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Research Article

Pharmaceutics For Effective Drug Dosage

Development and Characterization of Acetazolamide Nanoemulsion for Effective Ocular Delivery

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Abstract: Nanoemulsion has the potential of releasing the drug continuously, and they may easily permeate via the intense layers of the eye structure due to nano-size droplets, which makes nanoemulsion an effective drug delivery system for ocular delivery. The objective of our work was to prepare a nanoemulsion of acetazolamide for glaucoma treatment with enhanced efficacy as well as for continuous effect. Based on different compositions of oil (Olive Oil), surfactants (Tween-20), and co-surfactants (Transcutol P), forty-five test mixtures were made, water titration technique was employed for preparing the pseudo-ternary-phase diagrams. On the basis of these phase diagrams, twenty-five acetazolamide loaded nanoemulsion were formulated and examined for their nanosized droplets, PDI, zeta potential, viscosity, pH, transmittance and *in-vitro* drug release. The formulated nanoemulsion showed all the properties within the desired range i.e., droplet size (15.6 to 21.18), zeta potential (-15.5 to- 24.71), PDI (0.140 to 0.361), viscosity (3.234 ± 0.063to 5.174 ± 0.023cps), pH (6.922 ± 0.026to 7.033 ± 0.012), RI (1.379 ± 0.007 to 1.404 ± 0.006) and % transmittance was found (94.96± 0.6% to 96.68± 0.6%) and also the release rate of acetazolamide from nanoemulsion was found very good i.e., 81.59± 1.04% to 92.46± 0.33% after 24 hrs. The top four formulations having good drug release were selected for further evaluation of droplet sizes and which also fall in the nano range (15.68 to 21.18 nm). The study showed that it is possible to develop nanoemulsion of phenytoin drug, and the *in-vitro* drug release study showed that the prepared nanoemulsion had good bioavailability, sustained release and ability to target eye as an effective ocular delivery system.

Keywords: Acetazolamide, Nanoemulsion, Physicochemical Properties, Pseudoternary-Phase Diagram, Ocular Delivery, Glaucoma.

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1. INTRODUCTION

The ocular drug delivery system is a common and challenging area in pharmaceutical science. The eye has lacrimal secretion and nasolacrimal drainage properties which is responsible for the poor bioavailability as well as for the poor therapeutic response for conventional ocular drugs.[1]The ocular drugs should have a good concentration in eye site, and to achieve this, frequent dosing is required because during the drug application a major portion of the drug is diminished from the precorneal region.[2]However, during tear drainage, the gastrointestinal tract may absorb some fraction of the drug which can be carried out through the nasolacrimal duct, and sometimes it may cause side effects also.[3]The prolonged drug retention time in the eye can enhance effectiveness of the drug, resulting the increased bioavailability and reduced systemic absorption. This may also rule out the frequent drug administration requirement.[4] Several ophthalmic drug carriers like suspensions, aqueous gels, nanoemulsion, inserts, ointments, etc., are employed for enhancing the retention time of medications in the eye.[5] Ocular drug delivery systems have not been accepted universally even though they may have some promising improvements as compared to traditional liquid dosage forms due to their several drawbacks like blurred vision or low patient acceptability. There is a vast research scope for improving the ocular bioavailability from topical delivery.[6]Glaucoma is a serious eye disorder which is the world's second-leading disease, causing blindness without symptoms. It damages the optic disc of the eye and slowly leads to blindness due to the increased intraocular pressure.[7]Various research shows that glaucoma disease is mainly caused by an imbalance between the ocular drainage process and aqueous humor emission.[8]The main visual nerve for eye is the optical nerve, which is located at the back side of eye and images flow from eye to brain. The delicate fibres of optic nerve makes it most vulnerable part of eye and may get damaged either by direct pressure on the nerve or decreased blood flow to the nerve.[9]Glaucoma are mainly of two types, one which is developed due to unknown causes is called primary type glaucoma and another which is developed due to any known reason like eye trauma, cataracts, diabetes, eye surgery or tumours, called secondary type glaucoma.[10] Acetazolamide is a drug of choice for glaucoma, belongs to the Carbonic Anhydrase Inhibitor category and falls in BCS class IV.[11] It is available in tablet, capsule and parenteral dosage form, and all dosage forms also possess various systemic side effects. To overcome these side effects and make an effective drug delivery system it was hypothesized to prepare an ocular drug delivery system because till date no topical delivery is available. The conventional delivery system is not able to retain the effective concentration of drug into the eye.[12]Loftssonet al., 1996, prepared cyclodextrin polymer co-complexeye drops for enhancing the drug solubility and ocular bioavailability of acetazolamide.[12]The topically applied ocular eye drops have drainage issue which decreases the residence time and accordingly less release of drug. Due to the effective drug release rates, the nanoemulsion can be a very effective ocular drugs delivery dosage forms for enhancing bioavailability and sustained release of drugs. Nanoemulsion is a thermodynamically stable formulation with a size range up to100 nm,[13] Nanoemulsion is composed of oils, surfactant and co-surfactants and have the properties of encapsulating a variety of drugs in it.[14]Being a nano dosage form it can go deeper into to the eye. Also the overall property of the nanoemulsion makes it perfect to cross the anatomical barrier of eyes. Our work aims to formulate acetazolamide loaded nanoemulsion for enhancing the ocular bioavailability, therapeutic efficiency and achieving sustained release for prolonging the effect of the acetazolamide in the eye. Also, to perform the physicochemical characterization, *In-vitro* drug release analysis as well as particle size analysis of formed nano formulations.

2. MATERIALS AND METHOD

2.1 Materials

Acetazolamide was procured from Polpharma S.A., Poland, and Transcutol P, Labrasol, and Capryol 90 samples were gifted by M/s Gattefosse (Mumbai). Tween-20, Tween-80, propylene glycol, PEG 200, glycerol, Isopropyl myristate, sunflower oil, linseed oil, and olive oil were made available by the Department of pharmaceutical science, Kumaun University Nainital. All chemicals were of analytical grade. The water used for experiments is Milli Q water.

2.2 Screening of excipients

The screening of various oils, surfactants, and co-surfactants was based on the maximum solubility of the drug in particular excipients for the selection of optimum combination. [13,14]

2.3 Screening of oil

To determine the maximum solubility of Acetazolamide in various oils like Capryol 90, Isopropyl myristate, Olive oil, Sunflower oil, and Linseed Oil, a surplus drug amount was mixed in 2 mL of the oils in a 5 mL vials by using a vortexes. After that, vial containing the mixture were placed in an isothermal shaker for three days between 24 to 26 °C to achieve the equilibrium. After three days the samples were centrifuged for 15 minutes at 3,000 rpm and filtered with the help of 0.22-µm filter and analyzed in UV spectrophotometer at 263 nm for determining the drug solubility.[13,14]

2.4 Screening of surfactant and co-surfactant

The surfactant and co-surfactant were screened out on the basis of acetazolamide solubility in surfactant and co-surfactant, [13,14] similarly as the oil was selected. For the experiment Tween-20, Tween-80 and Labrasol were taken as surfactants, and Polyethylene glycol 200, Transcutol-P, glycerol and Propylene glycol were taken as co-surfactants.

2.5 Phase studies

The screened oil, surfactant and co-surfactant were further taken for phase study to achieve their optimum ratios for nanoemulsion formation. The water used for aqueous phase formation was MilliQ. The pseudo ternary phase diagram was prepared to know the nanoemulsion region for different concentrations of oil, surfactant & co-surfactant employing the water titration method (spontaneous emulsification method). The S_{mix} (combination of surfactant and co-surfactant) was formed in different volume combinations of surfactant and co-surfactant like 1:1, 1:2, 1:3, 2:1 & 3:1. For each phase diagram, oil and specific S_{mix} were mixed well in different ratios of increasing $S_{mix}$from 1 to 9 and decreasing oil ratios from 9 to 1. Accordingly, a total of nine combinations of S_{mix} and oil (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1) where to achieve a wide range for nanoemulsion region.[11,12,13,14]All the combinations of S_{mix} and oil mixture were slowly titrated with an aqueous phase under medium stirring, and the visual observation was done for checking the

phase inversion/separation with transparency/opaqueness and easily flowable nanoemulsion.

2.6 Selection of nanoemulsion

Based on the visual observation (phase inversion and transparency), a number of nanoemulsion having different compositions were chosen and plotted on pseudo ternary phase diagrams which showed the maximum nanoemulsion region. Selected formulations were taken further for preparing the acetazolamide loaded nanoemulsion.[13,14]

2.7 Development of Acetazolamide loaded nanoemulsion

From the constructed pseudo ternary phase diagram, the selected nanoemulsion with a predetermined ratio of oil and S_{mix} (Surfactant + co-surfactant) in the aqueous phase is further taken for loading the acetazolamide. The predetermined ratio of oil and S_{mix} are mixed and 1% (w/v) of acetazolamide drug was then incorporated into that mixture. The mixture is blended until the complete dissolution of the drug occurs. Then the aqueous phase was added drop wise under continuous stirring to achieve a transparent mixture after spontaneous phase inversion.[15]

2.8 Physicochemical characterization

Selected drug-loaded nanoemulsion formulations were characterized for the various parameters e.g. thermodynamic stability, viscosity, pH measurement, Refractive index, Percentage Transmittance, *In-vitro* drug release analysis Particle size, polydispersity index, and zeta potential and droplet size.[13, 14,]

2.9 Thermodynamic stability studies

The nanoemulsion should be thermodynamically stable, for this three stress condition tests are performed which include heat and cooling cycles, freeze-thaw cycles, and centrifugation.[14,15,16]

2.10 Heating-cooling cycles

Nanoemulsion formulations were exposed to 6 cycles involving refrigerating at 4 °C and heating at 45 °C and finally stored for 72 hrs. to check the stability. [13,14]

2.11 Centrifugation

In this step, nanoemulsion formulations were exposed to centrifugation studies. To detect any kind of phase separation in nanoemulsion, a centrifuge was used for centrifugation for half-hr. at 3500rpm. [13,14]

2.12 Freeze-thaw cycle

In this step nanoemulsion were placed in a deep freezer at -21°C for a day and after that back to room temperature at 25°C. To be thermodynamically stable the nanoemulsion formulation was observed to attain its original appearance within 2-3 minutes at room temperature. A repetition of such a process was done 2-3 times.[17, 18] After the aforementioned tests, all formulations were checked for physical instability like phase separation, turbidity, etc. The experiments were done in triplicate. The formulations which pass the above stress test were then further investigated for another test as described below.

2.13 Viscosity

Viscosity of nanoemulsion gives two important factors whether the formulation is stable or not and having efficient drug release. The viscosity affects the stability and drug release of the drug from nanoemulsion. Brookfield DVIII viscometer (Brookfield Engineering Laboratories Inc., USA) with spindle No. 4 at 25.0 ± 0.5 °C was used to measure the viscosity of nanoemulsion. The viscosity was determined using a rheogram by plotting the shear stress (dynes/cm^2) against the shear rate (s^{-1}) at a fixed speed of rotation up to 150 rpm.[19, 20]

2.14 pH measurement

The pH meter (Mettler Toledo, Switzerland) was employed for the determination of the pH of nanoemulsion.[21]For the ocular preparation, the pH of the formulation should be in the ranges of 7.11±1.5. Accordingly the pH of nanoemulsion formulations was expected to be within this range.[22]

2.15 Refractive index

Abbe-type refractometer (Shanghai Optical Instrument Factory, China) was used to know the refractive index of samples at 25°Cin triplicates. It indicates the isotropic nature of the formulation.[23]

2.16 Percentage Transmittance

The transparency of prepared NEs was analysed by using UV spectrophotometer (UV-6100 PC, EMC lab, Germany) in triplicate.[24] The percent transmittance quantifies how much light passed through a sample, whereas the absorbance in a quantitative indication of light absorbed by the sample and both the terms are contrary to each other. The following equation shows the relationship between both the terms:

$$Absorbance = -\log\log\left(\frac{Percent\ Transmittance}{100}\right)$$

$$\text{Percent Transmittance} = 10^{(-\text{absorbance})} \times 100\ \%$$

2.17 In-vitro drug release analysis

The release behaviour of acetazolamide from nanoemulsion was determined using the dialysis bag technique. In this method, 1 ml of prepared nanoemulsion was filled in a dialysis bag (MW cut-off 12,400 Da) rinsed by distilled water for 1 day at 4 °C. The bag was tied up to the paddle of the USP apparatus which rotates at a speed of 50 rpm and dipped in 900 ml of phosphate buffer (pH=7.4) release medium and the temperature of the USP apparatus was maintained at 34± 0.2 °C similar to eye environment.[25] For release studies, at different time intervals i.e. 15 min, 30 min, 1hr, and then after every one hr. For the next 12hr, and then finally at 24 hrs. 2 ml sample was withdrawn from the dissolution medium and the same amount was replaced with a fresh phosphate buffer. The sample was analyzed in UV spectrophotometer at 263 nm to determine the amount of acetazolamide released in given time of period and percentage cumulative drug release was calculated.[26]

2.18 Particle size, polydispersity index, and zeta potential

Four preparations having the best acetazolamide *in-vitro* drug release rate were taken further to study the size of particles; to assure that the formed emulsion was in the nanorange. The particle size, polydispersity index (PDI) and zeta potential of nanoemulsion was measured with the help of Malvern Zetasizer NanoZS (Malvern, United Kingdom). The acetazolamide loaded nanoemulsion was diluted to 1:50 v/v in HPLC grade water for the same testing.Based on droplet size, it was decided that the emulsion particles are in the range of nanoparticles or not. The polydispersity index (PDI) is a method to show the droplet size uniformity dispersed in nanoemulsion. For better uniformity of droplet size in nanoemulsion, the PDI value should be low. Zeta potential is the amount of repulsion force between particles within the bulk solution. For minimizing aggregation and flocculation the zeta potential value between particles should be on the higher side.[27]

2.19 Transmission Electron Microscopy (TEM)

To further confirm the droplet size of nanoemulsion, the transmission electron microscopy (TEM) of best NE formulations was also done using Morgagni 268D electron microscope (Fei Company, Netherlands) 70 kV. The TEM captures high-resolution images increasing the magnification to identify the form and size of droplets and their distribution in nanoemulsion. A water-diluted drop of nanoemulsion formulation was marked with a drop of 2% w/v phosphor tungstic acid solution for 30 seconds on a grid coated with carbon. After drying the carbon grid was observed using TEM. The TEM image analysis gives the true radius which are more precise as compared to the hydrodynamic radius provided by Zetasizer.[28]

3. STATISTICAL ANALYSIS

The data were represented as Standard deviation (n=3), students (paired) t-test was performed using MS excel, the difference considered significant for a p-value $p<0.05$.[27]

4. RESULTS

The most important factor to be considered during the screening of excipient is that they should be pharmaceutically acceptable for application i.e., generally-regarded-as-safe category.

4.1 Screening of excipients

a. Screening of oil

In the development of a drug-loaded nanoemulsion system, the main factor is to test the solubility of the drug in a variety of formulation components. Better solubility means the volume of drug-loaded nanoemulsion will also be reduced and provide a better therapeutic dose. The solubility test graph of acetazolamide drug in different oils is shown in figure-1. It depicts the solubility of acetazolamide was found highest in olive oil (8.2±0.075 mg/ml) in comparison with other oils, accordingly olive oil was selected as the oil base for the preparation of acetazolamide nanoemulsion.

b. Screening criteria for surfactants

As per industrial standards, the ocular o/w nanoemulsion formulations shall have good *in-vivo* stability. Therefore, it becomes more important to select a proper surfactant. The HLB value of surfactant should also be chosen properly as in o/w base nanoemulsion higher HLB value of a surfactant is required so that during the mixing of water phase it may easily break its bonding with oil phase and join with aqueous phase results a phase inversion.[29]After analyzing it was found that out of three surfactants Tween-20 showed the best solubility of acetazolamide drug i.e., 13.1±0.041 mg/ml (figure-1).

c. Screening of co-surfactants

Co-surfactants are used to reduce the interfacial tension and increase the fluidity of the interface. These are generally medium-chain alcohols (C3-C8). The co-surfactants are also allowed better penetration of oil by increasing the mobility of the hydrocarbon tails. As depicted in figure-1 the Transcutol P showed the best solubility of acetazolamide drugs i.e., 21.4±0.097 mg/ml.

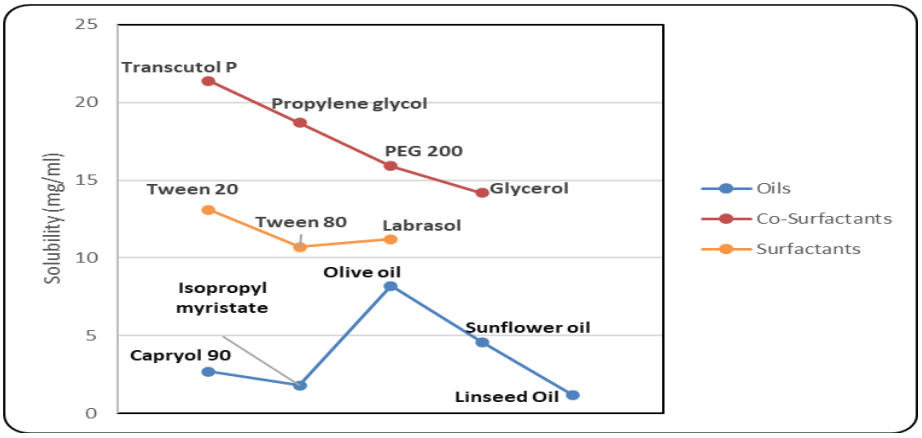


Fig-1: Solubility of acetazolamide in different oils, surfactants & co-surfactants at 25°C (mean± SD, n=3)

d. Preparation of pseudo ternary phase diagram

The formation of nanoemulsion is due to phase inversion caused by breaking the bonding of surfactant from oil to water. Due to this nanoemulsion required very less free energy. The co-surfactant helps to reduce the surface tension between oil and water because the nanoemulsion, having both surfactant and co-surfactant, exhibits better nanoemulsion region as compared to alone use of surfactant. On the phase diagram, an o/w nanoemulsion region was observed on the high-water ratio side. The range of concentration between which nanoemulsion are formed is shown in the phase diagram. The oil concentration higher than the nanoemulsion region was formed a phase separation scenario, as there was much oil in the mixture which was unable to disperse in the water phase. Similarly, the oil concentration lower than the nanoemulsion region was formed turbid mixture as less oil and high S_{mix} did not allow phase inversion in the aqueous phase. A good nanoemulsion region was observed by mixing an equal amount of both surfactant and co-surfactant i.e.,S_{mix} 1:1 (Figure 2a). Further increase in co-surfactant ratio as compare to surfactant i.e., S_{mix}1:2 increases the nanoemulsion region (Figure 2b). On further increase in co-surfactant concentration i.e., S_{mix}1:3 the nanoemulsion ratio did not affect much but the ratio of oil concentration was decreased (Figure 2c) which is not good, because less oil concentration means less acetazolamide loading. So, there is no need to increase the co-surfactant concentration. Similarly, when the concentration of surfactant was increased as compare to co-surfactant i.e. at S_{mix} 2:1 (Figure 2d), the nanoemulsion region again increased as compared to S_{mix} 1:1 region. However, it decreased by a further increase in surfactant concentration i.e. S_{mix} ratio of 3:1 (Figure 2e). Therefore, there was no need to attempt more tests with a further increase in surfactant concentration.

Table 1: Nanoemulsion formulations by different combinations of oil S_{mix}& Water

S. No.	Smix Ratio	Formulation No.	Drug (% w/v)	OIL	Smix	Water	Transparency
1	1:1	FACZ1	1	39.22	26.14	34.64	Clear
2	1:1	FACZ2	1	31.45	31.45	37.11	Clear
3	1:1	FACZ3	1	24.54	36.81	38.65	Clear
4	1:1	FACZ4	1	15.54	36.27	48.19	Turbid
5	1:2	FACZ5	1	45.75	19.61	34.64	Clear
6	1:2	FACZ6	1	36.81	24.54	38.65	Clear
7	1:2	FACZ7	1	30.49	30.49	39.02	Clear
8	1:2	FACZ8	1	21.98	32.97	45.05	Clear
9	1:2	FACZ9	1	15.63	36.46	47.92	Clear
10	1:3	FACZ10	1	43.48	18.63	37.89	Clear
11	1:3	FACZ11	1	37.50	25.00	37.50	Clear
12	1:3	FACZ12	1	26.88	26.88	46.24	Clear
13	1:3	FACZ13	1	20.73	31.09	48.19	Clear
14	1:3	FACZ14	1	16.39	38.25	45.36	Clear
15	1:3	FACZ15	1	9.71	38.83	51.46	Turbid
16	2:1	FACZ16	1	50.72	21.74	27.54	Clear
17	2:1	FACZ17	1	40.82	27.21	31.97	Clear
18	2:1	FACZ18	1	33.56	33.56	32.89	Clear
19	2:1	FACZ19	1	25.48	38.22	36.31	Clear
20	2:1	FACZ20	1	16.76	39.11	44.13	Clear
21	3:1	FACZ21	1	51.09	21.90	27.01	Turbid
22	3:1	FACZ22	1	41.10	27.40	31.51	Clear
23	3:1	FACZ23	1	35.46	35.46	29.08	Clear
24	3:1	FACZ24	1	24.54	36.81	38.65	Clear
25	3:1	FACZ25	1	18.52	43.21	38.27	Clear

%=Percentage; w/v= weight per volume; S_{mix}= Surfactant:Co-surfactant; ACZ= Acetazolamide

4.2 *Analysis Of Data*

The nano formulations obtained by different combinations of Oil, S_{mix} and Water were examined on the basis of their transparency as well as phase separation and then the pseudo ternary phase diagrams at different ratios of S_{mix} were plotted with the help of CHEMIX School software version 8.0.

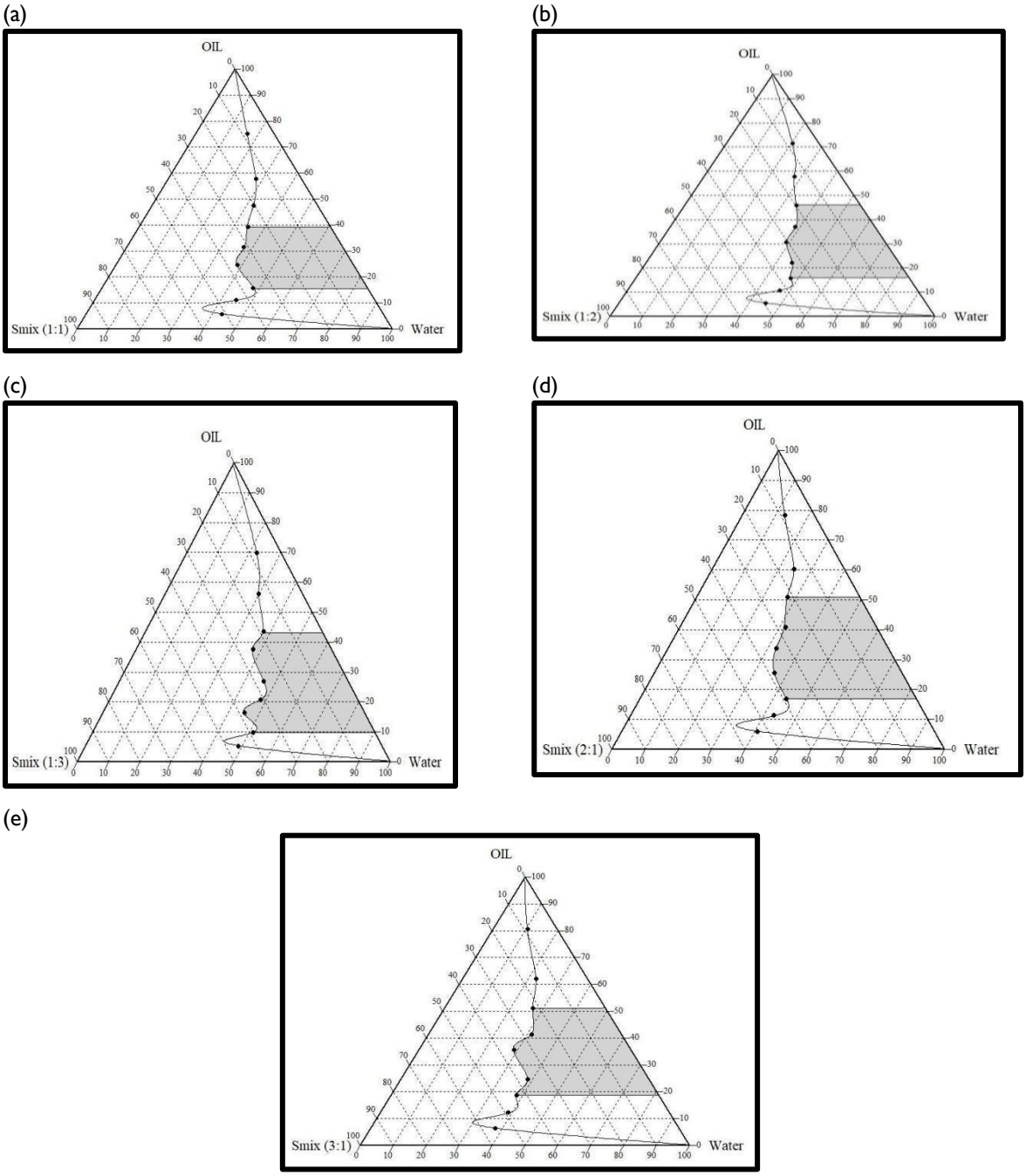


Fig-2: Pseudo ternary phase diagram with nanoemulsion region

4.3 *Thermodynamic stability tests of drug-loaded nanoemulsion*

Based on visual observation some optimized combinations of nanoemulsion formulations were selected and loading of 1% (w/v) of acetazolamide drug was done. For further stress testing of these drug-loaded nanoemulsion the heating-cooling cycle, freeze-thaw cycle, and centrifugation analysis was conducted under thermodynamic stability tests. The results of thermodynamic stability of formulations are shown in (Figure-3). In bar diagram all three stability tests were represented by different color bars. Any formulation succeeded in any test shall contain that color bar. So, the formulations having all three-color bars means got succeeded in all three tests and were thermodynamically stable. The thermodynamic stable nanoemulsion provides assurance of long shelf life in comparison to normal emulsions. The thermodynamically stable nanoemulsion was taken for further studies.

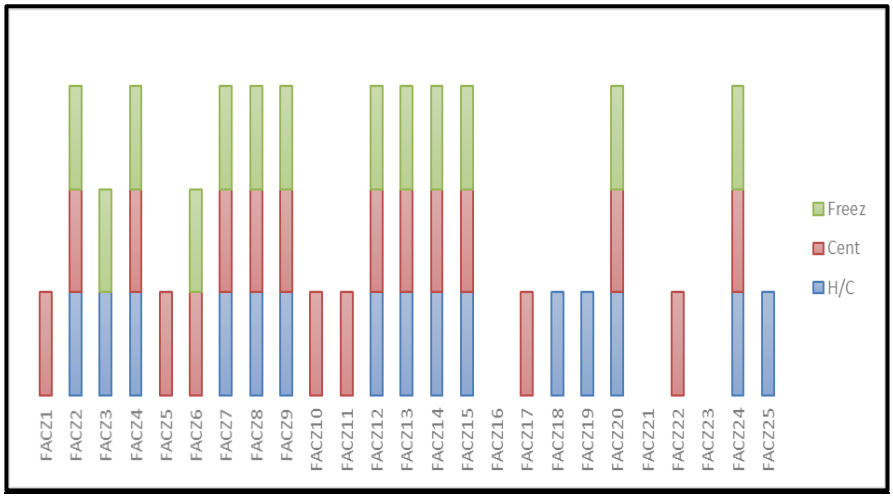


Fig-3: Thermodynamic stability testing of acetazolamide loaded nanoemulsion

4.4 Characterization of the selected nanoemulsion

The characterization of each nanoemulsion qualified from thermodynamic stress testing was done.

4.5 Viscosity

The viscosity was measured in triplicate at 25 ± 1 °C. The viscosity of all formulations was found between 3.234 ± 0.063to 5.174 ± 0.023cps (Table-2).

4.6 pH Measurements

The pH meter is used to measure pH of all formulations in triplicate at 25 ± 1 °C and found to be between 6.922 ± 0.026to 7.033 ± 0.012(Table-2).

4.7 Refractive index

The value of the refractive index for all the formulations was measured in triplicate and given in (Table-2). The RI of formulations ranged between 1.379 ± 0.007 to 1.404 ± 0.006.

4.8 Percentage Transmittance

Percentage Transmittance was found closer to 100% (above 94.96± 0.6) which indicated that the formulation absorbed very less light and a maximum of light easily possessed through the formulations. It also indicated the formulations were clear and transparent. Formulation FACZ24 had the least transmittance percentage of 94.96± 0.6% and highest absorbance of 0.0225± 0.0027 on the other hand formulation FACZ15 has a maximum transmittance percentage of 96.68± 0.6% and the lowest absorbance of 0.0147± 0.0027.

Table No. 2:Viscosity, pH Measurements, Refractive index, % Transmittance and absorbance

S. No.	Formulation	Viscosity (cps) ± SD	pH ± SD	Refractive Index ± SD	Transmittance (%)	Absorbance
1	FACZ2	5.174 ± 0.023	7.033 ± 0.012	1.403 ± 0.006	95 ± 1.3	0.0223 ± 0.0059
2	FACZ4	3.564 ± 0.039	6.95 ± 0.022	1.387 ± 0.004	96.08 ± 0.9	0.0174 ± 0.0041
3	FACZ7	4.824 ± 0.081	7.032 ± 0.015	1.399 ± 0.002	95.3 ± 0.6	0.0209 ± 0.0027
4	FACZ8	3.928 ± 0.05	6.986 ± 0.019	1.39 ± 0.007	95.89 ± 1.1	0.0182 ± 0.0050
5	FACZ9	3.594 ± 0.089	6.95 ± 0.011	1.385 ± 0.002	96.22 ± 0.4	0.0167 ± 0.0018
6	FACZ12	3.763 ± 0.075	7.028 ± 0.018	1.389 ± 0.009	95.96 ± 0.8	0.0179 ± 0.0036
\7	FACZ13	3.548 ± 0.048	6.987 ± 0.024	1.385 ± 0.004	96.22 ± 1.2	0.0167 ± 0.0054
8	FACZ14	3.905 ± 0.065	6.948 ± 0.01	1.387 ± 0.001	96.1 ± 0.8	0.0173 ± 0.0036
9	FACZ15	3.234 ± 0.063	6.922 ± 0.026	1.379 ± 0.007	96.68 ± 0.6	0.0147 ± 0.0027
10	FACZ20	4.066 ± 0.092	6.947 ± 0.02	1.395 ± 0.004	95.56 ± 1.1	0.0197 ± 0.0050
11	FACZ24	4.9 ± 0.086	6.985 ± 0.015	1.404 ± 0.006	94.96 ± 0.6	0.0225 ± 0.0027

SD=Standard Deviation; %=Percentage; Cps=Cinti poise; Values are mean ± SD; (n=55); (p<0.001)

4.9 In-Vitro drug release studies

The results of the *in-vitro* drug release profile of all prepared batches are shown in Table no. 3 & their graph in Figure-4.It was observed that initially the release of the drug from all formulations was very high and reached 41.03% to 71.95% in the first 6 hrs. After that, the release rate was slightly decreased and after 12 hrs. The release range was found to be 63.19% to 79.23%. The release of the drug was observed for 24 hrs. and the release rate of formulations was found in the range of 81.59% to 92.46% which is a very good release rate of the drug from nanoemulsion. It was observed that after 24 hrs. FACZ2 had the highest release and FACZ15 had the lowest. Formulations having top four release statistics i.e., FACZ2, FACZ7, FACZ20 & FACZ24 had been taken further for their droplet size analysis.

Table No. 3:In-Vitro Drug Release statics

	Time (hrs)	Absorbance	Concentration (µg)	% Drug Release
FACZ2	0.25	0.133 ± 0.0059	0.551 ± 0.094	4.96 ± 0.85
	0.5	0.198 ± 0.0070	1.586 ± 0.111	14.30 ± 1.01
	1	0.272 ± 0.0063	2.764 ± 0.100	24.99 ± 0.91
	2	0.351 ± 0.0031	4.022 ± 0.049	36.45 ± 0.46
	3	0.454 ± 0.0053	5.662 ± 0.084	51.41 ± 0.78
	4	0.52 ± 0.0036	6.713 ± 0.057	60.87 ± 0.53
	5	0.561 ± 0.0049	7.366 ± 0.078	67.03 ± 0.72
	6	0.595 ± 0.0073	7.908 ± 0.116	71.95 ± 1.07
	12	0.643 ± 0.0064	8.672 ± 0.102	79.23 ± 0.94
	24	0.735 ± 0.0021	10.137 ± 0.033	92.84 ± 0.33
FACZ4	0.25	0.119 ± 0.0047	0.328 ± 0.075	2.95 ± 0.67
	0.5	0.165 ± 0.0027	1.061 ± 0.043	9.56 ± 0.39
	1	0.202 ± 0.0079	1.650 ± 0.126	14.92 ± 1.14
	2	0.261 ± 0.0023	2.589 ± 0.037	23.45 ± 0.34
	3	0.315 ± 0.0037	3.449 ± 0.059	31.32 ± 0.54
	4	0.359 ± 0.0057	4.150 ± 0.091	37.63 ± 0.83
	5	0.402 ± 0.0051	4.834 ± 0.081	43.96 ± 0.75
	6	0.437 ± 0.0027	5.392 ± 0.043	49.01 ± 0.41
	12	0.549 ± 0.0015	7.175 ± 0.024	65.33 ± 0.24
	24	0.691 ± 0.0017	9.436 ± 0.027	86.04 ± 0.27
FACZ7	0.25	0.131 ± 0.0073	0.519 ± 0.116	4.67 ± 1.05
	0.5	0.187 ± 0.0017	1.411 ± 0.027	12.72 ± 0.25
	1	0.244 ± 0.0078	2.318 ± 0.124	20.96 ± 1.13
	2	0.32 ± 0.0073	3.529 ± 0.116	31.97 ± 1.06
	3	0.425 ± 0.0026	5.201 ± 0.041	47.19 ± 0.39
	4	0.495 ± 0.0023	6.315 ± 0.037	57.23 ± 0.35
	5	0.506 ± 0.0022	6.490 ± 0.035	59.06 ± 0.34
	6	0.533 ± 0.0029	6.920 ± 0.046	62.99 ± 0.44
	12	0.63 ± 0.0034	8.465 ± 0.054	77.24 ± 0.51
	24	0.728 ± 0.0017	10.025 ± 0.027	91.70 ± 0.27
FACZ8	0.25	0.126 ± 0.0048	0.439 ± 0.076	3.96 ± 0.69
	0.5	0.176 ± 0.0039	1.236 ± 0.062	11.14 ± 0.56
	1	0.232 ± 0.0059	2.127 ± 0.094	19.23 ± 0.85
	2	0.298 ± 0.0064	3.178 ± 0.102	28.80 ± 0.93
	3	0.351 ± 0.0042	4.022 ± 0.067	36.55 ± 0.62
	4	0.399 ± 0.0017	4.787 ± 0.027	43.43 ± 0.26
	5	0.465 ± 0.0037	5.838 ± 0.059	53.09 ± 0.55
	6	0.508 ± 0.0043	6.522 ± 0.068	59.29 ± 0.63
	12	0.601 ± 0.0021	8.003 ± 0.033	72.94 ± 0.32
	24	0.712 ± 0.0066	9.771 ± 0.105	89.25 ± 0.97
FACZ9	0.25	0.117 ± 0.0037	0.296 ± 0.059	2.67 ± 0.53
	0.5	0.161 ± 0.0031	0.997 ± 0.049	8.99 ± 0.45
	1	0.194 ± 0.0026	1.522 ± 0.041	13.77 ± 0.38
	2	0.244 ± 0.0033	2.318 ± 0.053	21.01 ± 0.48
	3	0.308 ± 0.0020	3.338 ± 0.032	30.29 ± 0.30
	4	0.353 ± 0.0034	4.054 ± 0.054	36.74 ± 0.50
	5	0.371 ± 0.0036	4.341 ± 0.057	39.49 ± 0.53
	6	0.405 ± 0.0019	4.882 ± 0.030	44.40 ± 0.29
	12	0.564 ± 0.0059	7.414 ± 0.094	67.43 ± 0.86
	24	0.677 ± 0.0026	9.213 ± 0.041	83.99 ± 0.39
FACZ12	0.25	0.123 ± 0.0068	0.392 ± 0.108	3.53 ± 0.97
	0.5	0.174 ± 0.0038	1.204 ± 0.061	10.85 ± 0.55
	1	0.228 ± 0.0016	2.064 ± 0.025	18.65 ± 0.24
	2	0.281 ± 0.0064	2.908 ± 0.102	26.35 ± 0.93
	3	0.357 ± 0.0070	4.118 ± 0.111	37.39 ± 1.02
	4	0.404 ± 0.0017	4.866 ± 0.027	44.12 ± 0.26
	5	0.43 ± 0.0045	5.280 ± 0.072	48.06 ± 0.67
	6	0.455 ± 0.0019	5.678 ± 0.030	51.68 ± 0.29
	12	0.595 ± 0.0022	7.908 ± 0.035	72.02 ± 0.33
	24	0.71 ± 0.0053	9.739 ± 0.084	88.90 ± 0.78

FACZ13	0.25	0.114 ± 0.0015	0.248 ± 0.024	2.24 ± 0.21
	0.5	0.149 ± 0.0045	0.806 ± 0.072	7.26 ± 0.65
	1	0.176 ± 0.0079	1.236 ± 0.126	11.17 ± 1.14
	2	0.238 ± 0.0047	2.223 ± 0.075	20.12 ± 0.68
	3	0.283 ± 0.0030	2.939 ± 0.048	26.68 ± 0.44
	4	0.313 ± 0.0071	3.417 ± 0.113	30.98 ± 1.03
	5	0.362 ± 0.0054	4.197 ± 0.086	38.15 ± 0.79
	6	0.389 ± 0.0025	4.627 ± 0.040	42.04 ± 0.38
	12	0.525 ± 0.0076	6.793 ± 0.121	61.76 ± 1.11
	24	0.664 ± 0.0054	9.006 ± 0.086	82.02 ± 0.80
FACZ14	0.25	0.121 ± 0.0039	0.360 ± 0.062	3.24 ± 0.56
	0.5	0.173 ± 0.0068	1.188 ± 0.108	10.71 ± 0.98
	1	0.225 ± 0.0063	2.016 ± 0.100	18.22 ± 0.91
	2	0.265 ± 0.0032	2.653 ± 0.051	24.05 ± 0.47
	3	0.332 ± 0.0023	3.720 ± 0.037	33.79 ± 0.35
	4	0.382 ± 0.0056	4.516 ± 0.089	40.95 ± 0.82
	5	0.448 ± 0.0043	5.567 ± 0.068	50.60 ± 0.63
	6	0.487 ± 0.0051	6.188 ± 0.081	56.23 ± 0.75
	12	0.574 ± 0.0036	7.573 ± 0.057	69.01 ± 0.54
	24	0.69 ± 0.0041	9.420 ± 0.065	86.01 ± 0.62
FACZ15	0.25	0.112 ± 0.0061	0.217 ± 0.097	1.95 ± 0.87
	0.5	0.14 ± 0.0070	0.662 ± 0.111	5.97 ± 1.01
	1	0.174 ± 0.0056	1.204 ± 0.089	10.88 ± 0.81
	2	0.232 ± 0.0050	2.127 ± 0.080	19.25 ± 0.73
	3	0.271 ± 0.0042	2.748 ± 0.067	24.95 ± 0.62
	4	0.325 ± 0.0030	3.608 ± 0.048	32.69 ± 0.45
	5	0.355 ± 0.0059	4.086 ± 0.094	37.12 ± 0.87
	6	0.382 ± 0.0020	4.516 ± 0.032	41.03 ± 0.31
	12	0.535 ± 0.0036	6.952 ± 0.057	63.19 ± 0.54
	24	0.661 ± 0.0071	8.959 ± 0.113	81.59 ± 1.04
FACZ20	0.25	0.129 ± 0.0067	0.487 ± 0.107	4.39 ± 0.96
	0.5	0.18 ± 0.0058	1.299 ± 0.092	11.72 ± 0.84
	1	0.238 ± 0.0059	2.223 ± 0.094	20.10 ± 0.86
	2	0.306 ± 0.0032	3.306 ± 0.051	29.95 ± 0.47
	3	0.385 ± 0.0023	4.564 ± 0.037	41.44 ± 0.35
	4	0.436 ± 0.0031	5.376 ± 0.049	48.75 ± 0.46
	5	0.475 ± 0.0015	5.997 ± 0.024	54.57 ± 0.23
	6	0.527 ± 0.0068	6.825 ± 0.108	62.06 ± 0.99
	12	0.611 ± 0.0080	8.162 ± 0.127	74.44 ± 1.17
	24	0.717 ± 0.0078	9.850 ± 0.124	90.04 ± 1.15
FACZ24	0.25	0.132 ± 0.0028	0.535 ± 0.045	4.82 ± 0.40
	0.5	0.193 ± 0.0027	1.506 ± 0.043	13.58 ± 0.39
	1	0.258 ± 0.0037	2.541 ± 0.059	22.97 ± 0.53
	2	0.334 ± 0.0048	3.752 ± 0.076	33.99 ± 0.70
	3	0.389 ± 0.0071	4.627 ± 0.113	42.06 ± 1.03
	4	0.479 ± 0.0032	6.061 ± 0.051	54.96 ± 0.47
	5	0.512 ± 0.0039	6.586 ± 0.062	59.92 ± 0.58
	6	0.575 ± 0.0050	7.589 ± 0.080	69.02 ± 0.73
	12	0.619 ± 0.0077	8.290 ± 0.123	75.71 ± 1.12
	24	0.733 ± 0.0062	10.105 ± 0.099	92.46 ± 0.91

hrs = Hours;µg= Micro Gram; %=Percentage; Values are mean ± SD; (n=330); (p < 0.001)

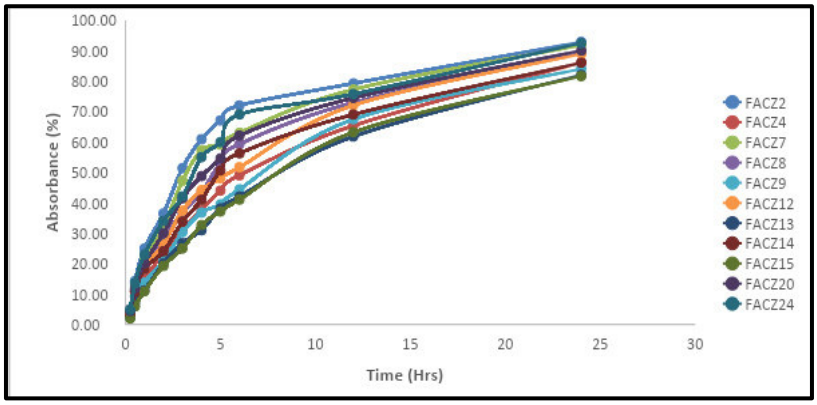


Fig-4: In-Vitro drug release

4.10 Particle size, polydispersity index, zeta potential

The particle size, polydispersity index, and zeta potential values are shown in table-4 and their corresponding Malvern zetasizer graphs are in figure 5

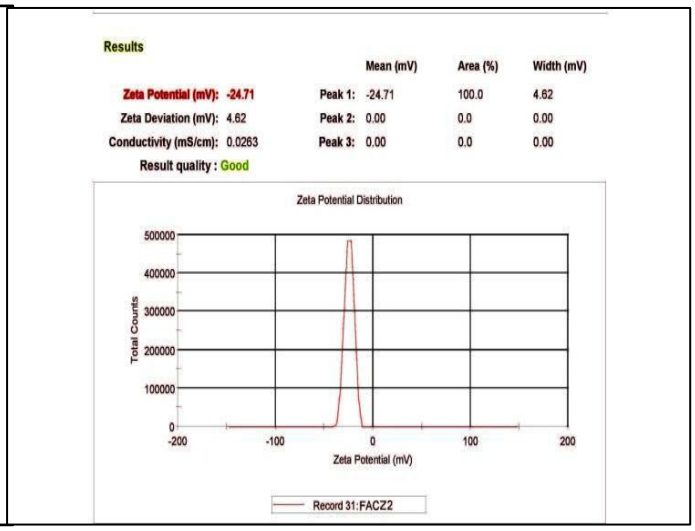
Table No. 4: Particle size, polydispersity index, and zeta potential of nanoemulsion

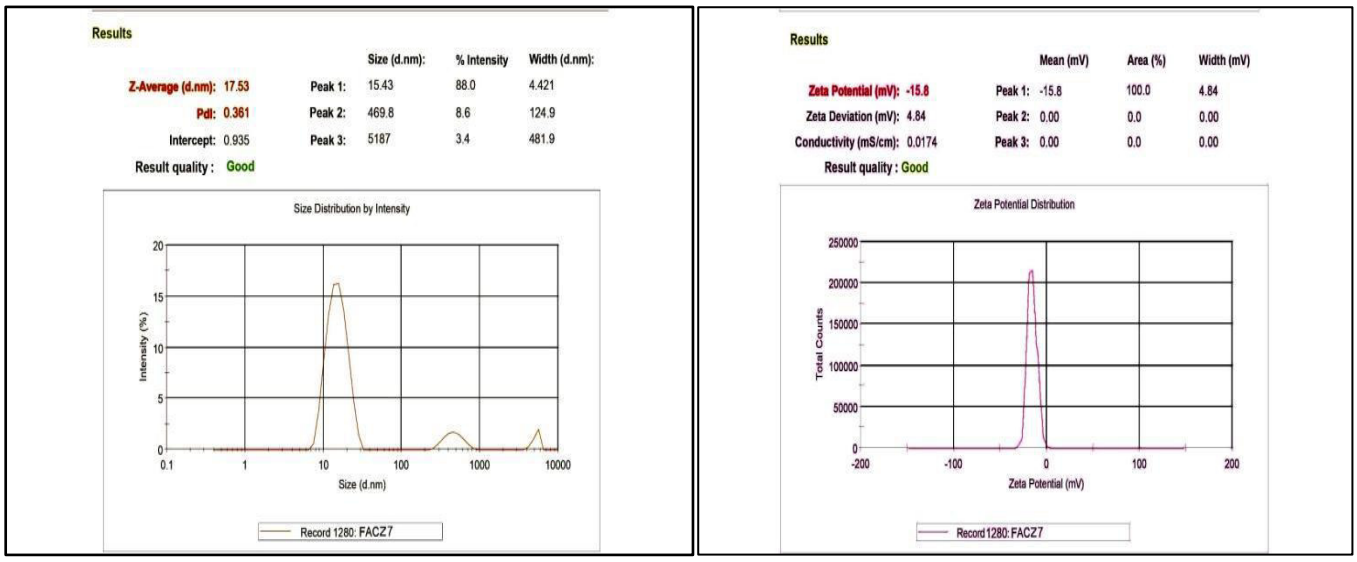
S. No.	Formulation code	Droplet Size (d.nm)	Polydispersity Index	Zeta Potential (mV)
1	FACZ2	16.23	0.140	-24.71
2	FACZ7	17.53	0.361	-15.8
3	FACZ20	21.18	0.175	-18.81
4	FACZ24	15.68	0.141	-15.5

d.nm = diameter in Nano Meter; mV = Milli Volts; (n=12); (p < 0.01)

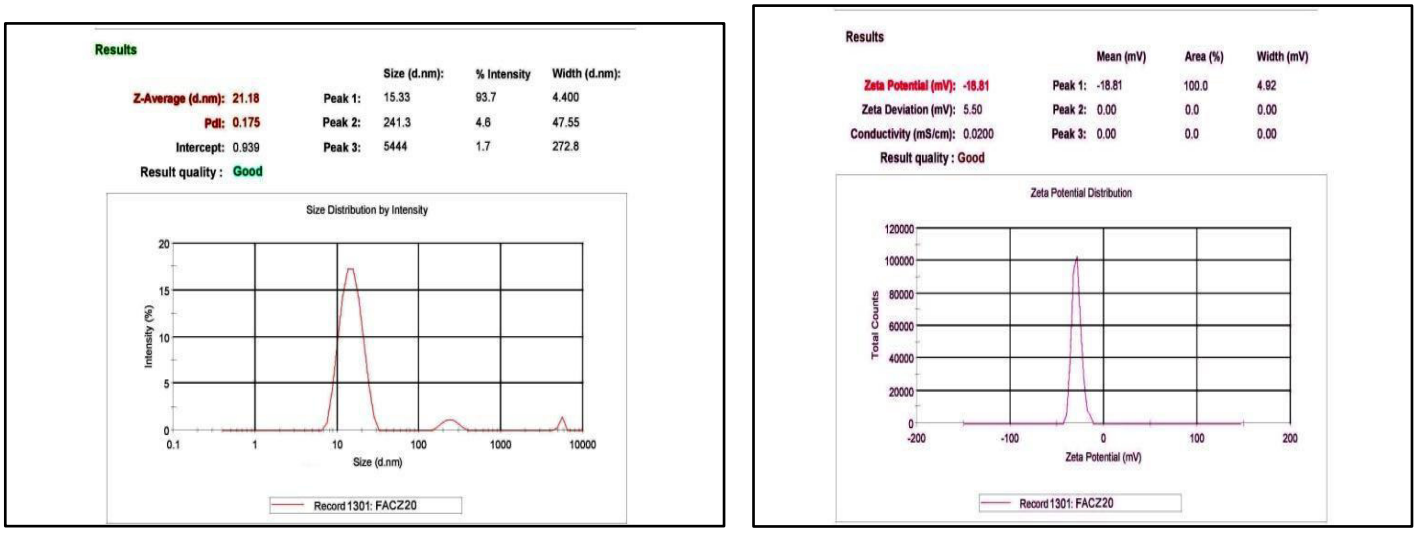
The data of top four formulations obtained from Malvern Zetasizer Nano ZS (Malvern, United Kingdom) were analyzed using Malvern Zetasizer software 7.13 for their droplet size, polydispersity index and zeta potential. The droplet size of formulations was in nanoform, the droplets were uniform as polydispersity index is less than 0.5 and zeta potential shows good repulsion force between droplets. The probability value (p) was less than 0.05 hence considered as statically significant.

FACZ2:

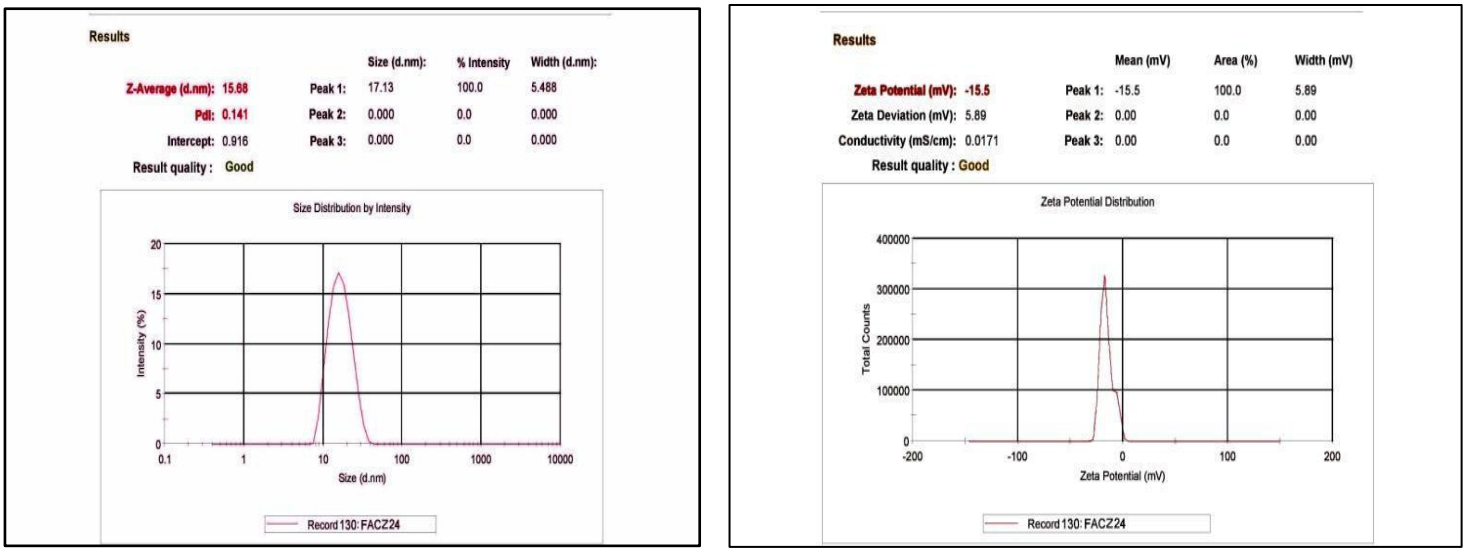




FACZ7:



FACZ20:



FACZ24:

Fig-5: Droplet Size & Zeta Potential Graphs

4.11 Transmission Electron Microscopy

To further confirm the nano-droplet formation of formulations, the morphology of the best selected NE formulations (FACZ2) was determined by TEM (Figure-6). The TEM provides a better measurement of particles as it is capable of calibrating point-to-point dimension. The distribution analysis of figure 4 shows the uniformity of particle size. The result obtained from TEM was in accordance with the result of the zeta sizer.

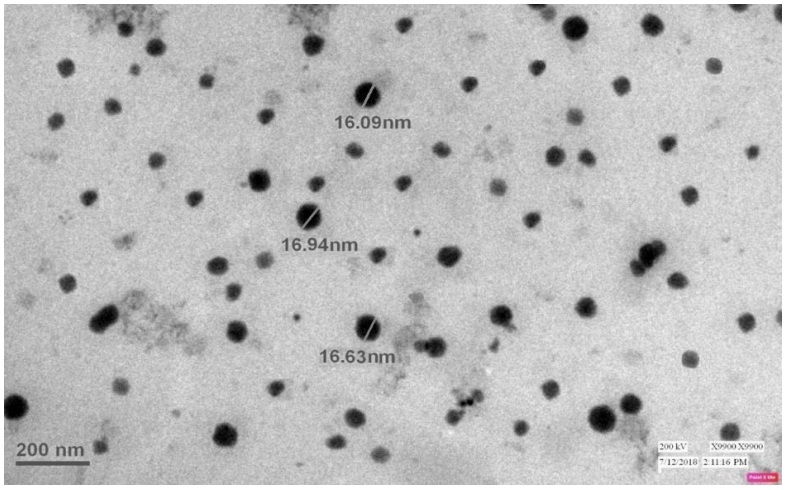


Fig-6: TEM image of optimized nanoemulsion FACZ2

The data obtained for Viscosity, pH, Refractive Index, Transmittance & Absorbance were presented as mean ± standard deviation (SD). The probability value (p) was less than 0.05 hence considered as statically significant.

5. DISCUSSION

The previous studies showed that oil in water (o/w) nanoemulsion are preferred with oil having high solubility of the drug. Due to the high solubility more amount of drug may be carried by a less amount of oil. Therefore, to achieve oil solubilization less surfactant concentration will be required, which makes nanoemulsion safe in terms of toxicity of the system.[30]In this study acetazolamide showed highest solubility in olive oil and the olive oil was selected for the preparation of nanoemulsion. The major problem with ocular drug delivery of nanoemulsion systems is that the surfactant and co-surfactant should be in a small amount as these may cause irritation to the eyes. So, for proper selection of surfactant, the solubility of the drug in surfactant becomes essential.[28] Accordingly, in this study it was focused that the amount of surfactant and co-surfactant should be minimum thus Tween-20 and Transcutol P were chosen as surfactant and co-surfactant respectively on the basis of their best solubility results of acetazolamide. Viscosity plays an important role in topical formulations; high viscous formulations may spread in less target areas as compare to less viscous formulations. On the other side less viscosity may lead to less contact time in case of ocular drug delivery.[31]So for ocular drugs the viscosity in the range of normal human tear fluid is preferred i.e. 4.4 to 8.3 cps.[32]During the thermodynamic stability test of drug loaded nanoemulsions, it was observed that formulations were less viscous i.e. 3.234 ± 0.063to 5.174 ± 0.023cps which leads to high drug release efficiency also the viscosity was alike to the viscosity of normal human tear fluids. The pH values of nano formulations were found in optimum range i.e. 6.922 ± 0.026 to 7.033 ± 0.012 as for ocular formulation the pH should falls in the desired range of 7.11±1.5 so that it should be safe for human eyes.[32]The refractive index (RI) of formulations were found closer towards RI of water i.e., 1.334 which shows the high-water content in formulations and more safe for human eyes in terms of toxicity of the system.[29] The result of particle size analysis confirms that the diameter of droplets in formulations varies between 15.68 to 21.18 nm which were in nano size range.[34]The Polydispersity index (PDI) valueless than 0.5 denotes the uniformity of droplet size in formulation.[35]The PDI values for all top four formulations exhibit the same as it ranges between 0.140 to 0.361. Also, the zeta potential of the formulation was found between -24.71 to -15.5 which showed good repulsion force between nanoparticles results in a good stability property of nano sized droplets in the aqueous phase.[36, 37] After analyzing top four formulations i.e., FACZ2, FACZ7, FACZ20, and FACZ24 which showed best drug release within 24 hrs. time frame, it was noticed that in all four formulations the water percentage varies between 37.11% to 44.13%, the formulations FACZ2, FACZ7 with S_{mix} ratio 1:1 had higher concentration of oil as compare to the other two formulations i.e., FACZ20 and FACZ24 with S_{mix} ratio 1:3. In other words we can say that the higher oil concentration requires higher surfactant concentration to achieve the desired results. The drug release pattern in these top four formulations found directly proportional with oil concentration.

6. CONCLUSION

As per our objective, we successfully prepared nanoemulsion by spontaneous phase inversion technique (surfactant particles that have a higher HLB value break their weak bonds with oil particles and form a strong bond with water particles). During phase inversion, the head of the surfactant attaches with water particles and the tail covers oil particles to form a surface layer around oil particles which were dispersed in the aqueous phase. The oil particles are loaded with drugs as the drug has good solubility in oil. The results showed the particle size of the nanoemulsion is in accordance with our work objective. Also, the optimized formulations were passed through a number of physicochemical characterization studies which represent the good stability of these formulations. The *in-vitro* drug release studies also fulfil our objectives ,after analyzing the result it can be concluded that topical acetazolamide loaded nanoemulsion are possible to make for effective treatment of glaucoma. Further, the *in-vivo* studies are also required to confirm the bioavailability, sustained release and effectiveness of the nanoemulsion formulation for the treatment of glaucoma.

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9. AUTHOR CONTRIBUTION STATEMENT

Professor Vijay Juyal and Neha Joshi conceptualized the study. Neha Joshi has done the experimental part under the guidance of Professor Vijay Juyal and Dr. Rajeshwar Kamal Kant Arya, Neha Joshi has drafted the manuscript supervised by Professor Vijay Juyal, Dr. Himanshu Joshi, Dr. Rajeshwar Kamal Kant Arya. All authors finally approved the manuscript for publication.

10. CONFLICT OF INTEREST

Conflict of interest declared none

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