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Research Article

EFFECT OF *PIPER NIGRUM* ON *IN-VITRO* RELEASE OF RIFAMPICIN MICROSPHERES

PINGALE PRASHANT L.*, RAVINDRA R.P.

SVKM's NMIMS, School of Pharmacy & Technology Management, Shirpur-425 405 Dist: Dhule Maharashtra, INDIA.
Email: prashant.pingale@gmail.com

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ABSTRACT

Tuberculosis therapy remains a challenge even today, mainly due to the problems of bacterial resistance, side effects and low patient compliance, associated with anti-tubercular drugs. A bioenhancer is an agent capable of enhancing bioavailability and bioefficacy of a particular drug with which it is combined, without any typical pharmacological activity of its own at the dose used. Microspheres are one of the multiparticulate drug delivery systems prepared to obtain prolonged drug delivery, to improve its bioavailability or stability and to target drug to specific sites. Rifampicin is an important component of fixed dose combination used in first line therapy of tuberculosis. In the present study, we attempted an innovative approach to overcome the above-mentioned problems of tuberculosis therapy by combining both these approaches of microspheres and bioenhancers. Small amount of herbal extract of *Piper nigrum* were incorporated as a bioenhancer in variable amount for each dose of rifampicin. The drug loading efficiency and *in-vitro* release behavior of loaded microspheres were found to be satisfactory. Prolonged release of the drug from the microspheres was demonstrated in a simulated intestinal fluid. *In-vitro* release of rifampicin from the microspheres containing variable fractions of bioenhancer showed significant increase in release profile i.e. 80.24 and 86.16% in formulation containing bioen hancer against 44.54% for the formulation without bioenhancer prepared by complex coacervation method.

Keywords: Tuberculosis, Microspheres, Drug release, Bioenhancer, Rifampicin, *Piper nigrum*

INTRODUCTION

Tuberculosis is an infectious disease caused by the *Mycobacterium tuberculosis*. The disease was called "consumption" in the past because of the way it would consume from within anyone who became infected. TB is a major cause of illness and death worldwide, especially in Africa and Asia. Each year the disease kills almost 2 million people. The disease is also prevalent among people with HIV/AIDS. Currently available treatment regimens are prolonged, placing unmanageable demands on indigent populations from the perspectives of both supervision and adherence. The result is the burgeoning tide of drug resistance. Repeated inadequate courses of therapy in patients with relapsing TB generate incremental increases in the degree of drug-resistance[1].

In 2011, there were 8.7 million new cases of active tuberculosis worldwide (13% of which involved co-infection with the human immunodeficiency virus [HIV]) and 1.4 million deaths, including 430,000 deaths among HIV-infected patients1 representing a slight decrease from peak numbers in the mid-2000s. It has been estimated that there were 310,000 incident cases of multidrug-resistant tuberculosis, caused by organisms resistant to at least isoniazid and rifampin, among patients who were reported to have tuberculosis in 2011. More than 60% of these patients were in China, India, the Russian Federation, Pakistan, and South Africa[2]

Rifampicin works by killing the bacteria that are causing the infection. It does this by targeting and inactivating a bacterial enzyme called RNA-polymerase. The bacteria use RNA-polymerase to make essential proteins and to copy their own genetic information (DNA). Without this enzyme the bacteria cannot reproduce and they die [3,4,5].

Rifampicin inhibits DNA-dependent RNA polymerase activity in susceptible *Mycobacterium tuberculosis* organisms. Specifically, it interacts with bacterial RNA polymerase, but does not inhibit the mammalian enzyme[3].

C.K. Atal, the Director of the institute scrutinized a list of ancient Indian Ayurvedic formulations used in the treatment of a wide range of diseases. He observed that a majority of Ayurvedic formulations contained either *Trikatu* or else one of the ingredients of *Trikatu*, namely *Piper longum* (*P. longum*) (210 formulations out of 370 reviewed) which is used in a large variety of diseases. He formed the working hypothesis that *Trikatu* increased the efficacy of formulations. *Trikatu* has three ingredients: black pepper (*Piper nigrum*), long pepper (*P. longum*) and ginger (*Zingiber officinale*). Based on this hypothesis, these ingredients were studied, which found that one of the ingredients, '*P. longum*', 'Piper' increased the bioavailability of many drugs[6].

Piperine, the active principal present in *P. nigrum* or *P. longum* was isolated and its bioavailability enhancing action was established. Further research on several classes of drugs including anti-tubercular, anti-leprosy, antibiotics, non-steroidal anti-inflammatory drugs, CVS and CNS drugs showed similar results. Piperine was found to increase bioavailability of different drugs ranging from 30% to 200%. Subsequent research has shown that it increases curcumin bioavailability by almost ten-fold with the use of piperine[7,8]. The release of isoniazid was increased almost double (from 45 to 90%) in microspheres if piperine were used as bioenhancer in the preparation[9].

The drug delivery system offers several potential advantages: drug release rates can be modified to the needs of a specific application; it provides a constant rate of delivery or pulsatile release. It provides protection of drugs, especially proteins, which are otherwise rapidly destroyed by the body. The controlled release systems of microspheres can increase patient comfort and compliance by replacing frequent (e.g., daily) doses with infrequent (once per month or less) injection.

Hence the effective formulation strategy for the optimization of the pharmacokinetic characteristics of anti-tubercular agent would be to use bioenhancers in combination therapy to improve the release performance and ultimately maximize their effectiveness along with the microspheres as a drug delivery system.

In this study, we have attempted a collective approach consisting of microspheres and bioenhancer in order to overcome the problems associated with the anti-TB drug therapy.

The objectives of this study were to

- Develop sustained release microspheres of rifampicin using various methods;
- Study the effect of various concentrations of bioenhancer (extract of *Piper nigrum*) on *in-vitro* drug release from microspheres; and
- Evaluate the effect of various processing variables on characteristics of microspheres.

MATERIALS AND METHODS

Rifampicin was obtained as a gift sample from Lupin Pharmaceuticals Ltd., Aurangabad, Maharashtra. *Piper nigrum* (Black pepper) was purchased from local market and authenticated from Dept. of Botany, Dr.P.R.Ghogrey College of Science, Dhule, Maharashtra. All other chemicals / polymers were of analytical grade.

Extraction and isolation of *Piper nigrum* used as bioenhancer[10,11,12]:

Macroscopy of Black pepper: The entire fruit was almost globular in shape, with 4- 6.5 mm of diameter, brownish to black in color. The surface was found to be uneven. The seeds were almost brown or black in color, aromatic with a pungent taste (Figure 1).

Figure 1: *Piper nigrum* seeds and powder

*Phytochemical evaluation of Black pepper[*12]**:** The phytochemical evaluation of fully mature, dried fruits of *Piper nigrum* Linn, family *Piperaceae* was carried out as per the provisions of Ayurvedic Pharmacopoeia for various parameters, the results of which are mentioned in Table 1.

Table 1: Evaluation of Black pepper (powder)

Sr. No.	Parameter	Standard (% w/w)	Observed value (% w/w)
1	Total ash	Not more than 5.5	4.10
2	Water soluble ash	Not more than 4.5	3.76
3	Acid insoluble ash	Not more than 1.0	0.57
4	Loss on drying	Not more than 4	3.05
5	Water soluble extractive	Not less than 15	23.11
6	Ethanol soluble extractive	Not less than 8	10.50

Evaluation of piperine (as an active constituent) from Black pepper seeds: It was determined by liquid chromatography as given in Indian Pharmacopoeia-2010. The amount of piperine was found to be 2.89 %w/w (IP-2010 limit not less than 2.50 %w/w).

Isolation of piperine from Black pepper: Black pepper (100 g) was ground to a fine powder and extracted with 95% ethanol (500 ml) in a Soxhlet extractor for 2 hours. The solution was filtered and concentrated in vacuum on a water bath at 60°C. 50 ml of 10% alcoholic KOH solution was added to it and after a while, the clear liquid decanted from the insoluble residue. The alcoholic solution was left overnight, Yellow needle shaped crystals of piperine were obtained. The melting point of the crystals was found to be 124-126°C.

Evaluation of piper nigrum hydro-alcoholic extract: The bioenhancers extract were evaluated against standards laid by IP-2010, and Ayurvedic Pharmacopoeia of India-2007 [12,13] as given in Table 2.

Table 2: Evaluation of Herbal Extract from Black pepper

Sr. No.	Test	Standard value	Observed value
1	LOD (% w/w)	< 5.0	2.10
2	Total Ash (% w/w)	< 20.0	7.61
3	Acid Insoluble Ash (% w/w)	< 3.0	2.5
4	Residual solvent analysis (MeOH)	< 3000 ppm	< 2291 ppm

PREPARATION OF RIFAMPICIN MICROSPHERES: Microspheres may be produced by several methods such as emulsion cross-linking method, multiple emulsion method, coacervation method, solvent evaporation method, spray-drying method etc. In this study, complex coacervation method was used to prepare the microspheres[14, 15, 16, 17,18,19].

The mixture of chitosan/gelatin B used as coating material. Chitosan and gelatin were dissolved in dilute acetic acid solution (1% v/v) together at concentrations of 1-4% w/v and adjusted to a certain solution pH 5.0. Rifampicin (150 mg) was dissolved in the above polymeric solution. The drug in polymeric solution was emulsified in 200 ml of liquid paraffin (1:1 mixture of light and heavy liquid paraffin) containing 1 ml Tween 80 (2% w/v). The emulsification time was allowed for 10 min under mechanical stirring (Remi Motors, India) at a variable rpm (1400-1600 rpm). Then 50 ml Na-TPP (2% w/v) with pH in the range 4-5 was added drop wise. Stirring was continued for 30 min. (cross linking time optimized 15-45 min.) to obtain cross-linked microspheres. Microspheres were collected by centrifugation and washed with double distilled water several times, then with acetone to remove water and dried at room temperature under vacuum. The prepared microspheres were stored in desiccator for further studies. The drug loaded microspheres with different polymer compositions (1:1, 1:2, 1:3 and 1:4) were prepared and studied for various parameters as shown in Table 3.

Optimization parameters: Polymer-copolymer ratio and concentration, drug-polymer ratio, concentration of cross-linking agent and time required for crosslinking were considered in the optimization of the formulation[20].

Table 3: Composition of RIF Microspheres by Complex Coacervation Method

Batch code	RIF (mg)	Chitosan: Gelatin B	Polymer Concentration (% w/v)	Na-TPP (% w/v)	RPM (±100)	Cross-linking time (min.)
Form.1	150	1	1:1	1	1400	15
Form.2	150	2	1:2	2	1500	30
Form.3	150	3	2:1	1	1600	45
Form.4	150	4	1:1	1	1400	30
Form.5	**150**	**4**	**1:2**	**2**	**1500**	**30**
Form.6	150	4	2:1	1	1600	45

Addition of bioenhancer extract in optimized batch of rifampicin microsphere: To the optimized batch of rifampicin microsphere prepared by complex coacervation method, the extract of black pepper was added. The detailed formulae are shown in Table 4.

Table 4: Optimized RIF Microspheres with Black peeper extract

Batch code	RIF (mg)	Chitosan: Gelatin B	Polymer Concentration (% w/v)	Na-TPP (% w/v)	RPM (±100)	Cross-linking time (min.)	BE extract (mg)
CRF_1	150	4	1:2	2	1500	30	--
CRF_2	150	4	1:2	2	1500	30	5
CRF_3	150	4	1:2	2	1500	30	10
CRF_4	150	4	1:2	2	1500	30	15

Evaluation of rifampicin microspheres:

Fourier Transform Infra-Red (FT-IR) analysis: Drug, physical mixture of drug, polymer and herbal extract and formulation were analyzed using Fourier Transform Infra-Red (FT-IR) spectroscopy. The purpose of the study is to check the compatibility of polymers and other excipients with the drug. 1 to 2 mg of drug, physical mixture (drug + polymer + herbal extract) and drug microsphere samples were weighed and mixed perfectly with potassium bromide (0.3-0.4 g) to a uniform mixture. A small quantity of the powder was compressed into a thin semitransparent pellet by applying pressure.

Differential Scanning Calorimeter (DSC) study: DSC analyses were carried out with a Mettler TA4000 Star^e software apparatus (Mettler Toledo, Switzerland) equipped with a DSC 25 cell. Samples of about 5-10 mg were weighed (Mettler M3 microbalance) in pierced aluminium pans and scanned under static air over a temperature range of 30° to 250°C at a heating rate of 10°C/min. Calibration of temperature and heat flow were performed with standard Indium samples.

Particle size: Particle size of the formulation has significant influence on the dissolution rate. An increase in the total effective surface area of formulation in contact with GI fluids causes an increase in its dissolution rate. The smaller the particle size, the greater the effective surface area exhibited by a given mass of drug formulation and the higher the dissolution rate. The microspheres were characterized for size by motic microscope (DMB series). Particle size of both - plain drug microspheres as well as microspheres with bioenhancer was measured using Motic microscope at 40 X magnification. In all measurements, at least 100 particles in each of 3 different fields were examined.

Percentage yield: The yield of microspheres was determined by comparing the whole weight of microspheres obtained against the combined weight of the polymer, drug and bioenhancers used for formulation. The percentage yield of the microsphere was determined using following formula: The percentage yield of prepared microspheres was determined by using the formula:

$$\text{Percentage yield} = \frac{\text{Wt. of microspheres obtained}}{\text{Total wt. of drug, polymer used for formulation}} \times 100$$

Percentage encapsulation efficiency: The drug content of the microspheres was determined spectrophotometrically (UV spectrophotometer Perkin Elmer, USA Lambda 25 model). Microspheres (10 mg) loaded with drug were dissolved in 10 ml of isotonic phosphate buffer pH 6.8 under sonication for 20 min. The solutions were filtered through 0.22 µm Millipore filters and the amount of drug was determined. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at a specific wavelength. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at 327 nm.
The percent drug entrapment was calculated using following formula:

$$\text{Percentage yield} = \frac{\text{Wt. of drug present in microsphere}}{\text{Total wt. of drug, polymer used for formulation}} \times 100$$

Percentage bioadhesion: *In-vitro* bioadhesion was determined for microspheres (in triplicate) by falling liquid film method.

Microspheres (50 mg) were placed on albino rat small intestine (area 2cm^2) and kept for 20-30 minutes in a humidity temperature controlled cabinet (Thermolab, India), maintained at 75 (±5)% relative humidity and temperature of 25 (±2)^{0}C to allow hydration of the microspheres. This was followed by thorough washing of the mucosal lumen with isotonic phosphate buffer pH 6.8, and then dried at 70^{0}C in a hot air oven[21]. Percent bioadhesion was determined by the following formula:

$$\text{Percentage yield} = \frac{\text{Wt. of adhered microsphere}}{\text{Wt. of applied microspheres}} \times 100$$

***In-vitro* drug release:** Dissolution studies were carried out using USP XXXI rotating basket method. The release profiles of rifampicin from microspheres were studied in simulated gastric fluid (SGF pH 1.2) and simulated intestinal fluid (SIF pH 6.8). The drug-loaded microspheres (equivalent to 20 mg of rifampicin) filled in empty capsule shells were put into the basket (50 rpm) and placed in 500 ml of the dissolution medium, thermostated at 37^{0}C. Samples of 2 ml each were withdrawn at regular time intervals, filtered, diluted suitably, analyzed using double beam UV spectrophotometer at 475 nm and an equal volume of fresh medium was immediately added to maintain the dissolution volume[23]. Dissolution studies were carried out up to 12 h. The drug release experiments were conducted in triplicate.

RESULTS AND DISCUSSION:

Fourier Transform Infra-Red (FT-IR) analysis: Pure drug RIF, placebo microspheres, RIF microspheres (2-5 mg) prepared with and without bioenhancer were weighed and mixed perfectly with potassium bromide (0.1 to 0.2 g) to form a uniform mixture. Potassium bromide pellet method was employed and background spectrum was collected under identical conditions. The IR spectrum of the pellet were recorded using FTIR (Perkin Elmer, USA, Spectrum RX1 Model) taking air as the reference and compared with each other to identify drug-excipient interaction, if any.

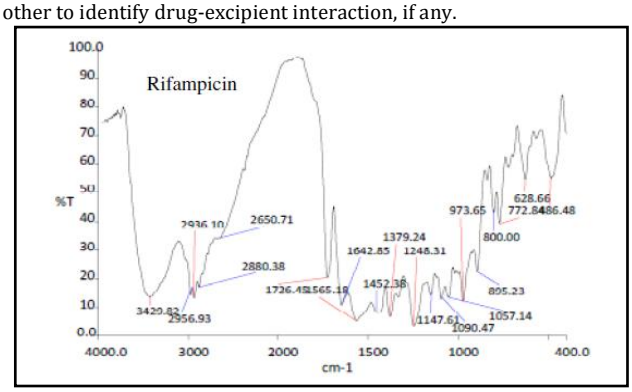

Figure 2 (a): FT-IR Spectra of Rifampicin Pure Drug

Each spectrum was derived from 16 single averaged scans collected in the region 400–4000 cm^{-1} at a spectral resolution of 2 cm^{-1}. FT-IR spectra of pure drug and rifampicin microsphere are shown in Figure 2a and 2b respectively.

Differential Scanning Calorimeter (DSC) study: Rifampicin powder sample (2-8 mg) was weighed into an aluminum pan and analyzed as sealed with pinholes and an empty aluminum pan was used as a reference. To determine the thermodynamic relationship of two forms, heat–cool–heat cycle was also used. The DSC

endotherm showed a sharp melting endotherm for rifampicin at 190^{0}C.

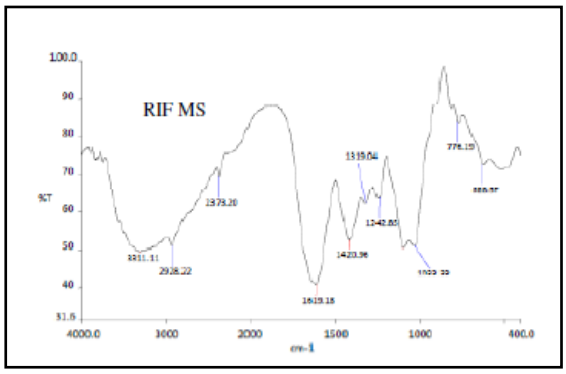

Figure 2 (b): FT-TR Spectra of Rifampicin Microspheres

Particle size: The microspheres had a smoother surface and were found to be discrete and spherical in shape (Figure 3a) there should not be any change in the morphology of drug-loaded microspheres (Figure 3b).

The mean particle size of the microspheres prepared by complex coacervation method was found to be 110-131µ as shown in Table 5.

Percentage yield: The yield of microspheres was determined by comparing the total weight of microspheres obtained against the sum of the weight of the drug, polymers and bioenhancer. The percentage yield of the optimized formulations was found to be 36.75-69.20% as shown in Table 5. The loss of the drug in the method may be due to loss accounted during hardening, washing and filtering processes of microspheres.

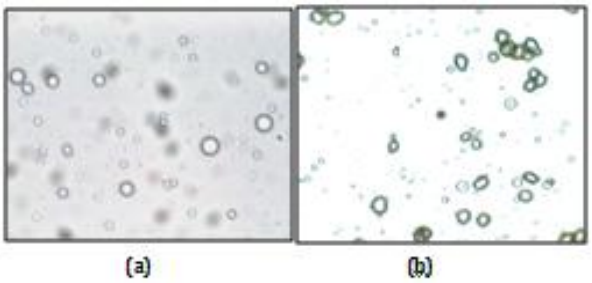

Figure 3 (a): Dummy Microspheres and (b) Microspheres Prepared by Complex Coacervation Method

Percentage entrapment efficiency: The entrapment efficiency was found to be in the range of 41.45-76.50%. Loss of the drug in these methods may be due to loss in the hardening, washing and filtering processes. During optimization of microsphere formulation, it has been observed that the concentration of polymer, cross linker concentration and cross-linking time may affect the entrapment efficiency of the microspheres as shown in Table 5.

The maximum values obtained are higher, or at least comparable to the highest value of entrapment efficiency reported in earlier studies using the sodium alginate method of preparation of microspheres[21]. The difference is due to the high aqueous solubility of RIF resulting in high concentrations of the drug present in the preparation medium in this method and possibly due to the use of bioenhancer in formulations.

Table 5: Particle Size, % Yield, % Drug Entrapment, % Bioadhesion and % Drug Release of Rifampicin Microspheres by Complex Coacervation Method

Formulation code	Mean particle size (µm)	Yield (%)	Drug Entrapment (%)	Bioadhesion (%) ± SD	Drug release (at 12th hour)
CRF_1	126-131	36.75 ± 1.89	41.45 ± 1.77	40.19 ± 1.21	46.81 ± 1.92
CRF_2	123-128	49.77 ± 1.89	56.71 ± 2.12	53.89 ± 1.79	59.67 ± 1.86
CRF_3	115-122	62.63 ± 1.87	68.34 ± 1.85	67.56 ± 1.19	73.23 ± 1.19
CRF_4	110-116	69.20 ± 1.43	76.50 ± 1.17	80.50 ± 2.04	86.16 ± 1.07

Percentage bioadhesion: The bioadhesion of the microspheres in the optimized formulations showed a significant change with the presence and quantity of bioenhancer. The bioadhesion study was performed (in triplicate) using a previously reported method[19]. The percentage bioadhesion was found to be 40.19 to 80.50% as shown in Table 5. The bioadhesive property (Figure 4) of the microspheres in which bioenhancers were used is higher as compared to microspheres without bioenhancer. The bioadhesive property of microspheres resulted in prolonged retention in the small intestine. It has been observed that, the microspheres containing comparably higher amount of bioenhancer showed significant increase in bioadhesion about 86%. It has also been observed that, the percentage bioadhesion increases as the amount of bioenhancer increases as shown in Table 5. The bioadhesive property of these particles resulted in prolonged retention in the small intestine.

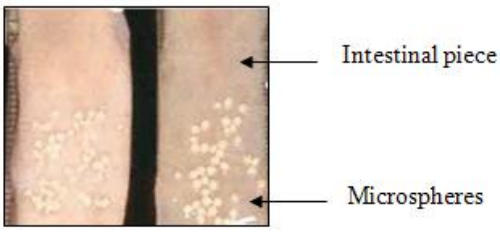

Figure 4: Bioadhesion study for microspheres

***In-vitro* drug release:** The *in-vitro* release study of rifampicin microspheres prepared by complex coacervation method in simulated gastric fluid (SGF), pH 1.2 and simulated intestinal fluid (SIF), pH 6.8, is shown in figure 5. Approximately, 8-10% of the drug was released in the SGF, pH 1.2 over a period of 2 h and about 35-80% in SIF, pH 6.8 up to 12 h. It has been found that the microspheres containing bioenhancer show greater increase in drug release as compared to microspheres without bioenhancer.

In Figure 5, CRF_1-formulation without bioenhancer and CRF_2, CRF_3 and CRF_4 are the formulations with bioenhancers prepared by complex coacervation method whereas the fraction of bioenhancer were used in 5, 10 and 15 mg respectively in 2nd, 3rd and 4th formulations in both the methods as shown in Figure 5.

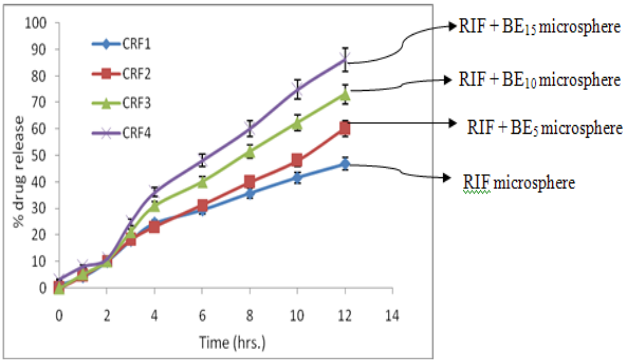

Figure 5: *In-vitro* drug release of Rifampicin Microsphere prepared by Complex Coacervation Method using *Piper nigrum* as a Bioenhancer

CONCLUSION

In this study, microspheres of rifampicin were prepared by complex coacervation method using chitosan and gelatin as a polymer and Na-TPP as a cross-linking agent. Effect of variables like drug-polymer ratio, cross linker concentration and the cross-linking time on *in-vitro* release of rifampicin was examined. The mean particle size of the microspheres increased with an increase in the concentration of polymer. The increase in the concentration of Na-TPP and chitosan-gelatin caused the increase in the entrapment efficiency and the extent of drug release. The cross-linking time shorter than 20 minutes resulted in higher entrapment efficiencies. The microspheres were spherical and well formed. The mean diameter, entrapment efficiency and bioadhesion of the optimized microspheres were found to be 103 -112 µm, 85.87 ± 1.75% and 87.42 ±1.51% respectively. The release profiles of rifampicin from microspheres were examined in simulated gastric fluid (SGF pH 1.2) and simulated intestinal fluid (SIF pH 7.4). About 10% of the rifampicin was released in the SGF in first 2 hours and released quickly about 85% in 10 hrs in SIF. The concentration of polymers and the presence of cross-linking agent had a great affect on the release of rifampicin. The most important finding of this study relates to the very significant enhancement in drug release (46.81 to 86.16%), due to co-administration of 15 mg bioenhancer along with each dose of rifampicin microspheres.

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