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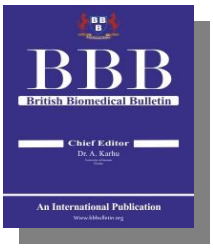

Original

Development *In Vitro* and Biological Evaluation of Chitosan Reinforced Eudragit® RL 100 Nanoparticles for the Improvement of Ocular Bioavailability of Acetazolamide

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ABSTRACT

Purpose of present study was to increase topical ocular bioavailability of Acetazolamide. Chitosan reinforced Eudragit® RL 100 nanoparticles were prepared by Nanoprecipitation Method with slight modifications. Particles Size and Zeta Potential were measured by Malvern Zetasizer, Surface Morphology was done by TEM, Drug Interaction was checked by FTIR spectroscopy, Entrapment Efficiency was determined by Centrifugation Technique, *In Vitro* Drug Release Study was done by using Franz Diffusion Cell, Intra Ocular Pressure lowering potential was measured in Normotensive Rabbits by using standardized Riester Tonometer. Short Term stability study was done. Particle Size measured was between 92.32±4.65 to 110±4.12 nm, all the formulations were cationic in nature and almost spherical in shape, no any interaction between drug to polymer was seen. Entrapment Efficiency was between 67.3±1.4 to 68.3±2.2 %. Drug Release was recorded for 8 hrs which means sustained delivery was achieved. The IOP lowering potential of plain drug solution in rabbit's eye was significantly lower than Eudragit Nanoparticles ($P<0.001$), which in turn was significantly lower than Chitosan reinforced Eudragit Nanoparticles ($P<0.001$). Formulations were stable for 6 months. Addition of Chitosan in Eudragit Nanoparticles increased the Intra Ocular Pressure lowering effect due to its penetration enhancing power.

Introduction

Eye is one of the sense organs of human body. Glaucoma is a disorder of eye and is characterized by optic nerve damage in a characteristic pattern, which may cause permanent blindness if left untreated. Increase in intra ocular pressure (IOP) > 21 mmHg is threatening [1, 2]. Reduction of Intra ocular pressure is the management of glaucoma[3]. Acetazolamide (ACZ) is used orally for the pre-operative management of closed angle glaucoma, or as an adjunct therapy in the treatment of open-angle glaucoma[4-7]. according to Biopharmaceutics Classification System, ACZ is class IV (low soluble and low permeable) so results in low ocular bioavailability[8-11]. Topical delivery of ACZ is not much successful because of its low solubility in tear fluid (1.9 mg/ml) and low permeability profile. Many researchers have tried many formulations to increase topical ocular bioavailability of ACZ like liposomes, niosomes, cyclodextrin complex etc[4, 10, 12-14]. Nanoparticle Suspension (NPs) unique size and other properties make it suitable for ocular delivery. Various polymers are being utilized for preparation of NPs[15, 16]. Eudragit® RL 100 is a copolymer of poly (ethylacrylate, methyl-methacrylate, and chloro trimethyl-ammonioethyl methacrylate) containing an amount of quaternary ammonium groups between 8.8% and 12%. It is insoluble at physiologic pH values and capable of limited swelling, thus representing a good material for the dispersion of drugs.

Various drugs have been studied to deliver at ocular site via Eudragit Nanoparticles (E-NPs) [17-28]. Chitosan (CH) is a cationic, natural polymer used numerously in pharmaceutical science. CH is also a mucoadhesive polymer but one unique feature which makes it superior then other polymer is its penetration enhancing effect[29, 30]. In present study Chitosan reinforced Eudragit nanoparticle suspension (CH-E-NPs) is to be prepared to enhance the potential of Eudragit polymer by combining with CH (penetration enhancer). The aim of our study is to achieve improvement in topical ocular bioavailability of ACZ and to release the drug for an extended period of time.

Materials and Methods

Acetazolamide (ACZ) was obtained as a gift sample from Intas Pharma Pvt Ltd (Dehradun, India), Eudragit was kindly provided as a gift sample by Evonik Röhm, GmbH (Darmstadt, Germany). Chitosan (CH) (99% pure), Poly vinyl alcohol (PVA), Acetone, Methanol and Acetic acid were purchased from Sigma Aldrich (India). Dialysis membrane cut off 12000 Da was purchased from Himedia Laboratories (India). Diffusion cell was purchased from Bombay Chemicals (Mumbai, India). Riester Tonometer No. 5113 schiotz C, eye tonometer spec. 3, with inclined scale was purchased from Rudolf Riester, GmbH (Jungingen, Germany).

Preparation of ACZ loaded CH-E-NPs

Nanoprecipitation Method was employed for preparation of NPs (Table I). Briefly, ACZ and Eudragit (1:10) were dissolved in 20 ml portion of organic phase consisting of acetone and methanol (3:1). This organic phase was poured drop wise into aqueous phase (containing 1% w/v of PVA with/ without various concentrations of CH dissolved in acetic acid) at room temperature (RT) with magnetic stirring (10,000 rpm) to form CH-E-NPs. Organic to aqueous phase ratio was 1:2. NPs were spontaneously formed and turned the solution slightly turbid. Finally, the organic solvents were evaporated under reduced pressure at 58°C. The resulting particle suspension was filtered through 1.2 µm cellulose nitrate membrane filter in order to remove larger particle aggregates[20, 25, 28].

Particle size analysis and zeta potential measurement

The Particle Size and Zeta Potential (ZP) of NPs were determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern Inst. UK; Nano ZS). The measurements were conducted in triplicate, in a multimodal mode of 200 s each at medium stable count rate[20].

Transmission electron microscopy

Transmission Electron Microscope (TEM) (Morgagni 2680 FEI, Holland) was used as a visualizing aid for NPs. Samples were dried on carbon-coated grid and negatively stained with aqueous solution of phosphotungstic acid. After drying, specimen was viewed under the microscope[25].

Fourier Transform Infrared spectroscopy (FTIR)

In order to check any possible Drug-Excipient Interaction, FTIR spectroscopy of formulation F5, ACZ, Eudragit, PVA and CH was done. Infrared Spectroscopy of the samples was performed in IR-Prestige 21 FTIR Spectrophotometer (Shimadzu, Zapan). Solid materials were analysed by preparing pellets. To prepare the pellets, a few milligrams of the samples were ground in a mortar with about 100 times the quantity of potassium bromide (KBr). The finely ground powder was introduced into a stainless steel die. The powder was then pressed in the die between polished stainless steel anvils (Pressure: about 9 t / in^2). The IR spectrum of the liquid formulation (NPs) was recorded in DLS Mode by direct introduction[31].

Drug entrapment efficiency

Entrapment Efficiency (EE) of NPs was determined by using cooling centrifuge [remi instrument Ltd. Mumbai India]. NPs suspension (10 ml) was taken and centrifuged at 10,000 rpm for 1 hr, after centrifugation supernatant was collected from tube by decantation method. The concentration of ACZ was determined spectrophotometrically at 292 nm using UV Visible double beam spectrophotometer 1800 (Shimadzu, Zapan).

$$EE= [Qt-Qs/Qt]*100$$

EE is the entrapment efficiency, Qt is amount of ACZ in suspension added, and Qs is amount detected only in the supernatant[21].

In vitro drug release study

ACZ release from NPs suspension was evaluated using diffusion cells, whereby a dialysis membrane with a molecular weight cut off of 12,000 Da, separated the acceptor from the donor compartment, consisting of 20 ml of formulation. The acceptor compartment was filled with 20 ml Simulated Tear Fluid (STF) and stirred magnetically at 200 rpm. Temperature was maintained at 37 ± 0.5ºC. At regular time intervals within 24 hours, samples (1 ml) were withdrawn from the acceptor compartment and replaced by the same volume of fresh STF solution. The concentration of ACZ was determined spectrophotometrically at 292 nm using UV Visible double beam spectrophotometer 1800 (Shimadzu, Zapan) [21].

In vivo studies

Adult male normotensive rabbits (3 - 4 kg) were used to measure IOP. The rabbits were provided balanced diet pellets and kept at 20ºC to 24ºC before the experiments. The experiments were done according to Animal ethical guidelines approved by Rungta College of Pharmaceutical Sciences and Research (Bhilai, India). The IOP was measured using a standardized Riester Tonometer (Jungingen, Germany). The rabbits were divided into 11 groups, each consisting of 3 rabbits: Group I received

plain drug solution and Group II to Group XI received formulations F1 to F10 respectively. Formulations were so diluted that all the rabbits received dose equivalent to 0.5 % ACZ. All the rabbits were given dose in right eye ad left was treated as a control. In this way the possible intersubject variability may be minimised. After administration of doses, IOP readings in both eyes of each rabbit were measured immediately before drug administration (zero reading), 30 minutes after instillation of the different drug formulations, and then every hour for a period of 8 hours. All the measurements were done in triplicate. Variables like time of start of experiment and investigator was kept constant[4, 13]. The ocular hypotensive activity is expressed as the difference in Intra ocular Pressure (ΔIOP) between the treated and control eye of the same rabbit, according to the following equation:

ΔIOP = IOP Control eye - IOP Treated eye

Statistical analysis

The statistical significance of differences in IOP lowering effect between drug solution and various formulations was tested by two way analysis of variance (ANOVA) followed by multiple comparison with Bone ferroni post test using GraphPad Prism 6 (GraphPad Software, Inc., California) software[25].

Short term stability study

To assess the physical stability of prepared NPs, formulations (F1, F2 and F5) were allowed to store at RT (25ºC) and freeze temperature (FT) (4ºC) for 6 months. Formulations were stored in glass vials. Samples (500 µl) were withdrawn after every 30 days interval and analysed for Particle Size, surface charge, surface morphology and EE as described earlier[20].

Results & Discussion

CH-E-NPs was prepared by Nanoprecipitation Method[24, 29, 32]. ACZ to Eudragit ratio and organic phase to aqueous phase ratio were kept constant. The amount of CH and the volume of CH solution in acetic acid were altered (Table I). The effects of these variables in physicochemical properties of NPs suspension were studied. Acetic acid was added to improve the solubility of CH. After evaporation of acetic acid, CH is available in water. Since the NPs prepared were intended to be delivered onto the ocular site so its size should not exceed 10 µm[32]. All the NPs prepared in this study were <110nm in size which is suitable for ophthalmic use. As the CH was added into the aqueous phase the slight difference in Particle Size was noted. The particles were slightly larger than formulations prepared without CH. Increase in Particle Size followed a linear pattern. As the CH solution (solution of CH in acetic acid) was increased, the Particle Size also increased (Table II). The PDI (poly dispersity index) was less than 0.7 for all the formulations so the particles were homogenous. Methanol and acetone were used as solvent system in preparation of E-NPs whereas for the preparation of CH-E-NPs, solution of CH in acetic acid was used. Larger particle size with the addition of CH solution (solution of CH in acetic acid) was seen. As the CH solution was increased, the Particle Size also increased. This may be due the lower dielectric constant of acetic acid. Dielectric constant of acetic acid is 6.2 which is much lower than methanol (32.7) and acetone (20.7). According to previous studies, larger particle sizes were seen with lower dielectric constant of solvents[33]. Other reason for larger Particle Size may be the concentration build up in aqueous phase due to addition of CH, which inhibits the diffusion of particles from organic phase to aqueous phase to form the NPs. So that larger particles were seen with the increase in CH amount.

These data are in accordance with earlier studies[34]. But still the Particle Size was less enough for effective ocular topical delivery (Table II). All the formulations showed positive surface charge (Table II). This is because of Eudragit polymer. It is cationic polymer having quaternary ammonium groups. Positive surface is essential for mucoadhesion of NPs onto the corneal surface of eye. Slight shift of surface charge towards more positive side was noted in formulations with the addition of CH and with the increase in amount of CH. This is because of cationic character of CH itself (Table II). To study the surface morphology, Transmission Electron Microscope (TEM) was used as a tool. The NPs were negatively stained and images were taken at various magnifications. The images revealed that all the NPs prepared were almost spherical in shape. None of any perforations or imperfections was seen onto the surface of NPs. It is good to have uniform surface properties of all the NPs because it may be helpful in topical application of drug onto the eye. Addition of CH did not impart any major changes in shape of NPs (Fig.1). This is due the reason that CH is available in aqueous phase, and it is itself cationic so it will not adhere to surface of positively charged E-NPs. So that it can be said that, it did not interfere with morphological characters of E-NPs. EE is drug loading capacity of any formulations. In present study centrifugation method was used to determine EE[21]. Good entrapment efficiency of ACZ was noted in Eudragit matrix. The addition of CH didn't impart any remarkable changes in EE (Table II). Hydrophobic drug like ACZ is a good candidate for hydrophobic polymers. So that in present study ACZ entrapment was good in Eudragit matrix. Any possible drug polymer interactions were checked by FTIR spectroscopy. ACZ showed peaks at 3587.60 cm^{-1} (-NH_2 str), 1157.29 (-C=N str), 1654.92 (-C=O str), 1382.96 (-CH_3 str), (-C-S str), 1037.70 (-O=S=O str), Eudragit showed peaks at 1654.92 (-C=O str), 1382.96 (-CH_3 str), 2133.27 cm^{-1} (4^0 ammonium compund), PVA showed peaks at 3639.68 cm^{-1} (-OH str), 3043.67 (-C-H str), 1125.43, (-C-C str) and CH showed peaks at 3206.76-3000 cm^{-1} (-OH str overlpimg –NH str), 1658.78 (-CH str of methylene), 1568.13(-C-O-C of –CH_2OH) , 1423.47 (-C-C str), 1373.32, 1070.49 (-Vas C-O-C and Vs C-O-C), 462.92 (-C-H small wegging). These all peaks were present in FTIR spectra of F5 which showed peaks at 3612.67 – 2976.16 cm^{-1} (-OH overlapping to the –NH of ACZ, PVA and CH), 2133.27 cm^{-1} (4^0 ammonium compund of E), 1718.58 cm^{-1} (-CH str of methylene group of CH), 1560.41 (-C-O-C of –CH_2OH of CH), 1421.54 cm^{-1} (-C-C str of CH), 1288.48 cm^{-1} (-CH_3 Str of ACZ, PVA and E), 844.82 cm^{-1} (-C-H small wegging of CH) (Fig.2). Presence of all important peaks in FTIR spectra of formulation F5 advocates no interaction of drug with excipients.

Diffusion cell was used to study the drug release profile of formulations. Cumulative % drug release was recorded up to 8 hrs. None of the formulations showed burst release pattern. Incorporation of CH slightly increased the total amount of drug which reached to the receptor compartment (Fig.3). All formulations were compared with formulation 1 (formulation without CH), which showed that the incorporation of CH increased the cumulative % drug release of ACZ. Reason behind this change is the penetration power of CH.

IOP of Rabbits eye was measured with the use of standardized Riester Tonometer before and after the administration of dose. Clear distinction was observed from the data obtained from various formulations. ΔIOP value of group I was lower significantly ($P<0.001$) as compared to group II to group XI at each time point except first reading which was not significant ($P>0.05$). ΔIOP value of group II was lower significantly

(P<0.001) as compared to group III – group XI up to 4 hrs of study, after that it was not significant (P>0.05). The ΔIOP value of group III to group XI was not significantly different with each other (Table III). Due to low ocular bioavailability of ACZ the IOP lowering effect was low and ends within 5 hours of administration {as seen in Group I (treated with plain drug solution)}. Whereas the group II was administered with F1 (ACZ loaded E-NPs) and the ΔIOP was measured. The magnitude of IOP lowering potential was higher than what seen in group I, as well as the duration of effect was also higher. This was due the cationic nature of Eudragit polymer which allows the NPs suspension to adhere to anionic ocular site and release the drug for longer time. Group III- Group XI was administered with ACZ loaded CH-E-NPs. The results were much better than what seen in previous two groups. The magnitude of IOP lowering effect and duration of action both were significantly higher as compared to plain drug solution and F1. The difference was due the addition of CH, which is capable of penetration enhancement across the cornea. So that CH allowed the low permeable drug to cross the cornea in a very simple manner which results in higher ocular bioavailability. As the ACZ loaded E-NPs crossed the cornea, drug release started from Eudragit matrix which was maintained for longer time. This complex phenomenon results in higher topical bioavailability of ACZ as compared to previous studies[10, 35]. The effect of amount of CH was also checked in present study. Formulations were prepared with three different amount of CH and three different volume of CH solution in acetic acid. The magnitude of IOP lowering effect was increased when CH amount was increased from 50 to 100 mg but not significant according to statistical analysis (P>0.05). But from 100 mg to 150 mg not any increase in magnitude was noted. Further the volume of CH solution was also altered. But no any remarkable difference was seen with change in volume of CH solution (P>0.05) (Table III). The formulations (F1, F2 and F5) were kept at RT and FT for 6 months and were evaluated for Particle Size, ZP and EE. According to Particle Size and EE data no instability characters were noted after 6 months storage at RT and FT. Only ZP values were less in all the formulations then their parent data (Table IV). This may be due the development of counter ions inside the formulations which neutralized the surface charge to some extent (Table IV).

Conclusion

ACZ loaded Eudragit NPs were prepared by nanoprecipitation method. Further CH-E-NPs was prepared by incorporating CH as penetration enhancer. The in vivo studies revealed that the CH increased the amount of ACZ which crossed the cornea.

Authors' contributions

PV participated in design of idea, in formulation and evaluation and drafting of manuscript, RNG participated in analytical work and AKJ participated in drafting and review of manuscript.

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Table 1. Formulation Code and Composition

Formulation Code	Chitosan Amount (mg)	Volume of chitosan solution
F1	-	-
F2	50	5
F3	50	10
F4	50	15
F5	100	5
F6	100	10
F7	100	15
F8	150	5
F9	150	10
F10	150	15

Table 2. Average Particle Size, Zeta Potential and Entrapment Efficiency of Formulations

F	d (nm) ±SD	PDI ±SD	ZP (mV)	EE (%)
F1	92.32±4.65	0.359± 0.18	+17.7±0.19	67.8±1.9
F2	95.25±5.34	0.765±0.16	+21.4±0.76	68.3±2.2
F3	97.23±4.11	0.453±0.23	+21.1±0.23	67.7±1.8
F4	98.34±3.41	0.444±0.12	+21.2±0.32	67.6±1.6
F5	103.1±2.23	0.461±0.43	+21.9±0.11	67.3±1.4
F6	104±4.11	0.323±0.45	+22.1±0.43	68.1±2.1
F7	106±3.43	0.313±0.32	+22.4±0.11	67.3±1.4
F8	108±3.33	0.441±0.12	+23.1±0.43	67.3±1.4
F9	108±4.56	0.321±0.34	+22.6±0.54	68.2±1.3
F10	110±4.12	0.375±0.43	+22.9±0.42	67.7±2.1

* Values are mean ± SD (n=3),

† d (nm): average particle size, PDI: Polydispersity index, ZP: zeta potential, EE: entrapment efficiency

Table 3. Data of *In Vivo* Studies

t (Hr)	ΔIOP±SD After Topical Administration (mmHg)				
	DS	F1	F2	F3	F4
0.5	2.23±0.12	2.29±0.11	2.31±0.13	2.30±0.13	2.32±0.13
1	2.43±0.11	3.11±0.11	3.65±0.11	3.66±0.12	3.63±0.11
2	2.98±0.09	3.87±0.16	4.12±0.11	4.11±0.11	4.14±0.12
3	2.56±0.14	4.32±0.11	4.91±0.14	4.93±0.11	4.92±0.16
4	0.43±0.12	4.33±0.14	4.99±0.13	4.99±0.13	4.99±0.13
5	0.42±0.13	4.37±0.15	4.03±0.11	4.11±0.13	4.07±0.12
6	0.42±0.13	3.03±0.14	3.32±0.11	3.37±0.17	3.33±0.11
8	-	2.10±0.13	2.11±0.13	2.10±0.11	2.12±0.16
10	-	2.08±0.08	2.10±0.11	2.07±0.12	2.10±0.14

*Values are mean ± SD (n=3)

† ΔIOP: difference in intra ocular pressure, DS: drug solution

Table 3. (Continued). Data of *In Vivo* Studies.

t (Hr)	ΔIOP±SD After Topical Administration (mmHg)					
	F5	F6	F7	F8	F9	F10
0.5	2.39±0.09	2.41±0.11	2.37±0.13	2.43±0.13	2.44±0.13	2.42±0.11
1	3.91±0.16	3.87±0.16	3.86±0.12	3.93±0.11	3.93±0.14	3.93±0.11
2	4.41±0.12	4.43±0.16	4.44±0.17	4.47±0.16	4.46±0.14	4.47±0.08
3	5.33±0.13	5.38±0.13	5.36±0.16	5.41±0.15	5.41±0.16	5.41±0.98
4	5.30±0.21	5.31±0.14	5.30±0.14	5.37±0.14	5.39±0.13	5.36±0.12
5	4.41±0.11	4.41±0.13	4.41±0.13	4.61±0.17	4.63±0.11	4.61±0.13
6	3.24±0.13	3.22±0.12	3.25±0.12	3.27±0.14	3.29±0.15	3.28±0.11
8	2.22±0.11	2.21±0.13	2.21±0.11	2.33±0.14	2.32±0.11	2.34±0.13
10	2.18±0.12	2.17±0.11	2.19±0.13	2.30±0.16	2.30±0.14	2.31±0.15

*Values are mean ± SD (n=3)

† ΔIOP: difference in intra ocular pressure, DS: drug solution

Table 4. Stability Study Data of Formulations

Formulations Parameters	t (Months)	F1	F2	F5
Average Particle size (nm)	0	92.32±4.65	95.25±5.34	103.11±2.23
	6 (RT)	93.12±3.11	94.21±3.33	101.21±4.10
	6 (RT)	93.22 ±3.32	95.01±4.11	104.1±3.98
Poly Dispersity Index	0	0.359±0.18	0.765±0.16	0.461±0.43
	6 (RT)	0.332±0.15	0.711±0.15	0.501±0.12
	6 (RT)	0.331±0.13	0.691±0.13	0.498±0.16
Zeta Potential (Mv)	0	+17.7±0.19	+21.4±0.76	+21.9±0.11
	6 (RT)	+17.1±0.14	+20.2±0.13	+20.1±0.16
	6 (RT)	+17.3±1.3	+20.5±0.9	+20.1±1.1
Entrapment Efficiency (%)	0	67.8±1.9	67.3±1.4	67.3±1.4
	6 (RT)	66.3±1.4	67.2±1.6	67.2±1.6
	6 (RT)	67.2±1.3	66.3±1.7	66.3±1.4

*Values are mean ± SD (n=3)

† RT: room temperature, FT: freeze temperature

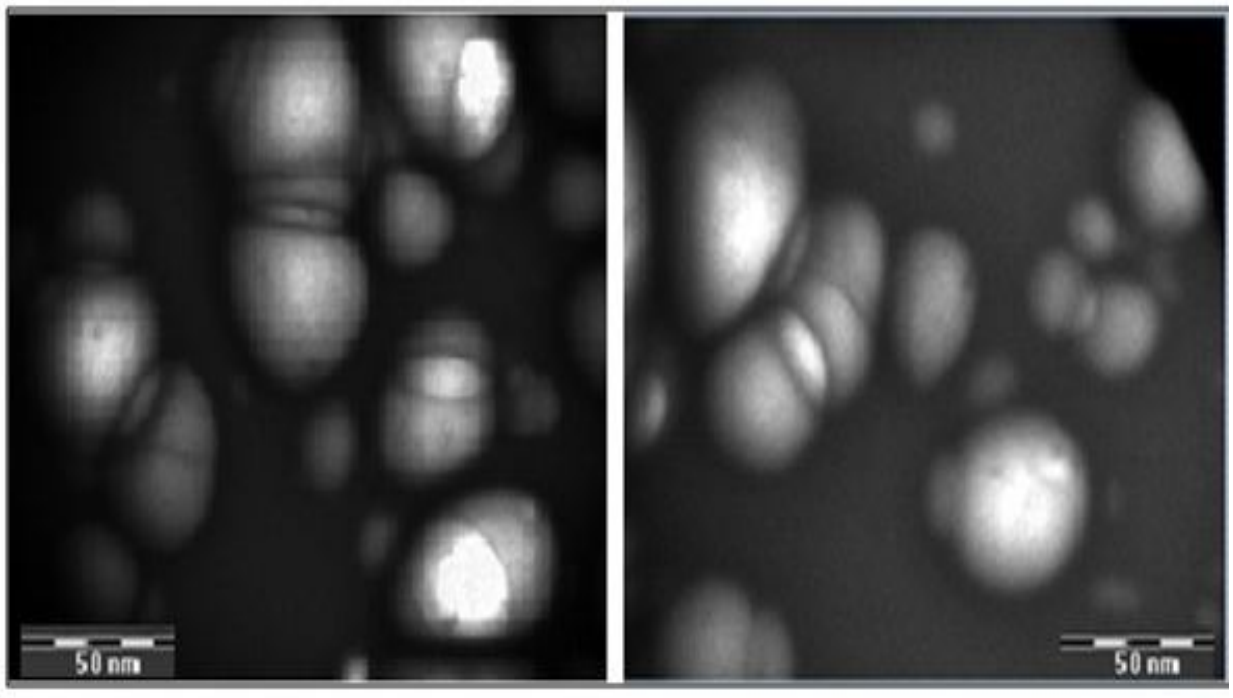

Figure 1. TEM Images of Nanoparticles, without Chitosan (left) and with Chitosan (right).

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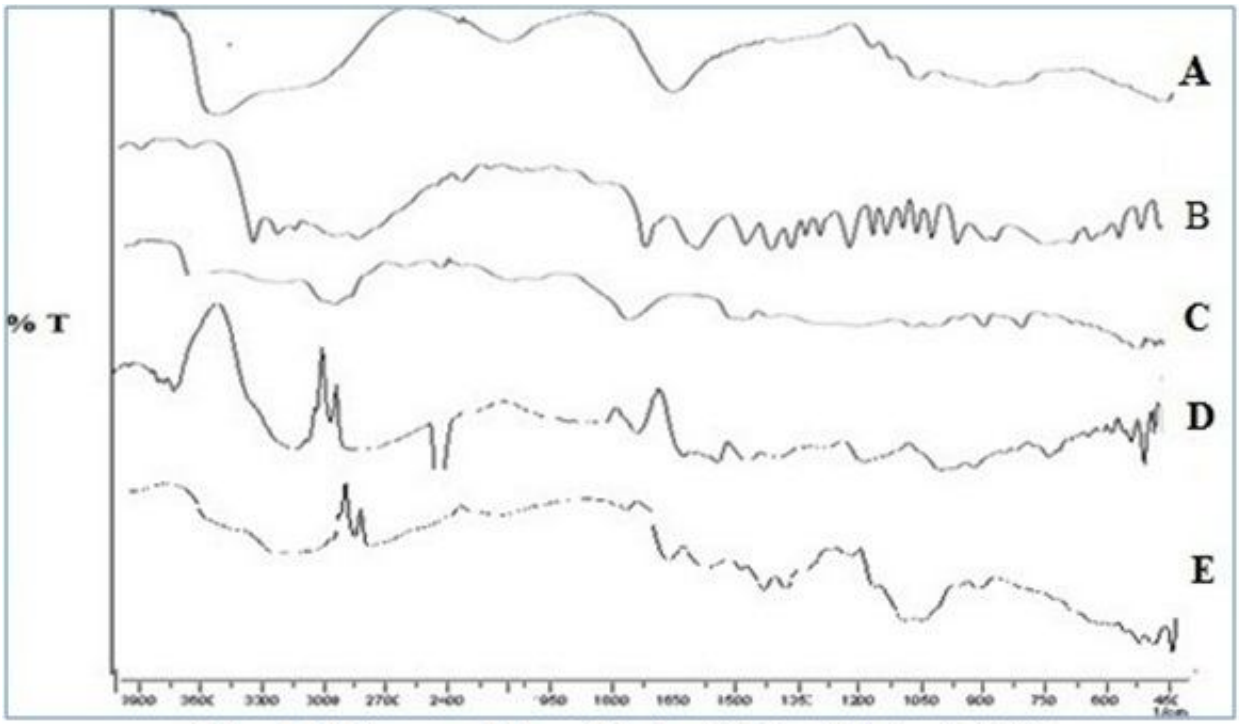

Figure 2. FTIR spectra of Chitosan Reinforced ACZ loaded Eudragit NPs (A), Acetazolamide (B), Eudragit RL 100 (C), Poly Vinyl Alcohol (D) and Chitosan (E).

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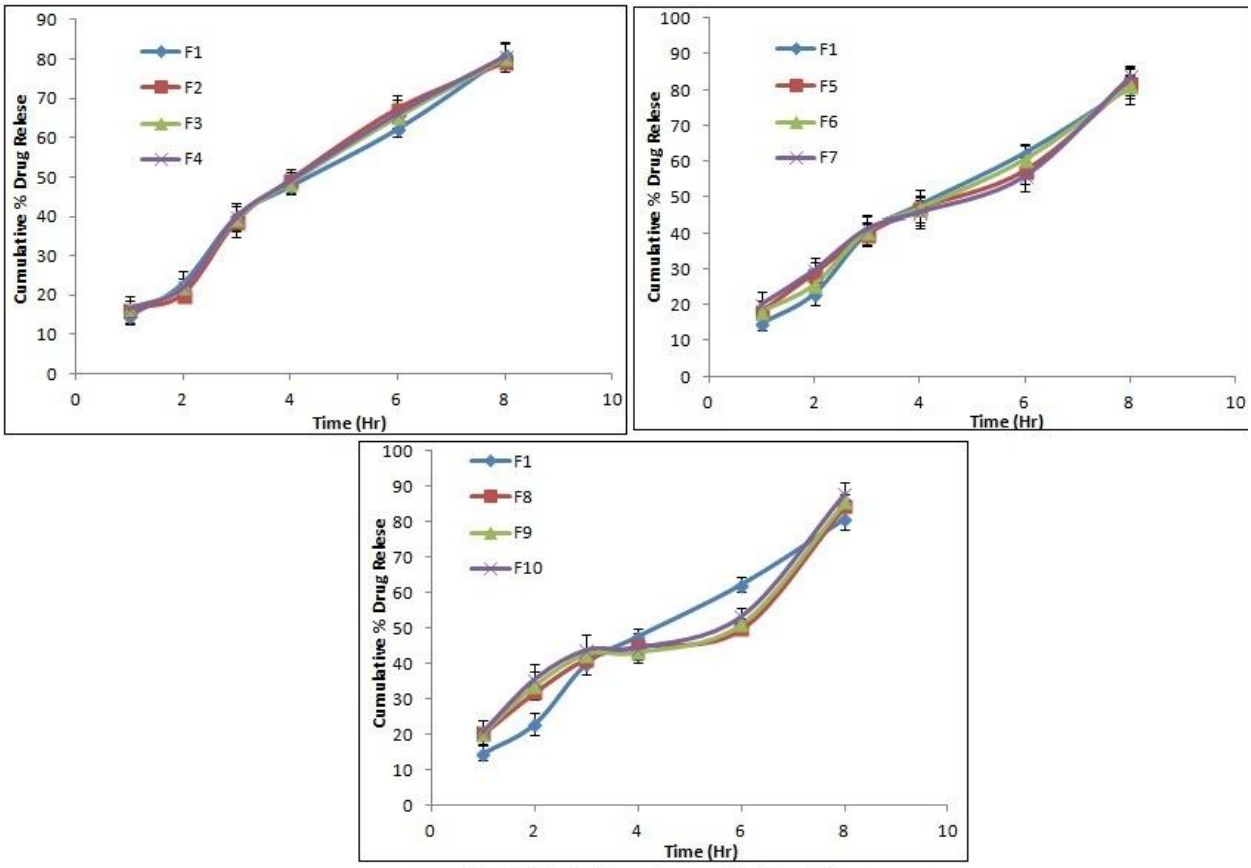

Figure 3. ***In vitro*** **drug release profile of formulations**

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