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FORMULATION AND CHARACTERIZATION OF NANOSUSPENSION OF LEFLUNOMIDE USING NANOPRECIPITATION TECHNIQUE

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ABSTRACT

The objective of the current research was to formulate and characterize leflunomide nanosuspension using nanoprecipitation method so as to achieve faster drug dissolution. In the present investigation, an attempt was made to prepare nanosuspensions of leflunomide using nanoprecipitation method. The developed leflunomide nanosuspensions were evaluated for particle size, zeta potential, poly dispersity index, % encapsulation efficiency and *in-vitro* drug release. Particle size range of leflunomide nanosuspensions were noticed in the range of 921.4 to 1439.6 nm which is based on the change in drug and the excipients concentrations. Formulations of nanosuspensions exhibited negative zeta potential in the range of -11.5 to +23.2 mv and polydispersity index 0.2 to 0.3. F2 & F6 formulations showed greater % entrapment efficiency i.e., 65.5% and 68.2%. *In vitro* drug release profile of leflunomide nanosuspension formulations F2 & F6 exhibited up to 90.76 % and 91.04% release in 8 h. Therefore, it is concluded that nanoprecipitation method have a capability to formulated consistent and uniform-sized nanosuspensions of leflunomide. The formulated nanosuspensions exhibited increased *in vitro* drug release profiles which can increase the oral bioavailability of drug.

Keywords: Leflunomide, nanoprecipitation, nanosuspension, % entrapment efficiency, *in vitro* drug release

INTRODUCTION

The oral route of drug administration is the most convenient and preferred route. However, there are certain limitations owing to a number of factors pertaining to the drug as well as formulation. The foremost reasons comprise first pass hepatic metabolism which leads to low oral bioavailability & high lipophilicity of API, therefore impeding the application of oral route of drug delivery.

Leflunomide is an immunomodulatory drug used in the treatment of rheumatoid arthritis as well aspsoriatic arthritis. Leflunomide belongs to class II of the bio-pharmaceutical classification systems (BCS) **[1, 2]**. Nanoprecipitation is a method used to incorporate APIs into colloidal drug delivery systems, patented by Fessi *et al.***[3, 4]**, significantly applicable for the development controlled release, target delivery pharmaceuticals mainly because of the simplicity of its procedure **[5]**.

The present research work was mainly focused to develop nanosuspensions containing leflunomide. The rationale for the choosing of nanosuspensions as the formulation approach was the ability of nanosuspensions to impart the attributes of enhanced biocompatibility in comparison with the polymeric nanoparticles. They were proven to exhibit controlled drug release profile in addition to reduced toxicity profile. Furthermore, the formulation of nanosuspensions have been reported hitherto for leflunomide and established to be favourable in terms of oral bioavailability.

MATERIALS AND METHODS

Materials

Leflunomide was received as a gift sample from Aurobindo Pharma Ltd., Hyderabad. Polyvinyl alcohol, poly ethylene glycol 400 and polyvinyl pyrrolidine K30 were purchased from Loba Chemie, Mumbai respectively. Acetone is procured from Thermo fisher scientific India Pvt. Ltd. All other chemicals and reagents used were of analytical grade.

Pre-formulation studies

Compatibility tests were used to determine for drugs, polymers and excipients which were involved in this study by looking at their physical appearance, drug content and FT-IRspectroscopic studies.

Drug-excipientcompatibility study

Physical mixtures of drug – excipients

Any colour change, flow or lump forming of samples prepared was observed using different physical mixtures.

Fourier Transform Infra-Red (FT-IR) analysis

Using a hydraulic press, approximately 2-4 milligrams of the physical mixture and heated potassium bromide were added and properly combined to form pellets. The produced pellet then placed in a FT-IR

sample holder for transmission mode analysis from 4000-400 cm^{-1} wave number range **[6]**.

Formulation development

UV scan spectrum of leflunomide

UV scan spectrum of leflunomide was constructed using UV-visible spectrophotometer.

Calibration of standard graph of leflunomide

The standard drug solution was made by dissolving 10 mg leflunomide into 2 ml of methanol, filling up the volume using phosphate buffer pH 7.4 and transferring it into a 100 ml volumetric flask to obtain 10 µg/ml of standard solution. From which, 3µg/ml, 6µg/ml, 9µg/ml, 12µg/ml and 15 µg/ml concentrations were prepared by adequate dilutions. The absorbance values of each solution were determined in triplicate by UV-visible spectrophotometer at the highest wavelength (λ_{max}) of 225 nm using phosphate buffer solution, pH 7.4.

Preparation of leflunomide nanosuspension

Nanosuspension of leflunomide was prepared using nanoprecipitation method. In water-soluble solvent acetone, necessary quantity of leflunomide was totally dissolved. Polymers of various types and concentrations were dissolved in water. On a magnetic stirrer with 1000 RPM speed, resulting drug solution was injected slowly into water containing polymer solutions - PVA, PEG 400, and PVP K30. After evaporation of solvent while mixing, solid drug particles get precipitated immediately. For 10 min, formulated nanosuspension was exposed to ultra-probe sonication at cooling conditions. Subsequently obtained nanosuspension was subjected to filtration, drying and stored in desiccators till its further usage **[7]**. Composition and concentrations of all formulations was illustrated in **Table 1**.

Table 1: Formulation table for leflunomide nanosuspension by nanoprecipitation method

Formulations	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Leflunomide (mg)	100	100	100	100	100	100	100	100	100	100	100	100
PVA (%)	0.25	0.5	0.75	1	-	-	-	-	-	-	-	-
PEG 400 (%)	-	-	-	-	0.25	0.5	0.75	1	-	-	-	-
PVP K30 (%)	-	-	-	-	-	-	-	-	0.25	0.5	0.75	1
Acetone (ml)	20	20	20	20	20	20	20	20	20	20	20	20
Water (ml)	20	20	20	20	20	20	20	20	20	20	20	20

Characterization of leflunomide nanosuspensions

Particle size analyser and zeta potential measurement

The particle size is determined using the Horiba scientific nano partica nano particle analyzer equipment and a dynamic light scattering approach. The prepared samples were diluted in distilled water to a 1:40 ratio and measured at a fixed angle at 165 ° at a temperature of 25 °. Particle size of the produced nanosuspension was

determined after the samples were placed in a glass cuvette. The zeta potential was calculated using electrophoretic mobility in the presence of an electric field. By introducing the diluted nanosuspension into a carbon electrode cuvette, samples are diluted with water **[8]**.

Drug entrapment efficiency

Using a cooling ultracentrifuge, freshly formed nanosuspension is centrifuged around 20,000 rpm for 20 minutes at 5 °C temperature. The quantity of unincorporated drug is determined by comparing the absorbance of the adequately diluted 25 ml supernatant liquid at 225 nm to that of a blank/control nanosuspension using a UV spectrophotometer. By deducting total of free drug with in supernatant from the total amount of drug consumed, drug entrapment efficiency was computed. For each batch, the experiment was repeated three times and thus the average was obtained **[9]**. The following equation could be used to calculate entrapment efficiency %.

$$Drug\ entrapment\ efficiency\ \left(\%\frac{w}{w}\right) = \frac{Total\ amount\ of\ drug\ -\ Amount\ of\ drug\ in\ supernatant}{Total\ amount\ of\ drug}\ x\ 100$$

***In vitro* drug release studies**

The technique employed was dialysis membrane diffusion. In a Franz diffusion cell with a receptor volume of 20 ml, 1ml of the nanosuspension was deposited in the dialysis bag (Mw cut-off 12,000–14,000 Hi-media). The entire system is regulated at 37 °C with magnetic stirring continuously. At predefined time intervals, 1 ml sample was removed from the receptor compartment and replaced with fresh medium. A UV spectrophotometer set to 225 nm was used to determine the amount of medication dissolved.

Differential scanning calorimetry

DSC analysis was conducted using pure leflunomide and in combination with polymers such as polyvinyl alcohol, polyethylene glycol 400 and polyvinyl pyrrolidine K30. The contents are precisely weighed within 4 mg and enclosed in a differential scanning calorimetry aluminium pan with a flat bottom (TA instruments). Data was collected utilizing O_2 as zero air for heat & N_2 gas for cooling at temperatures ranging from 30°C to 300°C. TA universal analysis software is used to examine transitions and melting point observations.

Stability studies

The stability of the nanosuspensions was studied at 30ºC ± 2ºC / 65% ± 5% RH for 90 days and were examined at periodic time intervals for the changes in particle size and % entrapment efficiency **[10]**.

RESULTS AND DISCUSSION

Pre-formulation studies of leflunomide

Physical appearance and FT-IR

spectroscopy analyses were used to evaluate drug - excipient interaction studies. Leflunomide and also the polymers used were studied for pre-formulation studies as follows

Analytical tests for drug/ API (Table 2)

Table 2: Analytical tests for leflunomide

S. No.	Pre-formulation study	Outcome
1.	Colour description	White
2.	Odour	Odourless
3.	Solubility	Insoluble in water, soluble in organic solvents like ethanol, acetone, methanol
4.	Drug category	Anti-rheumatic agent, immunomodulatory drug
5.	Melting point	245.75° C

FT-IR studies were carried out in order to find out the probable interactions between model drug leflunomide; polymers & other excipients. FT-IR of spectra of leflunomide with polymers and other excipients exhibited similar peaks as that of leflunomide pure drug. Based upon FT-IR spectra obtained, it was evident that there was no significant interaction of leflunomide with other excipients used in the formulation **(Table 3; Figure 1-4)**.

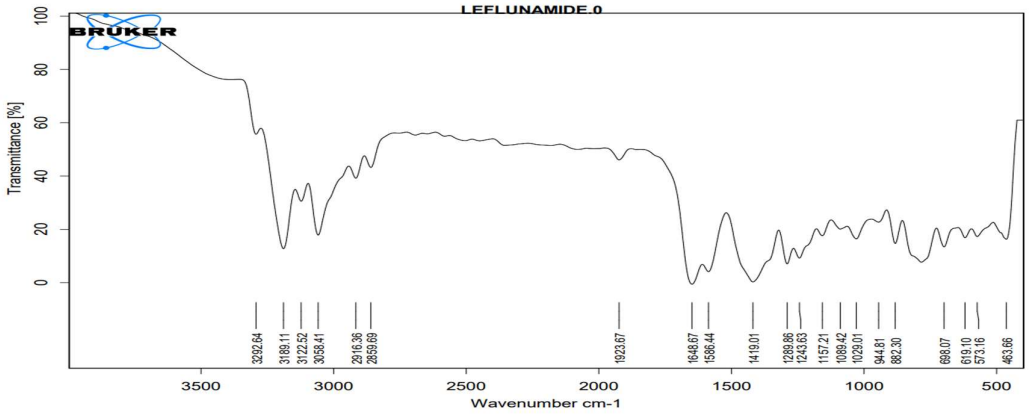


Figure 1: FT-IR spectra of leflunomide

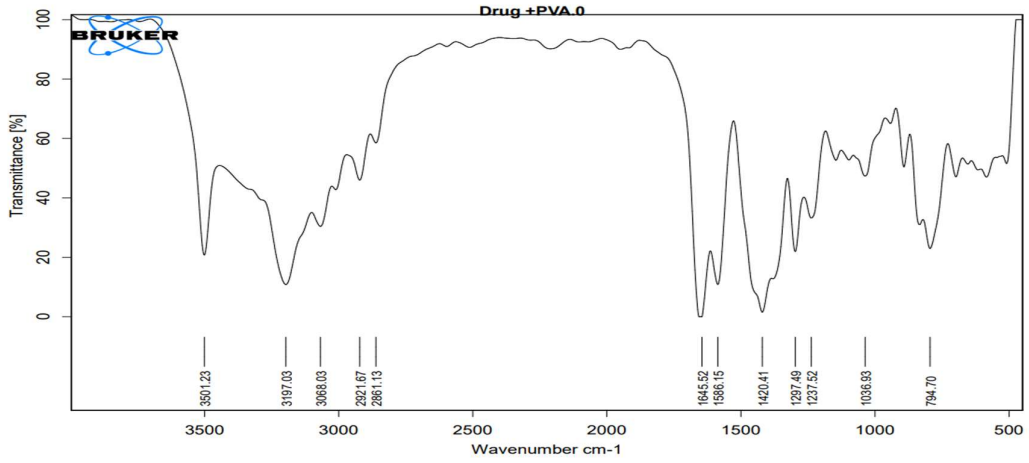


Figure 2: FT-IR spectra of leflunomide + PVA

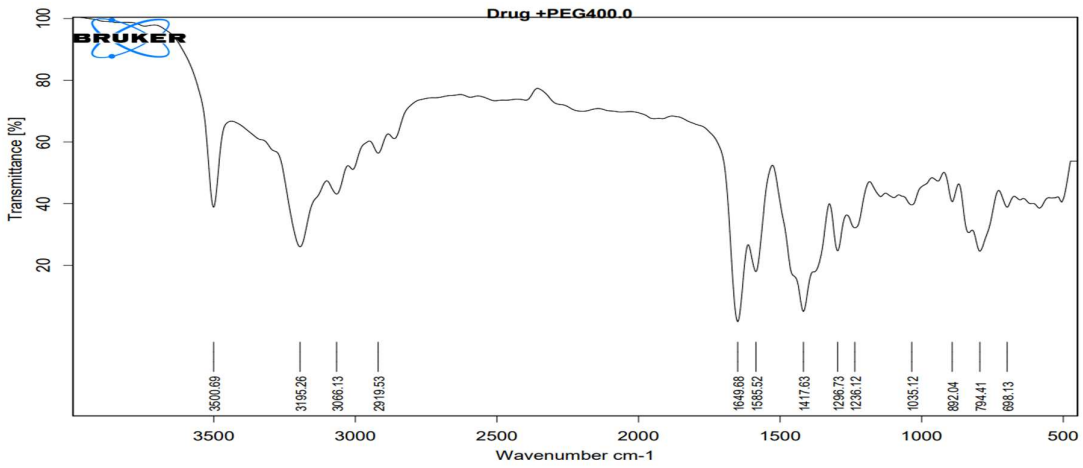


Figure 3: FT-IR spectra of leflunomide + PEG 400

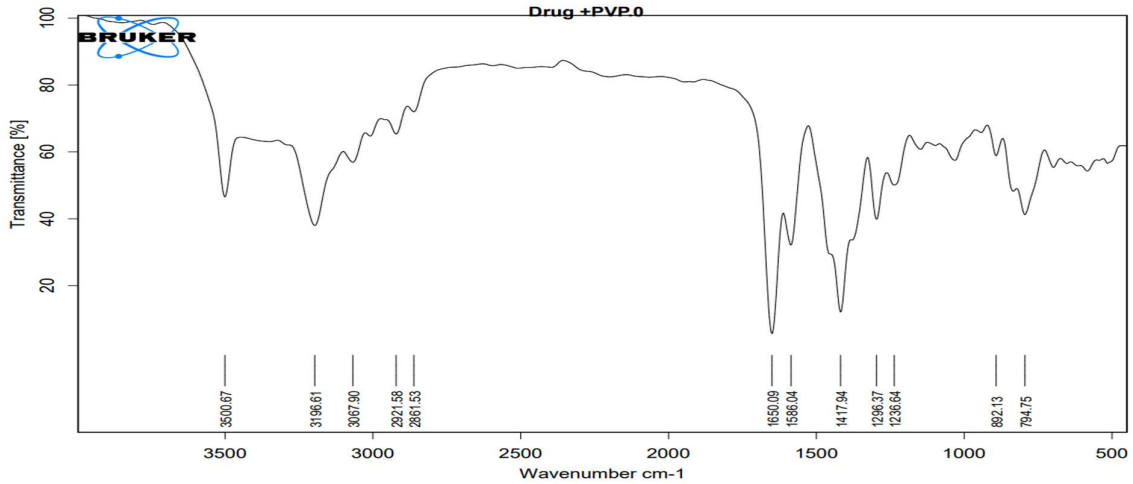


Figure 4: FT-IR spectra of leflunomide + PVP K30

Table 3: FT-IR spectra interpretation of leflunomide pure drug

S. No.	IR absorption peak (cm^{-1})	Interpretation
1.	3189.11	O-H stretching
2.	3058.41	C-H stretching
3.	1648.67	C=C stretching
4.	1586.44	N-H bending
5.	1419.01	O-H bending
6.	1289.86	C-X (Fluoride)

Inference

FT-IR experiments were carried out to see if there was any interaction between the chosen drug leflunomide and the excipients included in the nanosuspension formulation. Peaks were seen in the FT-IR spectra of leflunomide (pure drug) at 3189.11 cm^{-1} due to O-H stretching, 3058.41 cm^{-1} due to C-H stretching, 1648.67 cm^{-1} due to C=C stretching, 1586.44 cm^{-1} due to N-H bend, 1419.01 cm^{-1} due to O-H bending and 1289.86 cm^{-1} due to C-X bending (Fluoride). The characteristic peaksfor the drug were

retained in the combination of drug with the polymers. Therefore,it was evident that there was no interaction with the excipients involved in the formation of nanosuspensions, according to FT-IR wavelengths.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry is mainly necessary to detect the interactions between drug and excipients. The thermogram of leflunomide exhibited a sharp endotherm melting point range at 245.75°C and thermogram of leflunomide with excipients (PVA, PEG 400, PVP K30) exhibited sharp endotherm melting point range at 245.43°C, 244.85°C and 245.22°C respectively. By this it indicates that there are no chemical interactions between leflunomide and the excipients used in this formulation.

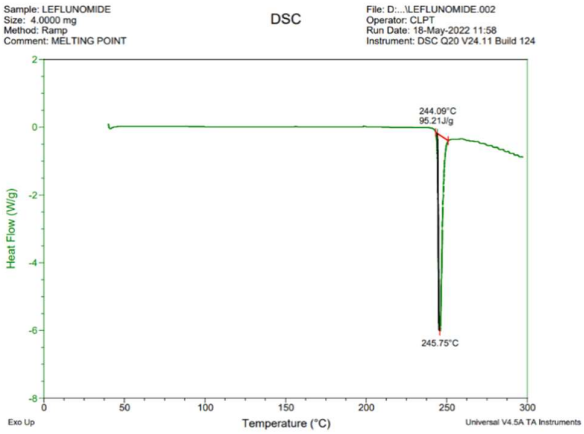


Figure 5: DSC thermogram of leflunomide

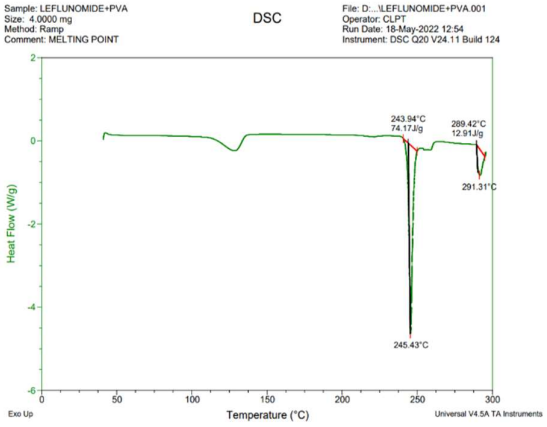


Figure 6: DSC thermogram of leflunomide + PEG 400

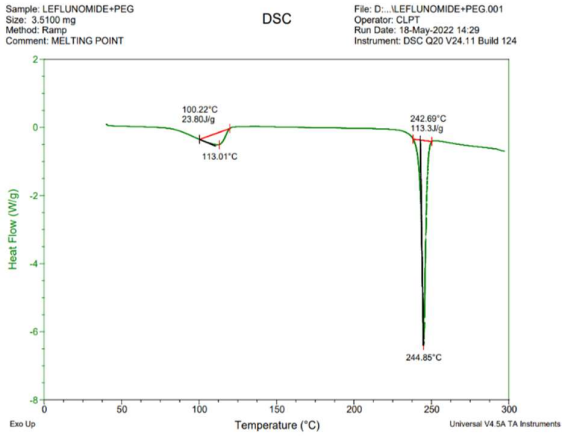


Figure 7: DSC thermogram of leflunomide + PEG 400

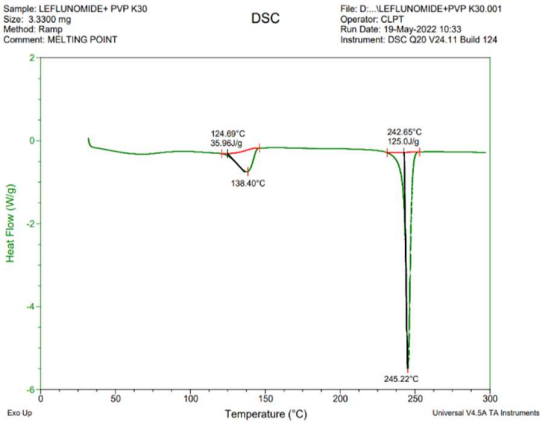
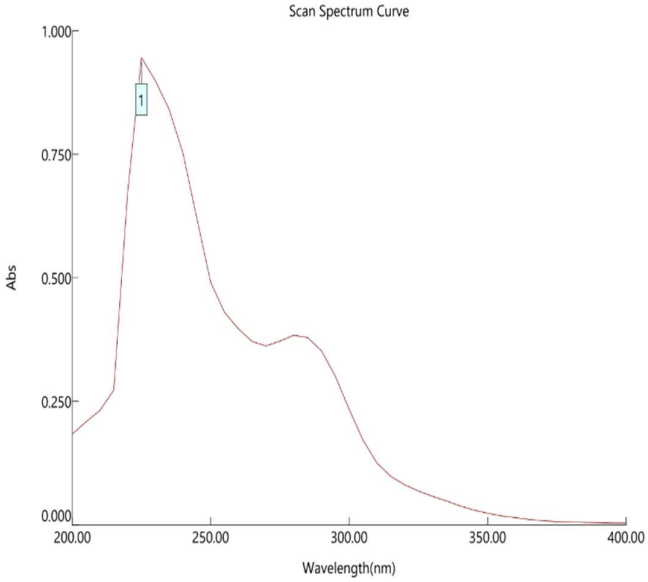


Figure 8: DSC thermogram of leflunomide + PVP K30

Formulation development



- **Instrument Performance**
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  Number : 20-1950-21-0001
  Spectral Bandwidth : 2.00 nm
- **File Information**
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  Create Date/Time : 12 May 2022 13:32:08
  Data Type : Original
  Method File:
- **Analyse Note**
  Analyser : Administrator
  Sample Name :
  Comment :

No.	ID	Mode	A 225.00 nm
1	1-1	Abs	0.220
2	2-1	Abs	0.411
3	3-1	Abs	0.620
4	4-1	Abs	0.804
5	5-1	Abs	0.980

Standard curve of leflunomide

UV scan spectrum of leflunomide

The UV-visible spectrophotometric method was used to determine the leflunomide calibration curve with phosphate buffer solution pH 7.4.The standard concentrationwas scanned over a wavelength range of 200-400 nm, yieldin g a peak at 225 nm which was taken as the absorption maximum (λ_{max}) for leflunomide.

By adequate dilutions, concentrations of 3µg/ml, 6 µg/ml, 9 µg/ml, 12 µg/ml and 15 µg/ml in phosphate buffer solution pH 7.4 were made and used to construct the calibration curve for leflunomide.

Table 4: Standard calibration graph of leflunomide

Concentration (µg/ml)	Absorbance (225 nm)
0	**0**
3	**0.220**
6	**0.411**
9	**0.620**
12	**0.804**
15	**0.980**

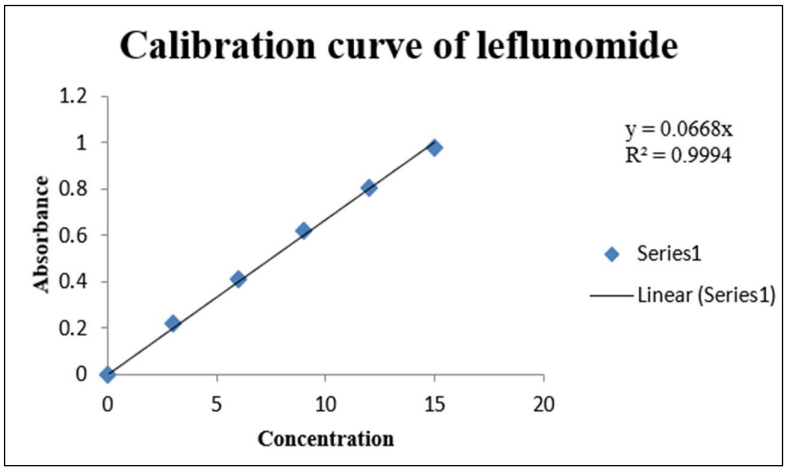

Figure 9: Standard calibration graph of leflunomide in phosphate buffer solution, pH 7.4 at $\lambda_{max}$of 225 nm

Using nanoprecipitation method, the total of 12 compositions of leflunomide nanosuspensions were developed by altering the concentrations of polymers like polyvinyl alcohol, polyethylene glycol 400 and polyvinyl pyrrolidine K30. The formulated nanosuspensions were subjected to further characterization.

Particle size, polydispersity index (PDI) and zeta potential:

The particle size of developed leflunomide nanosuspensions were measured before freeze drying by use of zeta sizer (Horiba Scientific). Results of particle size, PDI & zeta potential of different leflunomide nanosuspension formulations were summarized in **Table 5**.

Table 5: Particle size and zeta potential of leflunomide nanosuspension formulations (F1 – F12)

Formulation code	Particle size (nm) ± SD	PDI	Zeta potential (mv)
F1	1357.2 ± 24.5	0.284	-11.5
F2	921.4 ± 36.6	0.272	+11.7
F3	1285.2 ± 18.4	0.296	-19.6
F4	1830.5± 24.8	0.311	+17.4
F5	1244.5 ± 26.4	0.274	-15.5
F6	959.3 ± 29.2	0.228	-18.2
F7	1143.5 ± 22.5	0.255	+22.4
F8	1256.7 ± 28.4	0.267	+23.2
F9	1258.4 ± 20.6	0.299	-19.7
F10	1237.6± 32.5	0.320	-18.4
F11	1380.2 ± 28.4	0.313	+22.2
F12	1439.6 ± 34.5	0.249	-20.6

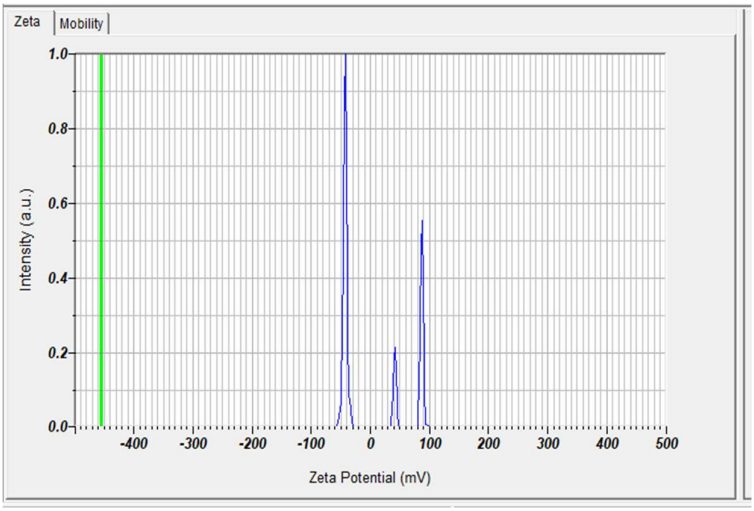

Figure 10: Particle size and zeta potential of leflunomide nanosuspension formulation

The particle size of the developed formulations, were found to be in the range of 921.4 to 1439.6 nm. The particle size of nanosuspension formulations was found to increase with the concentration of polymer.

Drug entrapment efficiency:

The %drug entrapment was observed to be in the range of 40.4% to 68.2%. The percentage entrapment efficiency was found to be greater for nanosuspension formulations prepared with polyvinyl acetate and poly ethylene glycol in the concentrations of 0.5% (i.e., formulations F2& F6) as shown in **Table 6**. It has been found that leflunomide-loaded nanosuspension formulationF2 & F6 showed greaterpercentage entrapment efficiency i.e., 65.5% and 68.2%, respectively, when compared to other formulations.

***In-vitro* drug release studies:**

The *in vitro* drug release studies were performed for the duration of 8 h. The cumulative percentage drug release from F1 to F12 formulations was summarized in the below mentioned **Table 7**.

Table 6: % EE of leflunomide nanosuspension formulations (F1 – F12)

Formulation code	% EE ± SD (n=3)
F1	46.5 ± 1.89
F2	65.5 ± 2.56
F3	44.8 ± 1.67
F4	40.4 ± 2.13
F5	50.1 ± 1.10
F6	68.2 ± 1.25
F7	47.3 ± 1.06
F8	44.7 ± 1.97
F9	45.2 ± 1.78
F10	46.4 ± 2.55
F11	48.3 ± 1.98
F12	40.5 ± 2.50

Table 7: *In-vitro* release studies of leflunomide nanosuspension formulations prepared by nanoprecipitation method (F1 to F12)

Time (h)	Cumulative % drug release											
	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
1	28.42 ± 0.44	30.45 ± 1.34	22.88 ± 0.58	29.47 ± 0.87	30.48 ± 0.18	35.24 ± 0.84	26.44 ± 0.25	28.90 ± 0.50	25.63 ± 0.46	26.96 ± 0.85	29.95 ± 0.17	29.65 ± 0.22
2	36.45 ± 0.40	37.54 ± 1.29	25.63 ± 1.53	31.22 ± 0.55	41.23 ± 0.35	39.81 ± 0.39	29.55 ± 0.48	32.18 ± 1.25	32.78 ± 1.71	29.78 ± 0.33	36.50 ± 1.47	33.82 ± 0.45
3	42.44 ± 0.18	55.28 ± 0.58	30.45 ± 1.45	36.48 ± 0.62	49.27 ± 0.49	42.69 ± 0.96	36.32 ± 0.75	39.49 ± 0.81	46.95 ± 1.32	36.75 ± 0.24	39.22 ± 0.55	39.70 ± 0.70
4	53.81 ± 0.24	63.74 ± 0.36	36.89 ± 1.24	40.85 ± 0.57	54.64 ± 1.14	49.54 ± 0.52	39.41 ± 0.96	45.85 ± 1.40	50.27 ± 0.98	43.80 ± 0.29	42.81 ± 1.25	43.52 ± 0.28
5	64.94 ± 0.89	68.85 ± 0.98	45.66 ± 1.08	47.64 ± 0.33	59.78 ± 0.57	59.60 ± 1.09	45.50 ± 1.08	53.81 ± 0.78	53.79 ± 1.24	49.74 ± 0.35	47.82 ± 0.87	49.19 ± 0.23
6	70.25 ± 0.33	77.25 ± 0.75	52.78 ± 1.36	50.82 ± 0.46	64.62 ± 0.96	68.47 ± 0.70	49.22 ± 1.29	59.28 ± 0.99	59.94 ± 0.34	53.87 ± 0.47	49.69 ± 0.59	53.09 ± 1.54
7	75.22 ± 0.40	80.88 ± 0.48	59.90 ± 0.87	56.90 ± 0.46	68.95 ± 0.70	79.88 ± 0.70	55.85 ± 1.33	60.80 ± 0.27	64.81 ± 0.67	59.35 ± 0.88	56.54 ± 0.48	59.67 ± 0.89
8	78.10 ± 0.56	90.76 ± 0.52	63.73 ± 0.78	59.40 ± 0.46	69.23 ± 0.29	91.04 ± 0.70	66.48 ± 1.20	67.46 ± 0.39	69.75 ± 1.26	62.48 ± 0.94	65.10 ± 0.96	62.63 ± 0.60

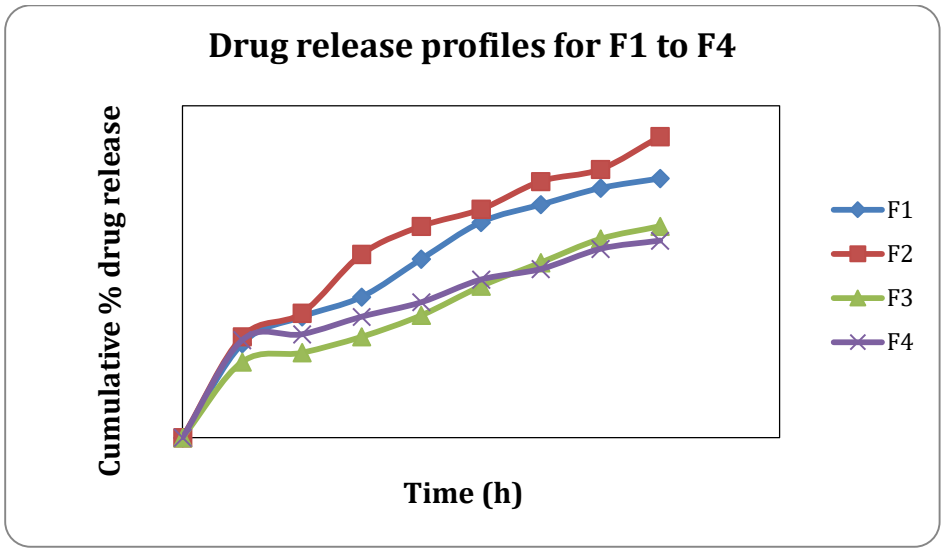

Figure 11: Drug release profiles for formulations F1 to F4

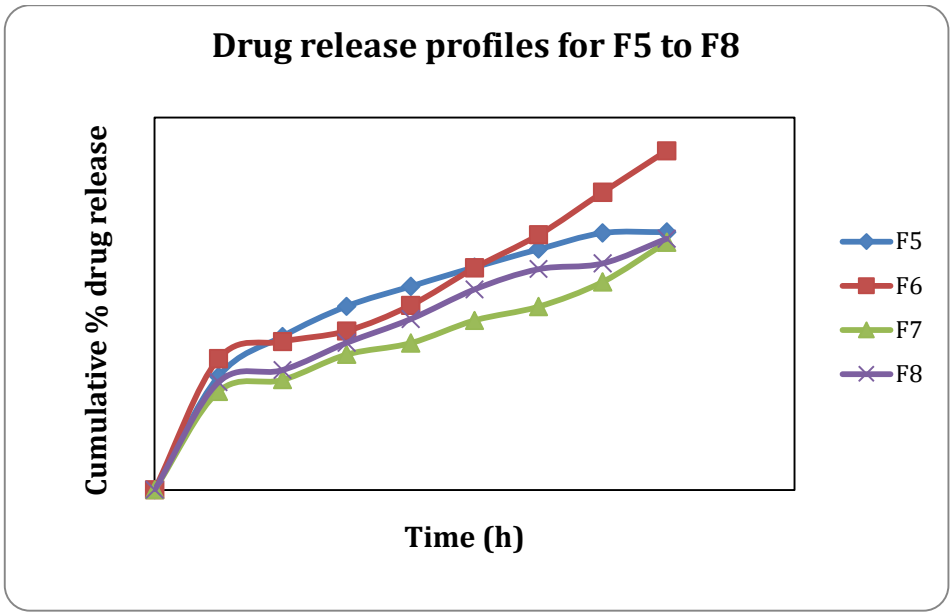

Figure 12: Drug release profiles for formulations F5 to F8

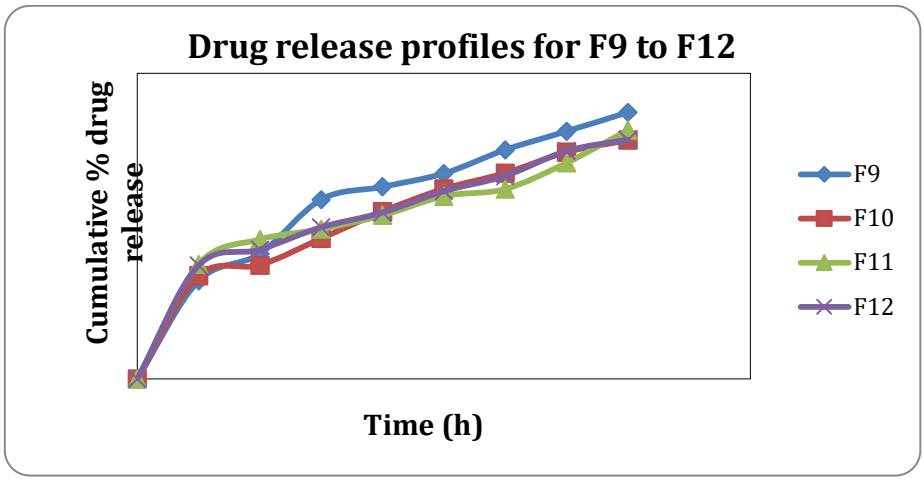

Figure 13: Drug release profiles for formulations F9 to F12

Kinetics of drug release:

From results obtained, kinetics of leflunomide release from the best leflunomide nanosuspension formulation developed by nanoprecipitation technique is shown in **Table 16**.

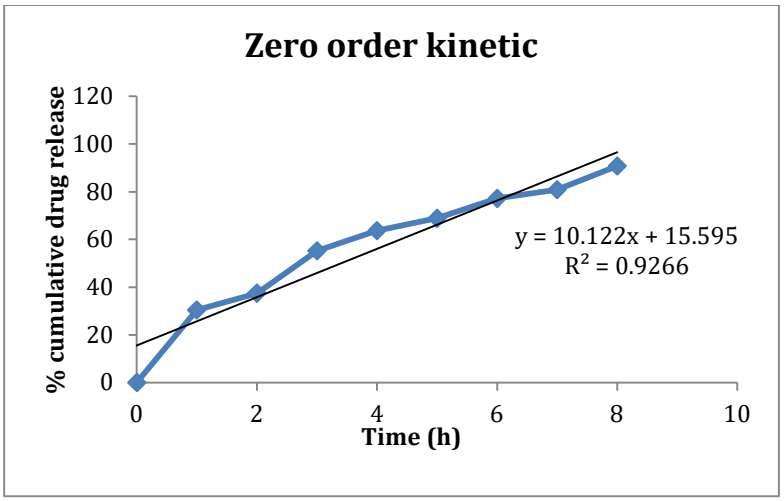

Figure 14: Drug release profile for zero order

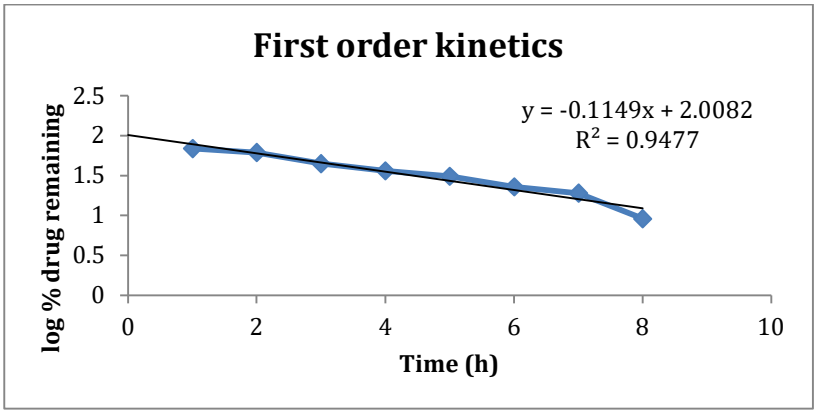

Figure 15: Drug release profile for first order

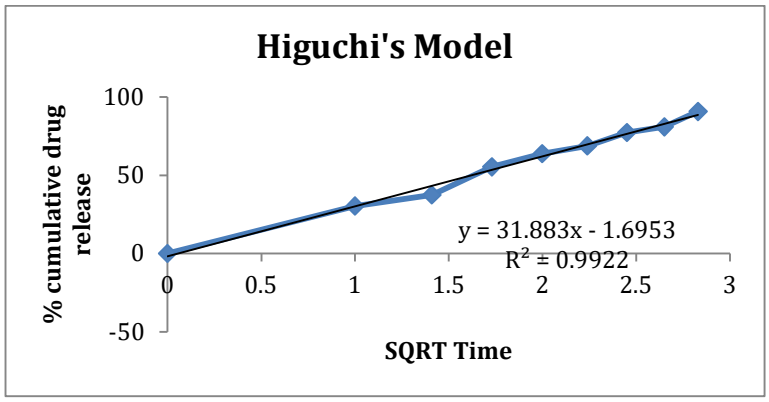

Figure 16: Drug release profile for Higuchi model

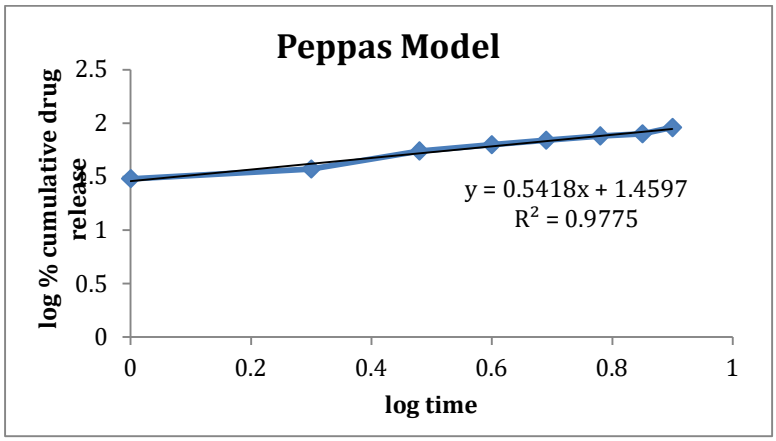

Figure 17: Drug release profile for Korsmeyer-Peppas model

Table 8: Drug release kinetics of leflunomide loaded nanosuspension F2

Zero order	First order	Higuchi	Korsmeyer-Peppas
0.926	0.947	0.992	0.977

Inference

For the formulation F2, drug release kinetics study was carried out to evaluate the kinetics and mechanism of drug release. The kinetic data of formulation F2 could be best expressed by first order kinetics as the plots showed linearity (R^2: 0.947) than zero order release kinetics (R^2: 0.926). From the release kinetics data, it was evident that the mechanism of leflunomide release from formulation F2 was non-fickian diffusion.

Stability studies

The stability studies were carried out at 30ºC ± 2ºC / 65% ± 5% RH for 90 days and were examined at periodic time intervals for the changes in particle size and % entrapment efficiency. The stability testing after 90 days led to the conclusion that there was negligible difference observed in the particle size and % entrapment efficiency of leflunomide nanosuspension formulation stored for 90 days.

Table 11: Stability studies for leflunomide nanosuspension formulation (F2)

Storage condition	Particle size (nm) Mean ± SD			Entrapment Efficiency (%) Mean ± SD		
	Initial	30 days	90 days	Initial	30 days	90 days
30 ºC ± 2 ºC / 65% ± 5% RH	968.2 ± 1.18	985.6 ± 1.95	985.3 ± 1.52	996.2 ± 1.15	985.3 ± 1.59	930.8 ± 1.32

CONCLUSION

Leflunomide nanosuspensions were prepared by nanoprecipitation method. It can be concluded that the developed nanosuspensions of leflunomide using PVA 0.5% and PEG 400 0.5% shown higher percentage of drug release and could enhance the oral bioavailability which has to be further confirmed with *in vivo* studies. Nanosuspensions could be one of the alternative approaches to other colloidal drug delivery systems and tablets in order to enhance the bioavailability of the drugs.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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