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Comparative Study of Herbal Extract of *Piper Nigrum*, *Piper Album* and *Piper Longum* on Various Characteristics of Pyrazinamide and Ethambutol Microspheres

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ABSTRACT

Bioenhancers are the 'bioavailability enhancers'; they do not show any therapeutic effect, but when used in combination enhances the activity of drug molecule. In a cited research paper, the effect of various species of piper used as bioenhancer singly and in combination in an equal ratio. The methods used for preparation of microspheres are Complex Coacervation and Modified Emulsion Method. The prepared microspheres were evaluated for various parameters like *in-vitro* release, drug entrapment efficiency, percent bioadhesion, permeability study using intestinal sac method. The *in-vitro* drug release of drugs from formulations where *Piper nigrum* was used as bioenhancers was found to be about 66-70% in 12 hrs. when used singly. When bioenhancers used in combination the *in-vitro* drug release of drugs was increased up to 85-90% for combination of *Piper album* and *Piper longum* in an equal proportion, the same was about 35-40% in case of formulations where no bioenhancers was used. The microspheres found to be less than 130 micron in size. The DEE was found to be in the range of 27-67%. The bioadhesion of the microsphere were found to be 20-76% (increased in formulations where bioenhancers incorporated). The *in- vitro* release study by USP paddle apparatus, the important results from *in-vitro* release study relates to the very significant enhancement in drug release, due to presence of bioenhancers.

Keywords: Microspheres, Bioenhancer, *Piper nigrum*, *Piper album, Piper longum,* Pyrazinamide, Ethambutol

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INTRODUCTION:

According to World Health Organization, Anti-TB agents are broadly classified as first line and second line agents based on their effectiveness and toxicity.[1, 2,3].

First line agents: These are the most effective, less toxic.

- Ethambutol (EMB or E), Isoniazid (INH), Pyrazinamide (PZA), Rifampicin (RIF).

Second line agents: These are considered as reserved therapy as they are less effective and more toxic for TB treatment. They are used if first line agents are not effective.

- Aminoglycosides: e.g., Amikacin, kanamycin
- Polypeptides: e.g., Capreomycin, viomycin, enviomycin
- Fluoroquinolones: e.g., Ciprofloxacin, levofloxacin, moxifloxacin
- Thioamides: e.g., Ethionamide, Prothionamide,

Third line agents: Other drugs that may be useful, but are not on the WHO list of second line drugs; they include rifabutin, macrolides antibiotics: e.g., clarithromycin, linezolid, thioacetazone, thioridazine.

Bioenhancers are the 'bioavailability enhancers'; they themselves do not show any typical drug activity, but when used in combination, they enhance the activity of drug molecule in several ways, including increasing bioavailability of the drug across the membrane, potentiating the drug molecule by conformational interaction, acting as receptors for drug molecule and making target cells more receptive to drugs [4].

Microsphere dug delivery consists of small particles of solids or small droplets of liquids surrounded by walls of natural and synthetic polymer films of varying thickness and degree of permeability acting as a release rate controlling substance and have a diameter up to the range of 0.1 to 200 µm. It is

one of the processes to provide the sustained and controlled delivery of drug for long periods of time [5,6].

The microspheres of the anti-tubercular agents were prepared by various methods of which the modified emulsion method and complex coacervation method were found to be most appropriate for our work. Variables like polymer concentration, drug-polymer ratio, concentration of cross-linking agent and time required for cross-linking were considered in the optimization of the formulation [7].

Hydro-alcoholic extract of 3 species of Piper namely *Piper nigrum, Piper album and Piper longum* used as herbal bioenhancers. Constant weight of bioenhancer extract (5 to 15 mg, singly or in combination) was incorporated into the drug loaded microspheres [8,9].

Figure 1: Fruits of *Piper nigrum*, *Piper album* and *Piper longum*

The prepared microspheres loaded with drug and bioenhancer/s were evaluated for various parameters including drug-excipient incompatibility, particle size, percentage yield, percentage entrapment efficiency, percent bioadhesion and intestinal permeability using intestinal sac method. Selected formulations were kept for stability studies as and no significant variation was found in physicochemical parameters of microspheres such as particle size, % drug entrapment efficiency (% drug content) and *in-vitro* drug release (% drug release in 12 hr.) was detected.

The most important finding of this study relates to the very significant enhancement in drug release from 35% (without bioenhancer) to 70% (with single bioenhancer) i.e. up to 90-100% increase in release rate and to 90% (with combination of bioenhancer) i.e. up to 100-120% increase in release rate of the anti-tubercular agents.

MATERIALS AND METHODS:

Pyrazinamide and Ethambutol was obtained as a gift sample from Lupin Pharmaceuticals Ltd., Aurangabad, Maharashtra. *Piper nigrum*, *Piper album* and *Piper longum* was purchased from local market and authenticated from Dept. of Botany, Dr. P. R. Ghogrey College of Science, Dhule, Maharashtra. Sodium alginate and Type B gelatin (bloom strength 220) were purchased from Loba Chemie, Mumbai. Sodium Tripolyphosphate (NaTPP) and Chitosan were purchased from Sigma Aldrich, Germany. All other chemicals / polymers used were of analytical grade.

EXPERIMENTAL WORK:

Extraction and isolation of Piper species used as bioenhancer: [10,11]

A. Macroscopy of *Piper nigrum (Black Pepper, Kali Meeri)*: The entire fruit was almost globular in shape, with 4 to 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.

Macroscopy of *Piper album (White Pepper, Safed Meeri)*: The entire fruit was almost rounding globular in shape, with 5 to 7.5 mm of diameter, white in color. The surface was found to be rough and uneven. The seeds were almost white to pale white in color, aromatic with a pungent taste.

Macroscopy of *Piper longum (Long peeper, Pimpli)*: The entire fruit was almost cylindrical, irregular in shape, 2 to 5 cm long and compact.

B. Phytochemical evaluation of piper species: The phytochemical evaluation of fully mature, dried fruits of *Piper nigrum, piper album and piper longum*, family *Piperaceae* was carried out as per the provisions of Ayurvedic Pharmacopoeia for various parameters, the results of which are mentioned in Table 1. [12,13]

Table 1: Phytochemical parameters of *Piper nigrum, P. album and P. longum*

Parameter (% w/w)	*Piper nigrum*		*Piper album*		*Piper longum*	
	Observed	Standard	Observed	Standard	Observed	Standard
Total Ash	4.10	NMT 5.5	3.88	NMT 5.0	NMT 5.5	NMT 5.0
Water soluble ash	3.76	NMT 5.5	3.89	NMT 5.0	NMT 5.5	NMT 5.5
Acid Insoluble ash	0.57	NMT 1.0	0.69	NMT 1.0	NMT 1.0	NMT 1.0
Water soluble extractive	23.11	NLT 15	18.75	NLT 12	18.75	NLT 15
Methanol soluble extractive	10.50	NLT 8	9.75	NLT 6	9.75	NLT 8
Loss on drying	3.05	NMT 4	2.79	NMT 4	2.79	NMT 4

(*NMT- Not More Than, NLT- Not Less Than)

C. Isolation of piperine from *Piper nigrum*, *Piper album* and *Piper longum:* Black pepper, White pepper and Pimpli (20 g) was ground to a fine powder and extracted with 95% ethanol (150ml) in a Soxhlet extractor for 2 hours. The solution was filtered and concentrated in vacuum on a water bath at 60°C. 10 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 122-128°C for various samples.

Preparation of Pyrazinamide and Ethambutol microspheres:

Sustained release 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, the double emulsification method [6, 14, 16, 18] and complex coacervation method [14, 15, 16, 18] were used to prepare the microspheres.

Method 1 - Double Emulsification Method (MEM)

Pyrazinamide (400 mg) and Ethambutol (400 mg) was separately dispersed in 3% aqueous solution of sodium alginate (10 ml). The aqueous phase was emulsified in light liquid paraffin (in the ratio 1:10) containing 1% (v/v) Span 80 using a mechanical stirrer (Remi Motors, India) at 1800 - 2200 rpm for 45 minutes. To it, 5 ml of 7.5% calcium chloride dissolved in a mixture of methanol and isopropyl alcohol (1:2) was added slowly to the emulsion and stirred to assure efficient crosslinking. Microspheres were collected by filtration in vacuum, washed with isopropyl alcohol thrice and finally dried at room temperature. Variables like concentration of polymer, drug - polymer ratio, cross-linking agent concentration and cross-linking time were considered in the optimization of the formulation. Finally, varying concentration of bioenhancer, piperine was added to the optimized formulation to study its effect on bioavailability of drugs as shown in Table 2.

Table 2: Formulae of Pyrazinamide and Ethambutol microspheres with variable polymer ratios and bioenhancer (Double Emulsification Method)

Formulation Code	PZA (mg)	EMB (mg)	Na. Alginate (%)	$CaCl_2$ (%)	Cross linking time (min.)	Bioenhancer (mg)
MPF1	400	--	3	7.5	45	--
MPF2	400	--	3	7.5	45	05
MPF3	400	--	3	7.5	45	10
MPF4	400	--	3	7.5	45	15
MEF1	--	400	3	7.5	45	--
MEF2	--	400	3	7.5	45	05
MEF3	--	400	3	7.5	45	10
MEF4	--	400	3	7.5	45	15

(*M- Modified Emulsion Method, P- Pyrazinamide, F- Formulation, E- Ethambutol)

Method 2 - Complex Coacervation Method (CCM)

Chitosan and gelatin were dissolved in dilute acetic acid solution (1% v/v) together at concentrations of 3% w/v in ratio 1:05 and adjusted to a pH 5.0. Pyrazinamide (400 mg) and Ethambutol (400 mg) was separately dissolved in the above polymeric mixture. The drug in polymeric mixture was emulsified in100 ml of liquid paraffin (1:1 mixture of light and heavy liquid paraffin) at 40°C containing 1ml Tween 80 (2% w/v). The emulsification was carried out for 15 min under mechanical stirrer (Remi Motors, India) at 1200 rpm. The w/o emulsion thus formed was cooled to 4°C to induce coagulation of gelatin. Then 50 ml Na-TPP (1.5% w/v) with pH 5 at 4°C was added drop wise. Stirring was continued for 30 min to obtain cross-linked microspheres. Microspheres were collected by centrifugation, washed with double distilled water thrice, then with acetone to remove water, and dried at room temperature under vacuum. The prepared microspheres were stored in desiccator for further studies. Concentration of polymer, polymer: copolymer ratio (Chitosan: Gelatin B), cross-linking time, rpm were considered as variables in optimization of the formulation. The composition of optimized formulation containing varying concentration of bioenhancer is shown in Table 3.

Table 3: Formulae of Pyrazinamide and Ethambutol microspheres with variable polymer ratios and bioenhancer (Complex coacervation method)

Formulation Code	PZA (mg)	EMB (mg)	Chitosan: Gelatin (ratio)	NaTPP (%)	Cross linking time (min.)	Bioenhancer (mg)
CPF1	400	--	1:0.5	1	30	--
CPF2	400	--	1:0.5	1	30	05
CPF3	400	--	1:0.5	1	30	10
CPF4	400	--	1:0.5	1	30	15
CEF1	--	400	1:0.5	1	30	--
CEF2	--	400	1:0.5	1	30	05
CEF3	--	400	1:0.5	1	30	10
CEF4	--	400	1:0.5	1	30	15

(*C- Complex Coacervation Method, P- Pyrazinamide, F- Formulation, E-Ethambutol)

Characterization of Microspheres:

Compatibility studies: Chemical interaction between the drug and the polymeric material, if any, during the preparation of the microspheres was studied by using Fourier Transform Infrared Spectroscopy (FTIR). Pure drug PZA and EMB, placebo microspheres, PZA and EMB 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. A small quantity of the powder was compressed into a thin semitransparent pellet by applying pressure. 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.

Particle size analysis: 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.

Determination of percentage drug entrapment: The drug content of the microspheres was determined spectrophotometrically (λmax = 270 nm and 292 nm for PZA and EMB respectively; PerkinElmer, USA Lambda 25 model). Microspheres (10 mg) loaded with PZA and EMB were dissolved in10 ml of isotonic phosphate buffer pH 6.8under sonication for 20 min. The solutions were filtered through 0.22 µm Millipore filter sand the amount of pyrazinamide and Ethambutol was determined. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at270 and 292 nm.

$$\text{Percentage drug entrapment} = \frac{\text{Mass of drug present in microparticles}}{\text{Mass of drug used in the formulation}} \text{ X } 100$$

The percent drug entrapment was calculated using following formula:

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 % yield of microsphere was determined using following formula:

$$\text{Percentage yield} = \frac{\text{Wt. of microspheres obtained}}{\text{Total wt. of drug, polymer used for formulation}} \text{ X } 1$$

Measurement of 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. Percent bioadhesion was determined by the following formula:

$$\text{Percentage bioadhesion} = \frac{\text{Wt. of adhered microspheres}}{\text{Wt. of applied microspheres}} \text{ X } 100$$

***In-vitro* drug release:** Dissolution studies were carried out using USP XXIV rotating basket method. The release profiles of pyrazinamide and Ethambutol 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 drugs) 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 λmax = 270 nm and 292 nm for PZA and EMB respectively and an equal volume of fresh medium was immediately added to maintain the dissolution volume. 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 PZA and EMB, placebo microspheres, PZA and EMB 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.

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 pyrazinamide and ethambutol microsphere are shown in Figure 2.

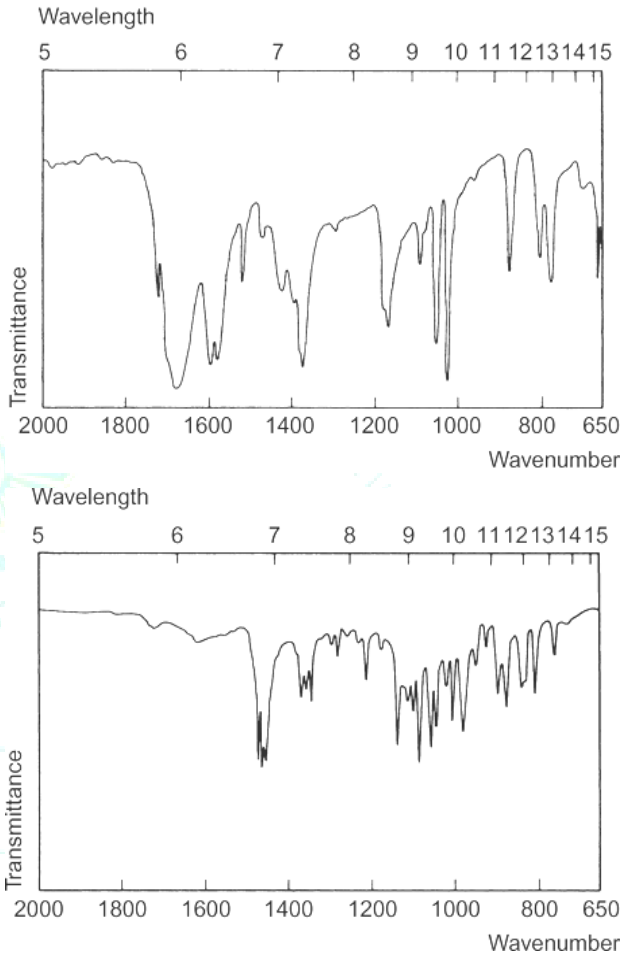

Figure 2: FT-IR spectra of Pyrazinamide and Ethambutol

Differential Scanning Calorimeter (DSC) study: Pyrazinamide and ethambutol 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 pyrazinamide and ethambutol at 190^{0}C.

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 4.

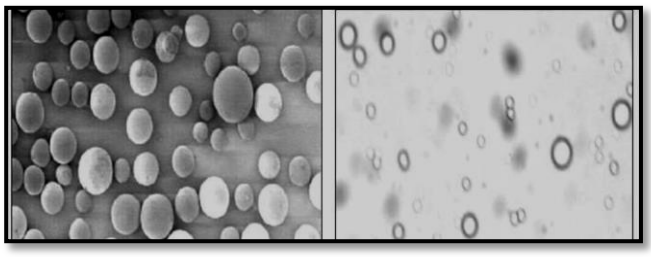

Figure 3a: Pyrazinamide microspheres prepared by MEM and CCM

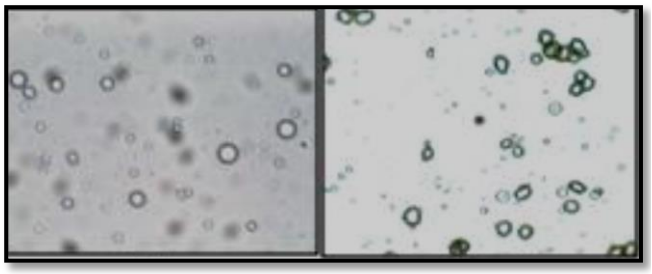

Figure 3b: Ethambutol microspheres prepared by MEM and CCM

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 4. The loss of the drug in the method may be due to loss accounted during hardening, washing and filtering processes of microspheres.

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 4. 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. The difference is due to the high aqueous solubility of PZA and EMB 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 [17].

Table 4: Evaluation Parameters for Pyrazinamide and Ethambutol Microspheres using Modified Emulsion and Complex Coacervation Method

Formulation Code	Mean particle size (µm)	Yield (%)	Drug Entrapment (%)	Bioadhesion (%) ±SD	Drug Release (at 12th hrs.) %
MPF1	129-135	41.50 ± 1.11	39.63 ± 1.13	40.12 ± 1.14	35.41 ± 1.11
MPF2	126-131	46.78 ± 1.19	51.52 ± 1.63	50.19 ± 1.17	48.12 ± 1.72
MPF3	122-128	56.95 ± 1.73	59.25 ± 1.47	61.24 ± 1.11	58.14 ± 1.37
MPF4	124-130	64.32 ± 1.03	67.21 ± 1.25	73.14 ± 1.36	68.17 ± 1.63
MEF1	126-134	39.63 ± 1.45	37.24 ± 1.24	41.25 ± 1.78	36.42 ± 1.14
MEF2	128-136	48.65 ± 1.23	46.85 ± 1.78	52.14 ± 1.14	47.78 ± 1.41
MEF3	124-128	58.32 ± 1.78	53.67 ± 1.34	62.13 ± 1.75	59.24 ± 1.52
MEF4	124-132	66.25 ± 1.13	68.12 ± 1.28	71.41 ± 1.18	68.22 ± 1.27
CPF1	124-134	43.21 ± 1.15	38.63 ± 1.44	39.15 ± 1.75	39.14 ± 1.29
CPF2	126-132	56.23 ± 1.29	49.12 ± 1.24	49.12 ± 1.16	49.17 ± 1.23
CPF3	130-134	61.58 ± 1.17	58.12 ± 1.18	60.01 ± 1.17	61.18 ± 1.44
CPF4	128-132	69.25 ± 1.36	71.14 ± 1.49	69.18 ± 1.01	69.14 ± 1.73
CEF1	122-126	38.12 ± 1.45	41.45 ± 1.73	41.13 ± 1.42	41.25 ± 1.18
CEF2	124-130	49.56 ± 1.23	56.71 ± 1.14	50.99 ± 1.45	49.21 ± 1.37
CEF3	126-132	64.50 ± 1.25	68.34 ± 1.19	61.24 ± 1.14	61.17 ± 1.27
CEF4	126-130	69.25 ± 1.08	72.40 ± 1.74	69.88 ± 1.19	69.09 ± 1.06

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. The percentage bioadhesion was found to be 40.19 to 80.50% as shown in Table 4. 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 4. The bioadhesive property of these particles resulted in prolonged retention in the small intestine [17].

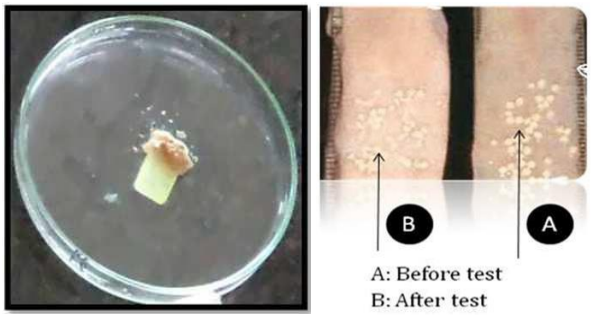

Figure 4: Bioadhesion study of Microspheres prepared by MEM and CCM

***In-vitro* drug release:** The in-vitro release study of pyrazinamide and ethambutol 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 a & b respectively. 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, CRF1-formulation without bioenhancer and CRF2, CRF3 and CRF4 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 a and b.

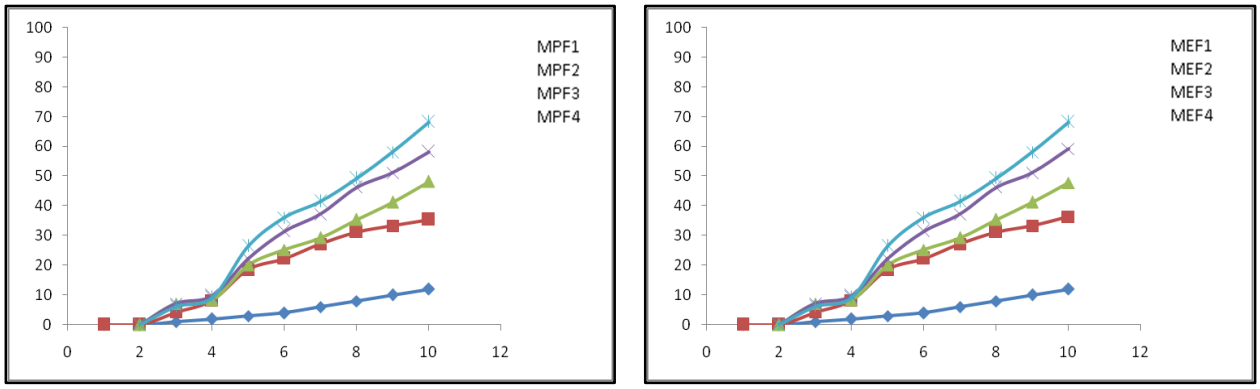

Figure 5a: *In-vitro* drug release from Pyrazinamide and Ethambutol Microspheres Prepared by Modified Emulsion Method

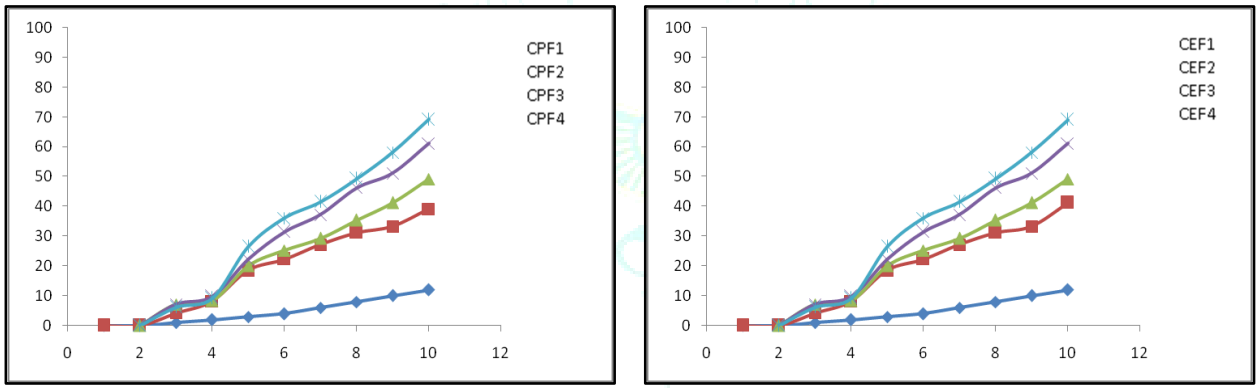

Figure 5b: *In-vitro* drug release from Pyrazinamide and Ethambutol Microspheres Prepared by Complex Coacervation Method

CONCLUSION:

In this study, microspheres of pyrazinamide and ethambutol were prepared by modified emulsion and complex coacervation method. Effect of variables like drug-polymer ratio, cross linker concentration and the cross-linking time on *in-vitro* release of pyrazinamide and ethambutol was examined. The mean particle size of the microspheres increased with an increase in the concentration of polymer. The cross-linking time shorter than 30 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 120-130 µm, 67.21 ± 1.25% and 68.12 ± 1.28% for pyrazinamide and 71.14 ± 1.49 and 72.40 ± 1.74 for ethambutol prepared by modified emulsion method and complex coacervation method respectively. . The release profiles of pyrazinamide and ethambutol from microspheres were examined in simulated gastric fluid (SGF pH 1.2) and simulated intestinal fluid (SIF pH 7.4). About 10% of the pyrazinamide and ethambutol 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 effect on the release of pyrazinamide and ethambutol. The most important finding of this study relates to the very significant enhancement in drug release (35 to 70%), due to co-administration of 15 mg bioenhancer along with each dose of pyrazinamide and ethambutol microspheres.

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