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Poloxamer Coated Injectable Nanoparticles Of Azithromycin For Reduced Protein Binding And Enhanced Bioavailability.

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ABSTRACT

Azithromycin, a broad-spectrum macrolide antibiotic has drawback of poor solubility and high protein binding in blood circulation. The present research work was to reduce protein binding of AZT by poloxamer coating of its nanoparticles to enhance the bioavailability. Nanoparticles were prepared successfully by solvent evaporation method and were in the range from 497.8nm to 138.6nm. The Formulation, AZY F7 was considered optimum due to its ideal characteristics such as particle size of 138.6nm and Polydispersibility index of 0.553 ± 0.0022 to 1.159 ± 0.0050 , % drug content of $97.42\% \pm 0.081$. The Protein binding studies of percent drug bound values of pure drug, AZY F6 & AZY F7, the pure drug has high percent bound value of 83.7% in 10 mins and 47.8% in 60 mins. Whereas AZY F6 shows 24.6% & AZY F7 shows 14.1% in duration of 60 mins. This indicates reasonable reduction in protein binding of azithromycin poloxamer coated nanoparticles. However, AZY F7 showing lowest value of 14.1% in duration of 60 mins is considered as promising formulation. This has evidenced good micromeritic properties without any incompatibility evidenced by FTIR and DSC analysis.

Key words: Azithromycin, Poor solubility, Nanoparticles, Poloxamer coating, Reduction in protein binding.

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INTRODUCTION

The role of antibiotics is crucial in prevention and treatment of bacterial infections. They act through various mechanisms like inhibition of cell wall synthesis, disruption of cell membrane function, inhibition of nucleic acid synthesis, interference with protein synthesis, and inhibition of metabolic pathways [1]. Over the decades, a wide variety of antibiotics have been developed, each belonging to different structural and functional classes with specific mechanisms of action and antimicrobial spectra.

Azithromycin is a broad-spectrum macrolide antibiotic of azalide subclass. It is derived from erythromycin by the insertion of a methyl-substituted nitrogen atom into the lactone ring, which improves acid stability and broadens its antibacterial spectrum. Azithromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, thereby preventing translocation during peptide chain elongation. It is primarily bacteriostatic, though it can exhibit bactericidal activity at higher concentrations [2].

Azithromycin is extensively used in clinical practice for the treatment of respiratory tract infections, otitis media, skin and soft tissue infections, and sexually transmitted diseases. It exhibits several favorable pharmacokinetic characteristics, including extensive tissue penetration and a long elimination half-life of 50 to 70 hours. However, despite these advantages, its clinical efficacy is limited by several formulation-related and pharmacokinetic challenges such as poor aqueous solubility (less than 1 mg/mL) and high plasma protein binding ranging between 7% and 51%. Due to these two reasons, AZT has limited dissolution rate and bioavailability. High binding affinity reduces the fraction of free, pharmacologically active drug available for antimicrobial activity. The drug is also available injection form also and chances for poor availability due to the two reasons of poor aqueous solubility and high protein binding.

Therefore, this present work is aimed at two objectives including preparation of AZT as nanoparticles and to carry out poloxamer coating of nanoparticles with Poloxamer 188 to reduces protein binding in an attempt to enhance bioavailability so that this approach is ideal for making AZT for parenteral administration with optimum bioavailability [3].

MATERIALS AND METHODS

In present work, azithromycin is first converted as nanoparticles by solvent evaporation method and selected nanoparticles are coated with poloxamer and finally the poloxamer coated nanoparticles are evaluated for reduced protein binding hence to possess enhanced bioavailability [4].

Preparation of Azithromycin Nanoparticles

Nanoparticles of azithromycin were prepared by the solvent evaporation method [5, 6]. Various trials containing 500 mg of azithromycin were tried and nanoparticles formulations, **Viz.**, AZY F1 to AZY F7 produced with clear yield are presented in Table 1. During preparation, accurately weighed azithromycin (500 mg) and hydroxypropyl methylcellulose (HPMC) were dissolved in ethanol to form the organic phase. Separately, polyvinyl alcohol (PVA) was dissolved in distilled water to obtain the aqueous phase. The organic phase was added dropwise into the aqueous phase under magnetic stirring (1000-1500 rpm) for 3-4 hours to facilitate solvent evaporation and nanoparticle formation. The obtained nanoparticles (AZY F1-AZY F7) were collected and used for next step.

Table 1: Ingredients of nanoparticles of azithromycin

Formulations	AZY (mg)	HPMC (mg)	PVA (mg)	Ethanol (ml)
AZY F1	500	450	400	10
AZY F2	500	350	350	10
AZY F3	500	320	300	10
AZY F4	500	300	250	10
AZY F5	500	250	220	10
AZY F6	500	220	200	10
AZY F7	500	200	170	10

Evaluation of azithromycin nanoparticles

Determination of Particle size

The prepared nanosuspension were evaluated for particle size using a Zeta sizer (Horiba). Particle size refers to the dimensions of individual particles in a substance. It can be measured in various ways, including diameter, volume, surface area and can be represented as an average or a distribution of sizes. A suspension of formulated azithromycin nanoparticles was prepared by adding it to 10 ml of distilled water. Ensured the particles are well-dispersed to avoid aggregation, which can affect the results.

Poly disperse index

The PDI is obtained along with the particle size to characterize the size distribution of drug particles or carriers. **PDI value of 1** indicates a mono disperse sample where all particles or molecules are of uniform size or weight. **PDI > 1** indicates a poly disperse sample with a broader distribution of sizes or molecular weights. The larger the PDI, the more heterogeneous the sample.

Percent drug content estimation

Accurately weighed quantity of lyophilized nanoparticle powder equivalent to 10 mg of Azithromycin was transferred into a 100 ml volumetric flask [7]. The powder was dissolved in a small quantity of ethanol and sonicated for 15 minutes to ensure complete drug extraction. The solution was then made up to 100 ml with ethanol to obtain a stock solution of 100 µg/ml. From the stock solution, serial dilutions were prepared to obtain concentrations of 2, 4, 6, 8, 10 and 12 µg/ml. The absorbance of each solution was measured at $\lambda_{\max}$ of 210 nm using a UV-Visible spectrophotometer, with ethanol as blank. A calibration curve was constructed by plotting absorbance versus concentration. The drug content present in the formulations was determined from the calibration curve, and the percentage drug content was calculated using the following formula:

$$\% \text{ Drug content} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard at the same dilution}} \times 100$$

Entrapment efficiency:

Nanosuspension was placed in the centrifuge tubes and supernatant was separated using high speed ultracentrifuge at 18000 rpm for 30 mins [8]. Separation of untrapped drug using a centrifuge and the clear supernatant obtained was analyzed spectrophotometrically for the presence of free drug at $\lambda_{\max}$ of 210 nm and % entrapment efficiency was calculated by using the following formula.

$$\% \text{ EE} = \frac{\text{Total amount of drug} - \text{free drug}}{\text{Total amount of drug}} \times 100$$

Preparation of poloxamer-coated nanoparticles of azithromycin:

Since all the trial formulations of nanoparticles of AZT are produced in nanoparticles range, they were all subjected to poloxamer coating. For the preparation of Poloxamer-coated nanoparticles, the previously obtained azithromycin nanoparticles were suspended in an aqueous solution of Poloxamer 188 (200 mg)⁹. The suspension was subjected to gentle stirring for 2 hours to allow uniform adsorption of the Poloxamer on the nanoparticle surface. The coated nanoparticles were then separated by centrifugation at 15000 rpm for 30 min, washed with distilled water to remove un adsorbed Poloxamer, and finally lyophilized to obtain poloxamer-coated azithromycin nanoparticles in dried powder form. The product was subjected to evaluation studies.

Protein Binding Studies Of Azithromycin Nanoparticles

Reagents

Preparation of Phosphate Buffer (pH 7.4)

Phosphate buffer of pH 7.4 was prepared according to IP 2010 by dissolving 2.38 g of disodium hydrogen phosphate 0.19 g of potassium dihydrogen phosphate 8 g of sodium chloride in 1000 ml of distilled water. The solution was mixed thoroughly and used as the buffer medium for the study

Preparation of 40 μ m Egg Albumin Solution

Egg albumin flakes 0.144 gm were dissolved in 50 ml of the prepared phosphate buffer. The mixture was shaken well and kept aside for further use.

Protein Binding studies of azithromycin

Poloxamer coated azithromycin nanoparticles equivalent of 500 mg of drug were accurately weighed and dissolved in prepared egg albumin solution. A boiling tube with both ends open was taken and one end was tied with a piece of egg membrane. The Azithromycin egg albumin solution was poured into the tube. The other end of the tube was kept in slight contact with phosphate buffer (pH 7.4) contained in a beaker, and the setup was placed in contact with sand to hold it in place. 1 ml samples were withdrawn from the buffer in the beaker at 10, 20, 30, 40, 50, and 60 minutes using a pipette. After each withdrawal, an equal volume of fresh phosphate buffer was replaced to maintain the volume. The collected samples were analyzed using a UV spectrophotometer at λ_{max} of 210 nm.

Assessment of poloxamer coated azithromycin nanoparticles

Flow properties

Since formulation, AZY F7 has lowest percentage of protein binding, the flow properties are evaluated for this formulation by determining the bulk density, tapped density, Carr's Index and Hausner's ratio [10].

Bulk density and tapped density

Bulk density and tapped density of nanoparticles was determined by 10ml graduated measuring cylinder. The parameters selected to study flow properties determined by following equations:

$$\text{Bulk density} = \frac{\text{Mass}}{\text{Volume}}$$

$$\text{Tapped density} = \frac{\text{Mass}}{\text{Tapped density}}$$

Drug excipient interaction studies

Compatibility of the drug with excipients used in the formulation was assessed by Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry for promising nanoparticles AZY F7 by comparing with pure drug data.

Fourier Transform Infrared Spectroscopy (FTIR)

In order to check the integrity (compatibility) of drug with excipients was done by FTIR (Bruker, Japan) studies using KBr disc method. The samples were thoroughly blended with dry powdered potassium bromide crystals, compressed to form a disc, placed in a sample holder and then the spectrum was recorded from 4000 to 400 cm^{-1} . The IR spectrum of the pure drug compared with the IR spectrum of the azithromycin nanoparticles compound.

Differential Scanning Calorimetry

In order to check the thermal behavior of the drug, Differential Scanning Calorimetry (DSC) studies were carried out using a DSC instrument, (METTLER). About 2-5 mg of azithromycin nanoparticles sample was accurately weighed and sealed in an aluminum DSC pan. An empty sealed pan was used as reference. The sample was heated from 30°C to 300°C at a rate of 10°C/min under a nitrogen purge (flow rate: 40 ml/min). The thermogram was recorded, and the thermal events such as melting point and possible degradation were observed.

RESULTS AND DISCUSSION

Particle size of azithromycin loaded nanoparticles

Particle size of prepared azithromycin loaded nanoparticles, AZY F1 to AZY F7 in the selected aqueous solution was determined by dynamic light scattering and the particle size was found to be in the range of 138.6 nm to 497.8 nm as shown in Table 2. Relevant figures obtained are presented in fig. 1 to fig. 7. As per the results it is observed that the present method is successful in producing the drug in the form of nanoparticles.

Poly disperse index data ranged from 0.553 ± 0.0022 to 1.159 ± 0.0050 is also in acceptable of the form range.

Table 2: Particle size data of azithromycin loaded nanoparticles

S.no	Formulation	Particle size (nm) mean (n)±S. D (n=3)	PDI mean(n)±S. D (n=3)
1	AZY F1	497.8±1.228	0.754±0.0015
2	AZY F2	387.3±1.266	0.710±0.0316
3	AZY F3	378.5±0.863	0.911±0.0024
4	AZY F4	412.8±2.074	0.767±0.0024
5	AZY F5	346.6±2.013	0.553±0.0022
6	AZY F6	186.7±1.550	1.159±0.0050
7	AZY F7	138.6±3.318	0.888±0.0022

Figure 1: Particle size of AZY F1

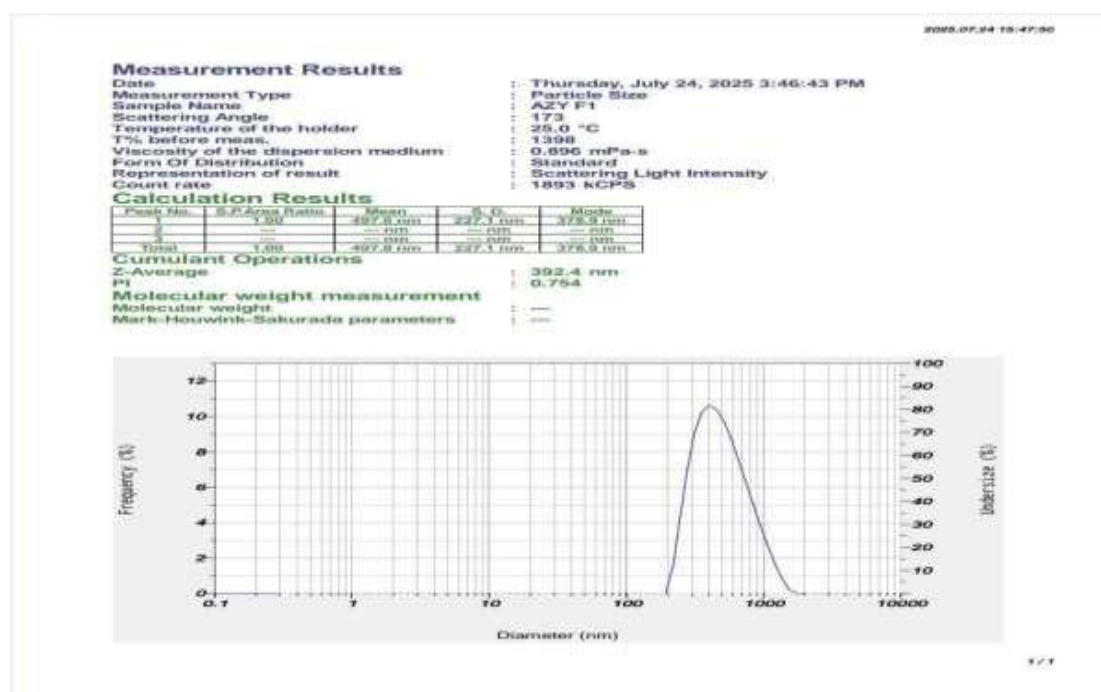


Figure 2: Particle size of AZY F2

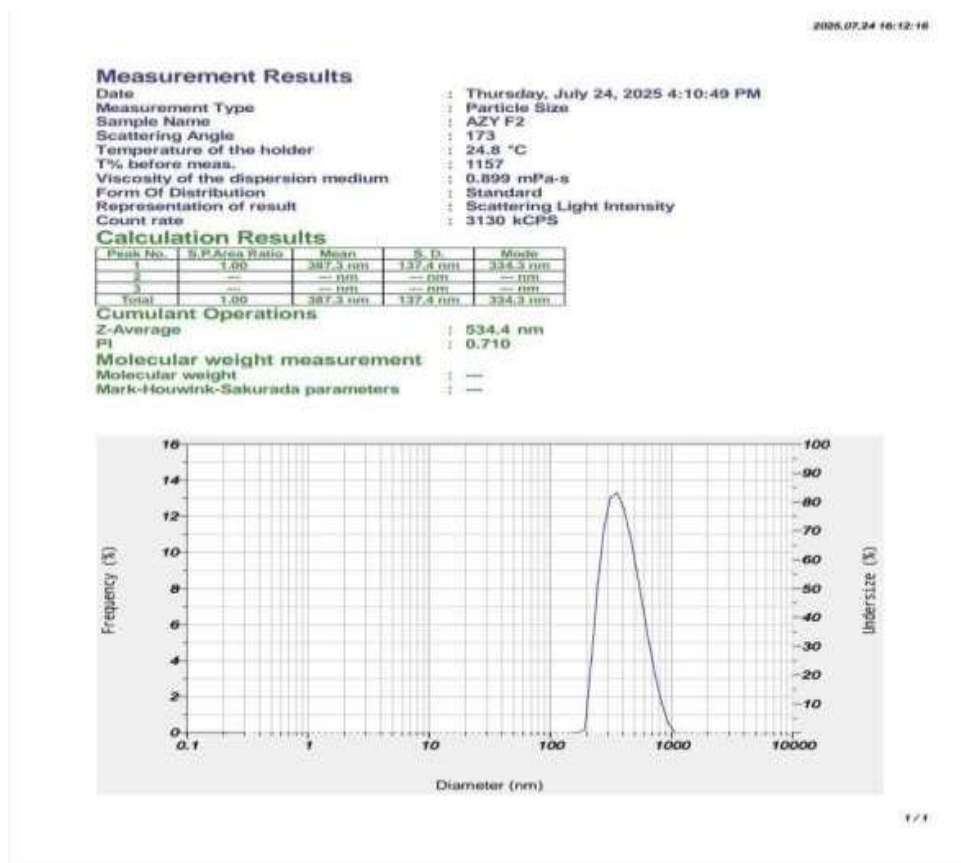


Figure 3: Particle size of AZY F3

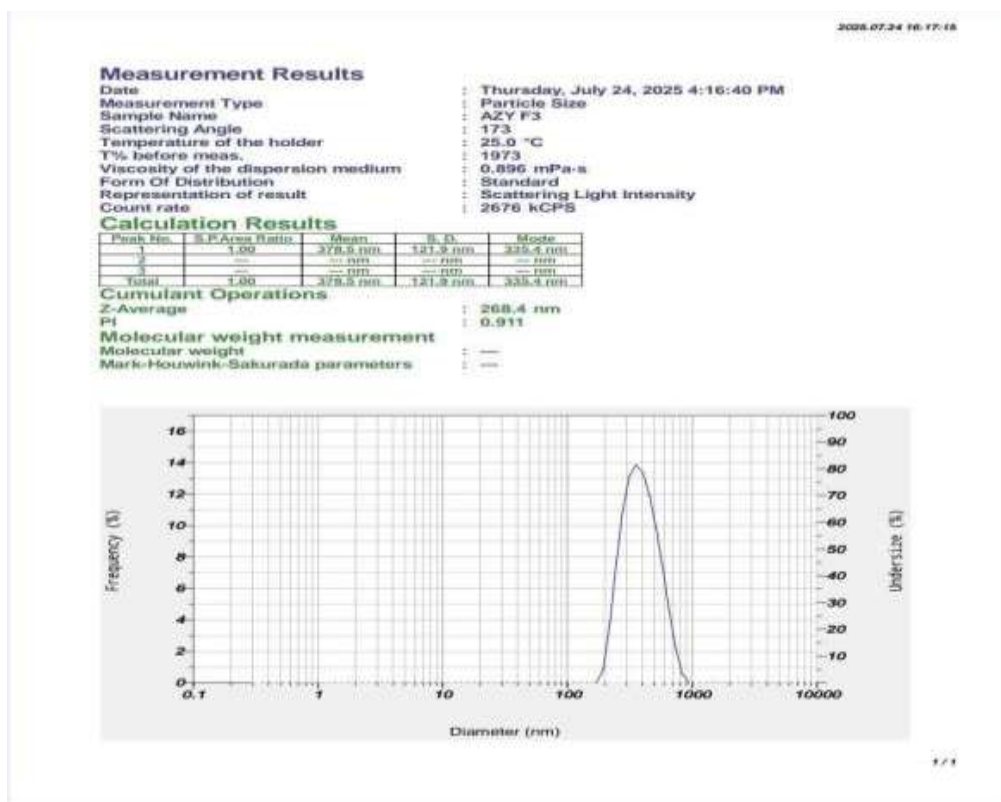


Figure 4: Particle size of AZY F4

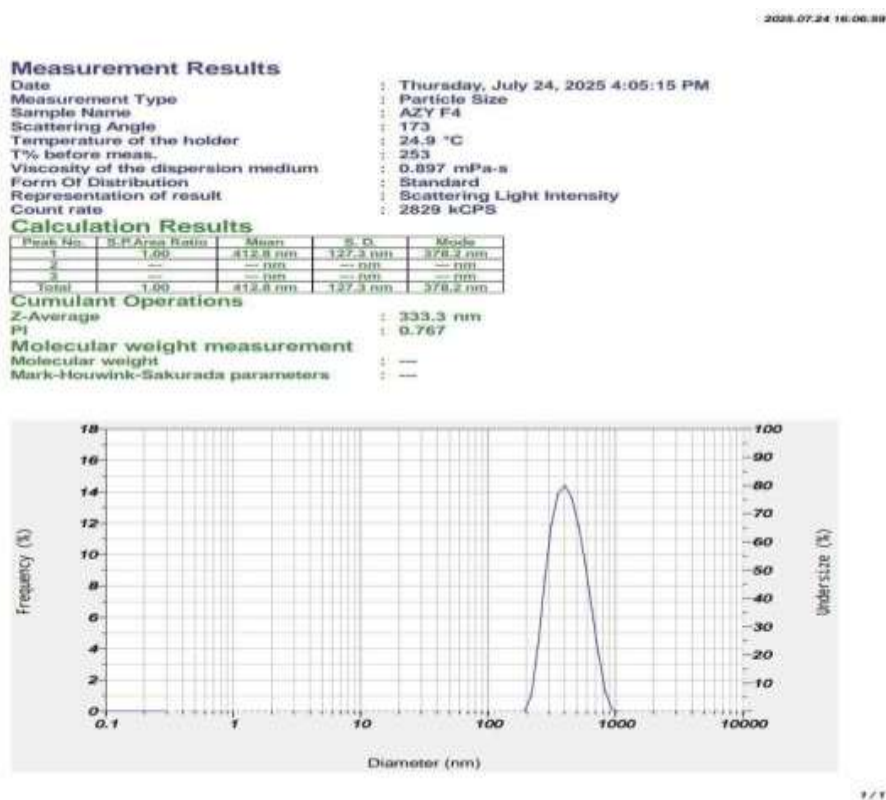


Figure 5: Particle size of AZY F5

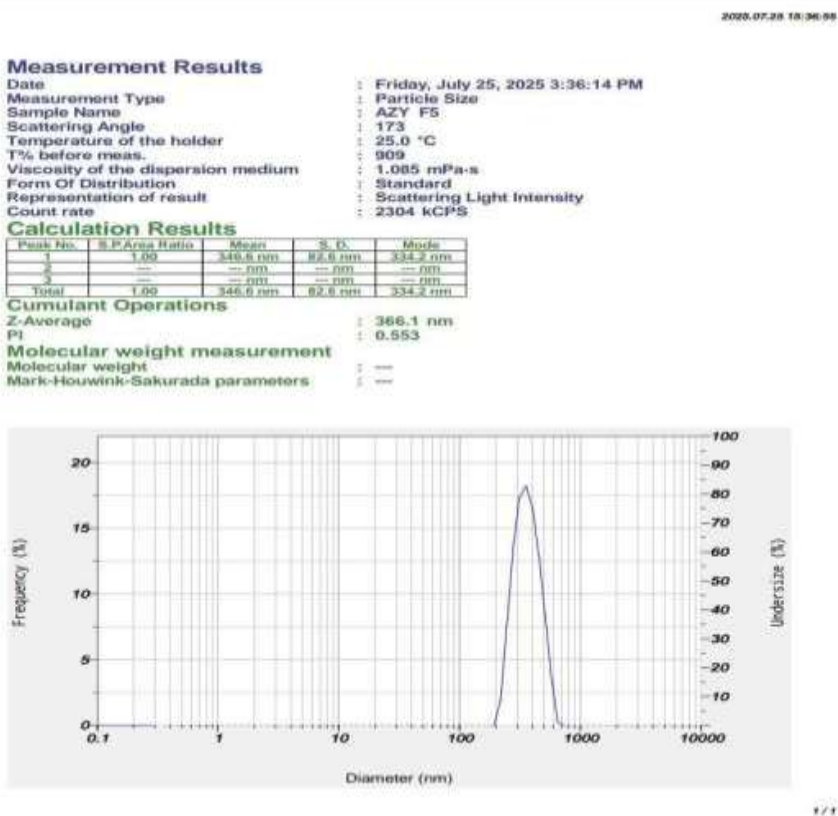


Figure 6: Particle size of AZY F6

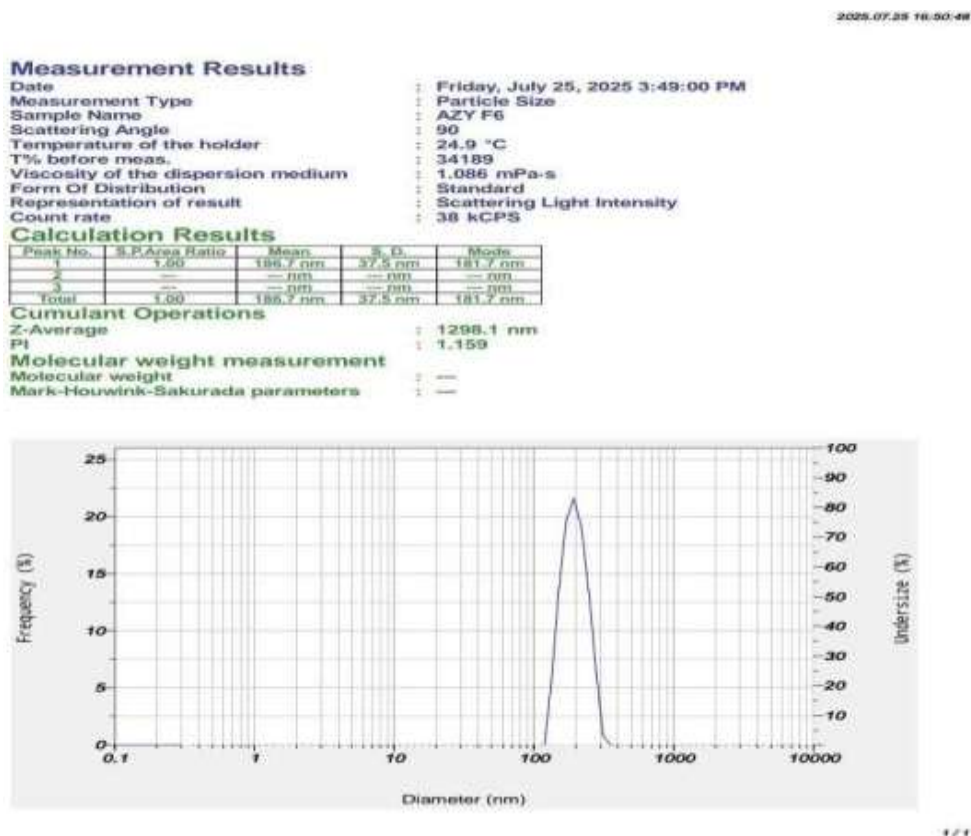
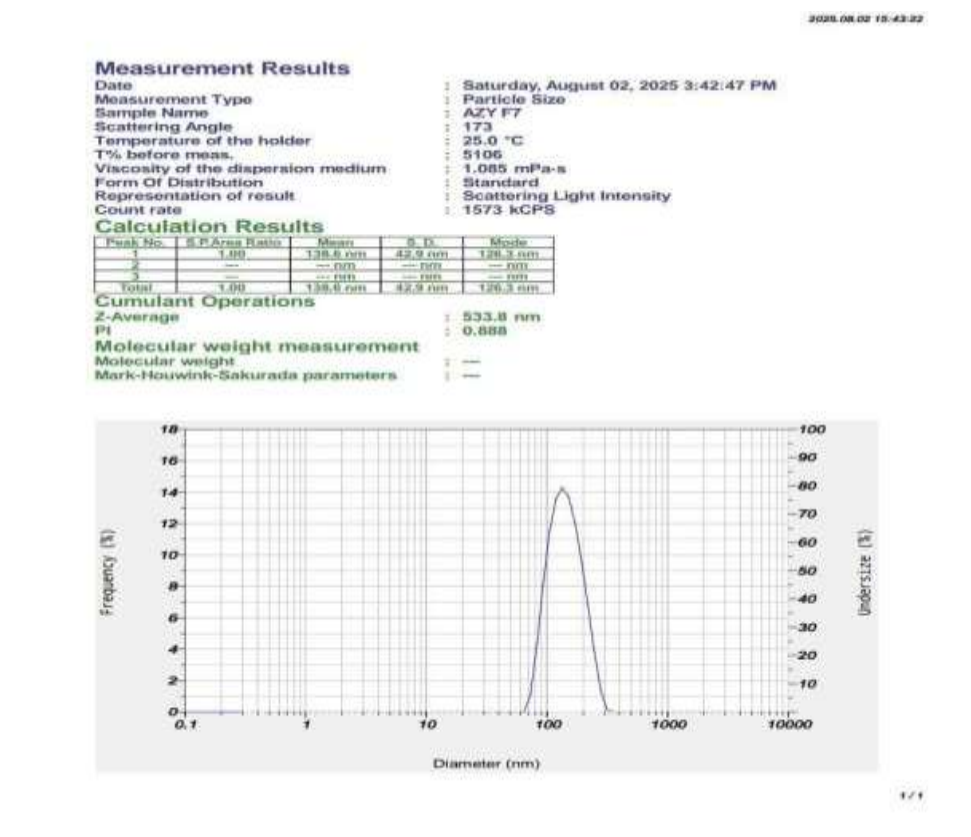


Figure 7: Particle size of AZY F7



Percent drug content

Percent drug content values of poloxamer coated nanoparticles of azithromycin are shown in Table 3. The values ranging from $80.5\% \pm 0.212$ to $97.42\% \pm 0.081$. and are found satisfactory.

Table 3: Percent drug content values of azithromycin loaded nanoparticles

Formulation	%Drug Content mean(n)±S. D (n=3)
AZY F1	80.5 ± 0.212
AZY F2	82.0 ± 0.208
AZY F3	91.75 ± 0.104
AZY F4	83.75 ± 0.160
AZY F5	92.25 ± 0.175
AZY F6	85.25 ± 0.071
AZY F7	97.42 ± 0.081

Entrapment Efficiency

The percent entrapment efficiency of prepared poloxamer coated nanoparticles of azithromycin. The following Table 4 shows the % entrapment efficiency of the 7 formulations. The ranged from $75.1\% \pm 0.360$ to $89.5\% \pm 0.264$ are found acceptable.

Table 4: % Entrapment Efficiency of azithromycin loaded nanoparticles

S.no	Formulation	% EE [mean(n)±S.D (n=3)]
1	AZY F1	78.2 ± 0.412
2	AZY F2	81.4 ± 0.412
3	AZY F3	85.9 ± 0.254
4	AZY F4	75.1 ± 0.360
5	AZY F5	88.0 ± 0.2
6	AZY F6	83.9 ± 0.212
7	AZY F7	89.5 ± 0.264

Protein binding studies of azithromycin nanoparticles:

When the particle size is observed in Table 2, the two formulations AZY F6 and AZY F7 are having low particle size of 186.7 and 138.6 and considering as ideal sizes these two formulations are subjected to protein binding studies.

The results of percent drug bound values of pure drug, AZY F6 & AZY F7 are shown in Table 5, 6, 7. When the results are observed table, the pure drug has high percent bound value of 83.7% in 10 mints and 47.8% in 60 mints. Whereas AZY F6 shows 24.6% & AZY F7 shows 14.1% in duration of 60 mins. This indicates reasonable reduction in protein binding of azithromycin poloxamer coated nanoparticles. However, AZY F7 showing lowest value of 14.1% in duration of 60 mins is considered as promising formulation.

Table 5: Protein binding ofAZY F6 nanoparticles

Time (min)	Absorbance	Concentration	Drug unbound	Drug bound	% Drug bound
10	0.2747	6.86	0.183	0.627	77.4%
20	0.4998	12.49	0.333	0.477	58.8%
30	0.5913	14.78	0.394	0.416	51.3%
40	0.7056	17.64	0.471	0.339	41.8%
50	0.8280	20.7	0.552	0.258	31.9%
60	0.9148	22.87	0.610	0.2	24.6%

Table 6: Protein binding ofAZY F7 nanoparticles

Time (min)	Absorbance	Concentration	Drug unbound	Drug bound	% Drug bound
10	0.5234	13.08	0.8733	0.9227	51.3%
20	0.6073	15.18	1.0135	0.7825	43.5%
30	0.7003	17.50	1.1684	0.6276	34.9%
40	0.8060	20.15	1.3453	0.4507	25%
50	0.8948	22.37	1.4935	0.3025	16.8%
60	0.9241	23.10	1.5423	0.2537	14.1%

Table 7: Comparison of protein binding values for pure drug azithromycin and poloxamer coated nanoparticles

Time (min)	% Drug bound of pure drug	% Drug bound of AZY F6	% Drug bound of AZY F7
10	83.7%	77.4%	51.3%
20	77.2%	58.8%	43.5%
30	71.6%	51.3%	34.9%
40	59.8%	41.8%	25%
50	52.5%	31.9%	16.8%
60	47.8%	24.6%	14.1%

Flow properties of the poloxamer coated nanoparticles were carried as per the procedures described in materials and methods shows a (4.7.2). The following Table 8 shows the values of various flow properties for the optimized formulations. These results suggests that micromeritic properties of optimized formulations were within the limit and they exhibited good flow properties. The values of Carr's index of 12.7% of indicates that the prepared nanoparticles are free flowing in nature.

Table 8: Flow properties of azithromycin loaded nanoparticles

Parameter	Result	Category
Bulk density	0.48 g/ml	Fairly good flow
Tapped density	0.55 g/ml	Used for compressibility
Carr's index	12.7%	Good ($\leq 15\%$ indicates good flow)
Hausner's ratio	1.15%	Good ($\leq 1.25\%$ indicates good flow)

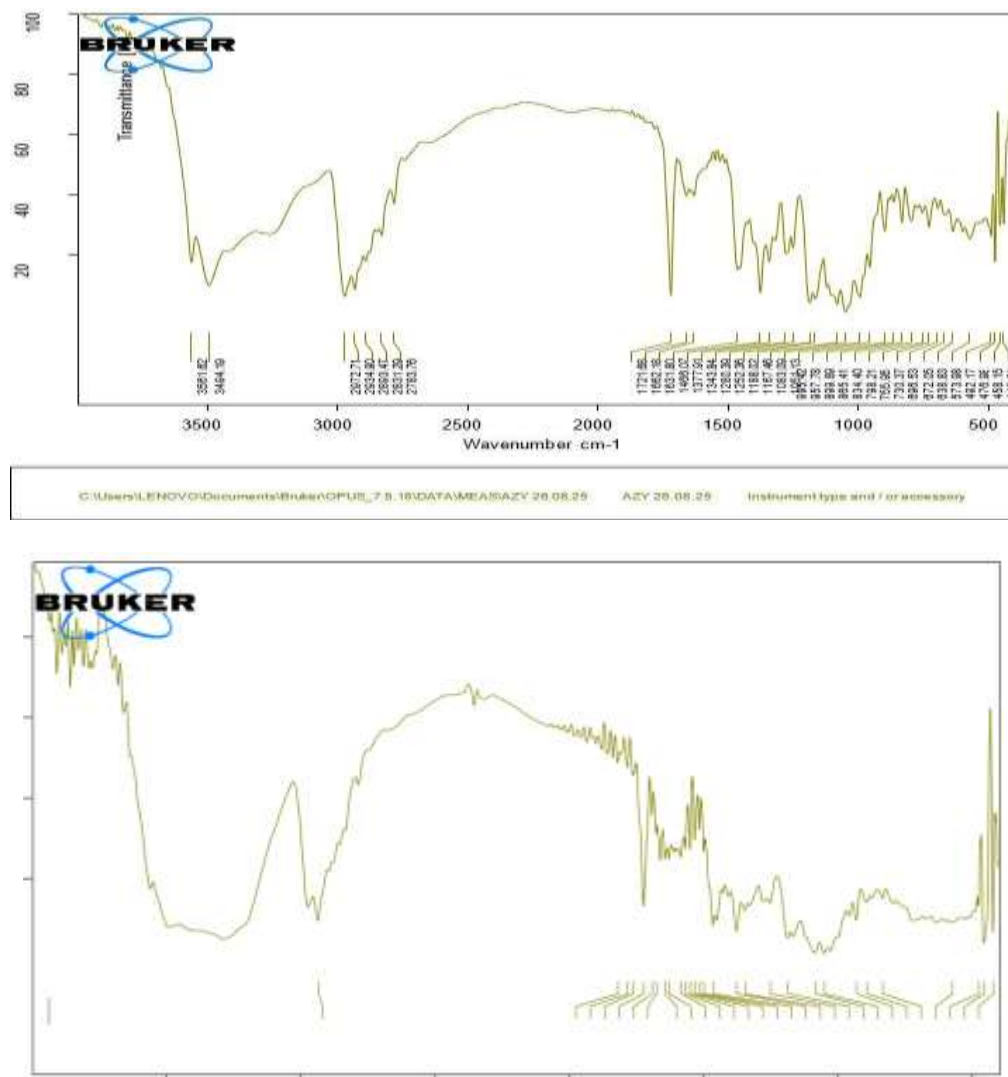
Drug Excipient Interaction Studies

FTIR Spectrum analysis

FTIR spectrum of physical mixtures of azithromycin nanoparticles formulation of AZY F7 showed in Fig & Table showed absorption peaks of its functional groups at 3557.79 cm^{-1} due to O-H stretch, 3285.04 cm^{-1} due to N-H stretch, 2971.35 cm^{-1} due to C-H stretch, 1628.94 cm^{-1} due to

C=O stretch, 1465.57 cm^{-1} due to CH_3 bending, 1187.56 cm^{-1} due to C-O stretch. FTIR spectrum of pure drug azithromycin in Fig & Table showed absorption peaks of its functional groups at 3561.62 cm^{-1} due to O-H stretch, 3494.19 cm^{-1} due to N-H stretch, 2972.71 cm^{-1} due to C-H stretch, 1662.18 cm^{-1} due to C=O stretch, 1466.07 cm^{-1} due to CH_3 bending, 1252.36 cm^{-1} due to C-O stretch. It is observed there is a slight modification in the peaks indicating the absence of any interaction between the azithromycin with polymer used for preparing poloxamer coated nanoparticles formulation.

Figure 8: FTIR Spectrum of pure drug azithromycin



Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) thermogram of azithromycin nanoparticles is shown in Fig and Table. The thermogram exhibited a sharp endothermic peak at 130.25°C, which corresponds to the melting point of reduced enthalpy, partial amorphization due to lyophilization. The onset temperature of melting was observed at 119.12°C, and the end set at 136.04°C. The thermogram of pure drug azithromycin shows in Fig and Table exhibit a sharp endothermic peak at 130.36°C. It is observed there is a slight modification when compared with the pure drug.

Figure 3: DSC thermogram of pure drug azithromycin

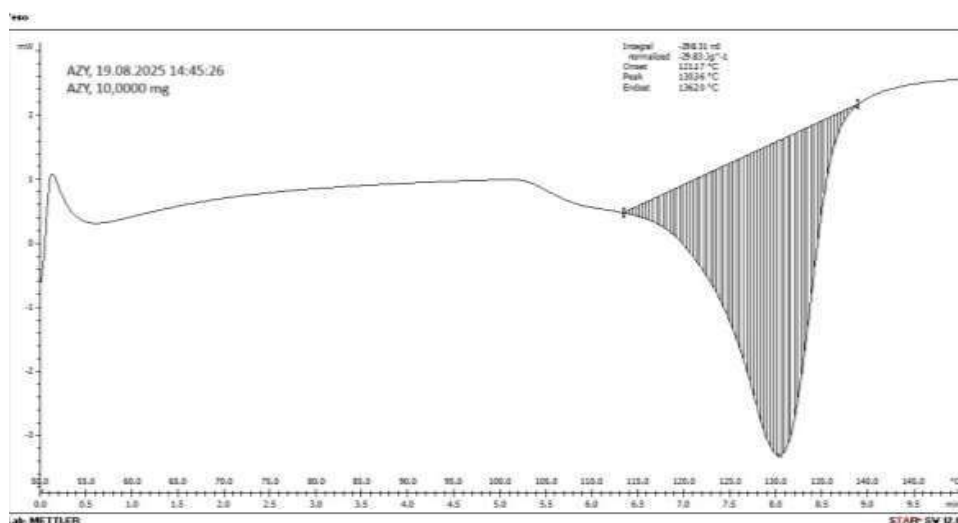
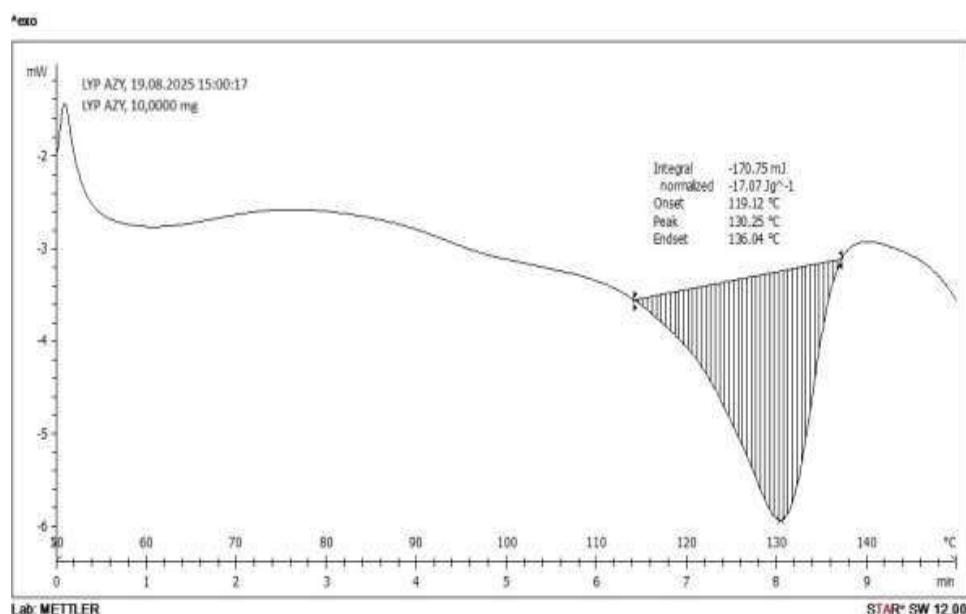


Figure 13: DSC thermogram of azithromycin nanoparticles



CONCLUSION

From the present study it is concluded that nanoparticles of azithromycin can be developed successfully by solvent evaporation technique using HPMC as polymer. In turn poloxamer coated nanoparticles evinced reduced protein binding from 14.1% against 83.7% for pure drug. Hence the studies are successful in proving reduced protein binding characteristics of poloxamer coated nanoparticles of azithromycin and showing ability to enhance bioavailability of azithromycin.

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