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Journal of Advanced Scientific Research

J. Adv. Sci. Res, 2010, 1(1); 46-54
Published on 10th Aug 2010

Research Article

Preparation and Evaluation of Controlled Release Acyclovir Microspheres Using Factorial Design

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ABSTRACT

Acyclovir, a synthetic purine nucleoside, is the most widely used antiviral agent. The short plasma half-life of oral acyclovir (3h) and low oral bioavailability (10-30%) with slow, variable and incomplete absorption through GIT requires high frequency dosing which may results in damaging side effects. Thus, the aim of present work is development of competent and efficient sustained release microspheres, to reduce the frequency of administration, to achieve patient compliance. Chitosan microspheres of acyclovir were prepared by ionotropic gelation where a 3^2 full factorial design was employed to study the effect of independent variables viz. chitosan concentration(X_1), tripolyphosphate concentration(X_1), and glutaraldehyde concentration(X_1) on entrapment efficiency, drug loading and t_{50}.

Smooth, spherical microspheres with 16 to 95µ median particle size were obtained. The regression equations obtained were as follows, $EE=32.332 + 8.033X_1 - 0.495X_2 + 1.343X_3 - 0.491X_1X_2 - 0.730X_1X_3 - 0.448X_2X_3 + 0.355X_1X_2X_3$, $DL=5.597 - 1.002X_1 - 0.062X_2 - 0.373X_3 - 0.512X_1X_2 - 0.241X_1X_3 + 0.125X_2X_3 + 0.005X_1X_2X_3$, $T_{50}=148.51 + 48.49X_1 + 27.72X_2 + 41.30X_3 + 15.11X_1X_2 + 16.32X_1X_3 + 10.16X_2X_3 + 6.63X_1X_2X_3$. ANOVA for results revealed that for obtaining controlled drug release with better microspheres properties relatively high levels of chitosan, tripolyphosphate and glutaraldehyde could be used.

Keywords: Acyclovir, Chitosan, Factorial design, ANOVA, Contour plots

INTRODUCTION

Genital herpes is the most commonly encountered cause of genital ulceration in the world and can involve the male and female genital tracts with equal prevalence. Herpes Simplex Virus type 2 (HSV 2) is the predominant viral type associated with herpes genitalis, and Herpes Simplex Virus type 1 (HSV 1) is most closely associated with oropharyngeal disease; however, each virus can cause infections in both anatomic area. Over the past decade, the incidence and severity of infections caused by HSV has increased due to the growth in number of immunocompromised patients, produced by aggressive chemotherapy regiments, expanded organ transplantation and a greater occurrence of human immunodeficiency virus infection.

Today, acyclovir, fanciclovir, and valacyclovir have been shown to be effective in the clinical management of genital herpes[1]. Acyclovir (acycloguanosine), 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy) methyl]-6H-purin-6-one, a synthetic antiviral agent. It is one of the most effective and selective agent against viruses of the herpes group. By going through pharmacokinetic and pharmacodynamic data of acyclovir, it is evident that acyclovir needs a potentially effective sustained release preparation. The mean plasma half-life ($t_{1/2}$) of elimination of Acyclovir is about 2.5 h. Upon oral administration, only 20% of the drug is absorbed and peak plasma

concentrations are reached in 1-2 hours. However due to short half-life (2.5 h) and low oral bioavailability (20%) of the drug, high frequency dosing is necessary for effective therapy. This often results in damaging side effects and leads to poor control of drug therapy. For this reason, it is necessary to prepare a sustained release dosage forms like microspheres which able to promote prolonged release of the drug[2-4].

The principal requirement for fabricating prolonged-action controlled-release drug delivery systems are viability of an appropriate material, which must be absolutely harmless to an organism and possess the necessary physical-mechanical and biomedical properties, including degradability in biological medium. These properties are absolutely fulfilled by a polymer known as chitosan[5,6]. It is a polycationic in acidic medium (pK_a 6.5) and can interact with negatively charged species such as TPP[7]. This characteristic can be employed to prepare biocompatible ionotropically gelled chitosan microspheres. The interaction of chitosan with TPP will be further cross-linked using glutaraldehyde which leads to formation of biocompatible cross-linked chitosan microspheres, which can be efficiently employed for drug delivery.

Thus in the present investigation sustained release chitosan microspheres containing acyclovir were prepared using ionotropic gelation method and the effect of independent variables viz. concentration of chitosan (X_1), concentration of TPP (Tripolyphosphate) (X_2) and concentration of glutaraldehyde (X_3) on dependent variables viz. percentage encapsulation efficiency, percentage drug loading and t_{50} were studied to optimize the formulation using 3^3 factorial design.

MATERIALS

Acyclovir- Cadila Pharmaceuticals Ltd.

Chitosan- Central Institute of Fisheries Technology, Cochin

Tripolyphosphate- Samir tech-chem Pvt Ltd, Vadodara.

Others: Loba Chemie Pvt Ltd. Mumbai

METHODS

Microspheres were prepared by w/o emulsion-ionotropic gelation method[8]. 1 % v/v acetic acid solution was prepared. 50 mg of Acyclovir drug was dispersed into 15 ml of 1 % acetic acid solution by sonication for 15 min using probe sonicator. Weighed quantity was dissolved into above dispersion using magnetic stirrer at low speed. Weighed amount of tripolyphosphate (TPP) was dissolved into 15 ml of distilled water to produce specific concentration of TPP solution. Specific quantity of glutaraldehyde was added in TPP solution. Chitosan solution was added drop by drop through 18# needle into 150 ml n-Hexane and 0.5 ml of Span 80 to prepare emulsion by continuous stirring at high speed using overhead stirrer. TPP solution was added drop by drop through 18 # needle in above emulsion with continuous stirring. Stirring was continued for 30 min. Once stirring stopped, mixture was put undisturbed for 5 min. Above separated organic phase was decanted and remaining microspheres was washed with 20 ml n-Hexane to remove excess span 80. Microspheres were allowed to dry at room temperature.

Factorial Design

3^3 full factorial design was used to optimize process variables, which were expected to affect formulation characteristics. As it was 3^3 factorial designs, 27 batches are prepared according to different combination of factors. Most critical factors selected for optimization were Chitosan concentration, Tripolyphosphate (TPP) concentration and Glutaraldehyde concentration. Responses of these independent variables were measured in

terms of Encapsulation Efficiency, Drug Loading and T_{50} (time required to release 50% of drug from the dosage form). Three levels were selected for each independent factor (Table 1 & 2) and the process parameters viz. stirring speed, volume of chitosan solution, volume of continuous n-Hexane solution, stirring time, cross-linking time and drying procedure were kept constant for all 27 batches.

Table 1: Transformation

Levels	Coded Value	Factors (Real values)		
		Chitosan Conc (% w/v)	TPP Conc (% w/v)	GLD Conc (ml/mg of Chitosan)
		X_1	X_2	X_3
1	-1	0.5	2	0.50×10^{-2}
2	0	1.0	6	1.25×10^{-2}
3	1	1.5	10	2.00×10^{-2}

Table 2: Factorial Design Layout

Batch No	Variable level in coded form			EE	DL	T_{50} (min)
	X_1	X_2	X_3			
C-1	-1	-1	-1	20.86	6.13	65.85
C-2	-1	-1	0	22.68	5.97	85.22
C-3	-1	-1	1	27.06	5.83	112.81
C-4	-1	0	-1	22.62	6.46	78.91
C-5	-1	0	0	23.30	6.42	100.47
C-6	-1	0	1	26.61	6.37	125.06
C-7	-1	1	-1	23.04	7.11	89.14
C-8	-1	1	0	23.68	7.09	113.07
C-9	-1	1	1	24.71	7.08	153.05
C-10	0	-1	-1	31.81	6.57	90.02
C-11	0	-1	0	34.55	5.97	125.46
C-12	0	-1	1	34.07	5.12	158.97
C-13	0	0	-1	31.81	6.68	103.35
C-14	0	0	0	35.64	6.19	138.29
C-15	0	0	1	35.66	5.70	164.11
C-16	0	1	-1	30.61	5.74	114.52
C-17	0	1	0	31.41	5.05	176.45
C-18	0	1	1	33.72	5.21	218.46
C-19	1	-1	-1	39.75	5.48	119.48
C-20	1	-1	0	40.84	4.82	146.03
C-21	1	-1	1	41.59	4.61	213.68
C-22	1	0	-1	39.30	5.34	143.27
C-23	1	0	0	40.14	4.61	184.39
C-24	1	0	1	40.40	3.45	237.77
C-25	1	1	-1	38.64	4.21	162.11
C-26	1	1	0	39.71	4.26	263.50
C-27	1	1	1	38.79	3.65	326.32

EVALUATION OF MICROSPHERES

Surface Morphology

Scanning electron microscopy was used to evaluate the quality of the microspheres obtained under the various conditions used. After being vacuum-coated (9 Torr) with a thin layer of gold, the microspheres were examined by means of a scanning electron microscope (Philips ESEM-XL-30TMP, Gaseous secondary electron detector) at an Acceleration range: 0.2 to 30 kV, using various magnifications to observe the surface morphology.

Encapsulation Efficiency (EE) and Drug Loading (DL)

A 50 mg drug loaded microspheres were dispersed in to 20 ml of Chloroform. Dispersed drug in organic phase was extracted with 10 ml of Phosphate Buffer Saline pH 7.4 (PBS) by sonication for 5 min using probe sonicator. Extraction process was again repeated twice using 10 ml of PBS. Acyclovir content was estimated in extracted liquid using UV-visible spectrophotometer at 251 nm after making necessary dilution (10 times dilution was carried out). EE was calculated using following equation.

$$\% \text{ Encapsulation Efficiency} = \frac{\text{Weight of Acyclovir loaded}}{\text{Weight of Acyclovir added}} \times 100$$

$$\% \text{ Drug Loading} = \frac{\text{Weight of Drug encapsulated in Microsphere}}{\text{Weight of Microspheres}} \times 100$$

Particle Size

It is possible to use the ordinary microscope for particle size measurement in the range of 0.2 µm to above 100 µm. to measure particle size of individual microspheres. Optical micrometer was calibrated using stage micrometer. According to microscopic method of size analysis, slides of dilute suspension of microspheres were prepared in liquid paraffin and slides were placed on mechanical stage of microscope. Size of 100 microspheres from each batch was measured for calculating average particle size.

***In-vitro* Release Study**

The release properties of beads were studied in Phosphate Buffer Saline pH - 7.4 (PBS) using a USP type-I dissolution apparatus (Scientific USP standards, Model DA). The dissolution medium 900 mL was maintained at (37 ± 0.5 ^{0}C) & stirring speed was set at 50 rpm. Microspheres (100 mg) were added to the dissolution medium and samples of 5 mL were taken and replaced with fresh medium at predetermined time intervals. The concentration of drug in each sample was determined by UV-visible spectrophotometer at wave length of 251 nm using corresponding blank.

RESULTS AND DISCUSSION

Polymer-Drug Compatibility study

It was carried out by using Differential Scanning Calorimeter (DSC). Chitosan did not show any peak in DSC spectra. Acyclovir showed one peak at 251.737^0 C. Physical mixture of Chitosan: Acyclovir (50: 50) was found to contain one peak at 255.785^0 C. This peak does not deviate much from peak of standard Acyclovir. Therefore polymer and drug were found compatible with each other.

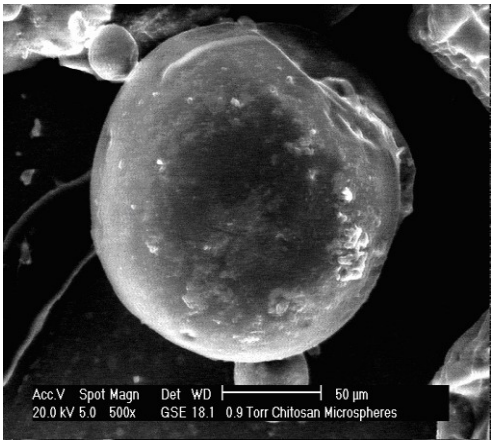
Preliminary study

First of all microspheres were prepared using liquid paraffin as continuous phase. TPP gelling agent, glutaraldehyde as cross-linking agent, amount of drug (50 mg) and chitosan concentration (1%) were kept constant for their batches. Two different surfactants (Span 80 and Tween 80) and two different stirrers (Magnetic stirrer and overhead stirrer) were used to prepare microspheres. Very few microspheres were formed in all batches as all chitosan solutions forms strong emulsion in liquid paraffin and were found difficult to harden. Further the batches were prepared with n-Hexane as continuous phase in the same way as mentioned above. Spherical microspheres with small particle size were formed in all batches. Batch prepared with magnetic stirrer

and tween 80 showed some aggregates. As span 80 is oil soluble surfactant, it was found to be easily washed off from prepared microspheres when washing was given with n-Hexane.

Further batches were prepared by 3^3 full factorial designs to study the effect of independent variables viz. Chitosan concentration, TPP concentration and glutaraldehyde concentration on dependent variables viz. EE, DL and T_{50}. The microspheres obtained under these conditions were found to be free flowing and without aggregation, and median size ranged from 16 to 95 µ with a satisfactory yield (86%). SEM of microspheres at magnification of 150X (Figure 1) revealed that microspheres were almost spherical in nature with slight smooth surface morphology.

Figure 1 SEM photomicroghraph (C23)



Summary of statistical analysis by ANOVA for selected dependent variables was depicted in Table 3.

Table 3: Summary of statistical analysis by ANOVA

EE							
Model	Source	df	SS	MS	F	Significance F	R
FM	Regression	7	1211.14	173.02	112.49	4.13×10^{-14}	0.9881
	Residual	19	29.22	1.54			
	Total	26	1240.36				
RM	Regression	2	1194.03	597.02	309.28	7.37×10^{-18}	0.9811
	Residual	24	46.33	1.93			
	Total	26	1240.36				
DL							
Model	Source	df	SS	MS	F	Significance F	R
FM	Regression	7	24.688	3.527	25.598	2.14×10^{-08}	0.9508
	Residual	19	2.618	0.138			
	Total	26	27.306				
RM	Regression	4	24.432	6.108	46.757	1.90×10^{-10}	0.9459
	Residual	22	2.874	0.131			
	Total	26	27.306				
T_{50}							
Model	Source	df	SS	MS	F	Significance F	R
FM	Regression	7	1211.14	173.02	112.49	4.13×10^{-14}	0.9881
	Residual	19	29.22	1.54			
	Total	26	1240.36				
RM	Regression	2	1194.03	597.02	309.28	7.37×10^{-18}	0.9811
	Residual	24	46.33	1.93			
	Total	26	1240.36				
Simple Model	Regression	3	86892.38	28964.13	64.04	9.93×10^{-14}	0.9832
	Residual	23	10402.86	452.30			
	Total	26	97295.24				

EE of all chitosan batches were found to be moderately varied between 20 to 42%. Full model equation derived for EE is given below as equation 1 as per Table 4.

Table 4: Summary of regression coefficients for EE:

Variable	Full Model			Reduced Model		
	Coefficient	t statistic	P-value	Coefficient	t statistic	P-value
Intercept	32.332	135.466	7.87×10^{-30}	32.332	120.922	6.05×10^{-35}
X_1	8.033	27.481	9.24×10^{-17}	8.033	24.530	1.66×10^{-18}
X_2	-0.495	-1.692	0.1069	-	-	-
X_3	1.343	4.594	0.0001	1.343	4.101	0.0004
X_1X_2	-0.491	-1.370	0.1866	-	-	-
X_1X_3	-0.730	-2.039	0.0555	-	-	-
X_2X_3	-0.448	-1.250	0.2263	-	-	-
$X_1X_2X_3$	0.355	0.811	0.4275	-	-	-

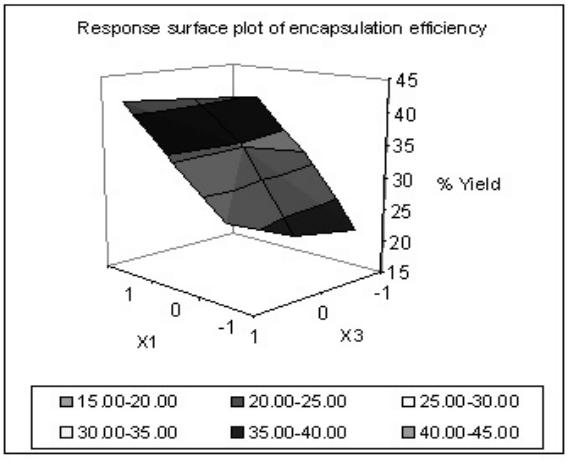
$$EE = 32.332 + 8.033X_1 - 0.495X_2 + 1.343X_3 - 0.491X_1X_2 - 0.730X_1X_3 - 0.448X_2X_3 + 0.355X_1X_2X_3 \text{-- (1)}$$

P-value of regression coefficients X_2, X_1X_2, X_1X_3, X_2X_3, and $X_1X_2X_3$ were found greater than 0.05, therefore they were proved as insignificant parameter for response (EE). Therefore these terms were eliminated from equation 1 to generate the reduced model. Remaining regression coefficients were found with significant for response EE hence they were retained in the reduced model.

$$EE = 32.332 + 8.033X_1 + 1.343X_3 \quad \text{---------- (2)}$$

It represents a simple model without any interactive term and it was found to contain only two independent variables. It is clear from equation 2 that high level of chitosan concentration and glutaraldehyde concentration favors higher EE. Batch C-21 having higher level of X_1 and X_2 and lower level of X_3 was found to contain highest EE (41.59%). The surface response plot of equation 1 (Figure 2) gave the impact of changing independent variable on EE.

Figure 2 Response surface plot of EE



As chitosan concentration increases, viscosity of solution increased significantly. This increased viscosity reduces the diffusion of acyclovir in the surrounding aqueous phase and also does not allow entrapped particle to escape easily. Therefore increase in concentration of chitosan, results in increased EE. Increase in

glutaraldehyde concentration results in increase cross linking between chitosan molecules. As a result a complex structure form which retains entrapped particle and ultimately increases EE.

DL of all 27 batches was found to be varied marginally between 3 to 7% by factors. Full model equation derived for DL is given below as equation 3 as per Table 5.

Table 5: Summary of regression coefficients for DL

Variable	Full Model			Reduced Model		
	Coefficient	t statistic	P-value	Coefficient	t statistic	P-value
Intercept	5.597	78.352	2.55×10^{-25}	5.597	80.467	1.13×10^{-28}
X_1	-1.002	-11.453	5.68×10^{-10}	-1.002	-11.762	5.84×10^{-11}
X_2	-0.062	-0.707	0.4882	-	-	-
X_3	-0.373	-4.269	0.0004	-0.373	-4.384	0.0002
X_1X_2	-0.512	-4.781	0.0001	-0.512	-4.910	6.55×10^{-05}
X_1X_3	-0.241	-2.253	0.0362	-0.241	-2.314	0.0304
X_2X_3	0.125	1.165	0.2583	-	-	-
$X_1X_2X_3$	0.005	0.035	0.9728	-	-	-

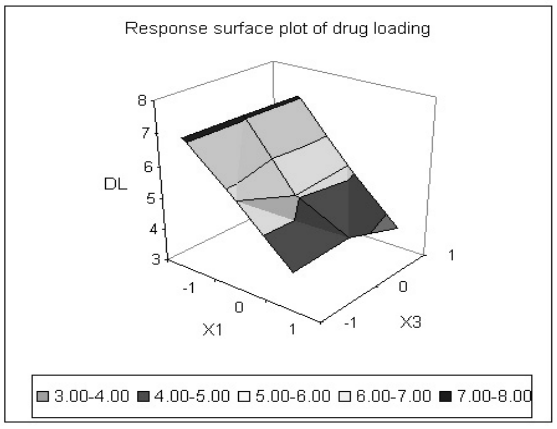
$$DL = 5.597 - 1.002X_1 - 0.062X_2 - 0.373X_3 - 0.512X_1X_2 - 0.241X_1X_3 + 0.125X_2X_3 + 0.005X_1X_2X_3 \text{---- (3)}$$

P-value of regression coefficients X_1X_3, X_2X_3, and $X_1X_2X_3$ were found greater than 0.05, therefore they were proved as insignificant parameters for response (DL). Therefore these terms were eliminated from equation 3 to generate the reduced model. Remaining regression coefficients were found with significant for response EE hence they were retained in the reduced model.

$$DL = 5.597 - 1.002X_1 - 0.373X_3 - 0.512X_1X_2 - 0.241X_1X_3 \quad \text{--------- (4)}$$

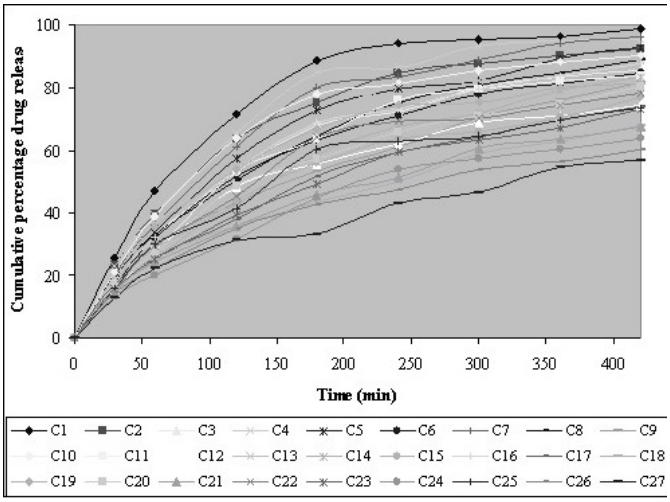
This reduced model equation has the two interactive terms i.e. X_1X_2 and X_1X_3. TPP concentration was found insignificant to affect DL. But its interactive term with chitosan shows significant effect on DL. It is clear from equation 4 that low level of chitosan concentration and glutaraldehyde concentration favors higher DL. Batch C-7 having low level of X_1 and X_2 and high level of X_3 was found to contain highest DL (7.11%). The surface response plot of equation 4 (Figure 3) showed impact of changing independent variable on EE. For this plot TPP concentration was fixed at higher level i.e. 1 since it exhibited most significant effect on DL.

Figure 3 Response surface plot of DL



The in-vitro drug release study was carried out for all batches (Figure 4).

Figure 4 In vitro drug release study



T_{50} of all batches of chitosan microspheres were found to be moderately varied between 65 to 330 min. Full model equation derived for T_{50} is given below as equation 5 as per Table 6.

Table 6: Summary of regression coefficients for T_{50}

Variable	Full Model			Reduced Model			Simple Model		
	Coeff	t stat	P	Coeff	t stat	P	Coeff	t stat	P
Intercept	148.51	62.75	1.70×10^{-23}	148.51	60.77	3.63×10^{-24}	148.51	36.28	8.31×10^{-22}
X_1	48.49	16.73	7.93×10^{-13}	48.49	16.20	5.75×10^{-13}	48.50	9.68	1.42×10^{-09}
X_2	27.72	9.56	1.07×10^{-08}	27.72	9.26	1.13×10^{-08}	27.73	5.53	1.26×10^{-05}
X_3	41.30	14.25	1.35×10^{-11}	41.30	13.80	1.11×10^{-11}	41.31	8.24	2.57×10^{-08}
X_1X_2	15.11	4.25	0.0004	15.11	4.12	0.0005	-	-	-
X_1X_3	16.32	4.59	0.0001	16.32	4.45	0.0002	-	-	-
X_2X_3	10.16	2.86	0.0099	10.16	2.77	0.0117	-	-	-
$X_1X_2X_3$	6.63	1.52	0.1435	-	-	-	-	-	-

$$T_{50} = 148.51 + 48.49X_1 + 27.72X_2 + 41.30X_3 + 15.11X_1X_2 + 16.32X_1X_3 + 10.16X_2X_3 + 6.63X_1X_2X_3 \text{--(5)}$$

P-value of regression coefficient $X_1X_2X_3$ were found greater than 0.05, therefore they were proved as insignificant parameter for response (T_{50}). Therefore these terms were eliminated from equation 5 to generate the reduced model. Remaining regression coefficients were found with significant for response (T_{50}) hence they were retained in the reduced model.

$$T_{50} = 148.51 + 48.49X_1 + 27.72X_2 + 41.30X_3 + 15.11X_1X_2 + 16.32X_1X_3 + 10.16X_2X_3 \text{--- (6)}$$

This reduced model was found to contain three significant interactive terms. Reduced model was tested in portions to determine whether the coefficients of interactive terms contribute significant information for prediction of T_{50} or not. This was done by testing the hypothesis that the three interactive terms were equal to zero. Therefore reduced model will become simple model.

Now, SS_{SM} - SS_{RM} = 10402.86 - 1370.07 = 9032.79

No of parameter omitted in reduced model = 3

F = (9032.79/3)/59.57 = 50.54

The critical value of F for 95% confidence level was equal to 3.10 (DF = 3, 20). Since the calculated value of F (50.54) was much greater than critical value (3.10), it may be concluded that the all three interactive terms of reduced model contributes significantly to the prediction of T_{50} and hence they were retained in reduced model.

Thus, it was found that the high level of chitosan concentration, glutaraldehyde concentration and TPP concentration favors the formation of sustained release formulation[9,10]. Batch C-27 having higher level of X_1, X_2 and X_3 was found to have better sustained release preparation (326.32 min). The surface response plot of equation 6 (Figure 5) showed impact of changing independent variable on T_{50}.

For this plot TPP concentration was fixed at higher level i.e. +1 since it exhibit most significant effect on T_{50}. It might be attributed to the increased concentration of chitosan. As chitosan concentration increases, particle size of microspheres increased which releases the drug at a slower rate from larger microspheres because of total effect of decreased surface area and increased diffusional path length. Similarly higher level of TPP and glutaraldehyde in the formulation favors the cross-linking reaction and resulted in sustained release effect.

CONCLUSION

The present study demonstrated the feasibility of efficiently encapsulating acyclovir into chitosan microspheres. The microspheres were prepared by ionotropic gelation method and full factorial design was employed. A statistical model with significant terms is derived to predict responses and their regression equations were found out. The results of multiple linear regression analysis revealed that for obtaining controlled drug release with better microspheres properties relatively high levels of chitosan, tripolyphosphate and glutaraldehyde could be used.

ACKNOWLEDGEMENTS

The authors wish thankful to SICART for availing the necessary facilities to carry out the practical work.

REFERENCES

1. Herfindal E, Gourley D. In Textbook of THERAPEUTICS - Drug and Disease Management; 7th edition. Lippicott Williams & Wilkins, 2000. p. 1546-49.
2. Brien J, Campoli-Richards D. *Drugs*, 1989; 37: 233-239.
3. Fletcher C, Bean B. *Drug Intell Clin Pharm*, 1985; 19: 518-523.
4. Vergin H, Kikuta C, Mascher H, Metz R. *Arzneimittelforschung*, 1995; 45: 508-515.
5. Singh UV, Udupa N. *J of Microencapsulation*, 1998; 15: 581-94.
6. Muzzarelli RAA, Baldassare V, Conti F, Gazzanelli G, Vasi V, Ferrara P, Biangini G. *Biomaterials*, 1988; 8: 247-52.
7. Lucia V, Silvia B, Dusan B. *Macro Bio*, 2007; 7: 629-34.
8. Dini E, Alexandridou S, Kiparissides C. *J of microencapsulation*, 2003; 20: 375-85.
9. Kashappa GH, Hyun JP. *Drug Dev Res*, 2005; 64: 114-28.
10. Anil KA, Willem FS, Carmen RL. *Int J of Pharm*, 2006; 312: 166-73.