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FORMULATION AND IN VITRO CHARACTERIZATION OF AMBRISENTAN NANOSUSPENSION USING SOLVENT EMULSIFICATION METHOD

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ABSTRACT
Ambrisentan is an orally active selective type A endothelin receptor antagonist indicated for the treatment of pulmonary arterial hypertension. The present investigation aimed at enhancing the oral bioavailability of Ambrisentan by improving its solubility and dissolution rate by preparing nanosuspensions. The nanosuspensions of Ambrisentan were prepared using emulsification solvent evaporation method. Various formulation as well as process parameters were optimized in order to achieve desirable size and saturation solubility. Characterization of the prepared nanosuspension was done with respect to particle size, zeta potential, saturation solubility, dissolution rate, morphology study (SEM), in-vitro dissolution study. The results indicated that increase in the stabilizer concentration of PEG 6000 shows 99.65% of drug release, so the formulations prepared by using PEG 6000 releases more drug release at the end of 30mins than the other stabilizers and follows first order kinetics. Nanosuspension seems to be a promising approach for bioavailability enhancement because of the simple method of its preparation and its universal applicability.
Keywords: Ambrisentan, PVP K30, zeta potential, SEM, solubility.

INTRODUCTION

Nanotechnology is a pertinent part of a more extensive territory of nanoscience(Sundar V.D, 2019) associated with these conventional approaches for solubility dissolution and bioavailability enhancement(Afrin.K,2019). More than 40% of the compounds being developed by the pharmaceutical industry are poorly water soluble or “insoluble” in water (Kiran.G.Sonkamble, 2021) and to overcome the problem of poor aqueous solubility and slow dissolution rate many approaches have been used such as particle size reduction, pH adjustment, complexation with cyclodextrins, salt formation, solid dispersion, use of surfactants, nanoparticles, use of co-solvents, use of polymorphs etc(Nakarani.M, 2010) .All those techniques have their own advantages and drawbacks(Bhalekar M R,2014). Nanotechnology can be used to improve the solubility as well as the

bioavailability of poorly soluble drugs. Reduction of the particles to nanometer range(Mastiholimath VS 2020) leads to the enhanced dissolution rate and increased surface area(Dr.Yasmin begum, 2009). These can be used to enhance the solubility of drugs that are poorly soluble in water(Manishaanjane, 2018). Hence particle size reduction up to several nanometres (Suvarna S ,2020) may prove to be a suitable method in order to enhance the solubility with the least possible limitations(K.V.gopaiah, 2021). Nanosuspensions can be prepared by a variety of techniques that are wet milling, high pressure homogenization, spray drying, solvent precipitation, supercritical fluid technology(Chotai et al, 2015) The nanosuspension drug delivery system can be employed as a liquid dosage form or transformed into solid dosage form such as powder, tablet, pellet, capsule, and film dosage forms (Patil O A ,2018). Thus, nanosuspension can be safely administrated by a variety of routes including oral, intravenous, ocular, dermal, pulmonary etc(Debjit Bhowmik, 2013).

Ambrisentan is an orally active selective type A endothelin receptor antagonist indicated for the treatment of pulmonary arterial hypertension. Endothelin-1 (ET-1) is an endogenous peptide that acts on the endothelin type A (ETA) and endothelin type B (ETB) receptors in vascular smooth muscle and endothelium. ETA-mediated actions include vasoconstriction and cell proliferation, whereas ETB predominantly mediates vasodilation, anti-proliferation, and ET-1 clearance. In patients with pulmonary arterial hypertension, ET-1 levels are increased and correlate with increased right arterial pressure and severity of disease. Ambrisentan is one of several newly developed vasodilator drugs that selectively target the endothelin type A (ETA) receptor, inhibiting its action and preventing vasoconstriction. Selective inhibition of the ETA receptor prevents phospholipase C-mediated vasoconstriction and protein kinase C-mediated cell proliferation. Endothelin type B (ETB) receptor function is not significantly inhibited, and nitric oxide and prostacyclin production, cyclic GMP- and cyclic AMP-mediated vasodilation, and endothelin-1 (ET-1) clearance is preserved(Michael G Risbano, 2017). The present study is aimed to formulate and evaluate Ambrisentan oral nanosuspension to improve the bioavailability of the drug by using sovent evaporation method.

MATERIALS AND METHODS

Ambrisentan of pharma grade was obtained from BMR chemicals ,Hyderabad. Urea was obtained from Rankem chemicals, Mumbai. Poly ethylene glycol (PEG) 6000 was obtained from Rankem chemicals Mumbai. Sodium lauryl sulphate and poly vinyl pyrrolidone ,PVP K 30 was obtained from Rankem chemicals, Mumbai. Methanol was obtained from Narmada chemicals, Hyderabad.

PRE-FORMULATION STUDIES:

Prior to the development of dosage form, it is essential that certain fundamental physical and chemical properties of the drug molecule alone and when combined with excipients are determined. This first learning phase is known as pre-formulation. The overall objective of the pre-formulation is to generate information useful to the formulator in developing stable and bioavailable dosage forms which can be mass produced. The goals of pre-formulation studies are:

- To evaluate the drug substance analytically and determine its necessary characteristics
- To establish its compatibility with different excipients.

IDENTIFICATION OF PURE DRUG:

1. Melting Point:

The temperature at which the first particle of the substance completely melts is regarded as melting point of the substance. The temperature at which the first particle starts to melt and last particle completely melts is regarded as the range of melting point.

2. Solubility studies:

Solubility of Ambrisentan was determined in Methanol, Ethanol, pH 1.2, pH 6.8 and pH 7.4 phosphate buffers. Solubility studies were performed by taking excess amount of Ambrisentan in different beakers containing different solvents. The mixtures were shaken for 48 hrs in rotary shaker. The solutions were centrifuged for 10mins at 1000 rpm and supernatant were analyzed at 262 nm by using UV Spectrophotometry.

3. Drug-Excipient Interactions Studies:

There is always possibility of drug- excipient interaction in any formulation due to their intimate contact. The technique employed in this study is IR spectroscopy. IR spectroscopy is one of the most powerful analytical technique, which offers possibility of chemical identification. The IR spectra was obtained by KBr pellet method. (Perkin-Elmer series 1615 FTIR Spectrometer)(Ibrahim et al, 2019).

METHOD OF PREPARATION OF NANOSUSPENSION:

Preparation of Ambrisentan Nanosuspension by Emulsification solvent evaporation method:

Nanosuspension was prepared by the Emulsification solvent evaporation technique. Ambrisentan was dissolved in methanol at room temperature (organic phase). This solution is followed by its emulsification into water containing different stabilizers of PVP K30, SLS, PEG 6000 and Urea maintained at room temperature. Addition of organic solvents by means of a syringe positioned with the needle directly into stabilizer containing water, and subsequently stirred on magnetic stirrer to allow the volatile solvent to evaporate. Evaporation leads to precipitation of the drug(Kiran G ,2021).

Table -1: Composition of Nanosuspension of Ambrisentan

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	**F12**
Ambrisentan	80	80	80	80	80	80	80	80	80	80	80	**80**
Urea	25	50	75	100	--	--	--	--	--	--	--	**--**
PVP K-30	--	--	--	--	25	50	75	100	--	--	--	**--**
PEG 6000	--	--	--	--	--	--	--	--	25	50	75	**100**
SLS	10	10	10	10	10	10	10	10	10	10	10	**10**
Methanol	5	5	5	5	5	5	5	5	5	5	5	**5**
Water (ml)	40	40	40	40	40	40	40	40	40	40	40	**40**

EVALUATION PARAMETERS OF NANOSUSPENSION AMBRISENTAN:

The Nanosuspension was evaluated for various parameters: -

1. Entrapment efficiency
2. Particle's size analysis
3. Zeta potential
4. In-vitro drug release studies
5. Scanning electron microscopy

1. Entrapment efficacy:

The freshly prepared nanosuspension was centrifuged at 20,000 rpm for 20 min at 5°C temperature using cool ultracentrifuge. The amount of un incorporated drug was measured by taking the absorbance of the appropriately diluted 5 ml of supernatant solution at 262nm using UV spectrophotometer against blank/control nanosuspensions. DEE was calculated by subtracting the amount of free drug in the supernatant from the initial amount of drug taken.

The entrapment efficiency (EE %) could be achieved by the following equation:

%Entrapment efficiency= Drug content *100/Drug added in each formulation

2. Scanning electron microscopy: The morphological features of Ambrisentan nanosuspension are observed by scanning electron microscopy at different magnifications.

3. Particle size and shape

Average particle size and shape of the formulated nanosuspensions was determined by using Malvern Zetasizer ZS using water as dispersions medium. The sample was scanned 100 times for determination of particle size.

4. In vitro drug release study:

In vitro dissolution study was performed by USP dissolution apparatus-type II using 900 ml of 6.8pH buffer as a dissolution medium maintained at 37 ± 0.5°C and stirring speed (50 rpm). The freshly prepared nanosuspensions were added to the dissolution medium, five-milliliter samples were withdrawn at specific intervals of time, then filtered through a 0.45 µm filter paper and analyzed for their drug concentrations by measuring at 262nm wavelength.

The results of in vitro release profiles obtained for the NDDS formulations were fitted into

Two models of data treatment as follows:

1. Cumulative percent drug released versus time (zero order kinetic model).
2. Log cumulative percent drug remaining versus time (first- order kinetic model)(Bhalekar M R, 2014).

Zeta potential:

There are three ways by which a solid particle (colloid) dispersed in a liquid media can acquire a surface charge. First, by the adsorption of ions present in the solution. Second, by the ionization of functional groups on the particle's surface. Third, due to the difference in dielectric constant between the particle and the medium. Attention should be paid to the formation of electric double layer at the solid-liquid interface. The zeta Potential

is defined as the difference in potential between the surface of the tightly bound layer (shear plane) and the electro-neutral region of the solution. The potential gradually decreases as the distance from the surface increases.

As the concentration of electrolyte increases in the medium, the zeta potential falls off rapidly due to the screening effect of the counter ions. The zeta potential cannot be measured directly; however, it can be calculated using theoretical models and from experimentally determined electrophoretic mobility data. The theory is based on electrophoresis and can be expressed as:

$$\mu = \zeta\varepsilon/\eta$$

Where (μ) is the electrophoretic mobility, (ε) is the electric permittivity of the liquid, (η) Is the viscosity and (ζ) us the zeta potential(K.V.Gopaiah ,2021).

RESULTS AND DISCUSSION

Determination of melting point

The melting point of Ambrisentan was found to be in range of 165-168° C which was determined by capillary method.

Saturation Solubility

Saturation solubility was carried out at 25^0C using Methanol, Ethanol, 0.1N HCL, 6.8 phosphate buffer, and 7.4pH buffer.

Table -2 : Solubility data

Solvent	Solubility(mg/ml)
Ethanol	22.06
Methanol	25.67
0.1N HCL	14.72
pH 6.8 phosphate buffer	19.13

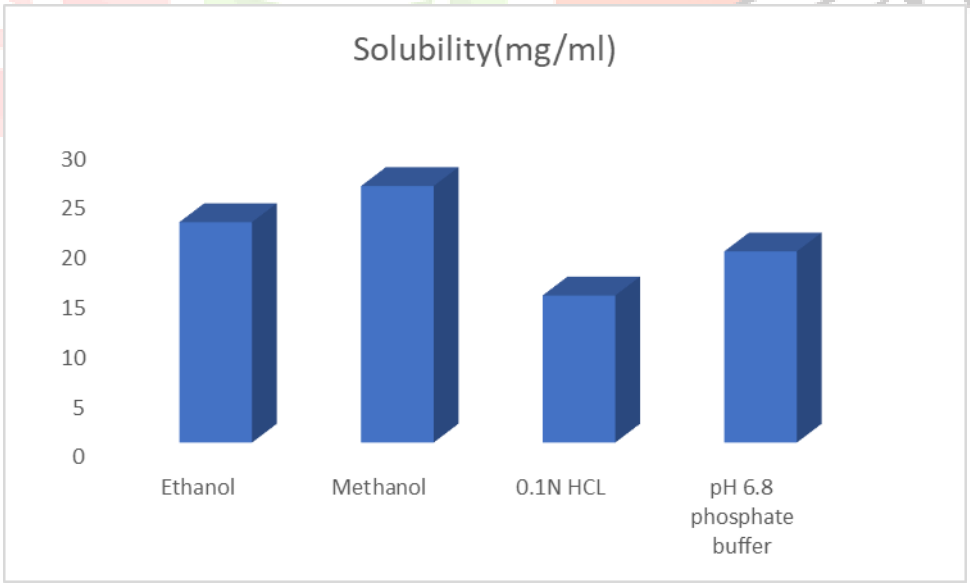

Figure -1: Solubility studies of Ambrisentan

Discussion: From the above conducted solubility studies in various buffers we can say that pH 6.8 phosphate buffer has more solubility when compared to other buffer solutions. So pH 6.8 buffer is used as dissolution medium, based upon the solubility studies on organic solvents, methanol has more solubility than others so methanol was used in the nanosuspension formulation.

Determination of absorption maximum (λmax):

Determination of Ambrisentan λ-max was done in pH 6.8 buffer medium for accurate quantitative assessment of drug dissolution rate.

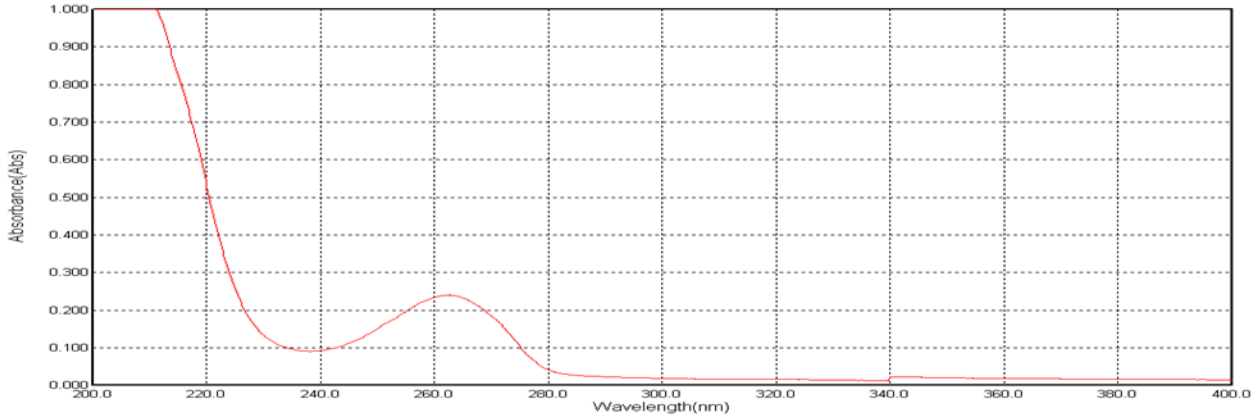

Figure -2 :UV spectrum of Ambrisentan

Table -3 : Standard graph of Ambrisentan in pH 6.8 (λmax 262 nm)

Concentration (µg/ml)	Absorbance
0	0
5	0.119
10	0.234
15	0.345
20	0.458
25	0.575
30	0.694

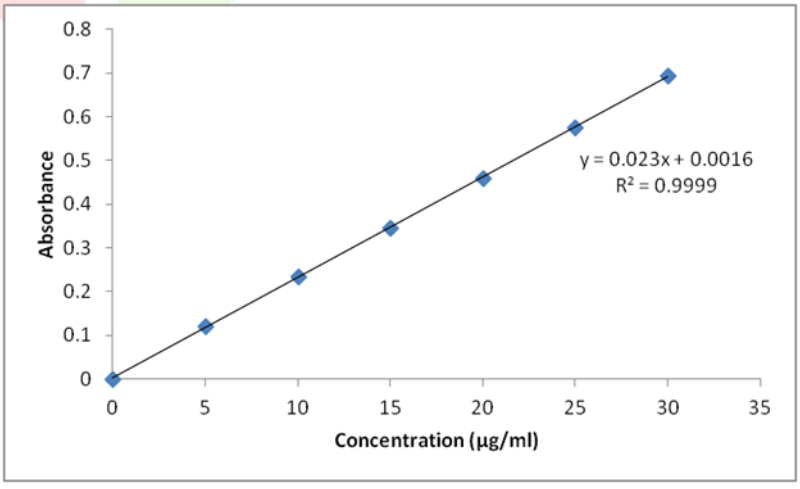

Figure -3 : Standard calibration curve of Ambrisentan in pH 6.8

Discussion:

The linearity was found to be in the range of 5-30 µg/ml in acetone, pH 6.8 buffer. The regression value was closer to 1 indicating the method obeyed Beer-lamberts' law.

Drug excipient compatibility:

Drug and excipient compatibility was confirmed by comparing spectra of FT-IR analysis of pure drug with that of various excipients used in the formulation.

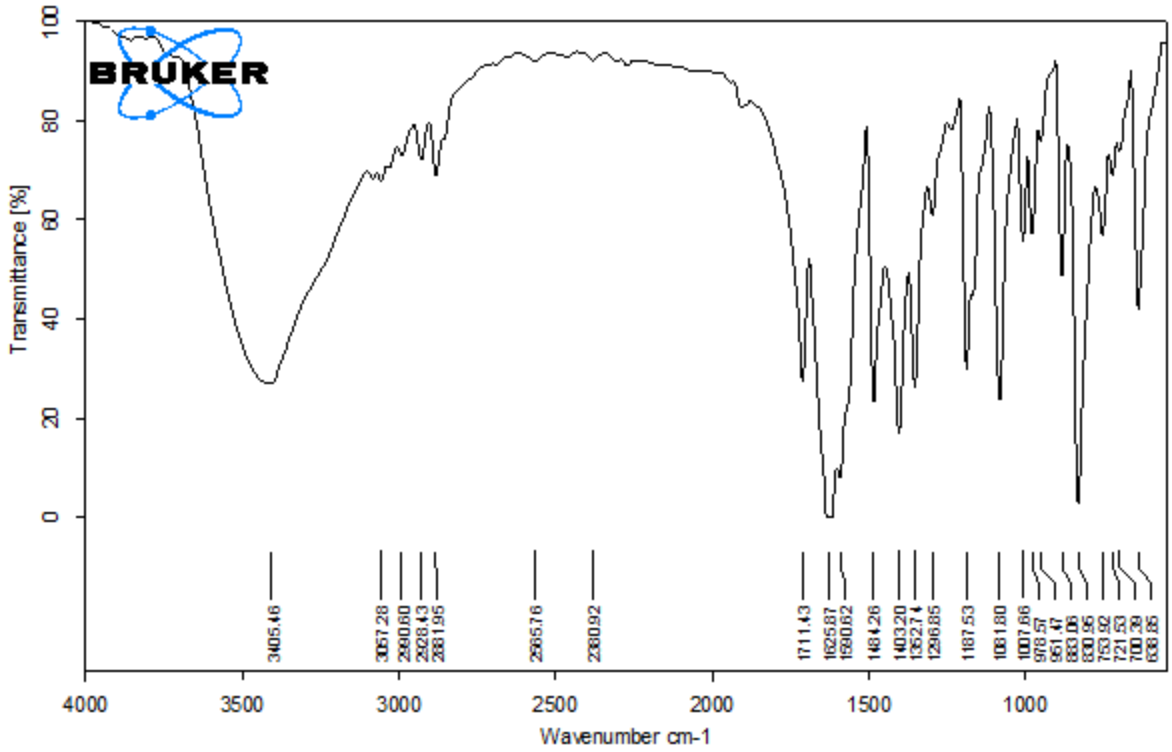

Figure -4 : IR spectrum of Ambrisentan

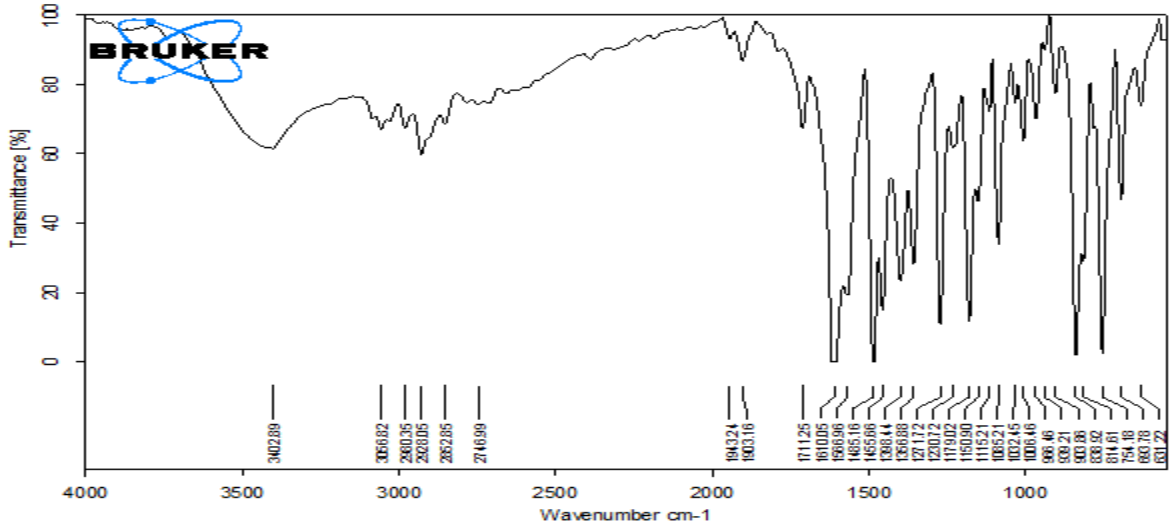

Figure -5 : IR spectrum of Ambrisentan Optimised Formulation

Discussion: Form the drug excipient compatibility studies we observe that there are no interactions between the pure drug (Ambrisentan) and optimized formulation (Ambrisentan+ excipients) which indicates there are no physical changes.

Entrapment efficacy:- The entrapment efficacy of the formulated Nanosuspension was found to be in the range of 82.46%-98.52% respectively.

Table -4 : Entrapment efficiency of formulated Nanosuspensions

Formulation code	Mean % entrapment efficiency
F1	74.84
F2	77.52
F3	79.15
F4	71.96
F5	88.21
F6	84.97
F7	82.64
F8	81.96
F9	95.67
F10	92.82
F11	94.78
F12	97.23

Discussion: The entrapment efficacy of formulation F1 was found to be 74.84%, formulation F2 was found to be 77.52%, formulation F3 was found to be 79.15%, formulation F4 was found to be 71.96%, formulation F5 was found to be 88.21%, formulation F6 was found to be 84.97%, formulation F7 was found to be 82.64%, formulation F8 was found to be 81.96%, formulation F9 was found to be 95.67%, formulation F10 was found to be 92.82%, formulation F11 was found to be 94.78%, formulation F12 was found to be 97.23%.

SCANNING ELECTRON MICROSCOPY:

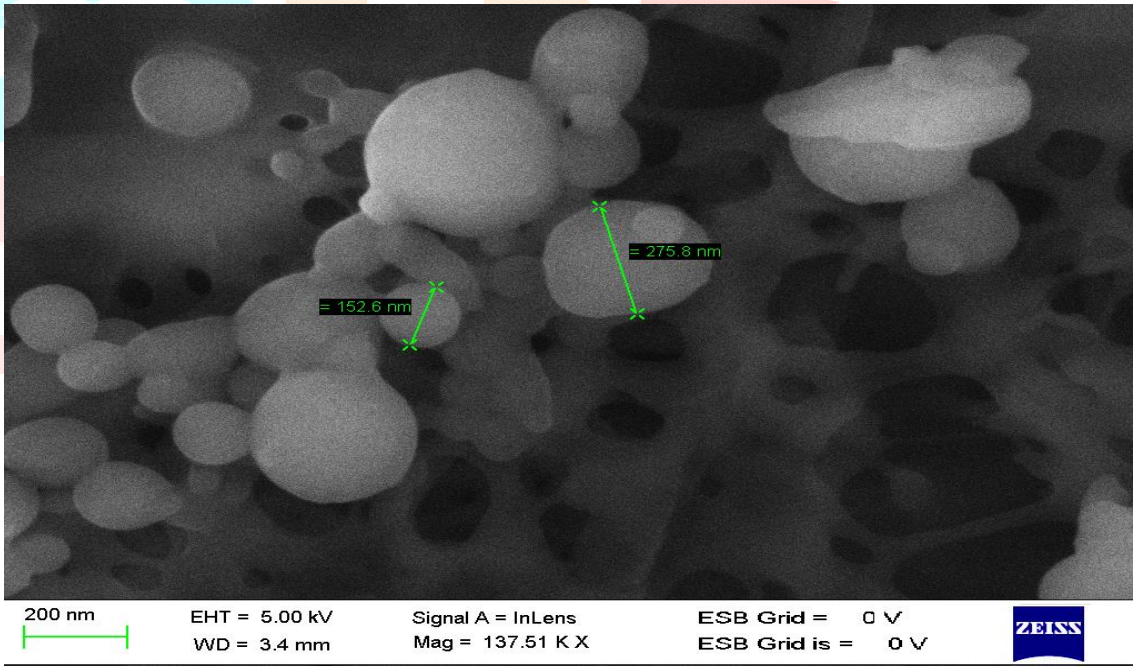

Figure - 6 : Scanning Electron Microscopy Of Optimized Formulation

Zeta Potential: The measurement itself is a particle electrophoresis, the particle velocity is determined via the doppler shift of the laser light scattered by the moving particles. The field strength applied was 20 V/cm. The electro phoretic mobility was converted to the zeta potential in mV using the Helmholtz-Smoluchowski equation. At standard measuring conditions (room temperature of 25°C, water) this equation can be simplified to the multiplication of the measured electro phoretic mobility (µm/cm per V/cm) by a factor of 12.8, yielding the ZP in mV.

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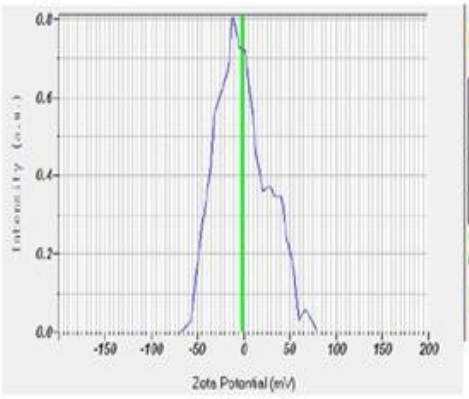
Measurement Results

Measurement Type	: Zeta Potential
Sample Name	: AGO
Temperature of the Holder	: 25.0 °C
Dispersion Medium Viscosity	: 0.894 mPa·s
Conductivity	: 0.067 mS/cm
Electrode Voltage	: 3.9 V



Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-7.6 mV	-0.000059 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -7.6 mV

Electrophoretic Mobility Mean : -0.000059 cm^2/Vs

Figure-7: Zeta potential value for the optimized formulation (F12)

Discussion: Zeta potential value for the optimized formulation (F12) was found to be within the acceptable limits.

Particle size analysis:

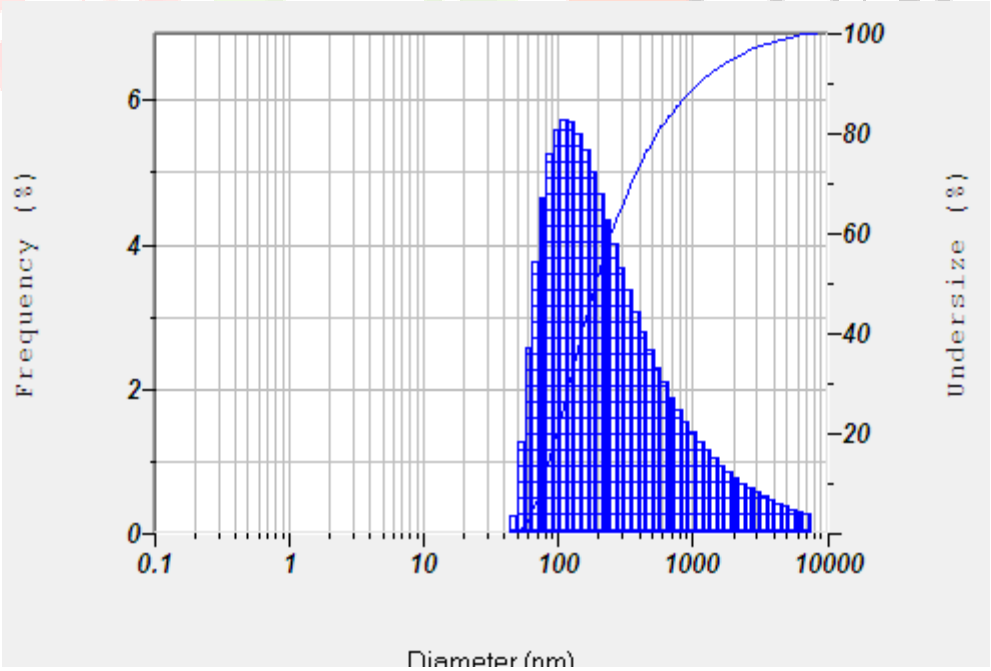


Figure -8 : Particle Size Analysis Of Optimized Formulation

Discussion: Average particle size of nanosuspension of optimized formulations (F12) was found to be having maximum particles at a range of 118 nm.

Dissolution results:

Table -5 : *In-vitro* drug release data of formulation F_{1to} F12

Time (min)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
0	0	0	0	0	0	0	0	0	0	0	0	0
5	22.65	31.53	35.78	38.45	36.08	40.64	43.56	53.09	42.84	45.78	55.08	62.64
10	29.63	38.18	42.67	45.85	43.63	47.53	50.86	61.78	49.48	52.77	63.63	73.03
15	34.86	43.26	47.54	50.68	48.04	52.61	55.75	67.09	54.49	57.54	69.04	79.91
20	39.46	48.03	52.19	55.49	53.48	57.49	60.94	72.49	59.52	62.19	74.48	86.49
30	48.68	57.35	61.65	64.62	62.65	66.65	69.85	82.29	68.34	71.65	84.15	99.65
45	62.65	71.58	75.45	78.68	76.05	80.06	83.38	96.64	82.53	85.45	98.05	
60	76.48	85.39	89.36	92.65	90.18	94.67	97.56		96.62	99.36		

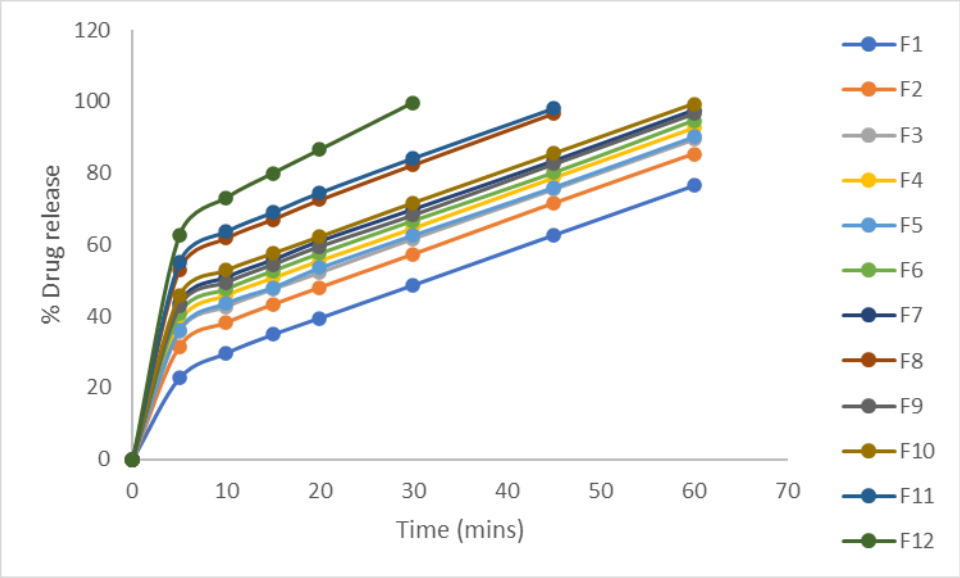


Figure-9 : Dissolution parameters for the formulations F1-F12

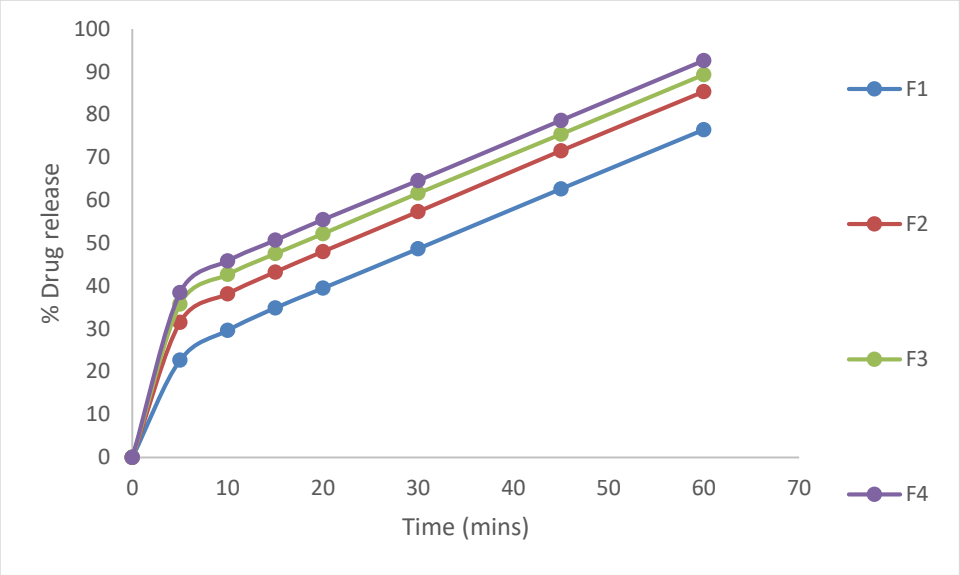


Figure -10 : Dissolution parameters for the formulations F1-F4

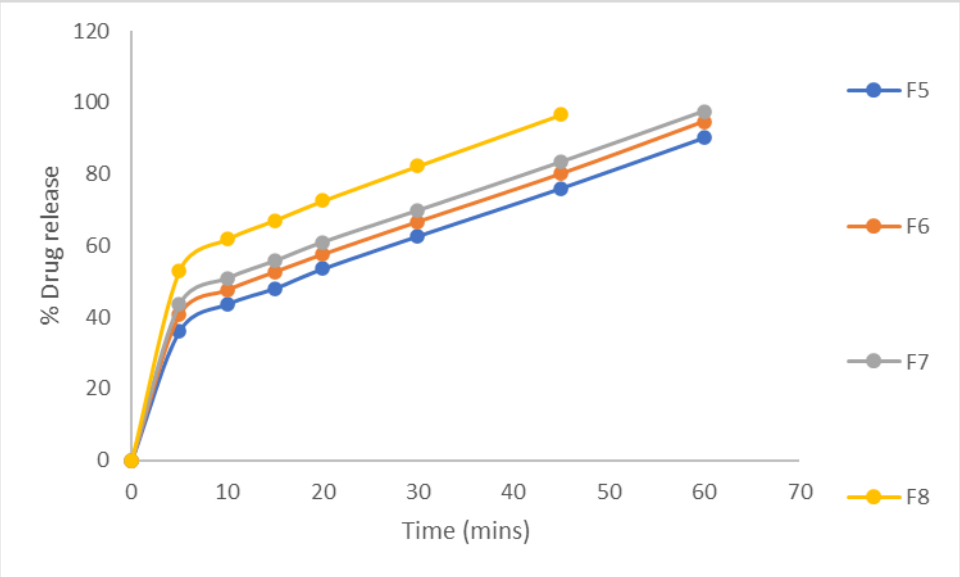


Figure -11 : Dissolution parameters for the formulations F5-F8

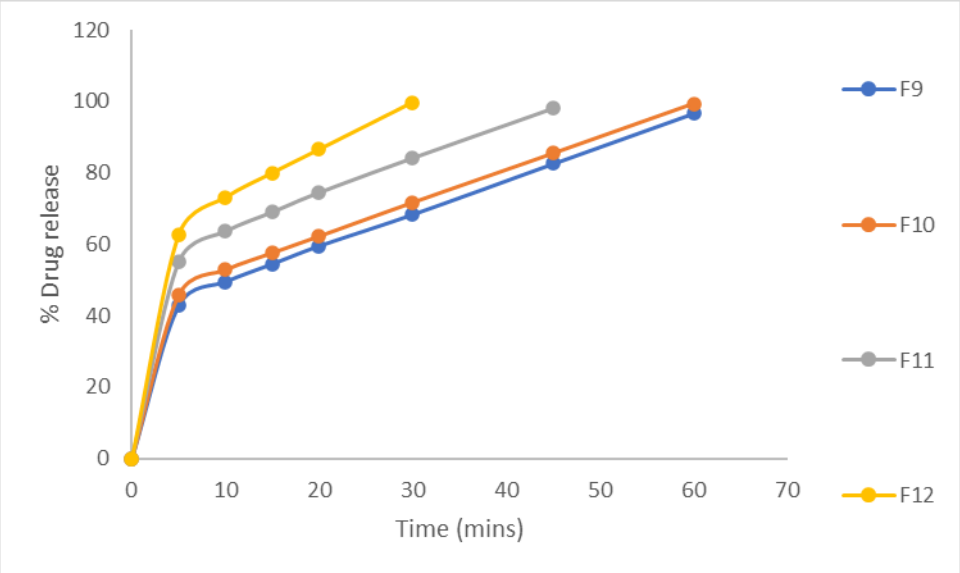


Figure -12 : Dissolution parameters for the formulations F9-F12

Discussion: From the above in vitro studies we can say that increase in the polymer concentration of polymers decrease in the dissolution time of all the formulations.

From the above in vitro studies we can say that at low polymer concentrations the drug release time was increased. So F12 is considered as optimized formulation as it shows drug release with in 30mins.

Among all the four stabilizers we have used F12 containing PEG 6000 at 1.0% concentration releases maximum drug release at the end of 30 mins when compared to the formulations prepared by using PVP K30 and Urea.

Increase in the stabilizer concentration of PEG 6000 shows 99.65% of drug release, so the formulations prepared by using PEG 6000 releases more drug release at the end of 30mins than the other stabilizers.

DRUG RELEASE KINETICS STUDIES: BEST FORMULATION F12

1. Zero order release kinetics:

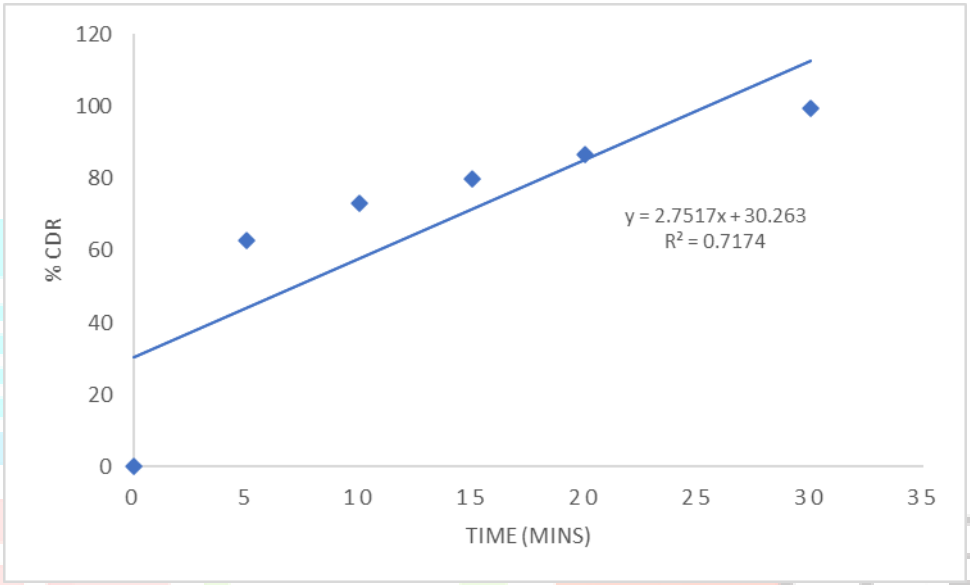


Figure -13 : Zero order release profile of formulation F12

2. First order release kinetics:

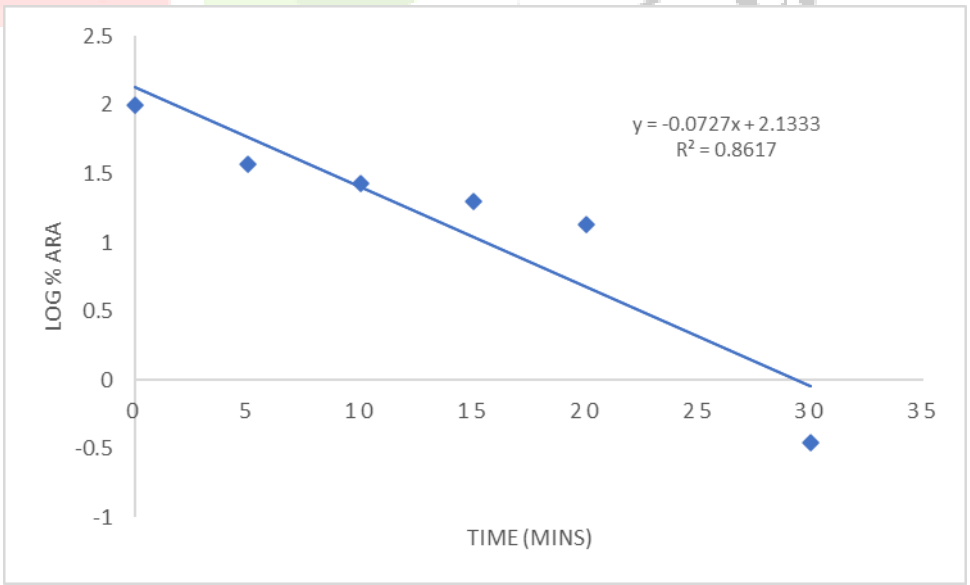


Figure -14 : First order release profile of formulation F12

Table -6 : Kinetic data of the formulation F12

ORDE OF KINETICS	ZERO ORDER	FIRST ORDER
REGRESSION	0.717	0.862

Discussion:

The drug release from the nanosuspension was explained by using mathematical model equations such as zero order, first order, and equation methods. Based on the regression values it was concluded that the optimized formulation F12 follows first order kinetics, indicating concentration dependent drug release.

SUMMARY AND CONCLUSION

In present investigation nanosuspension of Ambrisentan was prepared by emulsification solvent evaporation method. The nano suspensions are novel promising target and controlled released dosage form which is gaining importance because of ease of manufacturing and diversified applications. The present trend of pharmaceutical research lies in the usage of biodegradable polymer because of its availability and low toxicity. Nanosuspension containing drug was prepared by emulsification solvent evaporation method by using combinations of Urea, PEG 6000, PVP-K30, SLS, methanol and quantity sufficient water). Estimation of Ambrisentan was carried out spectrophotometrically at 262nm.The nanosuspension were evaluated for parameters such as drug content uniformity, scanning electron microscopy, particle size analysis, zeta potential, in-vitro release, drug excipient interactions (FTIR).The stability data was also subjected to statistical analysis.The melting point of Ambrisentan was found to be in range of 177°C which was determined by capillary method.Saturation solubility was carried out at 25^0C using 0.1N HCL, 6.8 phosphate buffer, methanol & ethanol. Form the drug excipient compatibility studies we observe that there are no interactions between the pure drug (Ambrisentan) and optimized formulation (Ambrisentan+ excipients) which indicates there are no physical changes.The entrapment efficacy of formulation F1 was found to be 74.84%, formulation F2 was found to be 77.52%, formulation F3 was found to be 79.15%, formulation F4 was found to be 71.96%, formulation F5 was found to be 88.21%, formulation F6 was found to be 84.97%, formulation F7 was found to be 82.64%, formulation F8 was found to be 81.96%, formulation F9 was found to be 95.67%, formulation F10 was found to be 92.82%, formulation F11 was found to be 94.78%, formulation F12 was found to be 97.23%. Zeta potential value for the optimized formulation (F12) was found to -7mv which was found to be within the acceptable limits. Average particle size of nanosuspension of optimized formulations (F12) was found to be 118nm. From the in vitro studies we can say that formulation F12 shows best drug release of 99.65% within 30 minutes whereas all the other formulations didn't release the drug.

The drug release from the nanosuspension was explained by the using mathematical model equations such as zero order, first order, and equation methods. Based on the regression values it was concluded that the optimized formulation F12follows first order kinetics.

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