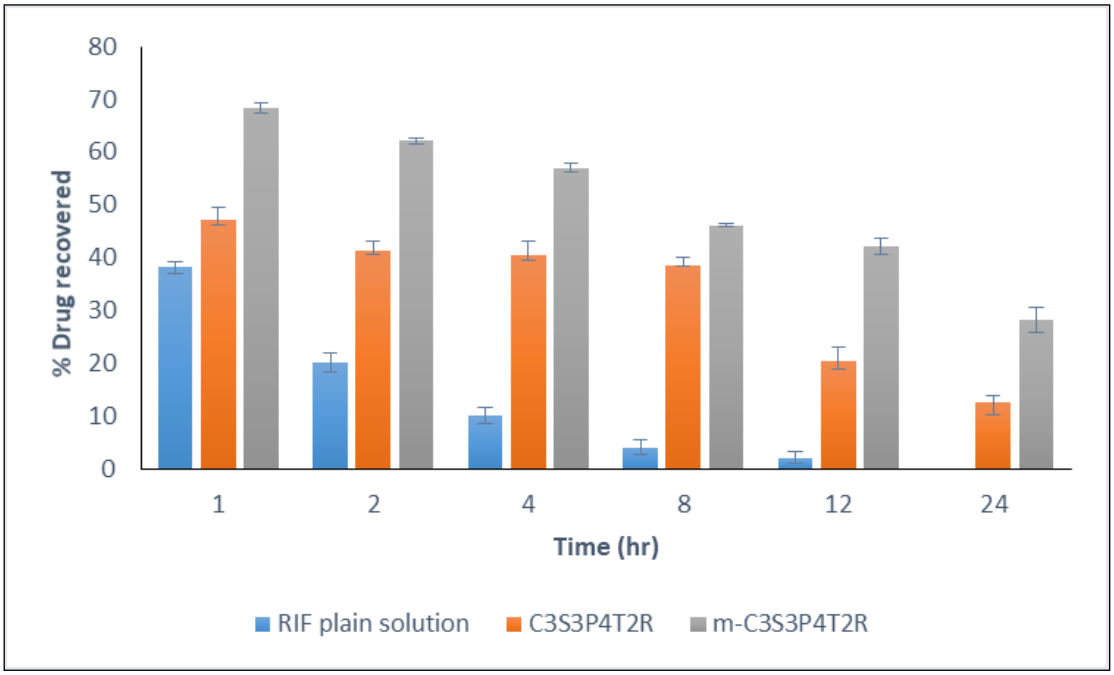


Fig.1: Organ distribution of RIF in Lungs after administration

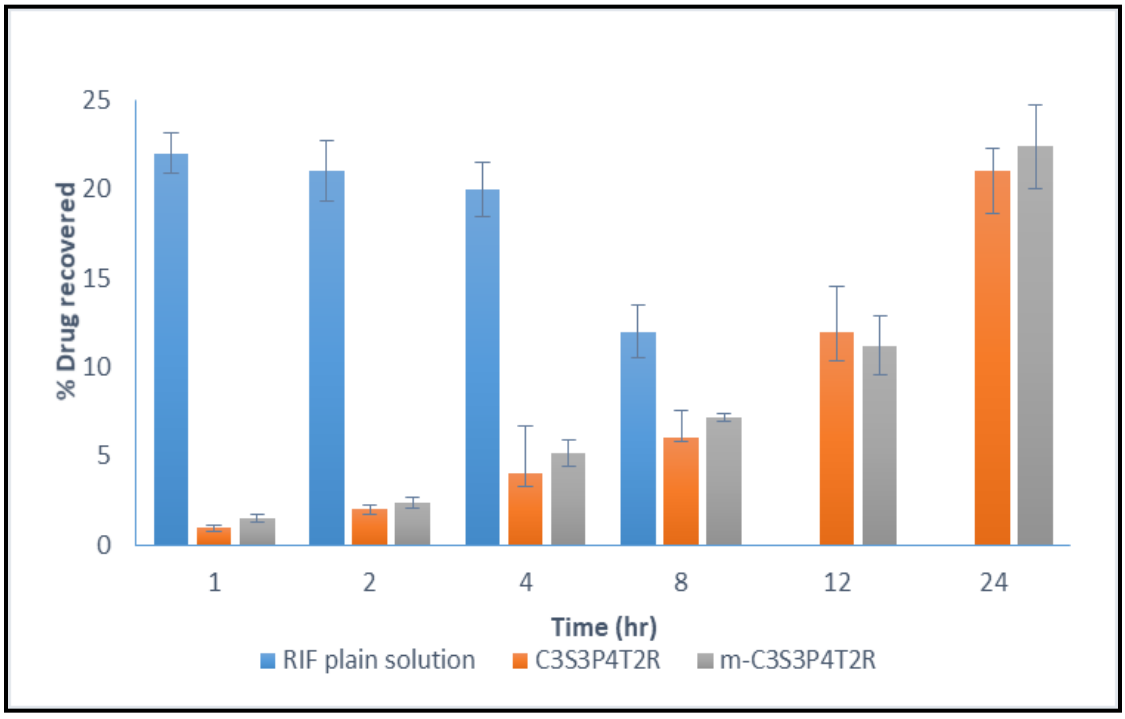


Fig.2: Organ distribution of RIF in Liver after administration

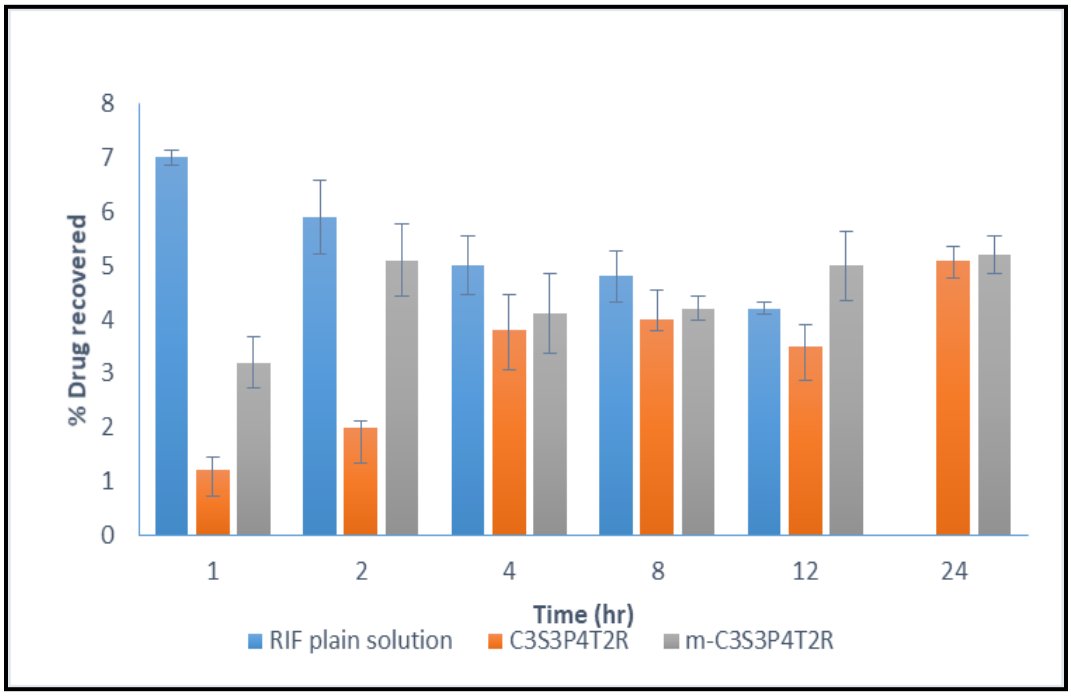


Fig.3: Organ distribution of RIF in Kidney after administration

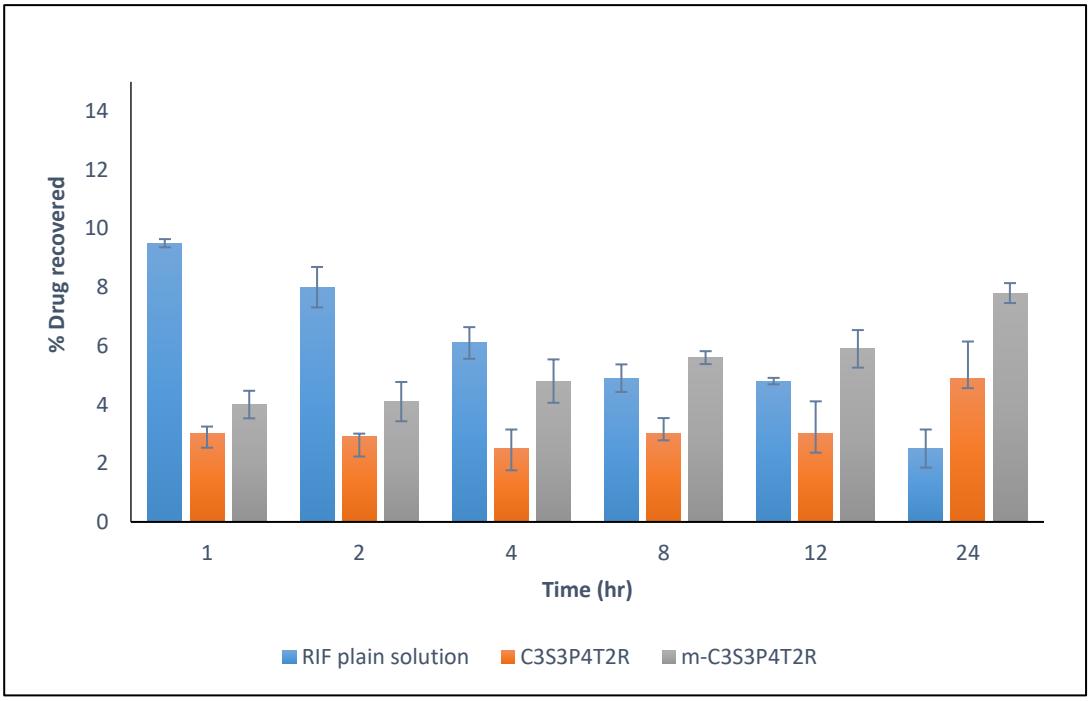


Fig.4: Organ distribution of RIF in Spleen after administration

RESULT AND DISCUSSION:

The plasma concentration v/s time data, obtained after intra-tracheal instillation of free antitubercular drug and optimized formulations were analyzed. Various parameters were calculated and compared with the free drug as described in Table 1.

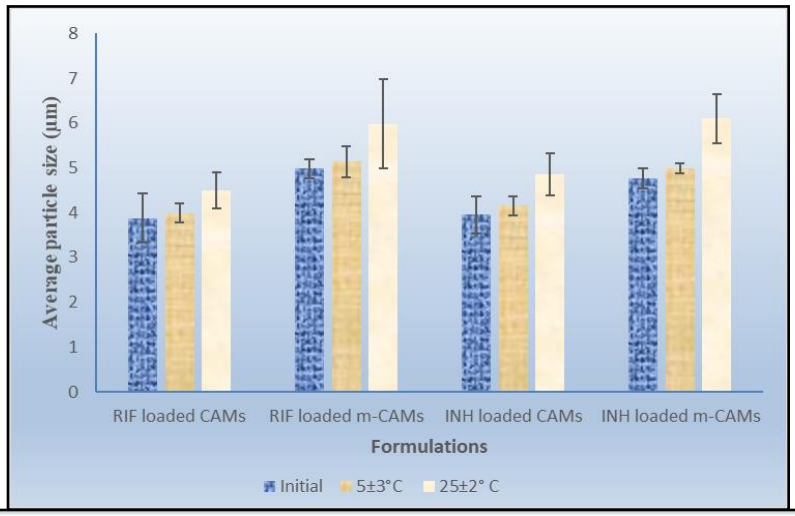
Pharmacokinetic activity revealed that the physico-chemical property of polymer plays an important role in determining the drug plasma profile of experimental carrier. Results showed Tmax of RIF (Free) was

2.89±0.31hr, whereas for chitosan ascorbate formulation Tmax was found to be 13.25±0.01hr and 16.41±0.81hr for C3S3P4T2R and C3S3P4T2I respectively. $AUC_{0\text{-}24h}$ was found to be 45.51±3.71µg.hr/mL for RIF. Whereas chitosan ascorbate microspheres showed 130.91±4. 10µg.hr/mL and 309.1±7. 10µg.hr/mL for C3S3P4T2R and C3S3P4T2I respectively. All experimental formulations exhibit higher Tmax and AUC over the plain drugs which can be attributed to significant drug release behavior of experimental formulation. Cmax was found to be a significantly lower in the chitosan ascorbate formulations indicated a substantial reduction in systemic toxicity in comparison to plain RIF.

In mannose conjugated chitosan ascorbate formulation, results showed Cmax was found to be 5.21±0.01µg and 5.30±1.74µg for m-C3S3P4T2R and m-C3S3P4T2I respectively. Tmax was found to be 18.22±0.14 hr and 19.48±.044 hr whereas $AUC_{0\text{-}24h}$ 145.41±4.75µg.hr/mL and 331.24±1.1µg.hr/mL for m-C3S3P4T2R and m-C3S3P4T2I respectively. It was concluded maximum Tmax and AUC over other formulation accounts a better utility of therapeutic entity. Higher Tmax and AUC in mannosylated chitosan ascorbate formulation could be attributed to its favourable physico-chemical behavior for pulmonary delivery and promote the uptake of carrier ensure better localization of experimental carrier at the target site.

The developed chitosan ascorbate formulations were studied for organ distribution. The percent (%) drug recovered from all experimental formulations in tissues of all the respective organs after 1, 2, 4, 8, 12 and 24 hr are shown in **Table 1, Fig. 1-4 a** bio-distribution study was conducted to determine the therapeutic efficacy of the developed formulations. Results of bio-distribution studies indicated that plain drugs show minimum lungs residence time in comparison to all other experimental formulations. RIF's shows higher lungs clearance rate which could be related to their inherent solubility. As RIF is more lipid soluble and RIF display higher lung clearance rate than INH. Among experimental formulations, mannosylated formulation shows maximum lungs residence time inferred better lungs targeting efficiency than other tested formulations. Targeting efficiency of mannosylated chitosan ascorbate formulation associated the multiple pharmaceutical aspects. Firstly, mannan due to its glactomannan subunit acts as specific ligand for mannose receptor located on the alveolar macrophage**.** Further, ascorbic acid due to its antioxidant nature provide the acidic pH at the site of granuloma**.** In addition to these, the aerodynamic diameter of the formulation plays an important role in passive localization of the carrier system at the target site**.** Experiment outcomes further support the hypothesis that units play an important role in active targeting of the carrier system which is further reinforced by ascorbic acid. From *in-vivo* studies, it was observed that there was a significant increase in drug accumulation within the desired cellular tropics when they were administered in microspheric form (CAMs and m-CAMs) formulations. As expected, the plasma and organ distribution studies had shown higher amount of drug recovery from lungs in the case of both the ligand conjugated formulations as compared to plain counterparts and free drug, indicating the efficacy of developed carrier for lung targeting for RIF.

Fig.5: Effect of temperature on particle size of formulation after 3 months



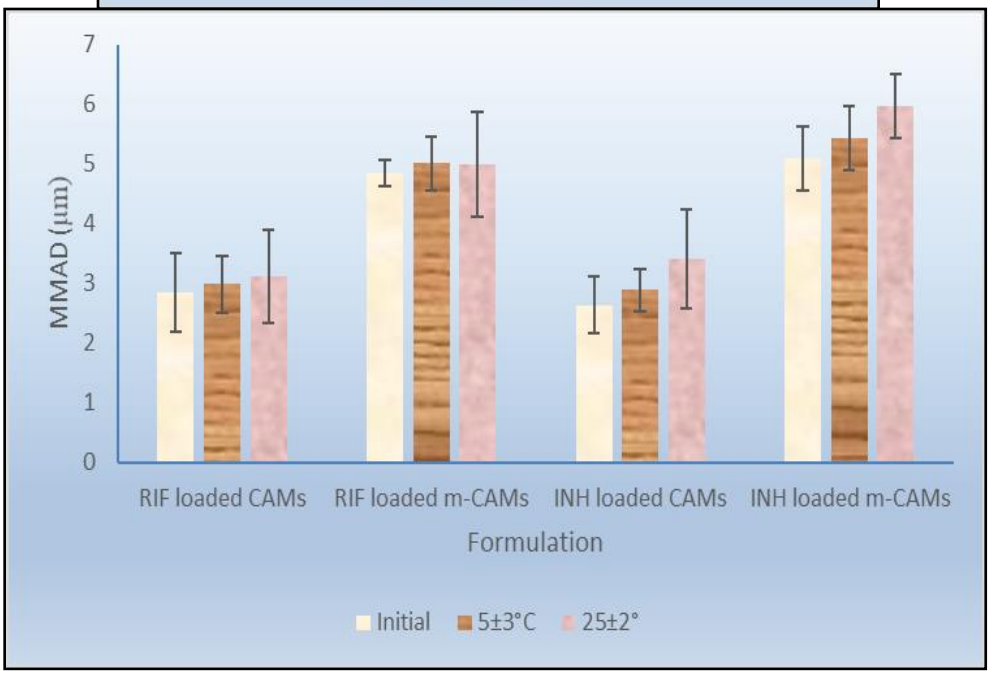


Fig. 6: Effect of temperature on MMAD of formulation after 3 months

To study the effect of storage temperature and duration on particle size and MMAD, optimized formulations were stored at refrigerated and at room temperature condition specified according to ICH guideline for 3 months. The formulations were then evaluated periodically for particles size and MMAD of formulation. The initial particle size during storage stability for C3S3P4T2R and m-C3S3P4T2R were 3.88±0.54µm and 4.98±0.21µm respectively whereas at refrigerated condition 3.99±0.22µm and 5.14±0.35µm while at room temperature condition 4.50±0.41µm and 5.98±0.99µm. For C3S3P4T2I and m-C3S3P4T2I initially 3.95±0.41µm and 4.76±0.22µm respectively and 4.15±0.22µm and 4.98±0.11µm, at refrigerated condition. While 4.85±0.47µm and 6.10±0.55µm at room temperature.

No significant change in particle size was observed for RIF containing microspheres after 90 days of storage at 5±3° whereas a marginal increase in particle size was recorded at room temperature (Table 9.1). It is evident that increase in average particle size was more pronounced when stored at room temperature in comparison to refrigerated conditions (Fig. 9.1 and Fig. 9.2). This may be attributed to the temperature induced fusion and

aggregation of microspheres at higher temperature. The initial MMAD for C3S3P4T2R and m-C3S3P4T2R were 2.86±0.66µm and 4.84±0.22µm respectively whereas at refrigerated condition 2.99±0.47µm and 5.01±0.44µm while at room temperature condition 3.12±0.78µm and 4.99±0.88µm. For C3S3P4T2I and m-C3S3P4T2I initially 2.64±0.48µm and 5.10±0.54µm respectively and 2.89±0.36µm and 5.44±0.54µm, at refrigerated condition. While 3.41±0.84µm and 5.98±0.54µm at room temperature.

It is clearly evident from the results that there is no significant change in MMAD when the formulations were stored at refrigerated conditions. The MMAD was, however slightly changed when the formulations were stored at room temperature but it still remained within the limit of respirable particles which can be taken up efficiently by the alveolar macrophages. The therapeutic effect of the formulation depends on the amount of drug that has been incorporated in the formulation; hence the residual drug content was monitored and compared with that obtained before stability testing period. The percent residual drug content of the various formulations was determined periodically after storing the formulations at refrigerated (5±3°) and room temperature (25±2°). The data revealed that refrigerated conditions are more suitable for the storage of formulations as after three months the residual drug content estimated was 95.2±1.4% and 96.1±1.8% for C3S3P4T2R and m-C3S3P4T2R and 95.7±2.1% and 96.2±1.6% for C3S3P4T2I and m-C3S3P4T2I. Whereas the residual drug content at room temperature was measured to be was 89.1±1.9% and 93.4±2.1% for C3S3P4T2R and m-C3S3P4T2R and 81.2±1.4% and 82.5±1.7% for C3S3P4T2I and m-C3S3P4T2I. The results suggest that the conjugation with ligand significantly reduced the leaching of drug from the formulations may be due to the presence of bulky group on the surface of microspheres which could have inhibited the leakage of drug offering slight protection against drug leakage.

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