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ORIGINAL STUDY

FABRICATION OF ACETAZOLAMIDE LOADED NASAL NANOSUSPENSION: AN IN VITRO AND EX VIVO CHARACTERIZATION

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ABSTRACT

The aim of the present study was to prepare chitosan nanosuspension of acetazolamide (AZM), to improve dissolution of poorly soluble AZM and thus to enhance bioavailability of the drug. The chitosan nanosuspensions were prepared by ionic gelation method by two techniques: by magnetic stirring technique and by magnetic stirring with sonication . The nanosuspensions prepared by these two techniques were evaluated for physical characterstics like visual appearance, particle size and shape. Chitosan nanosuspensions were optimized for chitosan concentration, surfactant concentration, sonication time and the optimized results were used for formulation of AZM loaded chitosan nanosuspension using ionic gelation method by magnetic stirring with sonication technique because this technique showed better results with respect to particle size (<1μm), shape (spherical) and settlement of particles after 24 hrs as compared to magnetic stirring technique. The prepared nanosuspensions were evaluated for characteristics like Shape and Surface Morphology, particle size, percentage yield, Drug Loading and Drug Entrapment Efficiency, in vitro drug release and nasal ciliotoxicity study. These prepared nanoparticles were fairly spherical in shape. The surface of the particles showed a characteristic smoothness with average particle size 153, 175.2, 203.1 and 277 of NSA4, NSA9, NSA14 and NSA19 respectively. Drug loading of the optimized batches was 66.60%, 86.92%, 88.36%, 89.49% respectively for NSA4, NSA9, NSA14, and NSA19 with drug entrapment efficiency of 68.20%, 62.02%, 51.52% and 48.37% respectively. Stability study of NSA4 showed no significant change in the physical appearance at 5 ± 10C and room temperature. The elimination rate constant (K) & Shelf life (T0.9) for NSA4 stored at 5 ± 10C and at room temperature were 1x 10-4 and 3 x 10-4 and 1040 and 346.66 days respectively. Microscopic investigation revealed that no marked damage on the goat nasal mucosa after nasal application of NSA4 for 6 hr. Results from 4 hrs In vitro release study revealed that all the optimized formulation showed 48.41 to 64% higher drug release than that of plain drug acetazolamide. Based on the results, NSA4 were stable and can target the drug to brain through intranasal route and thus can play as an alternative for conventional dosage form.

KEYWORDS*: nano suspension, ionic gelation, acetazolamide, nasal delivery.*

1.Introduction

Solubility is an important property for the drug formulation and their effectiveness. One of the major problem with the new molecular entities as drug formulation is their poor solubility. It is estimated that about 40% of active substances identified from High-throughoutput screening programs are poorly soluble in water. This bleak outlook has helped to drive the innovation of many novel techniques to administer poorly soluble compounds at safe and effective therapeutic drug levels. One recent, exciting area for improved drug solubility is the creation and

formulation of pharmaceutical nanosuspensions, where a two-phase suspension of nano-scale particles, or containing Active Pharmaceutical Ingredient (API) are suspended within a continuous liquid media. These pharmaceutical nanosuspensions have been shown to achieve a faster solubility rate, and as a consequence, a higher *in vivo* bioavailability for many poorly soluble drugs. Moreover, pharmaceutical nanosuspensions exhibit many other unique advantages for drug delivery including: passive targeting, ease of suspension, variable optical properties, and the ability to be functionalized [1-4]. For example, the small size of pharmaceutical nanoparticles allows for deep tissue penetration and the ability to travel to virtually any area of the body. However, the most notable advantage to pharmaceutical nanoparticles is the increased rate of solubility, and subsequent bioavailability exhibited both *in vitro* and *in vivo*[5-7]. The key goal for pharmaceutical nanotechnology is to increase the bioavailability of the drug, while simultaneously minimizing any potential side effects [8]. This can also result in a decreased dosage for drugs that are particularly potent, such as chemotherapy, where a great quantity of people suffer more from the side effects of the drug rather than for the cancer itself, and many poorly soluble drugs are dosed purposefully higher to compensate for poor solubility[9].

Many approches are used to solve the problems of poor solubility and poor bioavailability of drugs. The conventional approaches include [10,11] Micronization, Use of fatty solutions, Use of penetration enhancer or cosolvents, Surfactant dispersion method, Salt formation, Precipitation, Liposome, Dispersion of solids, Emulsion and microemulsion methods, Inclusion complexes with cyclodextrins. These techniques shows beneficial effect as drug delivery system but major problems of these techniques are lack of universal applicability to all drugs. Among the most promising solutions to this challenge are nanosuspensions. Nanosuspension technology can be used to improve the bioavailability of poorly soluble drugs and also provide stability to the drug.

Nanosuspensions (NS) are defined as biphasic systems consisting of submicron-sized crystalline drug particles dispersed in an aqueous vehicle in which the particles are stabilized by coatings of surfactant (surface-active agent which reduces surface tension) to produce stable pharmaceutical formulations.

AZM being very slightly soluble was investigated as the model drug for this study. Acetazolamide (AZM) is an anticonvulsant and mood stabilizing drug effective against absence seizures. It is sometimes useful also as an adjunct in the treatment of tonic-clonic, myoclonic, and atonic seizures, particularly in women whose seizures occur or are exacerbated at specific times in the menstrual cycle [12]. Its antiepileptic effect may be due to its inhibitory effect on brain carbonic anhydrase, which leads to an increased transneuronal chloride gradient, increased chloride current, and increased inhibition [13]. AZM may be the drug of choice when drug interaction is a problem, when rapid onset of effect is wanted, or when an additional drug is needed for a short period of time only. It is also used for adjunctive treatment of edema due to congestive heart failure; drug-induced edema; chronic simple (open-angle) glaucoma.

Because of having poor water solubility, its absorption is dissolution rate limited, which often results in irregular and delayed absorption. Reports in the literature reveal that AZM has got low oral bioavailability 25%. AZM has got very high plasma protein binding (98%) with the half-life is 3-9 hrs. AZM crosses the blood-brain barrier (BBB) [14,15]. Presently, AZM is available on the market in conventional tablet forms which can't increase the

oral bioavailability and have multiple of therapeutic effects. Therefore, an alternative route of drug delivery and dosage form that can selectively target the drug directly into various regions of the brain, including vasculature is needed for the treatment of epilepsy.

In the present study, an attempt was made to improve the dissolution of AZM using ionic gelation method.

2.Materials and Methods

AZM and chitosan was procured from Sigma Life Science, India. Sodium Tripolyphosphate and polyethylene glycol 400 (PEG 400) were obtained from Qualigens Fine Chemicals, India. Tween 80 and acetic acid were obtained from Central Drug House (P) LTD, India. All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Ionotropic gelation method was used for the preparation of acetazolamide loaded chitosan nanosuspension. Two types of techniques were used to prepare chitosan nanosuspension: (1) By magnetic stirring (2) By magnetic stirring with sonication. In magnetic stirring technique different concentration of chitosan (CS), ranging from 0.05 to 0.40% w/v was dissolved in 1.5% v/v acetic acid solution. Sodium TPP solution was also prepared in distilled water in concentration to reach final theoretical CS/TPP ratio of 3.5:1. The 0.5 ml of sodium TPP aqueous solution was added dropwise with a syringe into 10 ml of CS solution containing tween 80 (stabilizer) under mild magnetic stirring at room temperature. The CS/TPP nanoparticulate suspension was spontaneously formed.

The magnetic stirring with sonication technique was similar as former. In this technique we used probe sonication with magnetic stirring for further size reduction. In this procedure CS solution was kept on magnetic. The probe of the probe sonicator was applied into the solution and tween 80 was added. After 5 minutes of sonication, the TPP solution was added drop wise by a syringe into the CS-drug solution. The CS/TPP nanoparticulate suspension was spontaneously formed.

Optimization Of Formulation

Various process variables were used for the optimization of placebo formulation are optimization of chitosan concentration, Optimization of surfactant concentration, Optimization of stirring time, Optimization of sonication time.

For optimization of chitosan concentration different concentration (0.10%, 0.15%, 0.20%, and 0.25%) of chitosan was dissolved in 1.5%v/v acetic acid solution. To this add TPP solution to achieve final CS/TPP ratio of 3.5:1 to 6:1 in the formulation. The concentration of chitosan was optimized on the basis of visual appearance and particle size.

For optimization of surfactant concentration the chitosan was dissolved in 1.5%v/v acetic acid solution on a magnetic stirrer and to this solution different concentration of tween-80 in water was added. The concentration of surfactant was optimized regarding the particle size and aggregation after 24 hrs.

For optimization of stirring time, the stirring speed was kept constant at 2000 rpm, and the time of stirring was optimized. Six time points are used for the optimization (10-60 minutes), at a constant surfactant concentration of 2% and a sonication time of 60 min.

Preparation of drug loaded chitosan nanosuspension by magnetic stirring with sonication

AZM loaded nanosuspension were prepared by ionic gelation of chitosan (CS) solution with tripolyphosphate (TPP) anions. 0.20% w/v concentration of polymer (CS) was dissolved in 10 ml of 1.5% v/v acetic acid solution. Sodium TPP solution was also prepared in distilled water in

concentration ranging to reach final theoretical CS/TPP ratio of 3.5:1 to 6:1. CS solution was kept on magnetic stirrer and probe of the probe sonicator was applied into the solution. The 250 mg of AZM was dissolved in Polyethylene glycol 400 (PEG 400). This drug solution and tween 80 was added to chitosan solution. After few minutes of sonication, the TPP solution was added drop wise by a syringe into the CS-drug solution. The AZM loaded CS/TPP nanoparticulate suspension was spontaneously formed.

Characterization Of Css/Tpp Nanosuspension

Shape and Surface Morphology:

The morphological examination of nanoparticles was conducted by transmission electron microscopy (TEM). Samples were prepared from dilution in distilled water followed by sonication and dropped on to square of paper. After air drying, particles were coated with a negative staining material phosphor-tungstic acid (PT) (to make the sample conductive) and covered with a copper grid. After few minutes the grid was injected into the T.E.M. by grid injector and then examined by Transmission electron microscopy (Grenha et al, 2005).

Particle Size and Size Distribution:

1 ml of all optimized nanosuspension was diluted to 10 ml with distilled water and average particle size and polydispersity index were measured by Malvern zeta sizer.

FTIR spectroscopy of optimized formulation

FTIR spectroscopy of formulation was conducted to confirm the entrapment of drug in the nanoparticles and also for any interaction of drug with excipients of formulation.

Determination of nanoparticles production yield

The NP production yield was calculated by gravimetry. Fixed volumes of NP suspensions were centrifuged (16,000×*g*, 30 min, 15^0C) and sediments were lyophilized (24h at -34^0C and gradual ascent until 20^0C), using a Freeze Dryer (Optics Technology, India) (*n* = 3) (Wu et al, 2005). The process yield was calculated by equation 1.

$$\text{Process yield} = \frac{\text{Nanoparticle weight} \times 100}{\text{Total initial solids weight}} \quad (1)$$

Drug Loading and Drug Entrapment Efficiency:

A fixed quantity of AZM nanosuspension (10 ml) was taken with a pipette (10 ml, Borosil), and transferred into a centrifuge tube and centrifuged at 14000 rpm for 10 min at 20^0C (Remi, Scientific, India), the nanoparticles were isolated, and the absorbance of the drug in the supernatant was determined spectroscopically using UV-VIS Spectrophotometer (Shimadzu) at 266 nm. The concentration of drug was calculated from the calibration curve (Wu et al, 2005). The drug loading and entrapment of optimized nanosuspensions were calculated by the equation 2 and 3 respectively.

$$\%\ \text{Drug loading} = \frac{\text{Total AZM amount - Free AZM amount X 100}}{\text{Nanoparticle weight}} \quad (2)$$

$$\%\ \text{Drug Entrapment Efficiency} = \frac{\text{Total AZM amount - Free AZM amount x 100}}{\text{Total AZM amount}} \quad (3)$$

An in-vitro Drug Release Study in PBS (pH 6.4)

In vitro drug release study of AZM nanosuspension for a period of 4 hrs was carried out using self prepared assembly (shown in figure 4.3). To study the release behavior of formulation, nanosuspension was transferred into the open ended test tube tied at one end with 450 nm nanopore membrane filter (Cellulose nitrate, Rankem, Delhi). The test tube was dipped from membrane side in a beaker containing 200 mL phosphate buffer 6.4 (i.e.

pH of nasal mucosa). The temperature and stirring rate were maintained at 37 ± 2^0C and approx. 200 rpm, respectively. Samples (5 ml) were withdrawn periodically and replaced with an equal amount of phosphate buffer 6.4 to maintain the sink condition. After suitable dilution, samples were filtered through Whatman filter paper and then analyzed spectrophotometrically at 266 nm wavelength using double beam UV/Visible spectrophotometer (Shimadzu 1800). All measurements were performed in triplicate (Jain et al, 2011).

Drug Release kinetics

The drug release kinetics were studied by various kinetic models such as Higuchi plot, first order plot and zero order plot **(**Dash et al, 2010). The best fit model was confirmed by the value of correlation coefficient near to 1. The data was presented for the most appropriate model.

Statistical analysis

The means of *in vitro* release data of AZM from nanosuspension of formulations NSA4, NSA9, NSA14 and NSA19 were statistically analyzed by one-way analysis of variance (ANOVA) with post test (Newman-Keuls Multiple Comparison Test). Statistically significant differences between *in vitro* drug release of formulations were defined as $P<0.05$. Paired t test was also performed to analyze the effective formulation. Calculations were performed with Graph Pad InStat 3 software program.

Nasal ciliotoxicity studies

The nasal mucosa of goat was treated with formulation to evaluate the toxic effects of excipients used in the formulation. For nasal ciliotoxicity studies freshly excised goat nasal mucosa except for the septum were collected from the slaughter house in saline and treated with 0.5 ml of formulation for 6 hrs. The treated nasal mucosa was then fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Sections were cut on glass slides and stained with hematoxylin and eosin. Sections were examined under a light microscope to detect damage to the tissue.

Stability study

Five Batches of the optimized nanosuspension formulations were stored in screw capped amber color glass bottles at 5 ± 1^0C (Refrigerator), room temperature and 40 ± 1^0C and 75% Relative Humidity (RH) in the stability chamber. Samples were analyzed for drug content vs. time and log % drug content vs. time graph was plotted in order to evaluate shelf-life of the formulation.

Result and Discussion

In ionic gelation method with magnetic stirring technique, the particles were found to be bigger in micron range, the shape was irregular, and finally aggregation took place. The aggregation probably occurred due to lack of electrostatic stabilization. In magnetic stirring technique with sonication technique the particles were found to be smaller than micron range, the shape was spherical, and no aggregation took place.

Therefore**,** ionic gelation method by technique magnetic stirring with sonication was selected as the method of choice in the formulation of nanosuspension as it showed better results with respect to colour (transparent bluish colour), particle size (<1μm), shape (spherical) and settlement of particles after 24 hrs.) as compared to magnetic stirring technique.

From the various optimization process, the various optimization parameters selected were given in table I. Formula for optimized nanosuspension is given in table II.

Shape and surface morphology of prepared nanosuspension were evaluated by TEM. The study revealed that most of the nanoparticles were fairly spherical in shape. The surface of the particles showed a characteristic smoothness (figure 1). The

particle size and size distribution study by Malvern zeta sizer is given in figures 2-5. The particle size and polydispersity index was found to be given in table III. FTIR spectroscopy of AZM, chitosan and formulation is shown in figure 6, figure 7 and figure 8 respectively. From these FTIR studies it was clear that the formulation showed no interaction with excipients and drug was entrapped in the polymer. Nanoparticles Production yield of the optimized batches were found to be shown in Table IV. The loading capacity and drug entrapment efficiency of optimized nanosuspensions were found to be given in table V.

Table I. *Optimized parameter*

Parameter	Value
Chitosan concentration	0.20% w/v
Surfactant concentration	1 ml of 2%v/v
Stirring speed	2000
Stirring time	60 minutes
Sonication time	60 minutes

Table II. *Formula for optimized nanosuspension*

Conc. Of CS in 1.5% acetic acid (%w/v)	**0.20**			
Formula code	**NSA4**	**NSA9**	**NSA14**	**NSA19**
Ratio of CS:TPP	3.5:1	4:1	5:1	6:1
Conc. of Tween 80 (%) 1 ml	2	2	2	2
Stirring speed(rpm)	2000	2000	2000	2000
Stirring time (min)	60	60	60	60
Sonication time (min)	60	60	60	60

The in vitro drug release studies were carried with optimized formulation for their in vitro release pattern across cellophane membrane. The in vitro drug release profiles of optimized nanosuspension (NSA4, NSA9, NSA14, and NSA19) are shown in figure 9. From the figure 9, it is shown that all the batches showed an initial burst release due to the free drug, surface adsorbed drug on the nanoparticles or due to those drug molecules dispersing close to the nanoparticle surface, which was followed by controlled release varying from 62.81% (NSA19) to 78.40% (NSA4), as the drug slowly diffused through the nanoparticle core.

Table III . *Particle size and size distribution by zeta sizer*

Formula code	Average particle size	Polydispersity index
NSA4	153.3	0.350
NSA9	175.2	0.306
NSA14	203.1	0.311
NSA19	277	0.900

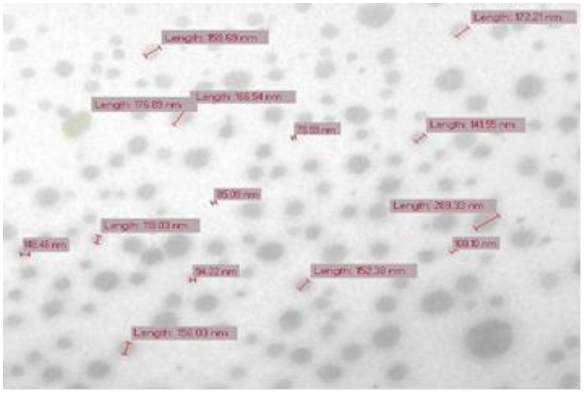

Figure 1. *TEM photograph of optimized nanosuspension*

One comparison of the release profile of four formulation it was observed that release from formulation NSA19 was found to be slow and constant manner. From the observed data it is shown that increase the concentration of chitosan decreases the cumulative percentage release. The cumulative percentage drug release was found in the order NSA4>NSA9>NSA14>NSA19. All the formulation showed increased in cumulative percentage drug release in comparison to pure AZM varying from 48.41 to 64% higher than pure AZM.

In order to investigate the release mechanism, the release data were fitted to zero order, first order and Higuchi model. The examination of coefficient of determination values (Table VI) indicated that drug release from the NSA4 and NSA9 formulation followed first order of release followed by diffusion control mechanism (Highuchi model) and formulations NSA14 and NSA19 follow higuchi model then followed by first order kinetics. Dissolution of the drug from an inert matrix can take place by two different processes: diffusion of the drug through the matrix into the solution or penetration of the solvent into the matrix and subsequent dissolution of drug into the penetrated solvent. In the formulation NSA4 and NSA9 the solvent penetrate into the matrix and dissolve the drug into penetrated solvent and then diffusion of drug solution occurs due to the thin nanoparticle core shell. Therefore these formulations follow first order kinetics after than diffusion mechanism. In the formulation NSA14 and NSA19 due to the increase in concentration of chitosan thickness of nanoparticle core shell increases which results in difficulty in the penetration of solvent thus the drug slowly diffuse through the nanoparticle core thus follow higuchi model of diffusion and when the drug reaches to the particle membrane it follow first order kinetics. Thus the formulation NSA14 and NSA19 firstly follow higuchi model and then first order.

The in vitro release data of formulations NSA4, NSA9, NSA14, and NSA19 compared with Pure drug by One way Anova and paired t test. All the formulation was found to be extremely significant ($P < 0.0001$) with the pure drug (table VII-X). When goat nasal mucosa was treated with the formulation NSA4, there was not found any damage to the nasal mucosa.

The elimination rate constant (K) & Shelf life ($T_{0.9}$) values for NSA4 stored at 5 ± 1^0C and room temperature were 1 x 10^{-4} and 1040 and 3 x 10^{-4} and 346.66 days respectively (Table XI). The $T_{0.9}$ obtained in case of formulation stored at 5 ± 1^0C was found to be higher as compared with formulation stored at room temperature. So it can be concluded that the formulation NSA4 was more stable at 5 ± 1^0C and tends to degrade faster at higher temperature. In the case of nanosuspensions stored at room temperature, the particle size increased from 153 to 288 nm in 45 days. However, under refrigerated storage conditions, there was a nominal increase from 153 to 249 nm indicating better stability under refrigerated conditions.

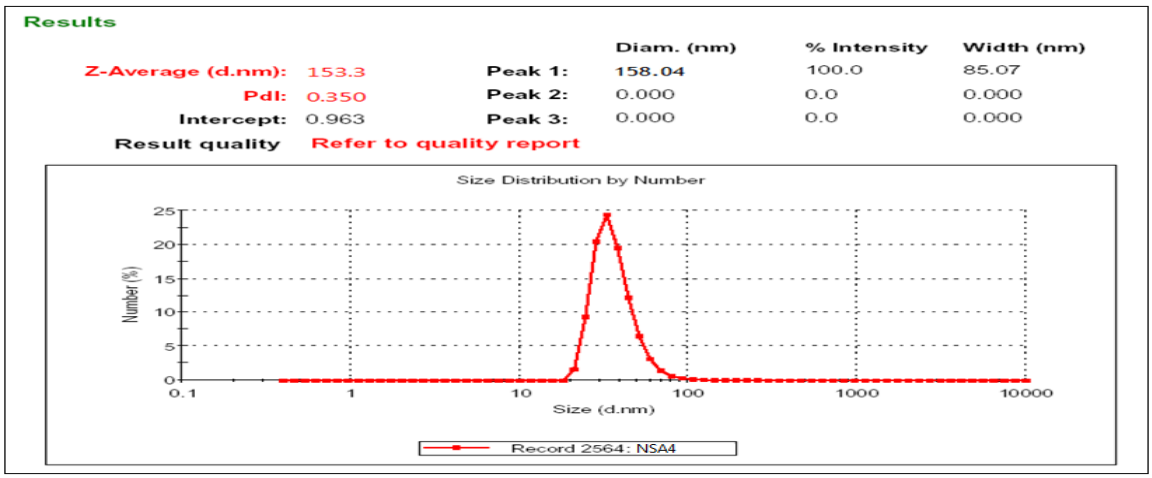

Figure 2. *Particle Size and Size Distribution of NSA4*

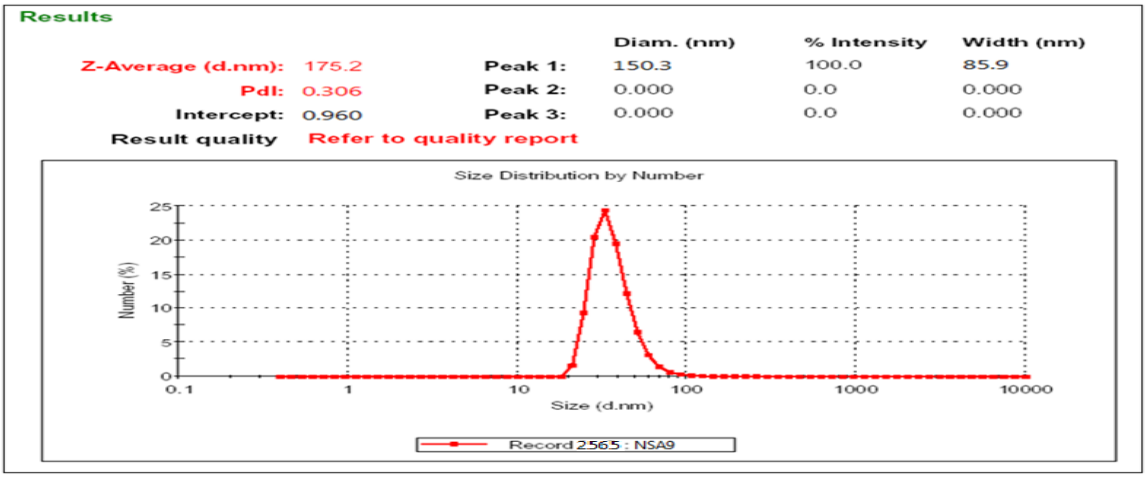

Figure 3. *Particle Size and Size Distribution of NSA9*

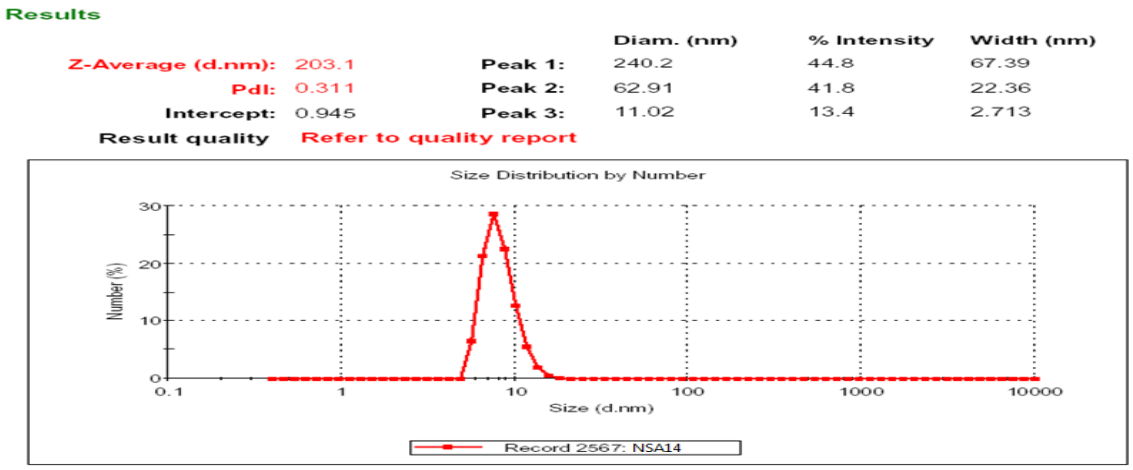

Figure 4. *Particle Size and Size Distribution of NSA14*

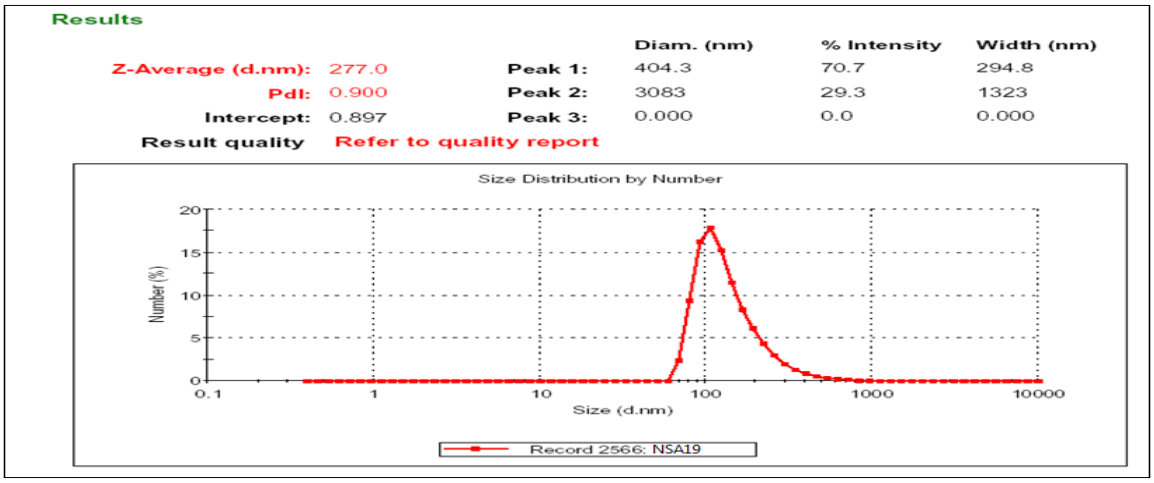

Figure 5. *Particle Size and Size Distribution of NSA19*

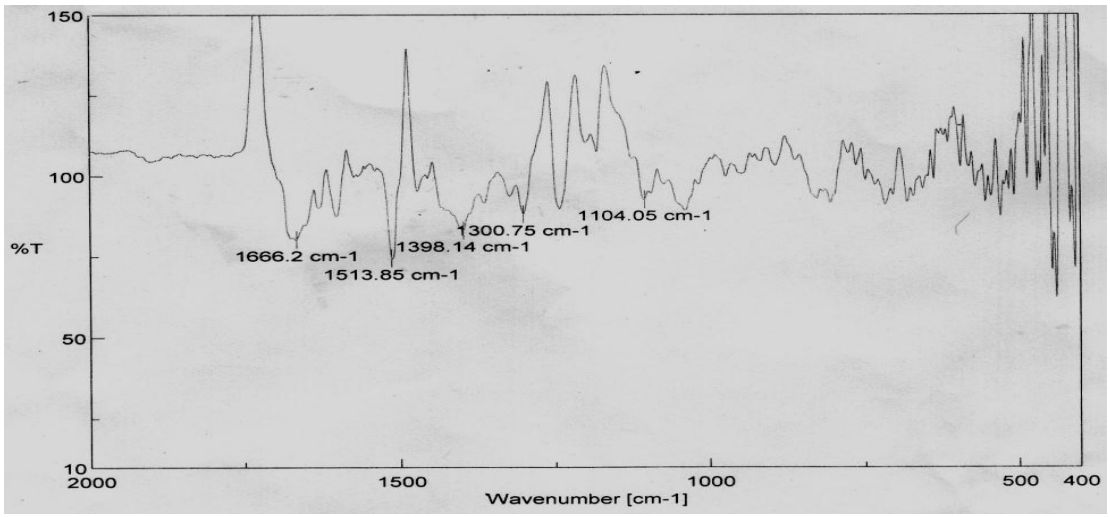

Figure 6. *FTIR spectra for Acetazolamide*

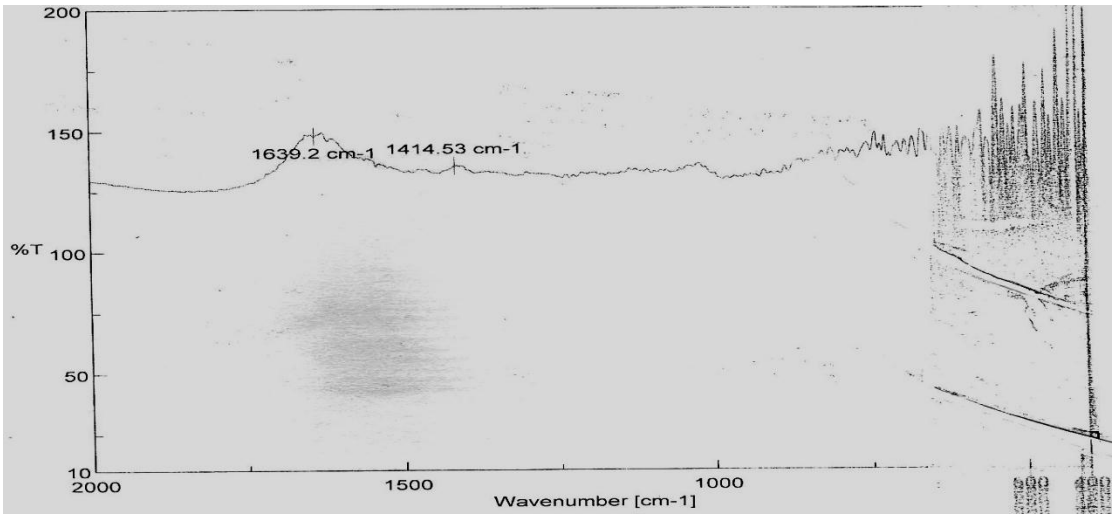

Figure 7. *FTIR spectra for polymer (chitosan)*

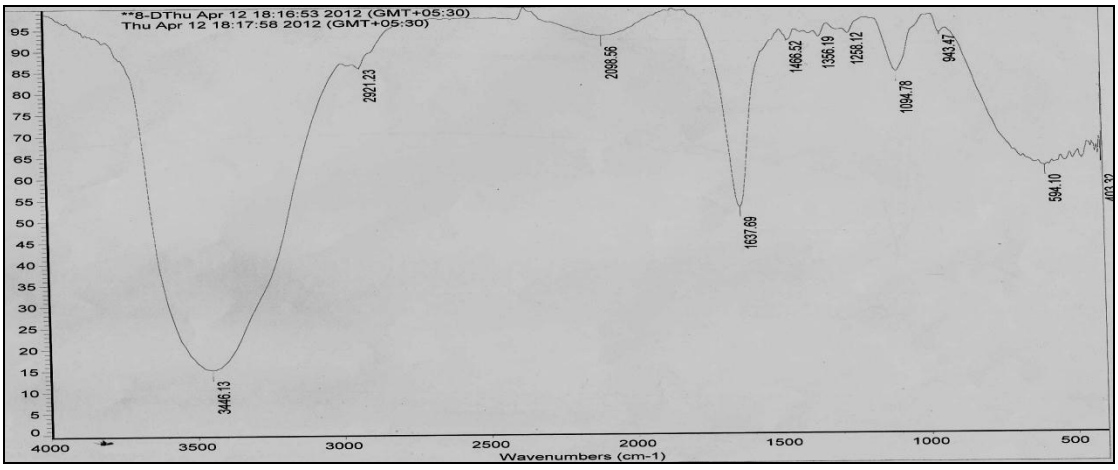

Figure 8. *FTIR spectra for acetazolamide nanosuspension*

Table IV. *Nanoparticles production yield of optimized nanosuspension*

Formula code	Nanoparticles weight	Process Yield (mean ± SD) (n= 3) (%)
NSA4	256.00	67.19 ± 0.01
NSA9	172.42	46.60 ± 0.02
NSA14	084.00	24.00 ± 0.07
NSA19	067.60	20.12 ± 0.01

Table V. *Drug loading and drug entrapment efficiency of optimized nanosuspension*

CS:TPP (w/w)	Abs. ± SD (n=3)	Dilution factor	Conc. (mg/ml)	Nanoparticles weight	Association efficiency	Loading capacity
NSA4	0.254 ± 0.002	1000	7.95	256	68.20	66.60
NSA9	0.303 ± 0.004	1000	9.495	172.42	62.02	86.92
NSA14	0.387± 0.001	1000	12.12	84.00	51.52	88.36
NSA19	0.413 ±0.003	1000	12.90	67.60	48.37	89.49

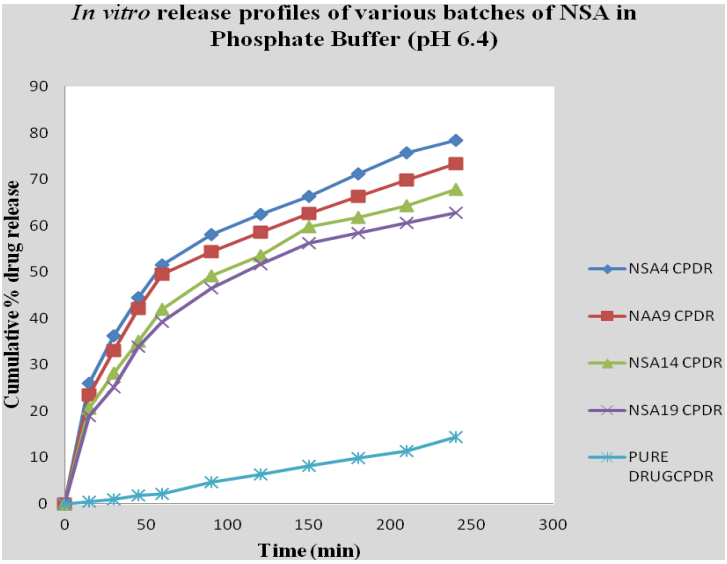

Figure 9 . *In vitro release profiles of various batches of NSA in Phosphate Buffer (pH 6.4)*

Table VI. *Drug Release kinetics of Optimized formulation and their comparison with pure drug and marketed formulation*

Release kinetics	Zero order		First order		Higuchi	
	K*	R^{2**}	K	R^2	K	R^2
NSA4	0.210	0.912	-0.002	0.981	4.312	0.977
NSA9	0.195	0.893	-0.001	0.965	3.059	0.936
NSA14	0.196	0.914	-0.001	0.967	4.036	0.980

NSA19	0.185	0.896	-0.001	0.948	3.829	0.970
Pure drug	0.061	0.993	-0.000	0.991	1.186	0.953

R^{2**} =coefficient of determination: K*= rate constant

Table VII. *One way ANOVA (Newman-Keuls multiple comparison) test for in vitro drug release of AZM nanosuspension and Pure drug*

Source of Variation	DF	SS	MS	F	P
Treatment (between columns)	4	15426	3856.5	**=MStreatment/MSresidual =9.48**	**P<0.0001**
Residual (within columns)	50	19384	387.67		
Total	54	34810			

Table VIII. *Student- Newman-Keuls Multiple Comparison Test*

Newman-Keuls Multiple Comparison Test	Mean Diff.	P value	Level of significance
Pure drug vs. NSA4	-46.346	P<0.0001	Extremely significant
Pure drug vs. NSA9	-42.990	P<0.0001	Extremely significant
Pure drug vs. NSA14	-38.340	P<0.0001	Extremely significant
Pure drug vs. NSA19	-35.729	P<0.0001	Extremely significant
NSA19 vs.NSA4	-10.617	P>0.05	Not significant
NSA19 vs. NSA9	-7.260	P>0.05	Not significant
NSA19 vs. NSA14	-2.610	P>0.05	Not significant
NSA14 vs. NSA4	-8.007	P>0.05	Not significant
NSA14 vs. NSA9	-4.650	P>0.05	Not significant
NSA9 vs. NSA4	-3.356	P>0.05	Not significant

Table IX. *Paired tests of the optimized formulations*

r.No.	Comparative Parameter	Test Applied	P value	Level of significant	Passed normality test
	Pure drug vs. NSA4	Paired t test	P<0.0001	Extremely significant	Yes
	Pure drug vs. NSA9	Paired t test	P<0.0001	Extremely significant	Yes
	Pure drug vs. NSA14	Paired t test	P<0.0001	Extremely significant	Yes
	Pure drug vs. NSA19	Paired t test	P<0.0001	Extremely significant	Yes
	NSA19 vs.NSA4	Paired t test	P<0.0001	Extremely significant	Yes
	NSA19 vs. NSA9	Paired t test	P<0.0001	Extremely significant	Yes
	NSA19 vs. NSA14	Paired t test	P<0.0001	Extremely significant	Yes
	NSA14 vs. NSA4	Paired t test	P<0.0001	Extremely significant	Yes

	NSA14 vs. NSA9	Paired t test	P<0.0001	Extremely significant	Yes
0	**NSA9 vs. NSA4**	Paired t test	P<0.0001	Extremely significant	Yes

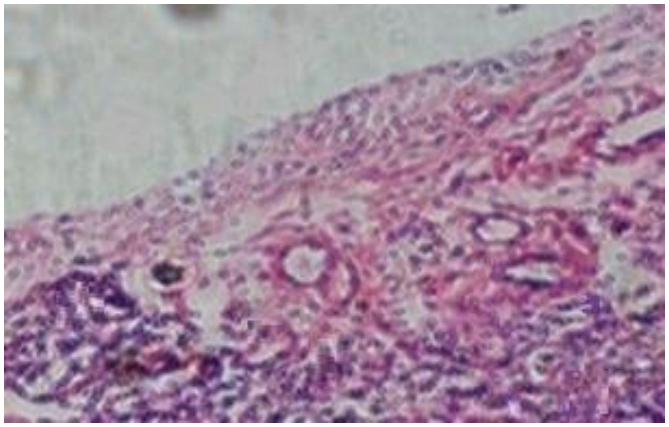

Figure 10. *Nasal mucosa treated with formulation (nanosuspension)*

Table X. *Stability data of acetazolamide loaded nanosuspension (NSA4)*

Sampling Interval (days)	**Drug content (%)***		**Physical Appearance**		**Mean Particle size ± SD**	
	5 ± 1^{0}C	**Room temperature**	**5 ± 1^{0}C**	**Room temperature**	**5 ± 1^{0}C**	**Room temperature**
0th	100	100	+	+	153	153
7th	99.80 ± 0.21	99.37 ± 0.22	+	+	166	171
14th	99.62 ± 0.07	98.67 ± 0.13	+	+	181	188
21th	99.40 ± 0.84	98.28 ± 0.08	+	+	195	212
28th	99.11 ± 0.12	97.70 ± 0.17	+	+	213	238
35th	98.70 ± 0.12	97.50 ± 0.12	+	+	232	259
45th	98.52 ± 0.03	96.66 ± 0.34	+	+	249	288

(*)- Mean ± SD (n=3), (+) - No change

Table XI. *Shelf-life of optimized formulation NSA4*

Sr. No.	**Parameters**	**Storage Conditions**	
		5 ± 1^{0}C	**Room Temperature**
1	**K (day^{-1})**	1 x 10^{-4}	3 x 10^{-4}
2	**$t_{½}$ (days)**	6842.11	2280
3	**$T_{10\%}$ (days)**	1040	346.667

4. Conclusion

AZM loaded chitosan nanoparticles were successfully prepared by ionic gelation method in four different CS: TPP ratios 3.5:1, 4:1, 5:1 & 6:1 giving the formulation NSA4, NSA9, NSA14 and NSA19. According to efficiency of yield and entrapment, 3.5:1 ratio (i.e. formulation NSA4) showed better yield compared to other 3 ratios. The entrapment efficiency was found of 68.20%. Average size of prepared NSA4 nanoparticles was found to be 153.3 nm with a polydispersity index 0.350. As the amount of polymer increased, size of the nanoparticles also increased. DSC and FTIR completely suggest the drug to polymer compatibility. In-vitro release studies showed highest release of drug from NSA4 formulation upto 78.40 ± 0.85 following first order kinetics and diffusion mechanism. Nasociliary study showed no nasal mucosa damage. From the present study, it is concluded that AZM loaded chitosan nanoparticles is an effective carrier for the design of controlled drug delivery of poorly water soluble drug like acetazolamide.

Thus the studies demonstrated that nanosuspension system comprising chitosan, sodium tripolyphosphate, tween 80 (1% v/v), and distilled water was optimal for intranasal delivery of AZM. The nanosuspension systems are transparent and stable at ambient conditions for 45 days. Enhanced rate and extent of AZM release following application on diffusion membrane from NSA formulations may help in decreasing the dose and frequency of dosing and possibly maximize the therapeutic index. The *in vitro* studies confirm the effectiveness and efficacy of the nanosuspension formulation in terms of better management of epilepsy.

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