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DESIGN AND CHARACTERIZATION OF ANTITUBERCULAR DRUG LOADED PLGA NANO PARTICLES

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Abstract:
The aim of the present work was to minimize or prevent the degradation of rifampicin,the antitubercular drug in gastric pH condition to improve the stability and therapeutic efficacy of the drug. The study was carried out by preparing Rifampicin loaded PLGA nanoparticles using ascorbic acid as an antioxidant. Dug loaded nanoparticles were fabricated by a multistep emulsion procedure and evaluations of the preparednanoparticles were then carried out by various methods. In this study four types of formulations were prepared. Formulation 1 (F1) is rifampicin alone loaded PLGA nanoparticles, formulation II (F2) is rifampicin – ascorbic acid (1:1) loaded PLGA nanoparticles, formulation III (F3) is rifampicin - ascorbic acid (1:2) loaded PLGAnanoparticles and formulation IV (F4) is rifampicin – ascorbic acid (1:3) loaded PLGA nanoparticles. The study concluded that ascorbic acid can minimize the degradation of rifampicin in acidic pH condition and thus improves the stability and bioavailability of rifampicin. The results also demonstrate that there is a statistically significant change in the percentage drug degradation profile when the concentration of ascorbic acid was increased.

Keywords: *Design, Charecterization, Nanoparticles, Antitubercular Drug, Plga Nanoparticles*

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INTRODUCTION:

In the last 50 years, material researchers have been extensively studying how to exploit nanoparticles and nanostructured materials in different biomedical and healthcare sectors [1]. The term "NP" usually defines minute particles of matter (1 to 100 nm in diameter), but other names can be used to describe larger particles (up to 500 nm in diameter). For example, nanorods, nanowires, and nanofibers are nanoparticles with a diameter in the 1–100 nm range but with one dimension outside the nanoscale dimension [2]. Nanostructured materials are nanomaterials with one dimension in the nanoscale range (<100 nm) and are made of a single material or multiple materials. Therefore, nanostructured materials are composed of interlinked parts in the nanoscale range [3]. Nanoparticles and nanostructured materials can be made of simple materials (e.g., metal, carbon, polymer) [4], of composites (e.g., polymer-metal, silica-metal, graphene-metal), or in the core-shell form [5,6,7,8].

Nanomaterials are typically synthesized by one of two main approaches, i.e., bottom-up approach and top-down approach. Among all the methods, recently, the synthesis of nanomaterials by physical vapor deposition, chemical vapor deposition, electrospinning, 3D printing, biological synthesis, and supercritical fluid have gained importance, which is mingled with other methods to improve the synthesis efficiency [9,10]. Nanomaterials display many interesting features, such as superior mechanical performance, the possibility of surface functionalization, large surface area, and tunable porosity, compared to their bulk materials [11,12,13]. These outstanding features explain why nanomaterials are the perfect candidates in the biomedical sector for the production of tissue-engineered scaffolds (e.g., blood vessels, bone), drug delivery systems (gene therapy, cancer treatments, drugs for chronic respiratory infections), chemical sensors [4,5], biosensors [6,7], and wound dressings [14,15]. Remarkably, several studies suggest that ancient civilizations in India, Egypt, and China used nanotechnology (metallic gold) for therapeutic purposes in 2500 BC [16]. Nanomaterials' discrete features can complicate the assessment of the effects and the toxicity risk associated with their use in a biological environment. Indeed, nanomaterials' chemical composition, size, shape, surface charge, area, and entry route in the body can influence their biological activities and effects [17].

Rifampicin is a bactericidal antibiotic drug of the rifamycin group. It is a semi synthetic compound derived from Amycolatopsis rifamycinica (formerly known as Amycolatopsis mediterranei and Streptomyces mediterranei).The literature survey thus reveals the importance of fixed dose combination of rifampicin, Isoniazid, Pyrazinamide and Ethambutol in the treatment of TB to overcome the drug resistance and to improve the patient compliances. Rifampicin appears to be the best choice for the treatment of TB however it has poor bioavailability from FDC formulation following oral administration due to degradation in the stomach. Rifampicin is well absorbed in the pH range of 1-2 even though it undergoes degradation in the acidic medium and the degradation of rifampicin increases in the presence of Isoniazid and thus affects bioavailability of rifampicin. Development of any method that can stabilize rifampicin against degradation in the stomach will be therapeutically beneficial. Since nanoparticles are known to cross the intestinal permeability barriers directly via transcellular / paracellular pathways, it offers better delivery of the encapsulated drug into the circulation. In this case they are expected to penetrate inside the infected cell, where TB is an intracellular infection. From the literature study it was found that PLGA is one of the most successfully used biodegradable nanosystem for the development of nanomedicine since it undergoes hydrolysis in the body to produce the biodegradable metabolite monomers, lactic acid and glycolic acid. Based on these considerations the present study attempted to improve the stability of rifampicin - PLGA nanoparticles usingascorbic acid as an antioxidant.

Rifampicin when administered alone or in combination with Isoniazid, Pyrazinamide and Ethambutol degrades in the stomach and results in poor bioavailability which makes difficulties in effectively controlling tuberculosis. Development of any method that can stabilize rifampicin against degradation in the gastric environment will be therapeutically beneficial.

MATERIALS:

Rifampicin in procured from Aastha Laboratories, India. Ascorbic acid P purchased from Qualigens Fine chemicals. LGA from Boehringer Ingelheim Pharma. Dichloromethane from Nice chemicals Pvt.Ltd. Mumbai, Polyvinyl alcohol from Molychem, India

METHODOLOGY:

Preparation of nanoparticles:

Rifampicin and ascorbic acid loaded PLGA nanoparticles were fabricated by an

Emulsification/solvent evaporation method, which involved the formation of stable emulsion and evaporation of organic solvent by continuous stirring. The study wascarried out by preparing four types of formulations.

Formulation 1 (F1) is rifampicin alone loaded PLGA nanoparticles, formulation II (F2)is rifampicin – ascorbic acid(1:1) loaded PLGA nanoparticles, formulation III (F3)is rifampicin -ascorbic acid (1:2) nanoparticles and formulation IV (F4)is rifampicin – ascorbic acid (1:3) loaded nanoparticles. In all the cases, drug: polymer ratio was taken as 1:1 and ascorbic acid was taken in three different ratios as shown in Table 1

Procedure:

Drug loaded PLGA nanoparticles were prepared by a multistep emulsion procedure.50mg of rifampicin and required quantities of ascorbic acid were accurately weighed and added to 10ml of dichloromethane containing the polymer [drug : polymer ratio was taken as (1:1)]. Distilled water was emulsified in the DCM containing drug and polymer to form w/o primary emulsion. It was then emulsified by sonication for 15 minutes. Primary emulsion was then poured into 8ml of 1%w/v aqueous Poly Vinyl Alcohol solution and stirred using a magnetic stirrer to form the second w/o/w multiple emulsion. The latter was then stirred continuously overnight for the complete removal DCM. The nanoparticles were then recovered by centrifugation (9000 -10,000 rpm for 15 minutes), washed thrice with distilled water and vaccum dried.

Table 1

FORMULATION CODE	INGREDIENTS
F0	PURE RIFAMPICIN
F1	RIFAMPICIN+PLGA(1:1)
F2	RIF+PLGA+ASC(1:1:1)
F3	RIF+PLGA+ASC(1:1:2)
F4	RIF+PLGA+ASC(1:1:3)

EVALUATION OF THE PREPARED NANOPARTICLES:

Characterization of the prepared nanoparticles was then carried out. It includes determination of particle size, size distribution, shape, surface morphology, Poly dispersity Index and zeta potential. Scanning electron microscopy was used to determine the shape and surface morphology of the nanoparticles. Average particle size and polydispersity index of nanoparticles were measured by Laser light scattering method. Zeta potential of the nanoparticles was determined using a zetasizer. Shape and surface morphology of nanoparticles.

The morphology of Rifampicin –ascorbic acid loaded PLGA nanoparticles were analyzedusing a scanning electron microscope. Samples were prepared from dilutions in distilled water of particle suspensions and dropped onto stubs using double sided sticking tape.

After air drying, particle were coated with a thin layer of platinum film and then examined by scanning electron microscopy.

Particle size characterization of the nanoparticles

The particle size, size distribution and poly dispersity index of the nanoparticles were measured by a laser particle size analyzer after suitable dilutions.

Zeta Potential Study [1]

The surface charge of nanoparticles was determined by the electrophoretic mobility of nanoparticles in a U type tube at 25˚C, using a zetasizer .

INVITRO RELEASE STUDY:

A solution of 0.1N HCL was placed in the vessel of USP dissolution apparatus type 2(US Pharmacopoeia XXIII, 1995) with rotating paddle at 100rpm and the temperature was maintained at 37±0.2°c.RIF loaded PLGA nanoparticles with ascorbic acid of different ratios were accurately weighed, dissolved in and diluted to 100ml with 0.1N HCL. The resulting solution was transferred immediately to the dissolution bath. Specimens were withdrawn at 15 min, 30min and 60min.An aliquot, 0.5ml, 1ml, 2 ml, 3ml, 4 ml and 5ml were extracted immediately with 100ml of pH 1.2 medium using a cyclomixer. Samples were analyzed spectrophotometrically at 475nm[1,7] and thepercentage degradation was calculated using the given formula

$$\% \text{ Degradation loss} = \frac{\text{Initial concentration - Final concentration}}{\text{Initial Concentration}} \times 100$$

Procedure for preparation of ph 1.2 buffer:
Preparation of pH 1.2: 50ml 0f 0.2M KCl is mixed with 85ml of 0.2M HCl and makeup to 200ml with water.
Note:
0.2M KCl: 14.911gm of KCl was dissolved in H_2O and dilute with water and made up to1000ml.
0.2M HCl: 17ml of HCl was mixed with 1000ml 0f H_2O.

Preparation of standard stock solution:

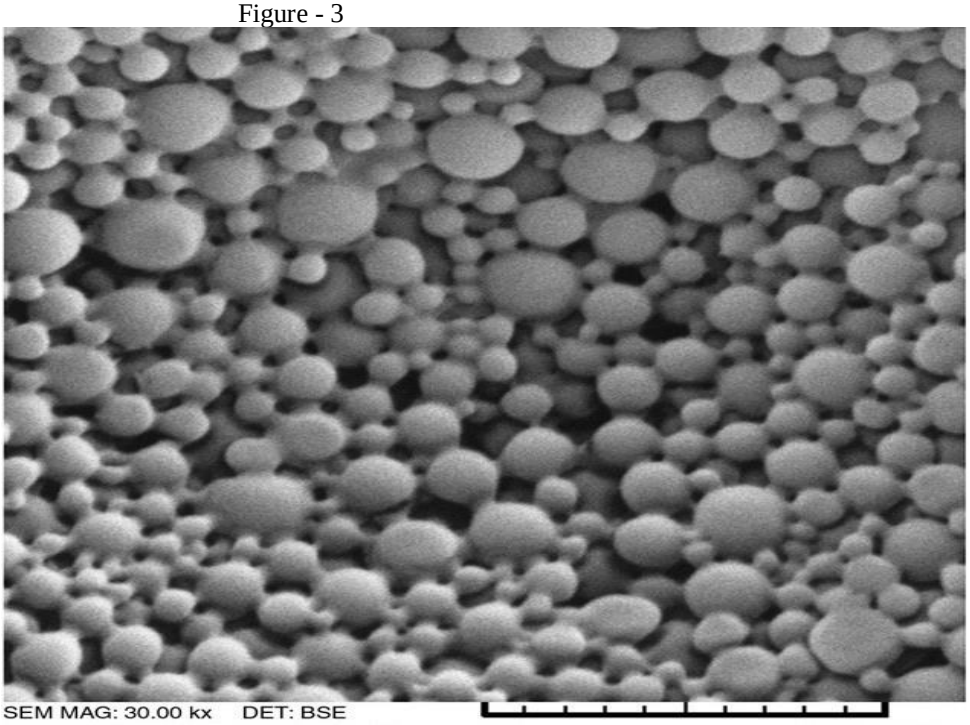
100mg Rifampicin PLGA nanoparticles were dissolved in 100ml pH 1.2 solution. From this required quantities were taken for further dilution process.

RESULTS:
SHAPE AND SURFACE CHARACTERIZATION OF THE PREPAREDNANOPARTICLES [SEM]

Scanning electron micrograph of the prepared Rifampicin - Ascorbic acid loaded PLGA nanoparticles are shown in Figure 3

SEM images revealed that the nanoparticles were spherical with smooth surface and theyare relatively mono dispersed.

Figure - 3



Particle size characterization of the nanoparticles:
The mean particle size and polydispersity Index of all the samples were determined(Table 2)

Table 2

S.NO	FORMULATIONS	MEAN DIAMETER(nm)±SD	PdI
1.	RIFAMPICIN+PLGA(1:1)	375± 20	0.312
2.	RIF+PLGA+ASC(1:1)	378 ± 22	0.316
3.	RIF+PLGA+ASC(1:2)	374 ± 18	0.308
4.	RIF+PLGA+ASC(1:3)	380 ± 23	0.317

Laser particle size analyzer yields the diameter of the bulk population. Particles were in the size range of 374-380 ± 18-23 (SD) nm. Polydispersity index is a measure of the distribution of particles in a given polymer sample. It gives the distribution range from 0.000 to 0.500.Polydispersity index greater than 0.5 indicates aggregation of particles. Here it is in the range of 0.308 - 0.317.

Zeta potential study:
Zeta potential is a term related to the stability of samples. For molecules and particles thatare small enough, high zeta potential will confer stability i.e. it resist aggregation. Here zeta potential of the prepared nanoparticle was found to be -46.6, which would not allow aggregation.

In-vitro stability study:
Standard curves of rifampicin alone and in combination with ascorbic acid indifferent ratios at pH 1.2 buffer (Table 3)

Table -3

Conc. in µgm	Rif	Rif + PLGA NPs	Rif+asc(1:1) NPs	Rif+asc(1:2) NPs	Rif+asc(1:3)NPs
0	0	0	0	0	0
5	0.082	0.084	0.090	0.052	0.054
10	0.160	0.162	0.140	0.103	0.093
20	0.305	0.320	0.280	0.192	0.181
30	0.456	0.473	0.422	0.280	0.273
40	0.607	0.633	0.561	0.381	0.352
50	0.759	0.801	0.701	0.475	0.446

Figure 4

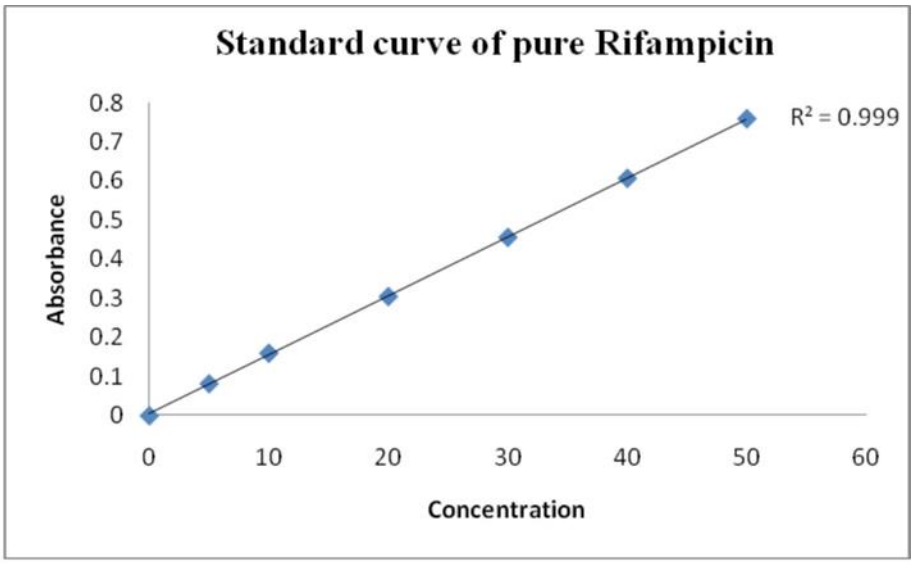


Figure 5

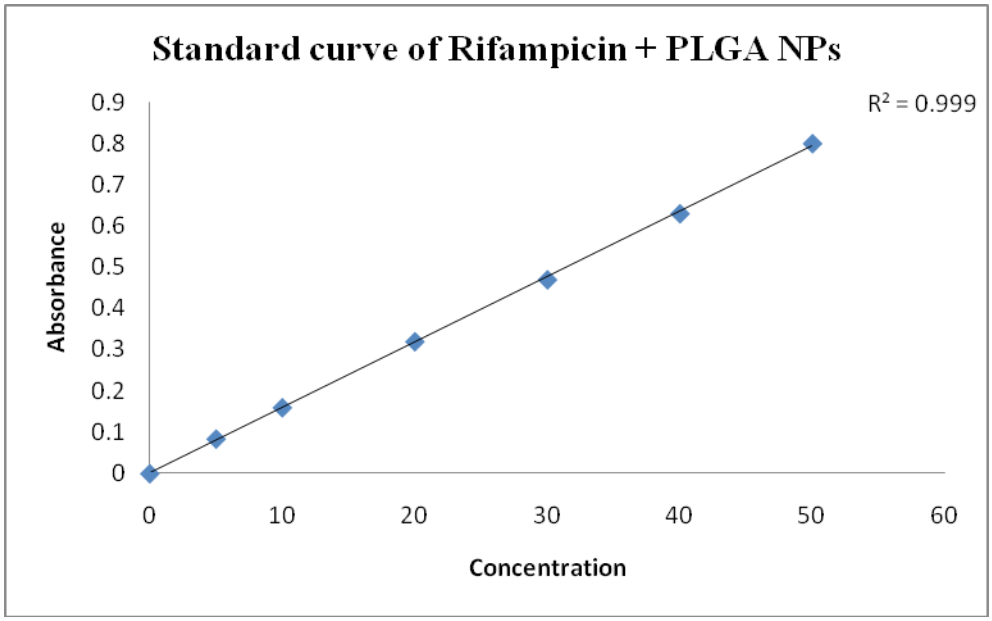


Figure 6

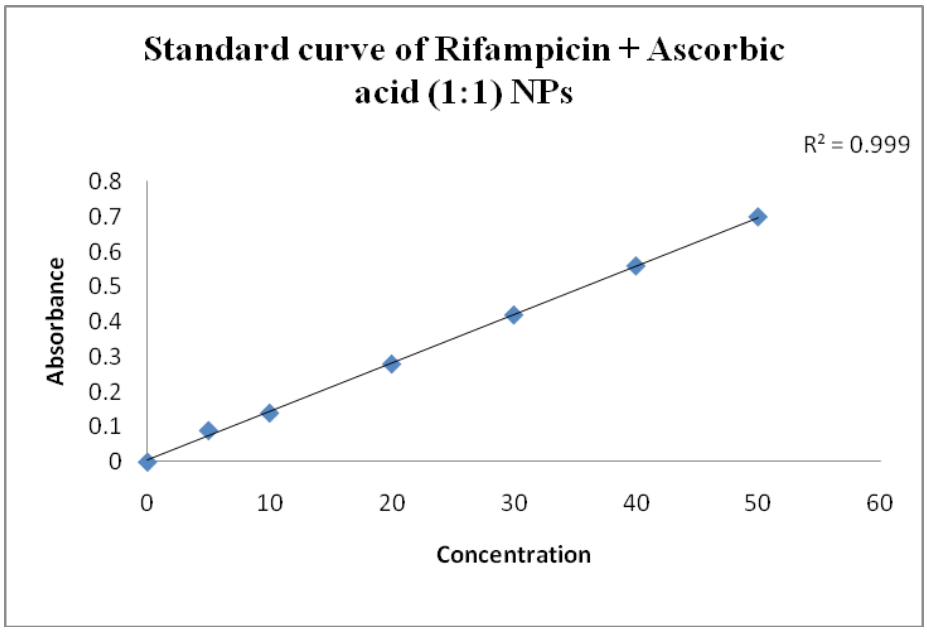


Figure 7

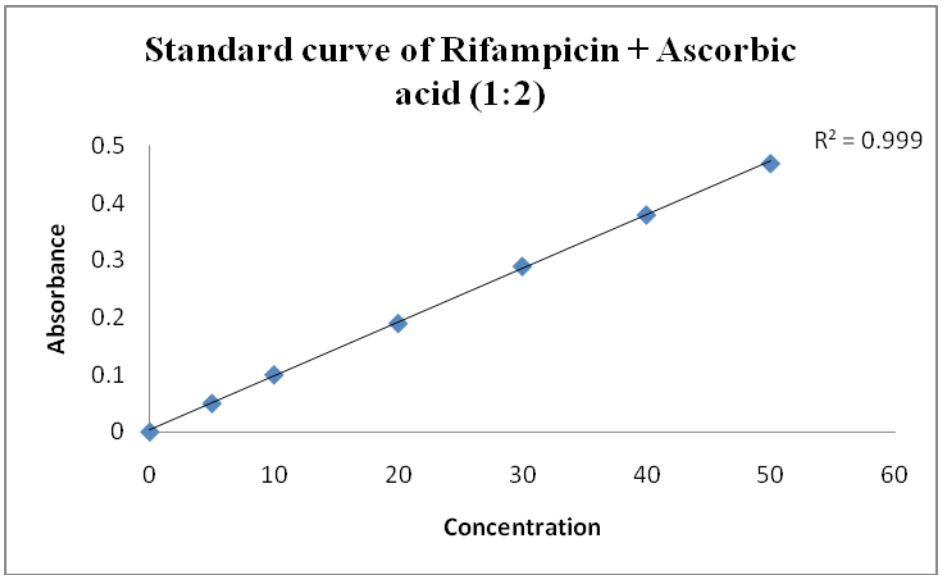
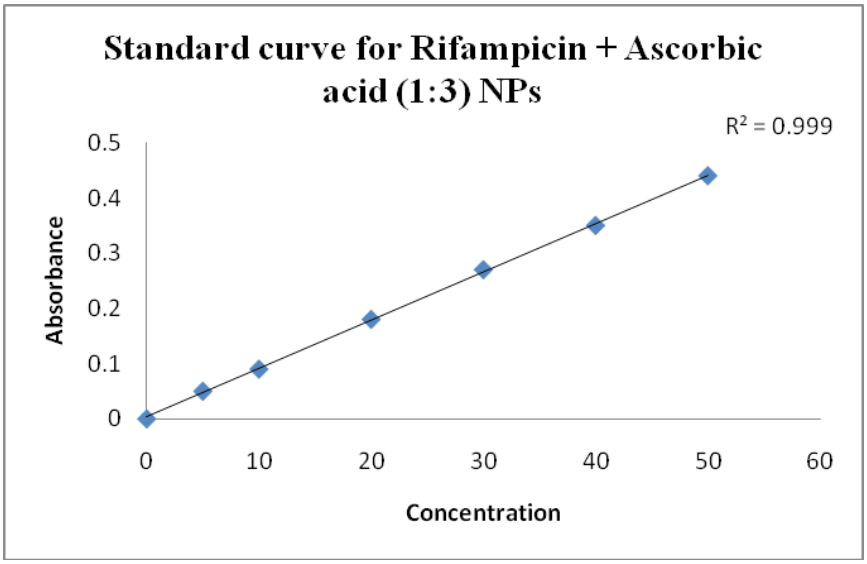


Figure 8

Invitro Stability study of rifampicin PLGA nanoparticles and rifampicin in combination with ascorbic acid in different

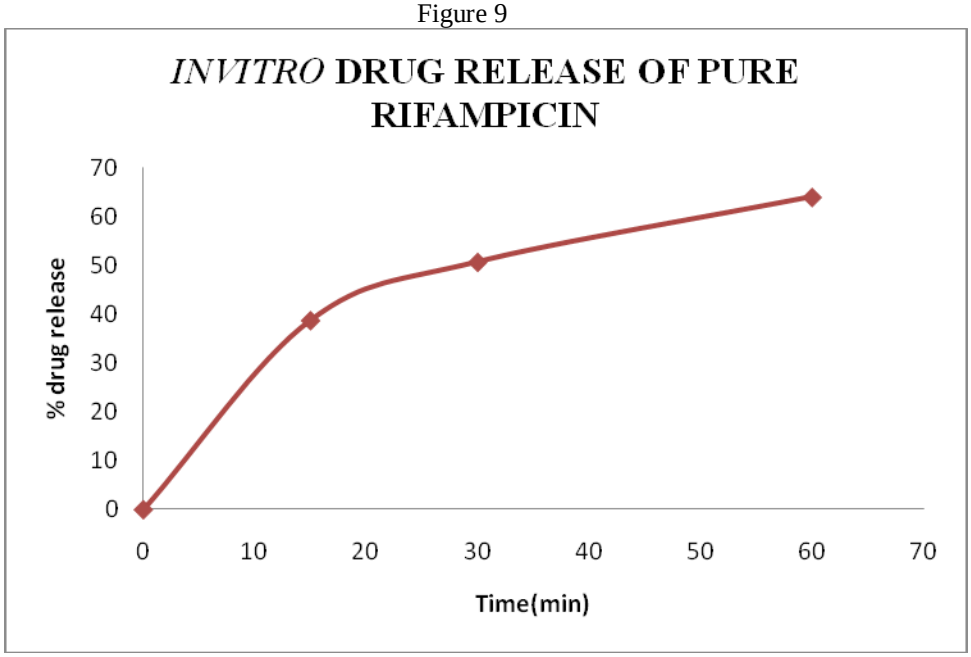


ratios at pH 1.2 buffer (Table 4 - 8)

Pure Rifampicin (Table 4)

Time (mins)	Absorbance			Concentration			% drug release			Mean % drug release±SD
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
0	0	0	0	0	0	0	0	0	0	0
15	0.620	0.621	0.619	43.20	43.21	43.19	38.8	38.81	38.79	38.80±0.010 %
30	0.861	0.860	0.859	56.41	56.40	56.39	50.77	50.76	50.75	50.76±0.010 %
60	0.980	0.980	0.981	71.20	71.20	71.21	64.08	64.08	64.09	64.08±0.005 %
P value										*0.0197

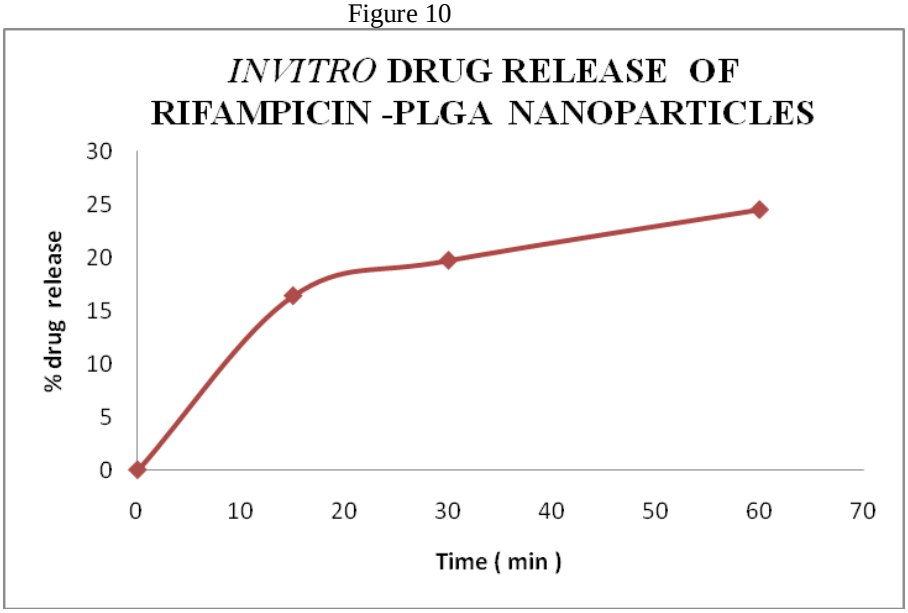
Figure 9



RIFAMPICIN + PLGA NANOPARTICLES (Table 5)

Time (mins)	Absorbance			Concentration			% drug release			Mean % drug release±SD
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
0	0	0	0	0	0	0	0	0	0	0
15	0.190	0.202	0.201	18.1	18.2	18.2	16.39	16.42	16.42	16.38± 0.07 %
30	0.335	0.334	0.335	21.90	21.8	21.9	19.71	19.70	19.71	19.71±0.005 %
60	0.420	0.421	0.422	27.20	27.20	27.21	24.48	24.48	24.50	24.48 ±0.011 %
P value										*0.0133

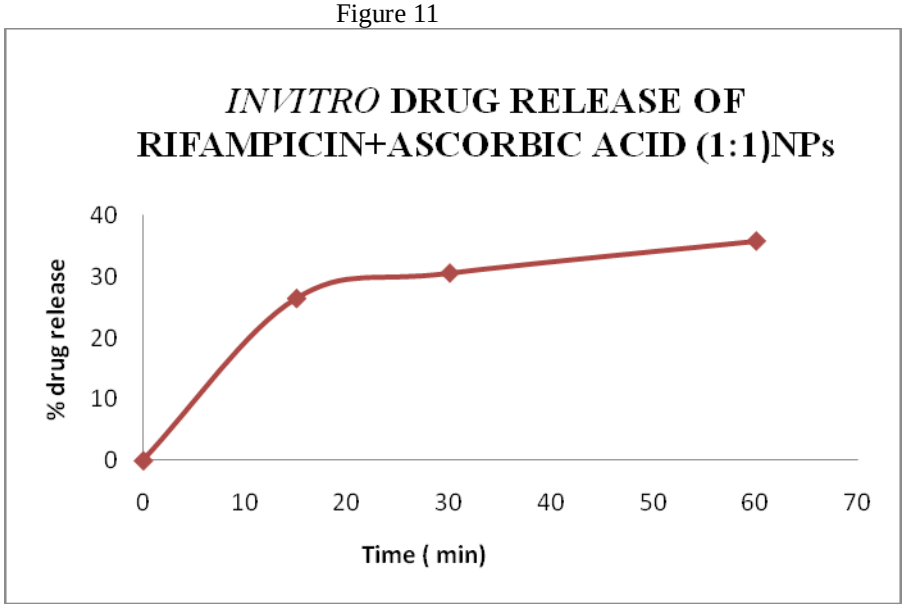
Figure 10



RIFAMPICIN + ASCORBIC ACID (1:1) Table 6

Time (min)	Absorbance			Concentration			% drug release			Mean % drug release±SD
	Trial1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
0	0	0	0	0	0	0	0	0	0	0
15	0.410	0.413	0.412	22.5	29.4	30.0	26.46	26.48	26.48	26.46±0.011%
30	0.472	0.471	0.472	34.20	34.11	34.20	30.61	30.59	30.61	30.60±0.010 %
60	0.540	0.542	0.541	39.80	39.82	39.82	35.82	35.83	35.83	35.82±0.005 %
Pvalue										**0.0076

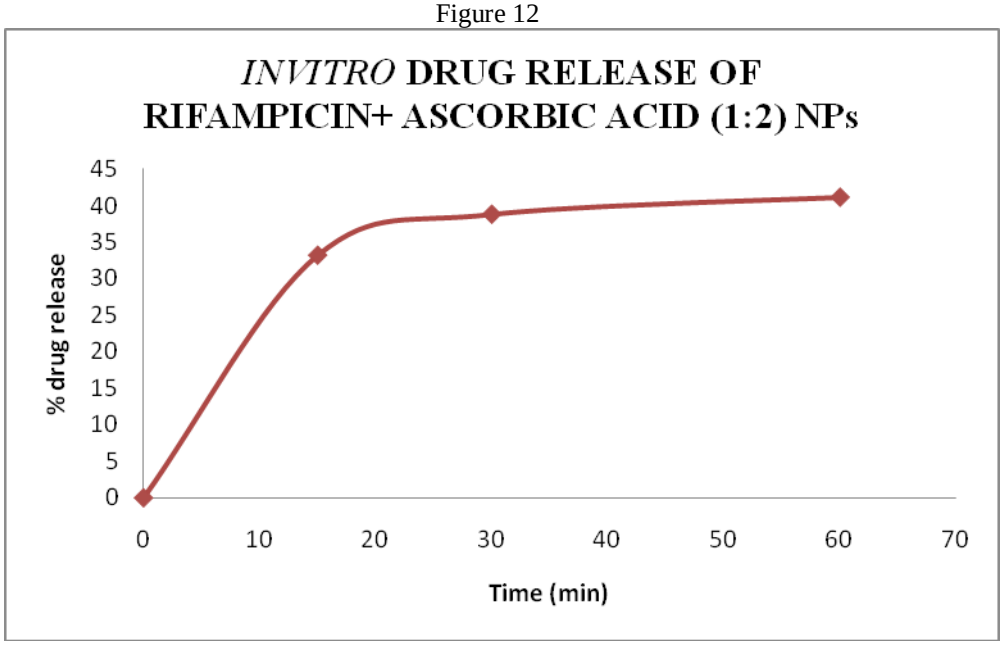
Figure 11



RIFAMPICIN + ASCORBIC ACID (1:2) Table 7

Time (mins)	Absorbance			Concentration			% drug release			Mean % drug release±SD
	Trial1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
0	0	0	0	0	0	0	0	0	0	0
15	0.360	0.359	0.361	37.00	36.90	37.10	33.20	33.19	33.21	33.20±0.010%
30	0.391	0.390	0.389	43.21	43.20	43.14	38.81	38.80	38.79	38.80±0.010 %
60	0.442	0.440	0.440	45.73	45.71	45.71	41.15	41.13	41.13	41.13±0.011%
P value										**0.0039

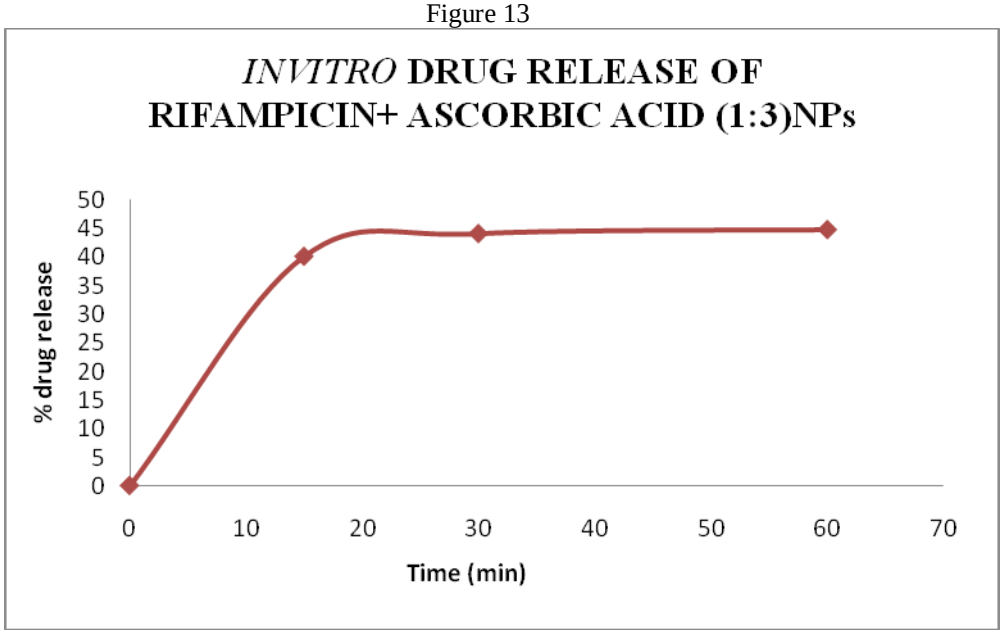
Figure 12



RIFAMPICIN + ASCORBIC ACID (1:3) Table 8

Time (mins)	Absorbance			Concentration			% drug release			Mean % drug release±SD
	Trial1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
0	0	0	0	0	0	0	0	0	0	0
15	0.351	0.349	0.350	44.71	44.69	44.7	40.21	40.19	40.20	40.20±0.010 %
30	0.442	0.440	0.444	49.20	49.00	49.22	44.20	44.19	44.22	44.20±0.015 %
60	0.446	0.445	0.447	49.90	49.89	49.90	44.89	44.88	44.90	44.89±0.010 %
P value										**0.0011

Figure 13



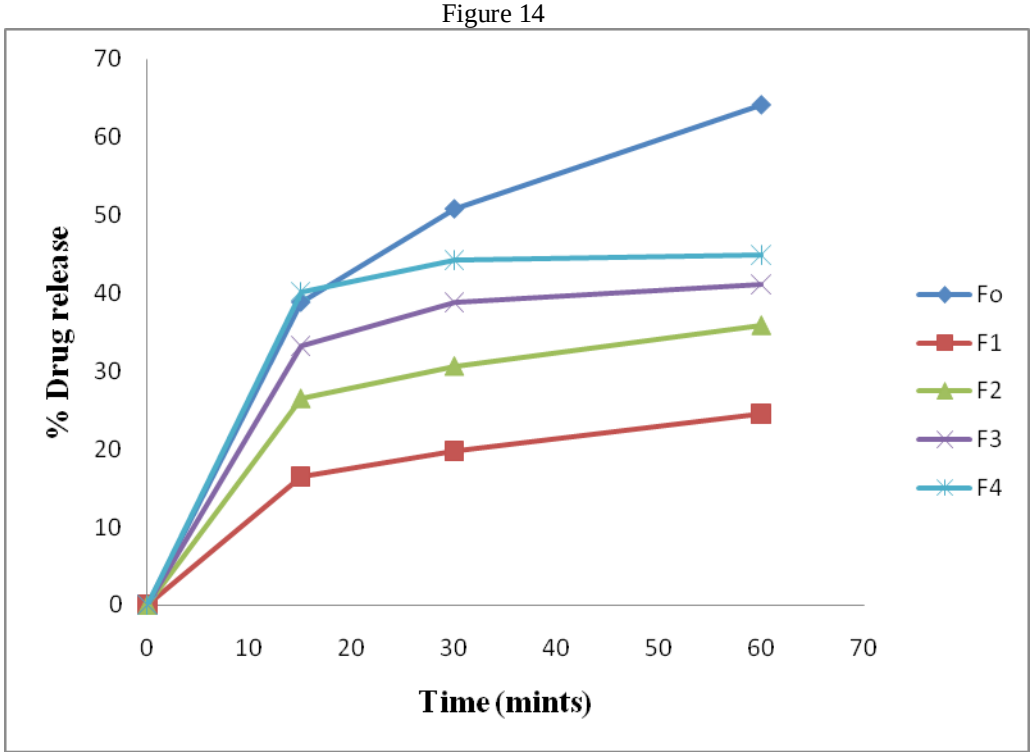
% DRUG RELEASE PROFILE OF THE FORMULATIONS

Table 9

Time (min)	F0	F1	F2	F3	F4
0	0	0	0	0	0
15	38.80±0.010 %	16.38±0.07%	26.46±0.011%	33.20±0.010%	40.20 ±0.010 %
30	50.76±0.010 %	19.71±0.005%	30.60±0.010%	38.80±0.010%	44.20 ±0.015%
60	64.08± 0.005%	24.48±0.011 %	35.82±0.005%	41.13±0.011%	44.89 ±0.010%

% DRUG RELEASE PROFILE OF THE FORMULATIONS

Figure 14



Statistical Analysis of all the formulations:
One way Analysis of Variance (ANOVA) : Tukey-Kramer Multiple ComparisonsTest

Table 10

Comparison	P value
F0 vs F1	** P<0.01
F0 vs F2	* P<0.05
F0 vs F3	ns P>0.05
F0 vs F4	ns P>0.05
F1 vs F2	ns P>0.05
F1 vs F3F1 vs F4	ns P>0.05
F2 vs F3F2 vs F4	* P<0.05 ns P>0.05ns
F3 vs F4	P>0.05 ns P>0.05
P value	** 0.0020

*considered significant
**considered very significant

Statistical Analysis of nanoparticles

One way Analysis of Variance (ANOVA): Tukey-Kramer Multiple Comparisons Test

Table 11

Comparison	P value
F1 vs F2 F1 vs F3 F1 vs F4	* P<0.05 ** P<0.01 *** P<0.001
F2 vs F4 F3 vs F4	* P<0.05 ns P>0.05
Overall P value	* * *0.0005

COMPARITIVE % DRUG DEGRADATION PROFILE

Table 12

FormulationCode	% drug degradation
F0	64.81 %
F1	49.77 %
F2	40.17 %
F3	23.54 %
F4	11.61 %
P value	* 0.0156

% DRUG DEGRADATION PROFILE OF F0 TO F4

DISCUSSION:

Rifampicin is a first line anti-tubercular drug, administered orally in fixed dose combination with Isoniazid, Pyrazinamide and Ethambutol in order to overcome drug resistance to tuberculosis arising from administration of these drugs separately. However bioavailability of rifampicin is reduced owing to degradation of the drug in the stomach. Rifampicin degrades in acidic condition of the stomach and the degradation of rifampicin is pH dependent.One report says that the problem of poor absorption of rifampicin fromcombination products is perhaps due to increased decomposition in stomach conditions and the decomposition of rifampicin is enhanced in the presence of INH.

Rifampicin is well absorbed in the pH range of 1-2 even though it undergoes degradation in the acidic medium. Rifampicin hydrolyses to 3 formyl rifampicin SV (3-FRSV)in acidic condition and it undergoes air oxidation in alkaline medium to form in active quinone derivative rifampicin quinine. It shows high antimicrobial activity but is inactive in *in-vivo* (USP DI,1996). Therefore formation of 3-FRSV in the acidic environment of stomach can be an important factor affecting bioavailability of rifampicin and cannot be overlooked.

Development of any method that can prevent or minimize degradation of rifampicin in the stomach either as a single drug or in combination of other anti tubercular drug is therapeutically beneficial and can achieve effective control of tuberculosis with improvedbioavailability of rifampicin.

Previous study shows that the degradation of rifampicin due to oxidative side reaction was prevented by the addition of ascorbic acid to the reaction media.[54]Another study reveals the protective effect of adding ascorbic acid on the stability of rifampicin in plasma and that the degradation can be effectively prevented by adding ascorbic acid thus

prolonging stability for 12 hours

Based on the above factors the present study aimed to prepare and evaluate Rifampicin loaded PLGA nanoparticles and an attempt was made to investigate the influence of ascorbic acid as an antioxidant on stabilizing rifampicin in the gastric environment by *invitro* study in pH 1.2 medium simulating the condition in stomach.

Evaluations of the prepared nanoparticles were then carried out by different methods. Shape and Surface characterization of the nanoparticles were done by Scanning Electron microscopy and the SEM images revealed that the nanoparticles were spherical with smooth surface and the nanoparticles were found to be relatively mono dispersed. Laser particle size analyzer yields the diameter of the bulk population and a polydispersity index gives the distribution range from 0.000 to 0.500. Polydispersity index is a measure of the distribution of particles in a given polymer sample. PdI greater than 0.5 indicates aggregation of particles. Here the polydispersity indexes of the nanoparticles were in the range of 0.308-0.317.

Particles were in the size range of 374 - 380 ± 18 - 23 (SD) nm. Particle size of the nanoparticle can be affected by processing parameters such as drug/polymer ratio, concentration of surfactant and stirring speed. Since in the present study these parameterswere maintained constant, their influence on the mean particle size of nanoparticles cannot be ascertained.

Zeta potential is a term related to the stability of samples. For molecules and particles thatare small enough, high zeta potential will confer stability i.e. it resist aggregation. When zeta potential is low, attraction exceeds repulsion. Therefore particles with high zeta potential (-ve or + ve) are electrically stabilized. Generally particle aggregation is less likely to occur for charged particles (high zeta potential) due to electric repulsion. Lower zeta potential facilitates aggregation. Here zeta potential of the prepared nanoparticleswas found to be -46.6, which would not allow aggregation.

The invitro dissolution study was conducted for 1 hour. It was carried out using the five formulations. First formulation is pure Rifampicin. Second is rifampicin alone loaded PLGA nanoparticles and in the next three formulations ratio of rifampicin and PLGA were same where as the concentration of ascorbic acid was increased. The dissolution study was carried out in pH 1.2 solutions to simulate the acidic gastric condition. At specific time intervals samples were withdrawn and analyzed by U.V spectrophotometer. Percentage release of drug from the nanoparticles at 15min, 30min and 60min were determined and the percentage degradation of the drug was also calculated.

The results of the invitro drug dissolution study indicates that the % drug release of the formulation F0, F1, F2, F3 and F4 at 60 minutes was found to be 64.08%, 24.48%, 35.82%, 41.13% and 44.89 % respectively and the percentage drug degradation of the formulation F0, F1, F2, F3 and F4 was found to be 64.81%, 49.77 %, 40.17 %, 23.54% and 11.61 % respectively.

From the data obtained it is understood that ascorbic acid minimized the degradation of rifampicin and the degradation was further reduced when the concentration of ascorbic acid was increased. Statistical analysis of the % drug degradation profile was done and it was found that there is a statistically significant change (statistically significant; *P 0.0156) in the percentage degradation as the concentration of ascorbic acid was increased. It can be hypothesized that ascorbic acid being an antioxidant prevents the oxidative side reactions of rifampicin in the gastric pH and minimized the degradation of rifampicin.

SUMMARY & CONCLUSION:

The results of the study demonstrate that ascorbic acid can minimize the degradation of rifampicin in gastric pH condition and thus improves the stability and therapeutic efficacy of rifampicin. The study also concluded that there is statistically a significant change in the percentage drug degradation profile when the concentration of ascorbic acid was increased. Further *invivo* studies are recommended to address the therapeutic efficacy of rifampicin –ascorbic acid loaded PLGA nanoparticles

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