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DEVELOPMENT AND OPTIMIZATION OF GASTRORETENTIVE MUCOADHESIVE MICROSPHERES USING 3^3 FACTORIAL DESIGN

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ABSTRACT: This experimental work aimed at formulating and systemically characterizing mucoadhesive amoxicillin microspheres for the eradication of *H.pylori* and its associated diseases. The microsphere batches were prepared by emulsion-solvent evaporation technique with the use of carbopol-934P as mucoadhesive polymer and ethyl cellulose as carrier polymer. Initially 27 formulations were prepared using 33 factorial design using Design Expert software. Results of preliminary trials showed that amount of drug-to-polymers ratio, emulsifying agent and stirring speed influenced the characteristics of microspheres. Prepared microspheres were discrete, spherical, free flowing with good percentage of drug entrapment. An *in-vitro* mucoadhesive test showed that amoxicillin microspheres adhered more strongly to the gastric mucous layer and could retain in the gastrointestinal tract for an extended period of time. A 33factorial design was employed to study the effect of independent variables, drug-to-polymer-to-polymer ratio (amoxicillin-ethyl cellulose-carbopol-934P) (X1), Emulsifying agent (X2) and stirring speed (X3) on dependent variables, drug entrapment (Y1), particle size (Y2). The best batch exhibited a high drug entrapment efficiency of 66% and *In vitro* mucoadhesion after 1 h was 79%. A sustained drug release was obtained for more than 12 h. The morphological characteristics of the mucoadhesive microspheres were studied under a scanning electron microscope. *In vitro* release test showed that amoxicillin released slightly faster in pH 1.2 hydrochloric acid than in pH 7.8 phosphate buffer. In conclusion, the prolonged gastrointestinal residence time and enhanced amoxicillin stability resulting from the mucoadhesive microspheres of amoxicillin might make a contribution to *H. pylori* complete eradication.

INTRODUCTION: Oral ingestion is the most convenient and commonly used method of drug delivery. More than 50% of drug delivery systems available in the market are oral drug delivery systems. These systems have the obvious advantages of ease of administration and patient acceptance. Attempts to develop a single – dose therapy for the whole duration of treatment have focused attention on controlled or sustained release drug delivery systems [1].

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The dosage forms that can control the release rates and target drugs to a specific body site have an enormous impact in the development of novel drug delivery systems. Among various novel drug delivery system microspheres as a drug delivery systems made from the naturally occurring biodegradable polymers have attracted considerable attention for several years in sustained drug delivery [2-3].

Although, the release from the microspheres is limited, because of their short residence time at site of absorption. So, it would, be advantageous to have means for providing an intimate contact of the drug delivery system with the absorbing membranes [4-7]. This can be achieved by using mucoadhesion properties to microspheres and developing mucoadhesive microspheres.

This mucoadhesive drug delivery system shows the advantage such as efficient absorption and enhanced bioavailability of drugs owing to a high surface-to volume ratio, a much more intimate contact with the mucus layer and specific targeting of drugs to the absorption site [8-11]. Carbopol-934P as an anionic polymer is used in mucoadhesive systems by several researchers [12-16]. Carbopol-934P was used as a polymer in the preparation of mucoadhesive microspheres owing to its good mucoadhesive and biodegradable properties and ethyl cellulose as carrier polymer for microspheres.

Amoxicillin is a broad-spectrum, beta-lactam antibiotic for the treatment of various bacterial infections, including Helicobacter pylori (*H. pylori*). It inhibits the cell wall biosynthesis of *H. pylori* at and is first line drug for the treatment of H. pylori induced peptic and duodenal ulcers. Clinical studies using amoxicillin showed least resistance compare to Clarithromycin or Metronidazole against H. pylori [17-18].

None of various combinations against *H. pylori*, have shown complete eradication of bacterium. The incomplete eradication of *H. pylori* may be due to sub-bactericidal concentration of antibiotics in the gastric mucosal region, both from the lumen of the stomach and from the gastric supply.

Hence local diffusion of drug into gastric mucosa is essential for therapeutic efficacy various delivery systems of Amoxicillin have been prepared in recent time for increasing its local availability and efficacy such as polymer matrix tablets, gastroretentive floating systems,. Most of these studies emphasised on increasing the retention time of drug in the stomach and increasing the stability of antibiotics in acidic environment of stomach. But these systems could not assist in the complete eradication of bacterium. Since last decade, the strategy for effective delivery of antibiotics to *H. pylori* has shifted to the use of mucoadhesive based delivery systems based on the fact that mucoadhesive particulate show longer retention in stomach and thus deliver the antibiotic locally in the stomach mucosa for longer duration. These systems provide an intimate contact with mucus membrane due to polyvalent adhesive interaction or electrostatic attraction, H-bond formation, van-der-Waal forces and other. The system has an additional advantage of protecting acid sensitive drugs against acid degradation and offers effective drug diffusion across the mucus layer. [19]

The *H. pylori* has a survives in the environment of the stomach by colonizing in the gastric mucosal layer and it adheres to the surface of mucus epithelial cells and penetrate using flagella.

Mucoadhesion helps in increasing the gastric residence time of particles, also thick viscoelastic mucosal gel do not allow antimicrobial drugs to penetrate through it easily [20]. The Swelling of the polymer hinders docking it in gastric mucus and strong mucoadhesion decrease the mobility and thus interpenetrate penetrability in to mucus. In addition, gastric motility and proteolytic activity make mucus turnover intense there by make gastric residence of formulation shorter [21]. Hence efficient adherence to mucus could make the system incapable of penetrating across the mucus layer and entering the underlying epithelia [22].

To overcome these limitations, the particulate system, are required to penetrate the mucus membrane and deliver the drug close proximity to the site of *H. pylori* infection. Many researchers reported the preparation of particulate systems capable of penetrating mucus membrane.

The present work is an attempt to develop a novel bi-specific, biodegradable, mucopenetrating system for delivery of Amoxicillin to deep mucus layers near the sanctuary of the *H. pylori*.

In context of the above principles, a strong need was felt to develop a dosage form that delivered amoxicillin in the stomach and would increase the efficiency of the drug providing sustained action. Thus, an attempt was made in the present investigation to use carbopol-934P as a mucoadhesive polymer and ethyl cellulose as a carrier polymer and prepare mucoadhesive amoxicillin microspheres.

MATERIALS: Amoxicillin was obtained as a gift sample from Cipla (Baddi) and Carbopol 934 was obtained as a gift sample from Loba chemie. All other chemicals used were of analytical grade.

Methods:

Preparation of Mucoadhesive Microspheres:

Mucoadhesive microspheres of Amoxicillin were prepared by emulsion solvent evaporation method using Carbopol 934 and ethyl cellulose as polymers. In the first step ethyl cellulose was dissolved in 200 mL of ethanol, then drug and polymer were dispersed in the solution of ethyl cellulose under stirring. The preliminary trial batches were prepared and optimized using 3^3 Factorial design earlier by varying the drug-to-polymer-to-polymer (amoxicillin-ethyl cellulose-carbopol-934P) ratio in range of 1:3:1 to 1:3:3 %. The final mixture was extruded through a syringe (gauge No. 20) in 500 mL of liquid paraffin (mixture of heavy and light, 1:1 ratio) containing Span 80 and stirring was carried out using a propeller stirrer (Remi, Mumbai, India) at 1000 rpm. The stirring was done for three hours. In preliminary trial batches the amount of emulsifying agent (1-3%), the drug: polymer concentration (1:3:1) to (1:3:3) % and stirring speed (500-1000 rpm) were varied.

For optimization Factorial design approach was used, total twenty seven formulations were prepared, the drug-to-polymer-to-polymer ratio, concentration of emulsifying agent and stirring speed were varied and all other parameters were kept same. The Microspheres prepared were filtered and washed several times with petroleum ether (80:20) to remove traces of oil. The microspheres were then dried at room temperature (25^{o}C and 60 % RH) for 24 hrs. Formulated Batches are shown in **Table 1.**

TABLE 1: FACTORIAL DESIGN BATCHES

Batch Code	Variables in coded form			Y_1	Y_2
	X_1	X_2	X_3		
	Polymer Conc	Emulsifying agent	Stirring Speed	Drug Entrapment (%)	Particle Size (µm)
A1	-1.00	-1.00	-1.00	28	35
A2	0.00	-1.00	-1.00	48	38
A3	1.00	-1.00	-1.00	63	54
A4	-1.00	0.00	-1.00	31	39
A5	0.00	0.00	-1.00	39	42
A6	1.00	0.00	-1.00	51	58
A7	-1.00	1.00	-1.00	36	41
A8	0.00	1.00	-1.00	48	52
A9	1.00	1.00	-1.00	56	57
A10	-1.00	-1.00	0.00	35	34
A11	0.00	-1.00	0.00	51	48
A12	1.00	-1.00	0.00	65	63
A13	-1.00	0.00	0.00	41	50
A14	0.00	0.00	0.00	56	51
A15	1.00	0.00	0.00	61	47
A16	-1.00	1.00	0.00	45	54
A17	0.00	1.00	0.00	62	62
A18	1.00	1.00	0.00	61	58
A19	-1.00	-1.00	0.00	52	45
A20	0.00	-1.00	1.00	51	47
A21	1.00	-1.00	1.00	54	51
A22	-1.00	0.00	1.00	54	57
A23	0.00	0.00	1.00	57	59
A24	1.00	0.00	1.00	64	43
A25	-1.00	1.00	1.00	54	47
A26	0.00	1.00	1.00	58	64
A27	1.00	1.00	1.00	66	99

Coded Values	Actual Values		
	X_1(mg)	X_2 (%)	X_3 (rpm)
Low (-1)	100	1%	800
Medium (0)	200	2%	1000
High (1)	300	3%	1200

Where, X_1= Polymer Concentration, X_2= Emulsifying Agent, X_3= Stirring Speed

Further Batch no: A12, A24 and A27 were selected for further study on the basis of good drug entrapment efficiency.

Optimization of Mucoadhesive Microspheres using 3^3 Factorial Design:

A response surface design model with 3 factors, 3 levels and 27 runs was selected for the optimization study. The polynomial equation generated by this experimental design (using Design Expert 7.1.6) was as follows:

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1 X_2 + b_{13}X_1 X_3 + b_{23}X_2 X_3 + b_{11}X_1 X_1 + b_{22}X_2 X_2 + b_{33}X_3$$

Where, Y is the dependent variable; b_0 is the intercept; b_1 to b_{33} are the regression coefficients; and X_1, X_2 and X_3 are the independent variable that was selected from the preliminary experiments.[23-24] On the basis of the preliminary trials a 3^3 full factorial design was employed to study the effect of independent variables, i.e. Polymer Concentration (X1) , Emulsifying cagent concentration (X2) and the stirring speed (X3) on dependent variables drug entrapment and Particle size. Further evaluations were performed on the selected formulations.

Drug entrapment efficiency:

Two hundred milligrams of accurately weighed microspheres were crushed in a glass mortar-pestle and the powdered microspheres were suspended in 10 mL phosphate buffer (pH 7.8). After 24 hrs the solution was filtered and the filtrate was analysed for the drug content. The drug entrapment efficiency was calculated using the following formula: Practical drug content/Theoretical drug content × 100. The drug entrapment efficiency for trial batches is reported in **Table 1**.

Particle size of microspheres:

The particle size of the microspheres was determined by using optical microscopy method. Approximately 300 microspheres were counted for particle size using a calibrated optical microscope (Labomed CX RIII, Ambala, India). The particle size of microspheres of trial batches is reported in **Table 1**.

***In-vitro* wash-off test for microspheres:**

The *in-vitro* wash-off test as reported by Lehr et al [25] was used for the evaluation of percent mucoadhesion. A 1x1 cm piece of rat stomach mucosa was tied onto a glass slide (-3 inch-by-1inch-) using thread. Microspheres were spread (~50) onto the wet rinsed tissue specimen and the prepared slide was hung onto one of the groves of a USP tablet disintegrating test apparatus with continuous oxygen supply. The disintegrating test apparatus was used where the tissue specimen was given regular up and down movements in the beaker of the disintegration apparatus, containing gastric fluid (pH 1.2), for 10 hrs, the number of microspheres still adhering onto the tissue was counted. The results of *in-vitro* wash-off test after 10 hrs of trial batches are shown in **Table 2** and **Fig.1.**

TABLE 2: PERCENT MUCOADHESION OF SELECTED BATCHES

Batch no	1h (%)	5h (%)	10hr (%)
A12	69	54	48
A24	79	68	62
A27	81	72	68

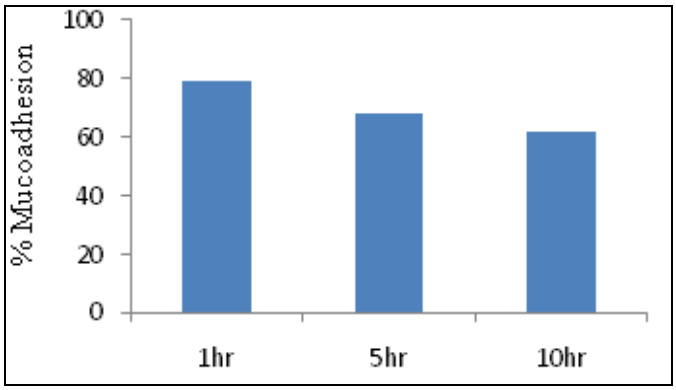

FIG. 1: % MUCOADHESION OF BATCH A27

FIG. 2*: IN-VITRO* MUCOADHESION TEST ASSEMBLY

Scanning electron microscopy:

Scanning electron photomicrographs of drug-loaded carbopol-934P mucoadhesive microspheres were taken. A small amount of microspheres was spread on glass slide. Afterwards, the slide containing the sample was placed in the scanning electron microscope (JSM 5610 LV SEM, JEOL, Datum Ltd, Tokyo, Japan) chamber. Scanning electron photomicrograph was taken at the acceleration voltage of 20 KV, chamber pressure of 0.6 mm Hg, at different magnification. The photomicrograph of batch is depicted in **Fig. 3** and **4**.

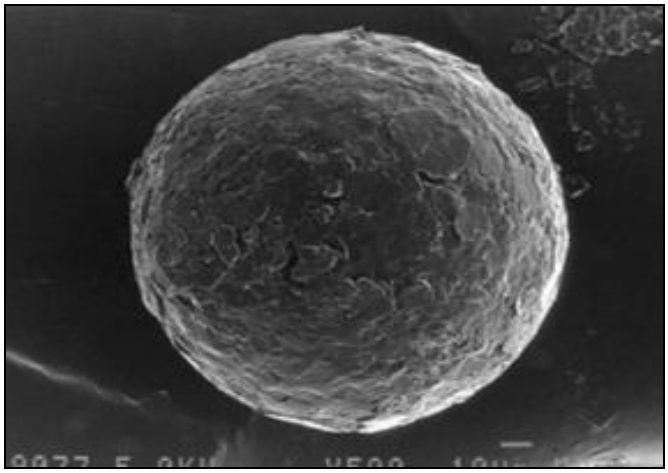

FIG.3: SEM (SCANNING ELECTRON MICROSCOPY) OF BATCH A27

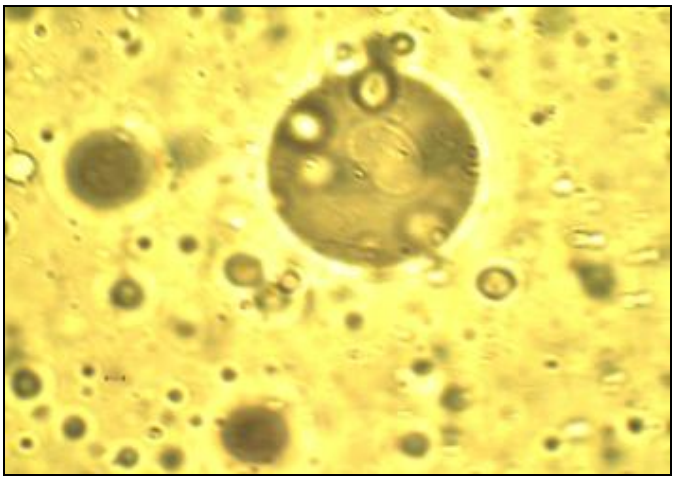

FIG. 4: OPTICAL MICROSCOPIC PHOTOGRAPH OF BATCH A27

Swelling index:

50 mg of microspheres were allowed for swelling in SGF (pH 1.2) for 4 h, the excess adhered liquid was removed by blotting with filter paper and weighed.[26-27]

The swelling Index was calculated using the following equation:

$$\text{Swelling Index} = \frac{X_s - X_0}{X_0} \times 100$$

Where,

X_s is the weight of the swollen microspheres after time t,

X_0 is the initial weight at zero time.

Swelling Index came out to be 1.45 (**Fig.5**).

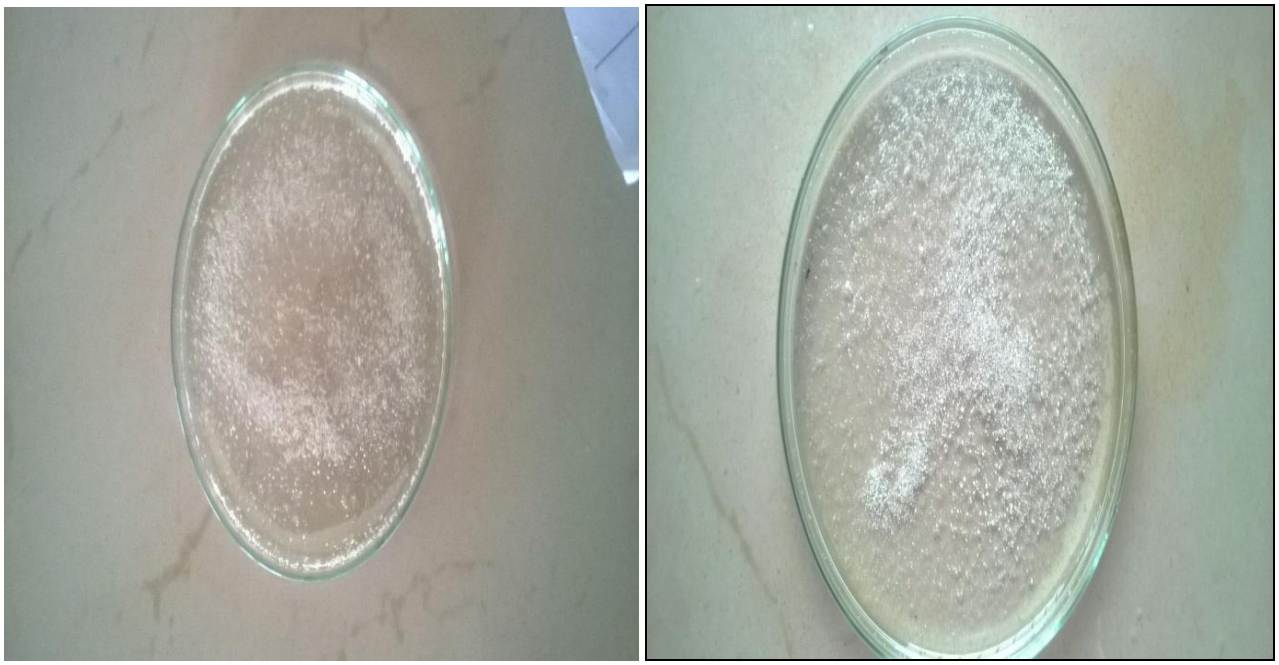

FIG.5: SWELLING INDEX OF MICROSPHERES INITIALLY AND AFTER 4 HRS

Drug release study:

The drug release study was carried out using USP XXIV basket apparatus (Electrolab, TDT-06T, India) at 37°C±0.5°C and at 100 rpm using 900 mL of phosphate buffer (pH 7.8) as a dissolution medium (n=5) as per USP XXVI dissolution test prescribed for amoxicillin tablets. Microspheres with the weight equal to 100 mg of amoxicillin were used for the test. 5 ml of sample from solution was taken at predetermined time intervals and were filtered through a 0.45 µm membrane filter, properly diluted, and analyzed spectrophotometrically. An equal amount of fresh dissolution medium was added immediately after taking of the test sample. Dissolution rate of drug dissolved at various intervals was calculated. The release profile is shown in **Table 3.**

TABLE 3: *IN-VITRO* DRUG RELEASE PROFILE

S. No	Time	% Cumulative Drug Release (pH-1.2)	% Cumulative Drug Release (pH-7.8)
1	0 min	0	0
2	30 min	7.96	9.56
3	1hr	15.91	23.54
4	2 hrs	21.42	39.27
5	3 hrs	26.47	45.36
6	4 hrs	38.13	57.37
7	5 hrs	51.02	61.02
8	6 hrs	58.43	63.42
9	7 hrs	69.17	72.36
10	8 hrs	82.48	79.15
11	9 hrs	88.14	83.24
12	10 hrs	90.02	91.27

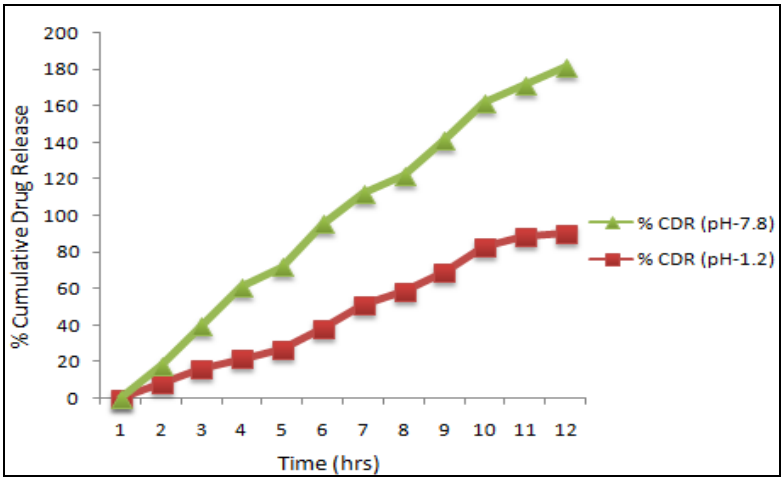

FIG.6: *IN VITRO* DRUG RELEASE PROFILE OF FORMULATION A27

Kinetics of Drug Release:

The kinetic of drug release from the gastro retentive films were established using the formula given by peppas, used to study the drug release behavior from the polymeric drug delivery systems. The release kinetic parameters were calculated according to peppas equation.

$$Mt/Mw = k\ t^n$$

Where,

Mt/Mw is the fractional release of the drug, t denotes the release time, k represents a kinetic constant, incorporating structural and geometrical characteristics of the controlled release device, and n is the diffusional exponent and characterizes the type of release mechanism during the dissolution process. Table 4 and 5 shows thr release kinetics of A27 batch in pH 1.2 and 7.4. **Fig. 7** to 14 shows the release kinetics graph of A27 batch in pH 1.2 and 7.4

TABLE 4: RELEASE KINETICS PROFILE OF FORMULATION A27 IN pH 1.2.

S.No	Time (hr)	Root T	Log T	Cum. (%) drug release	Cum. (%) drug retained	Log cum. (%) drug release	Log cum. (%) drug retained
1	30 min	0.54	-0.5	7.96	92.04	1.2	1.96
2	1	1	0	15.91	84.09	1.33	1.92
3	2	1.41	0.301	21.42	78.58	1.42	1.89
4	3	1.73	0.447	26.47	73.52	1.58	1.86
5	4	2	0.602	38.13	61.87	1.7	1.79
6	5	2.23	0.698	51.02	48.98	1.76	1.65
7	6	2.44	0.778	58.43	41.57	1.79	1.61
8	7	2.64	0.845	69.17	30.83	1.83	1.48
9	8	2.82	0.903	82.48	17.52	1.91	1.24
10	9	3	0.954	88.14	11.866	1.94	1.07
11	10	3.16	1	90.02	9.79	1.95	0.99

TABLE 5: RELEASE KINETICS PROFILE OF FORMULATION A27 IN pH 7.4.

S.No	Time (hr)	Root T	Log T	Cum. (%) drug release	Cum. (%) drug retained	Log cum. (%) drug release	Log cum. (%) drug retained
1	30 min	0.54	-0.5	9.56	90.44	0.98	1.95
2	1	1	0	23.54	76.46	1.37	1.88
3	2	1.41	0.301	39.27	60.73	1.59	1.78
4	3	1.73	0.447	45.36	54.64	1.65	1.73
5	4	2	0.602	57.37	42.63	1.75	1.62
6	5	2.23	0.698	61.02	38.98	1.78	1.59
7	6	2.44	0.778	63.42	36.66	1.8	1.56
8	7	2.64	0.845	72.36	27.64	1.85	1.44
9	8	2.82	0.903	79.15	20.85	1.89	1.31
10	9	3	0.954	83.24	16.76	1.92	1.22
11	10	3.16	1	91.27	8.73	1.96	0.9

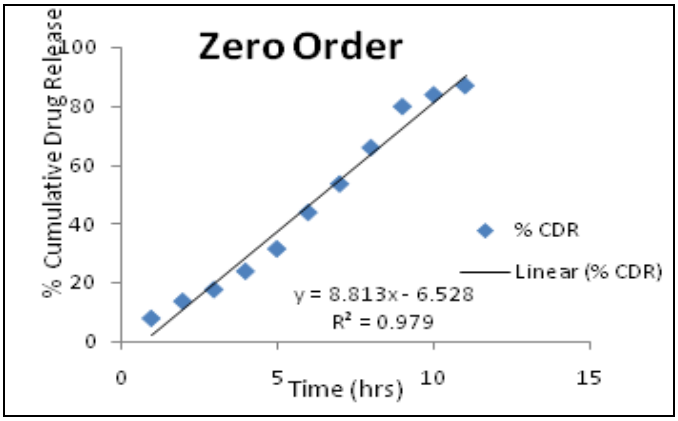

FIG.7: ZERO ORDER RELEASE OF A27 IN pH 1.2

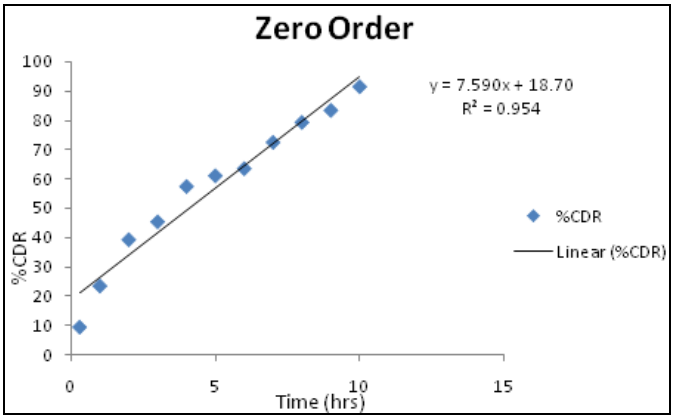

FIG. 11: ZERO ORDER RELEASE OF A27 IN pH 7.4

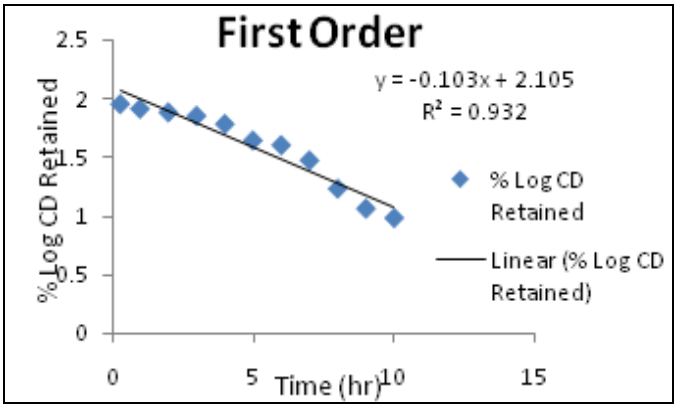

FIG.8: ZERO ORDER RELEASE OF A27 IN pH 1.2

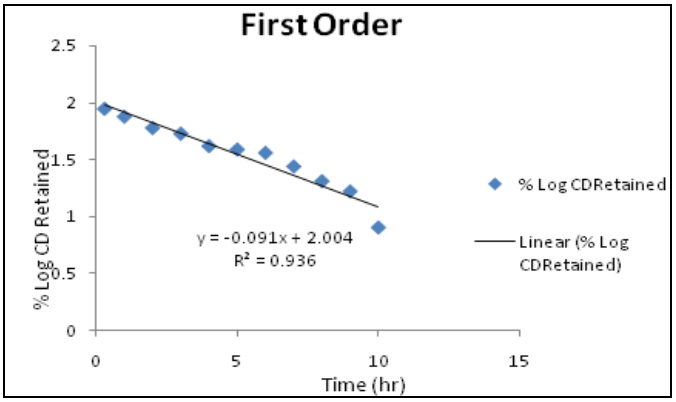

FIG. 12: FIRST ORDER RELEASE OF A27 IN pH 7.4

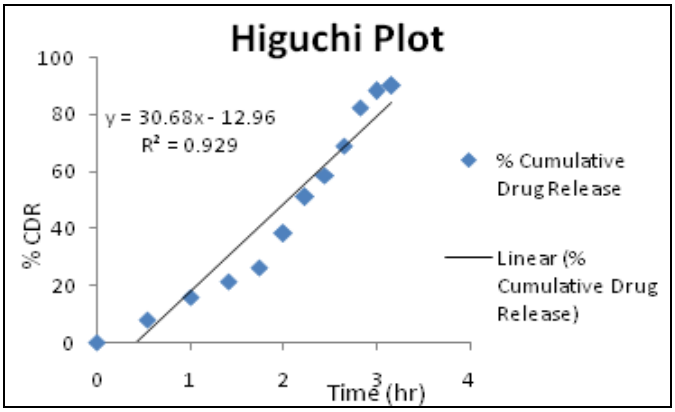

FIG. 9: HIGUCHI MODEL RELEASE KINETICS OF FORMULATION A27 IN pH 1.2

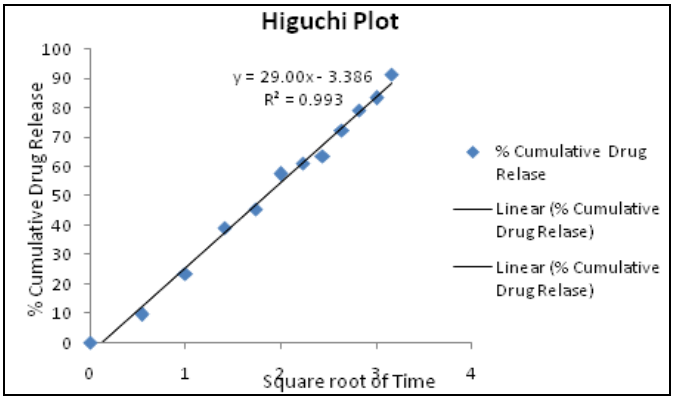

FIG. 13: HIGUCHI MODEL RELEASE KINETICS OF FORMULATION A27 IN pH 7.4

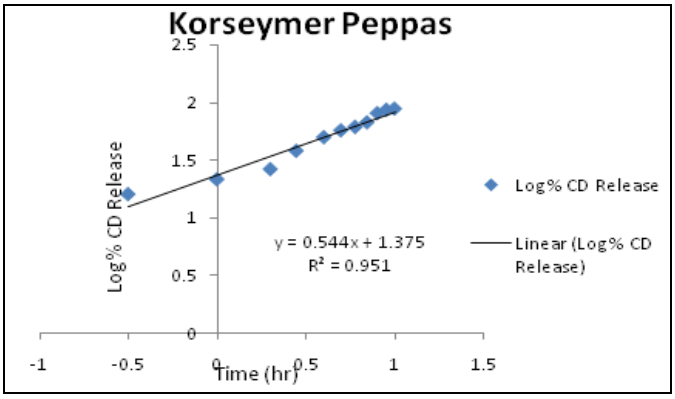

FIG.10: KORSEYMER PEPPAS RELEASE KINETICS OF FORMULATION A27 IN pH 1.2

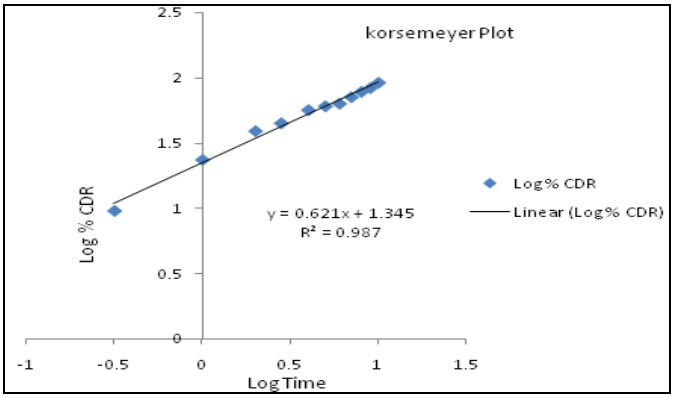

FIG. 14: KORSEYMER PEPPAS RELEASE KINETICS OF FORMULATION A27 IN pH 7.4

TABLE 6: MATHEMATICAL MODEL USED TO DESCRIBE THE DRUG RELEASE

	Zero-order	First order	Higuchi Kinetics	Korsemeyer Peppas
	Regression Coefficient (R^2)			
pH 1.2	0.9791	0.9328	0.9298	0.9517
pH 7.4	0.9541	0.9363	0.9934	0.9874

RESULTS AND DISCUSSION:

The mucoadhesive microspheres of Amoxicillin were prepared by emulsion-solvent evaporation technique using carbopol-934P and ethyl cellulose. The Carbopol-934P was used as a polymer for the preparation because of its biodegradable and mucoadhesive properties. Ethyl cellulose was used as carrier polymer.

Further 3^3 factorial design was used to study the effect of independent variables (polymer concentration [X_1], Emulsifying agent concentration [X_2] and stirring speed [X_3]) on dependent variables particle size, drug entrapment efficiency. The results clearly shows that all dependent variables are affected by the independent variables. The polynomial equations for each response with their high magnitude of the coefficients and mathematical sign indicate about the fit of the model.

Factorial Equation for Particle Size:

$Y = 52.26 + 6.28\ X_1 + 3.33\ X_2 + 2.67\ X_3 - 0.75\ X_1 X_2 - 2.25\ X_1 X_3 - 1.25\ X_2 X_3 + 0.05\ X_1 X_1 - 0.44\ X_2 X_2 - 3.11\ X_3 X_3$

The Model F-value of 3.61implies the model is significant. There is only a 1.10% chance that a "Model F-Value" this large could occur due to noise.

TABLE 7: ANOVA OF DEPENDENT VARIABLES

	for drug entrapment				
	df	**SS**	**MS**	**F**	**R^2**
Regression	9	2639.38	293.5	14.02	0.8813
Residual	17	355.46	20.91		
Total	26	2994.74			
	for particle size				
	df	**SS**	**MS**	**F**	**R^2**
Regression	9	1182.92	131.44	3.61	0.8898
Residual	17	618.94	36.41		
Total	26	1801.85			

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Results of the polynomial equation indicate that the effect of X_1 (drug polymer ratio) is positive and more significant than X_2(Emulsifying agent) and X_3 (stirring speed) i.e., as the drug polymer ratio was increased there was an increase in the polymer concentration, which lead to increased particle size, whereas as with the increase in stirring speed particle size was decreased. The contour plot and response surface graphs of Particle size response are given in **Fig. 15** and **16**.

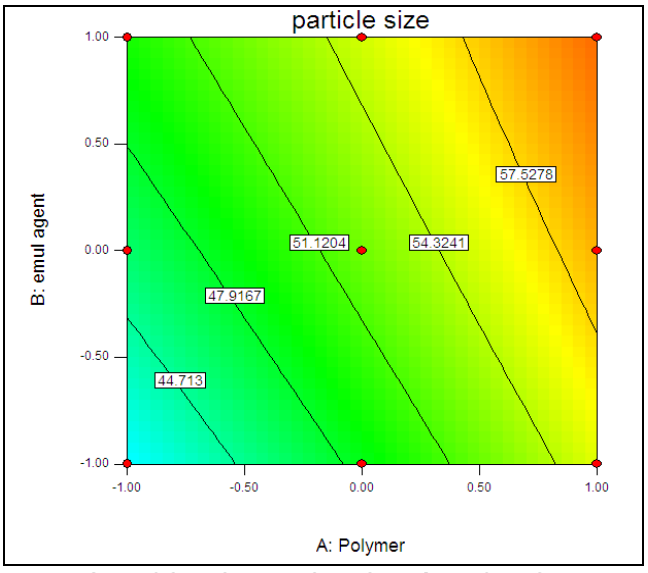

FIG.15: CONTOUR PLOT FOR PARTICLE SIZE

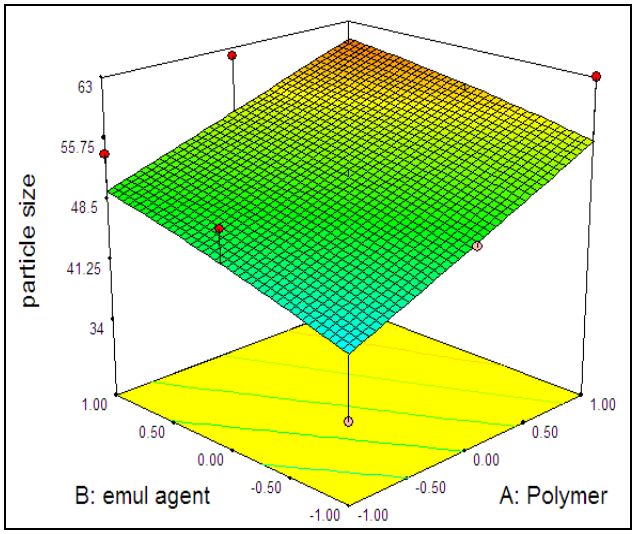

FIG.16: RESPONE SURFACE PLOT FOR PARTICLE SIZE

Factorial Equation for Drug Entrapment:

$Y = 53.37 + 9.17\ X_1 + 2.33\ X_2 + 6.11\ X_3 - 1.58\ X_1 X_2 - 4.25\ X_1 X_3 + 1.67\ X_2 X_3 - 1.61\ X_1 X_1 + 1.56\ X_2 X_2 - 2.78\ X_3 X_3$

The Model F-value of 14.02 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. Both variables have significant effect up to a level afterwards with the increase in both parameters the entrapment efficiency reduced. The contour plot and response surface graphs of Particle size response are given in **Fig.17** and **18**

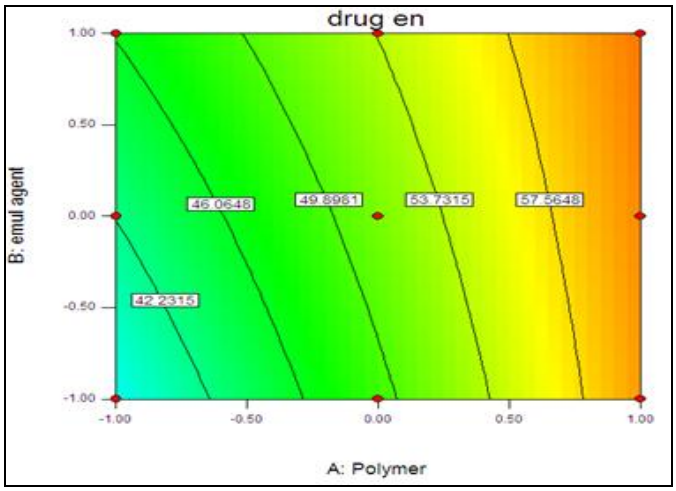

FIG.17: CONTOUR PLOT FOR DRUG ENTRAPMENT

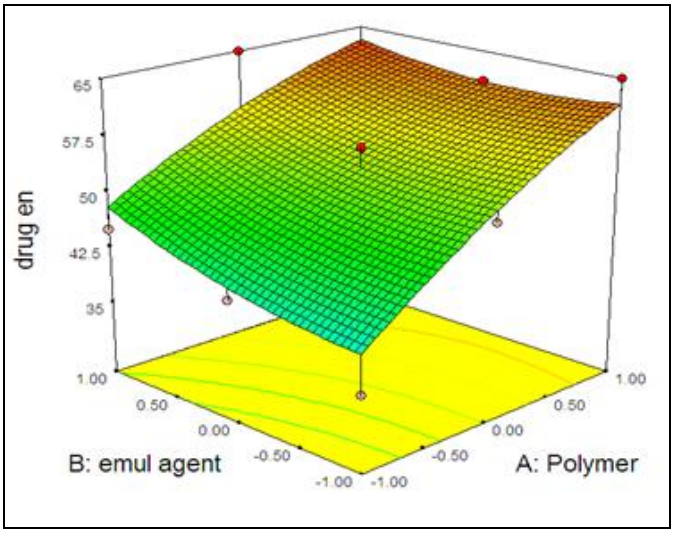

FIG.18: RESPONSE SURFACE PLOT FOR DRUG ENTRAPMENT

The results of a 3^3 full factorial design revealed that the polymer to-drug ratio, emulsifying agent and stirring speed significantly affected the dependent variables percentage drug entrapment efficiency and particle size. The microspheres of the best batch exhibited a high mucoadhesion of 68% after 10h, 66% drug entrapment efficiency. The *in vitro* release studies indicate that the mucoadhesive microspheres of Amoxicillin could sustain the release of the drug for more than 24 h.

CONCLUSION: The mucoadhesive microspheres of Amoxicillin prepared using 3^3 factorial design showed good percentage of mucoadhesion, drug entrapment and sustained release property. The Drug-to-polymer ratio, concentration of emulsifying agent and stirring speed showed a significant effect on dependent variables, which might show a potential use of mucoadhesive amoxicillin microspheres in *H. pylori* infection.

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