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Original Article

Formulation and *In Vitro* Evaluation of Acyclovir Mucoadhesive Microspheres for Intravaginal Application

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Abstract

The purpose of the research was to formulate microspheres of acyclovir (ACV) using mucoadhesive polymers, sodium alginate and chitosan. Calcium chloride was used as the ionotropic gelling agent. Sodium alginate was crosslinked by calcium chloride leading to a slower release of the drug. Chitosan which is a cationic polymer interacted with sodium alginate, an anionic polymer, to form an interpolymer complex, which also slowed the release and improved the mucoadhesion. Prior to the formulation, drug: excipient compatibility study was carried out for 12 weeks at 40 ºC ± 2 ºC /75% RH ± 5 RH. Then, FTIR was recorded to check for any chemical degradation of the drug ACV. The morphological properties, the drug encapsulation efficiency, the drug release profile and the *ex vivo* mucoadhesion strength were investigated. Based on these studies, P8 was found to be the best formulation. P8 had a % cumulative drug release (CDR) of 100.81% at the end of 12 h. High encapsulation efficiency of 80.92% and smaller average particle size of 596.74 µm further favored its selection as the best formulation. P8 followed Higuchi model. Further, SEM of P8 was recorded. Accelerated stability study of P8 for 6 months at 40 ºC ± 2 ºC /75% ± 5% RH indicated that it was stable.

Keywords: Acyclovir, Chitosan, Intravaginal, Microsphere, Mucoadhesive, Topical

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1. Introduction

WHO statistics state the alarming figure of 1 million cases of sexually transmitted diseases (STDs) every day. Globally, 500 million cases of STDs are reported every year. STDs impose a formidable challenge on the vulnerable female population [1]. Preventive measures provide the best defence against such diseases. The female population is susceptible and unable to negotiate the use of condoms with their male partners in many countries [2]. Hence, the basis of this research is to provide effective, biocompatible,

user-friendly, viable, stable and novel topical intravaginal formulation of microbicide for the pre–exposure prophylaxis of venereal diseases. Acyclovir (ACV) is known by its IUPAC name as 2-Amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one [3]. Acyclovir is a synthetic purine nucleoside analogue with *in vitro* and *in vivo* inhibitory activity against HSV-1, HSV-2 and Varicella Zoster Virus (VZV). Acyclovir is highly selective due to its affinity for the enzyme thymidine kinase (TK) encoded by HSV and VZV. This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleoside analogue. The monophosphate is further converted into triphosphate by a number of cellular enzymes. Under *in vitro* conditions, acyclovir triphosphate stops replication of herpes virus DNA. This is accomplished in three ways: competitive inhibition of viral DNA polymerases, incorporation into and termination of the viral DNA chain and inactivation of the viral DNA polymerases. The greater antiviral activity of acyclovir against HSV compared to VZV is due to its enhanced phosporylation by the viral TK [4].

HSV- type 2 infections are the most frequent cause of genital ulcer disease. Moreover, they are associated with a 3.1 fold increase in HIV acquisition in women [5]. Acyclovir is already marketed as a 5% cream for topical infections [6]. But the creams have the inherent problems of low mucoadhesive property and low residence time. The above reasons have prompted us into developing novel topical vaginal mucoadhesive formulations, especially targeting the vulnerable female population. Among the formulations developed are vaginal mucoadhesive microspheres of ACV. Microspheres can be employed as efficient carriers of drugs to the desired site of action. But they have limitations due to short residence time which can be improved by conferring mucoadhesion via incorporation of mucoadhesive polymers. Mucoadhesive microspheres comprise completely of mucoadhesive polymers or may possess a coating of the polymer [7]. Mucoadhesion can be defined as the adhesion of two surfaces, one which is the mucus, for an extended period of time. The adhesion between the two surfaces occurs due to interfacial forces [8]. Mucosa or the mucus membrane is the moist tissue that lines organs and body cavities such as oral cavity, gastro-intestinal tract, rectum, vagina, nose, and eye [9]. There has been a great interest in developing novel mucoadhesive drug delivery systems for various routes. For example, gastric mucoadhesive discs/microparticles of metformin hydrochloride were formulated and evaluated [10], mucoadhesive gels of *Quercus brantii* and *Coriandrum sativum* for periodontal drug delivery were prepared and evaluated [11] and mucoadhesive vaginal tablets of acriflavine were developed and investigated [12]. Many techniques have been reported to formulate mucoadhesive microspheres, such as, emulsion/solvent evaporation technique [13],

coacervation precipitation technique [14], spray-drying [15] and ionic gelation technique [16]. In this research work, ionic gelation technique was used due to its simplicity, reproducibility, avoidance of organic solvents and heat.

Sodium alginate and chitosan were employed as biodegradable and mucoadhesive polymers because of their biocompatibilty and safety.

2. Materials and Methods

The animal experiments were conducted after obtaining certification from the institutional animal ethics committee of Krupanidhi College of Pharmacy, vide letter, Ref No: KCP/IAEC-02/2012-13.

2.1. Materials

Acyclovir was a gift sample from Atul Limited, PP site, Atul- 396020, Gujarat. Chitosan was a gift sample from CIFT, Matsyapuri, Kochi – 682029. Sodium alginate and calcium chloride were purchased from Loba Chemie, 107, Jehangir Villa, Wodehouse Road, Colaba, Mumbai – 400005. All solvents used were of analytical grade.

2.2. Drug: Excipient Compatibility Studies

The physical mixtures comprising 100 mg drug ACV + 100 mg excipients (sodium alginate, chitosan, and calcium chloride) were kept in a glass vial at 40 ºC ± 2 ºC /75% ± 5% RH.for 12 weeks. At the end of 12 weeks, the sample was sent for FTIR analysis.

2.3. Preparation of Mucoadhesive Microspheres of Acyclovir

The mucoadhesive vaginal microspheres of acyclovir were prepared according to the table 1. Briefly, all the sodium alginate solutions were prepared by dissolving the respective amounts in

Table 1. Formulation chart for preparation of ACV microspheres.

Code	Sodium alginate concentration (% w/v)	Calcium chloride concentration (% w/v)	Chitosan concentration (% w/v)
P1	2	5	1
P2	4	5	2
P3	4	2.5	2
P4	2	5	2
P5	4	2.5	1
P6	4	5	1
P7	2	2.5	1
P8	2	2.5	2

distilled water. To the sodium alginate solutions, 1% w/v of acyclovir was added under homogenization for 5 min to yield smooth dispersions of acyclovir in sodium alginate solutions. The chitosan solutions (1% w/v or 2% w/v) were prepared in 5% v/v aqueous acetic acid. To the chitosan solutions, respective amount of calcium chloride was added. The acyclovir–alginate dispersions were taken into syringe fitted with 0.45 mm needle and dropped at the rate of 1ml/min into chitosan – calcium chloride solutions stirred at 100 RPM at room temperature to yield opalescent beads. The microspheres were allowed to harden for another 2 h, filtered, washed thrice with distilled water and kept for drying at 40 ºC in an oven for 24 h. After 24 h, the size of the beads reduced and they were kept in labeled self-sealing plastic bags.

2.4. Characterization of the ACV Micropsheres

2.4.1. Particle Size Determination by Optical Microscopy

All the eight batches prepared (P1 to P8) were analyzed for particle size by optical microscope. First the eye piece micrometer was calibrated using a stage micrometer and then on a clean glass slide a small quantity of microspheres were placed using a thin brush. Then they were covered carefully with a cover-slip and observed under 10X magnification. One hundred particles from each batch were counted and average particle diameter was found out by using the formula:

$$\text{Average particle diameter} = \frac{\sum n * d}{N}$$

Where, n = total no. of particles in that size range

d = Diameter of the particles of that size range

N = total no. of particles.

2.4. 2. Drug Content & Encapsulation Efficiency

20 mg of the microspheres from each batch were taken and digested in 100 ml of 0.1N HCl in a 100 ml volumetric flask and kept aside with intermittent shaking for 24 h. Then, the contents of the flask were filtered by using Whatman filter paper no.1. Then 1 ml of the filtrate was diluted with 50 ml of dimethyl sulfoxide (DMSO) in a volumetric flask and sonicated for 10 min to extract ACV. This was again filtered by using Whatman filter paper no.1; one ml from this was further diluted with methanol up to 10 ml and absorbance measured at 252 nm using methanol as blank. After recording the absorbance, the drug content and encapsulation efficiency were calculated. The readings were taken thrice and the average reading was taken for further calculation.

$$\text{calculated amount of drug} = \frac{\left(\frac{\text{Abs} - \text{intercept}}{\text{slope}}\right)(* 10 * 100)}{1000}$$

$$\text{Drug content} = \frac{\text{Calculated amount of drug}}{\text{total amount of microspheres}} * 100$$

$$\text{Encapsulation efficiency} = \frac{\text{Calculated drug content}}{\text{theoretical drug content}} * 100$$

2.4.3. Ex Vivo Bioadhesion Studies

The *ex vivo* evaluation of the mucoadhesive properties of the microspheres was carried out using cleaned vaginal tissue obtained from a freshly slaughtered sheep from slaughter house. The vaginal tissue was washed with SVF (simulated vaginal fluid) pH 4.2 and attached on a glass slide with cellophane tape. Thirty pre-swollen microspheres were brought in contact with the tissue by using a pressure of 25 g on the slide for 2 min. The mucoadhesiveness of the microspheres was measured by connecting the prepared slide to the arm of a USP disintegration test apparatus. The particles were given a reciprocating motion in 900 ml of SVF pH 4.2 medium at 37± 0.5 ºC. Then at the end of 60 min, the apparatus was stopped and the remaining adhered microspheres were recounted carefully. The readings were taken thrice and the average reading was taken for further calculation.

The percentage mucoadhesion was then calculated by using the formula:

$$1 - \frac{\text{Initial no.of microsphere} - \text{Microspheres remaining attached at T 60min}}{\text{Initial attached microsphere}} * 100$$

2.4.4. In Vitro Release Studies

The *in vitro* dissolution studies were carried using USP - 34 paddle type dissolution apparatus. Fifty mg ACV loaded microspheres were placed in a dialysis bag (Himedia LA 393) and introduced into100 ml dissolution medium of SVF pH 4.2 maintained at 37± 0.5 ºC at a rotation speed of 50 RPM. 1 ml of aliquots was withdrawn at predetermined time intervals and an equivalent volume of fresh medium was replaced to maintain sink condition. The aliquots were diluted and analyzed spectrophotometrically at 252 nm to determine the concentration of drug present. The readings were taken thrice and the average reading was taken for further calculation.

2.4.5. Accelerated Stability Studies for the Selected P8 Mucoadhesive Vaginal Microspheres

The selected P8 microspheres were subjected to accelerated stability studies for 6 months at 40 ºC ± 2 ºC /75% ± 5% RH.

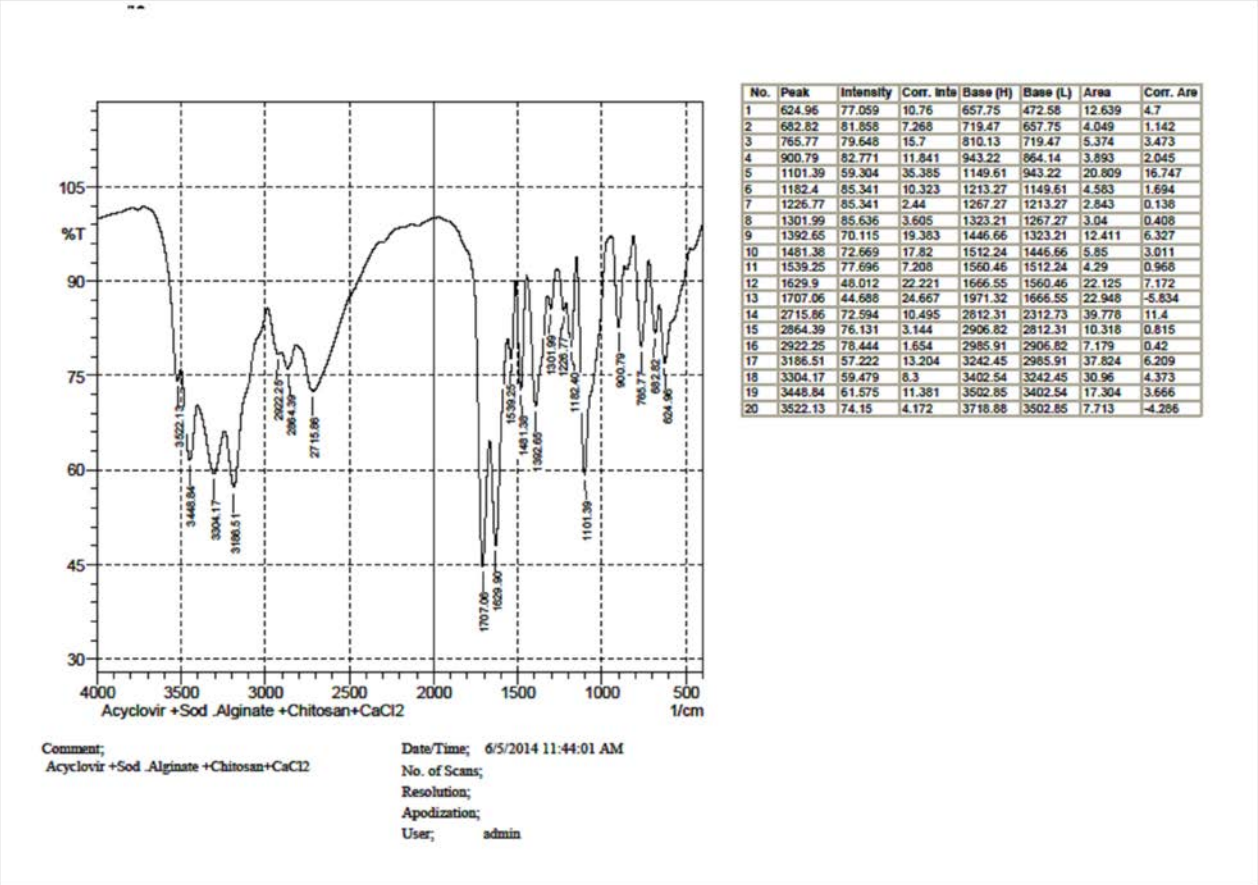
2.4.6. Statistical Analysis

Three readings were taken and the average was taken for further calculation. SD ± is reported. GraphPad Prism trial version 6.05 was used for statistical analysis.One way ANOVA was used to find out any significant differences. P value was 0.0001.

3. Results and Discussion

3.1. Drug: Excipient Compatibility Studies

The Drug: excipients compatibility study revealed no interaction as evident by the main bands of ACV corresponding to 3522 cm^{-1} (OH stretching), 3448 cm^{-1} (NH_2 stretching), 3186 cm^{-1} (CH aliphatic stretching), 1707 cm^{-1} (C = O stretching), and 1101 cm^{-1} (C-O stretching) depicted in Fig.1.



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	624.96	77.059	10.76	657.75	472.58	12.639	4.7
2	682.82	81.858	7.268	719.47	657.75	4.049	1.142
3	765.77	79.648	15.7	810.13	719.47	5.374	3.473
4	900.79	82.771	11.841	943.22	864.14	3.893	2.045
5	1101.39	59.304	35.385	1149.61	943.22	20.809	16.747
6	1182.4	85.341	10.323	1213.27	1149.61	4.583	1.694
7	1226.77	85.341	2.44	1267.27	1213.27	2.843	0.138
8	1301.99	85.636	3.605	1323.21	1267.27	3.04	0.408
9	1392.65	70.115	19.383	1446.66	1323.21	12.411	6.327
10	1481.38	72.669	17.82	1512.24	1446.66	5.85	3.011
11	1539.25	77.696	7.208	1560.46	1512.24	4.29	0.968
12	1629.9	48.012	22.221	1666.55	1560.46	22.125	7.172
13	1707.06	44.688	24.667	1971.32	1666.55	22.948	-5.834
14	2715.86	72.594	10.495	2812.31	2312.73	39.778	11.4
15	2864.39	76.131	3.144	2906.82	2812.31	10.318	0.815
16	2922.25	78.444	1.654	2985.91	2906.82	7.179	0.42
17	3186.51	57.222	13.204	3242.45	2985.91	37.824	6.209
18	3304.17	59.479	8.3	3402.54	3242.45	30.96	4.373
19	3448.84	61.575	11.381	3502.85	3402.54	17.304	3.666
20	3522.13	74.15	4.172	3718.88	3502.85	7.713	-4.286

Figure 1. FTIR spectrum showing drug: excipient compatibility studies.

3.2. Preparation Mechanism

The ACV microspheres were formed due to the ionotropic gelation of the monovalent sodium alginate, an anionic polymer by the divalent Ca^{++} ions emerging from calcium chloride. The gelling was further enhanced due to the formation of an interpolymer polyelectrolyte complex between the anionic alginate and cationic chitosan polymer. Thus the chitosan- alginate polyelectrolyte complex retards the diffusion of the drug [17].

3.3. Characterization of the ACV Micropsheres

3.3.1. Micromeritic Properties of the Microspheres

The particle size of the microspheres was found to be between 568.65 µm (SD ± 0.461) for P1 and 751.82 µm (SD ± 0.506) for P3. P2, P4, P5, P6, P7 and P8 had average particle sizes of 715.57 µm (SD ± 0.154), 636.12 µm (SD ± 0.297), 727.15 µm (SD ± 0.381), 747.19 µm (SD ± 0.308), 612.86 µm (SD ± 0.306) and 596.74 µm (SD ± 0.310) respectively. There was a statistically significant difference among the

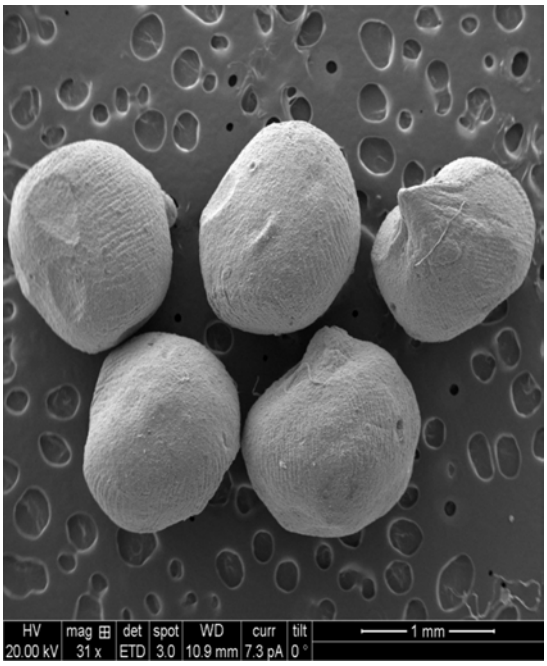


Figure 2. SEM of P8.

values (P < 0.0001). The shape was spherical with slightly elongated tips found in some microspheres. The SEM of P8 is shown in Fig. 2.

3.3.2. Drug Content & Encapsulation Efficiency

The drug content and encapsulation efficiency was 30.8477 mg and 80.2072% (SD ± 0.0015),for P1, 26.5948 and 82.4646% (SD ± 0.001) for P2, 30.3879 mg and 79.0118% (SD ± 0.001) for P3, 28.8649 mg and 80.8315% (SD ± 0.0035) for P4, 31.0057 mg and 80.6182% (SD ± 0.002) for P5, 26.1925 mg and 78.5854% (SD ± 0.002) for P6, 33.8075 mg and 74.3839% (SD ± 0.0015) for P7 and 33.7213 mg and 80.9246% (SD ± 0.002) for P8. There was a statistically significant difference among the values (P < 0.0001).

3.3.3. Ex Vivo Bioadhesion Studies

The *ex vivo* bioadhesion efficiency was highest for P8 (86.66667%, SD ± 0.578) which

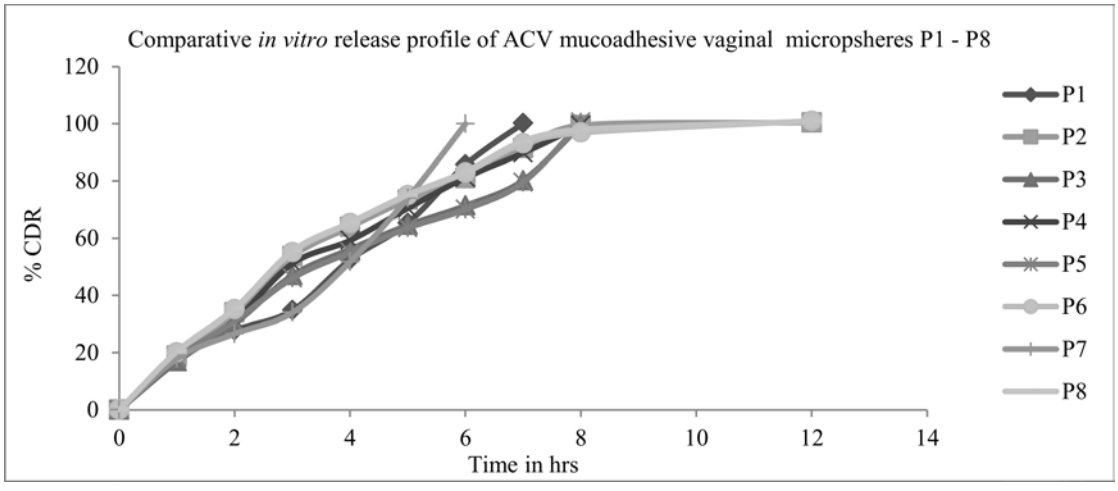


Figure 3. *In vitro* release profile of ACV mucoadhesive vaginal microspheres P1- P8.

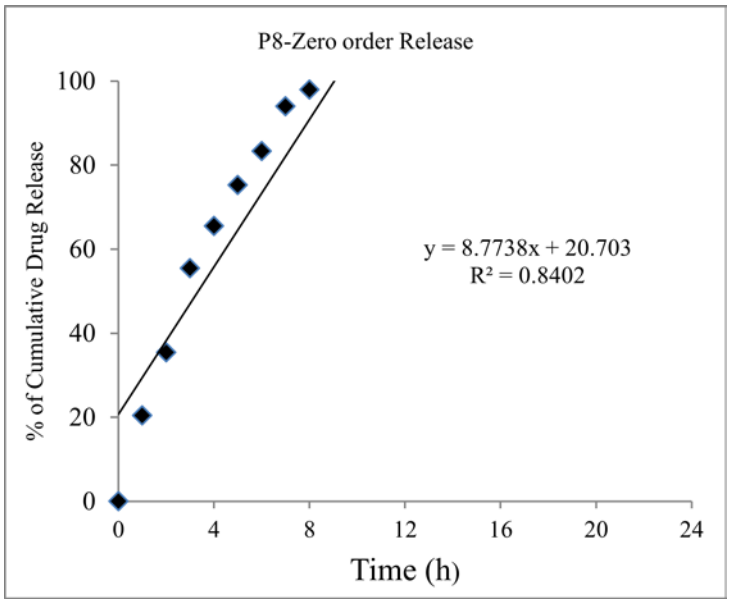


Figure 4. Zero-order model of P8.

had an equal proportion of sodium alginate and chitosan (2% w/v each) and lowest for P7 (70%, SD ± 0.576)) in which chitosan was half the concentration of sodium alginate (1% w/v chitosan and 2% sodium alginate). The remaining batches P1, P2, P3, P4, P5 and P6 had bioadhesion efficiencies of 73.33333% (SD ± 0.577), 80% (SD ± 0.577), 83.3333% (SD ± 0.578), 80% (SD ± 0.577), 76.66667% (SD ± 0.577) and 76.66667% (SD ± 0.5778) respectively.There was a statistically significant difference among the values ($P < 0.0001$).

3.3.4. In Vitro Release Studies

P7 had a % CDR of 99.93 at the end of the sixth hour, which was comparatively the fastest release rate among all the batches. This may be attributed to the low polymer content (2% and 1% w/v of sodium alginate and chitosan respectively) and low concentration of the crosslinking agent (2.5 % $CaCl_2$). P1 followed next with a % CDR of 100.14 at the end of the seventh hour. P3, P4 and P5 had % CDR values of 99.59, 99.41 and 100.51 respectively at the end of the eighth hour. P2, P6 and P8 had a % CDR of 100.41, 100.98 and 100.81 respectively which were the slowest release rates having release up to the twelfth hour. Such a slow release may be attributed due to the higher proportions of the polymers and the crosslinking agent. The graphical representation of the *in vitro* release study is presented in Fig. 3.

Based on results of the *ex vivo* bioadhesion study and *in vitro* release study, it was obvious that P8 had the maximum (86.66667%) bioadhesion and the desired sustained release up to 12 h. Hence, it was selected as the best formulation. P8 was subjected to release-

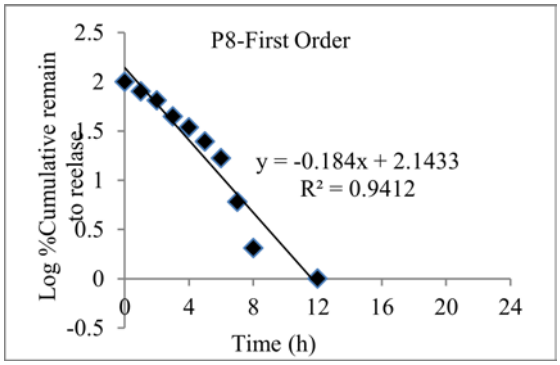


Figure 5. First-order model of P8.

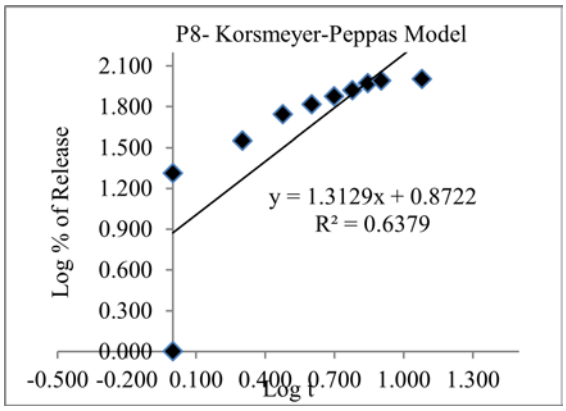


Figure 6. Korsmeyer- Peppas model of P8.

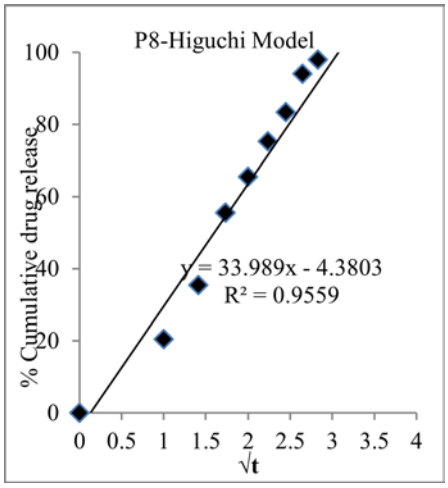
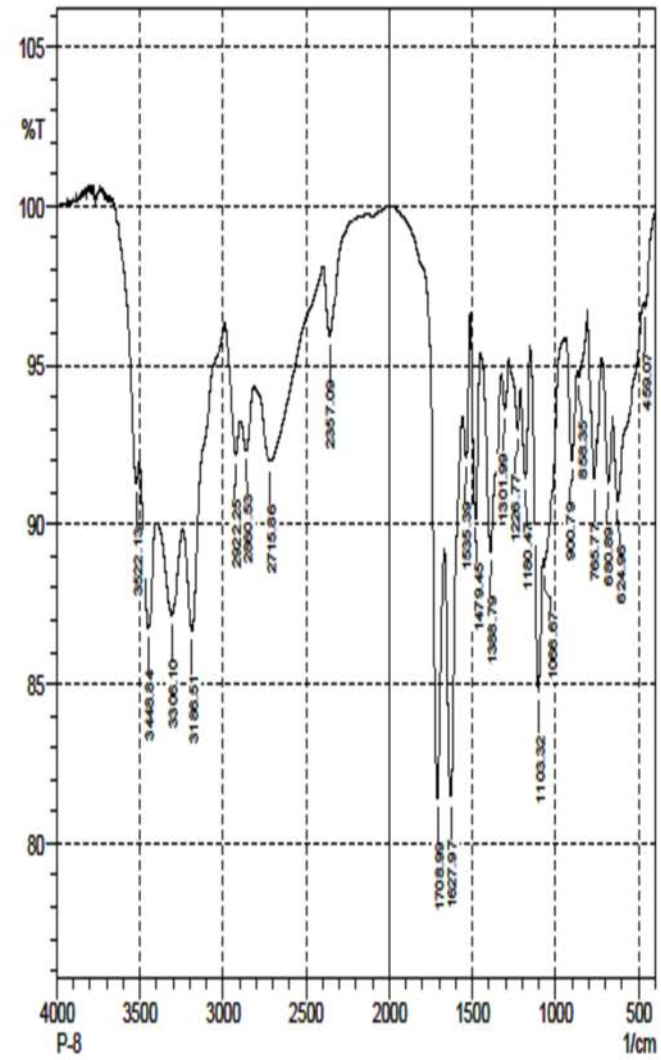


Figure 7. Higuchi model of P8.

kinetics study to understand its release mechanism. An MS excel based software was used to derive the various parameters. The R^2 values for P8 were 0.8402, 0.9410, 0.6370 and 0.9559 for zero- order, first- order, Korsmeyer-Peppas, and Higuchi models respectively. Based on R^2 values it was inferred that P8 followed Higuchi model. The graphs of the release-kinetics study are presented in Fig. 4-7.

3.3.5. Accelerated Stability Studies of P8 Mucoadhesive Vaginal Microspheres

The colour and shape of P8 microspheres remained white and spherical as before. The FTIR of P8 microspheres showed the major



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	459.07	96.801	0.325	462.93	399.28	0.403	0.03
2	624.96	90.745	3.205	653.89	472.58	5.283	1.375
3	680.89	91.344	2.813	709.83	655.82	1.829	0.424
4	748.41	91.441	2.166	808.2	752.26	1.678	0.321
5	858.35	94.65	0.332	864.14	810.13	1.134	0.111
6	900.79	92.034	3.303	931.65	866.07	1.886	0.512
7	1066.67	88.694	0.296	1074.39	1055.1	0.988	0.015
8	1103.32	84.803	6.468	1151.54	1076.32	3.807	1.12
9	1180.47	91.441	3.481	1209.41	1153.47	1.766	0.499
10	1226.77	92.997	1.421	1280.78	1211.34	1.833	0.184
11	1301.99	93.646	1.275	1321.28	1282.71	1.006	0.135
12	1388.79	89.173	3.645	1448.59	1359.86	3.266	0.653
13	1479.45	90.171	5.78	1512.24	1450.52	1.969	0.866
14	1535.39	92.082	2.817	1554.68	1514.17	1.201	0.306
15	1627.97	81.459	9.249	1666.55	1556.61	6.426	2.106
16	1708.99	81.402	10.704	1791.93	1668.48	5.775	2.248
17	2357.09	95.916	2.489	2397.6	2185.42	1.621	0.635
18	2715.86	91.986	3.185	2810.38	2399.53	9.85	2.919
19	2860.53	92.314	1.376	2895.25	2812.31	2.562	0.252
20	2922.25	92.205	1.893	2987.84	2897.18	2.507	0.381
21	3186.51	86.675	4.561	3240.52	2989.76	9.196	1.432
22	3306.1	87.151	2.764	3404.47	3242.45	8.472	1.008
23	3448.84	86.763	4.178	3500.92	3406.4	4.927	1.064
24	3522.13	91.325	1.793	3635.94	3502.85	2.648	0.274

Figure 8. FTIR spectrum of P8 after the accelerated stability study.

peaks of the drug acyclovir [(3522 cm^{-1} (OH stretching), 3448 cm^{-1} (NH_2 stretching), 3186

cm^{-1} (CH aliphatic stretching), 1707 cm^{-1} (C = O stretching), 1101 cm^{-1} (C-O stretching)], indicating stability of the formulation. The FTIR scan is presented in Fig. 8. The drug content of P8 was 80.9246% w/w before the accelerated stability study and 80.4074% w/w after the study which indicated a negligible change. The results of *ex vivo* bioadhesion study (pre-study: 86.66667% and post-study: 83.3333%) also indicated that the bioadhesion property of the P8 microspheres did not change appreciably.

4. Conclusion

The basic aim of this research was to develop mucoadhesive formulation for topical vaginal delivery for the prevention of venereal diseases. Mucoadhesive efficiency is the primary goal of this research along with acceptable residence time. In this regard, P8 was selected as the best formulation as it had the highest mucoadhesive efficiency of 86.66667% and appreciable *in vitro* release of 100.81% CDR at the end of 12 h. Further *in vivo* research is necessary to confirm the promising results of the *in vitro* study.

Acknowledgements

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