RESEARCH ARTICLE

Formulation and Evaluation of Microspheres Drug Delivery for Multiple Sclerosis Disease Condition

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ABSTRACT

Multiple sclerosis (MS) is chronic inflammatory disease that affecting the central nervous system (CNS) which leads to the degeneration of oligodendrocytes, neurons, and myelin over time. Being the foremost reason of neurological disability multiple sclerosis has affected about 2.5 million humans around the world. MS is mainly seen in early adulthood and adults in their middle age. Disease modifying agents are being used in treatment of relapsing multiple sclerosis. Medications that reduce the severity of the disease are frequently utilized as standard therapies. The immune system is the primary focus of modern MS treatments. However, researchers are putting more effort into designing novel CNS-focused treatments. Therefore, under the current scenario, an alternative route for drug delivery is required, one that provides a speedy onset of action and then maintains the drug release over an extended period. The desired prolong-acting intramuscular injecting stable fingolimod HCl microspheres to treat MS were formulated using solvent evaporation technique. Further, based on the number of factors to be optimized, formulation was prepared using solvent evaporation. Additionally, the formulated fingolimod microspheres were characterized for the size distribution of particle, particle surface morphology, and lyophilization of formulated microspheres. Post lyophilization, the formulated microspheres evaluation was done for efficiency of entrapment, drug release *in-vitro*, and stability parameters. Furthermore, *in-vivo* studies were performed on suitable animal models for the optimized formulation.

Keywords: Formulation, Multiple sclerosis, Microsphere, Lyophilization, Particle size, Drug release, Drug content.

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INTRODUCTION

Multiple sclerosis (MS) a chronic autoimmune disorder of the central nervous system (CNS), identified as MS, causes inflammation that causes demyelination, astrogliosis, and the death of the neurons and oligodendrocytes.[1] Multiple sclerosis being the front-line neurological disability killing over 25 lakh population in their early adulthood and mid – aged adults globally. High incidence rates have been documented in the countries such as USA, Europe, Australia, Canada, northern Asia and New Zealand. The frequency is highest in Caucasians.[2]

A secondary progressive MS (SPMS) clinical history is followed by a progressive deterioration in function for most MS patients (85%). 10% of patients with primary progressive MS (PPMS) do not relapse and instead have an accelerated course from the beginning. About 10 to 15% of RRMS individuals experience a modest illness history and can maintain clinical stability for decades.[3-5]

Initiating regulatory T-cell responses may be one way that immature dendritic cells support peripheral tolerance maintenance and innate immune responses. Immunological tolerance is lost in MS patients, and their antigen-presenting dendritic cells are abnormally activated and mature.[6]

Systems for delivering medications are crucial in treating multiple sclerosis. Currently, various forms of relapsing MS are treated by means of disease-modifying therapies, and the treatment option depends on factors related to patients such as comorbidities, lifestyle, and personal preferences, as well as drug factors such as cost, availability, convenience, effectiveness, and side effects. Drugs that treat diseases and reduce their severity constitute traditional remedies. Most MS medications currently available target the immune system; however, research towards creating novel treatment plans that concentrate on the CNS is gaining momentum. Several oral MS medications are in the last stages of development.[7-9]

Research into drug delivery methods, such as microspheres, is crucial for treating MS. A recent study tested dendritic

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polyglycerol microspheres (DPGlyM) as a potential long-term drug delivery system for treating multiple sclerosis.[10-13] Microspheres are minute, spherical particles with sizes ranging from 1 to 1000 µm. They are made of polymeric, waxy, or other protective substances such as starches, gums, proteins, and lipids.

According to the relevant findings, the preparation of PLGA as a surfactant in nanoparticles preparation provides a dependable platform for a variety of antigen crosslinking techniques with in the context of providing immunological inability to prevent experimental autoimmune encephalomyelitis, the widely used pre clinical study model for MS.[14]

After four years of therapy, 85.7% of patients were found with non-relapsing of condition, no progression of disability was found in 86.3% of the patients, and 85.7% were devoid of clinical activity (no relapses, no worsening of disability), according to a study by the Spanish Gilenya Registry of patients receiving fingolimod in Spain.[15-17]

Hence, the present study focuses on developing prolong-acting intramuscular injecting stable fingolimod HCl microspheres for improved patient compliance and reduced toxicity.

MATERIALS AND METHODS

The fingolimod hydrochloride was free sample from Shilpa Medicare Ltd, Bengaluru. All the ingredients are as per I.P specifications.

Methods

The methods adopted while formulating and evaluating fingolimod-loaded microspheres are described below.

Preformulation studies

Preformulation studies are indispensable for developing safe, effective, and stable dosage forms. Preformulation data provide us with the complete depiction of properties and handling parameters of the drug, polymer, process, and processing conditions. Hence pre-formulation studies are performed to obtain a clinically safe dosage form which is effective and, stable.

Identification tests

- *Raw material appearance*

Fingolimod hydrochloride was physically examined for color, odor, and texture.

- *Determination of melting point*

The melting temperature of fingolimod HCl recorded applying the differential scanning calorimetry (DSC) method. The samples were kept in a sealed aluminium pan to determine the melting point. The pan containing the drug was kept in the DSC apparatus and ran to detect the melting point.[18]

- *Solubility studies*

Solubility of fingolimod HCl at saturation was determined in 0.1 N HCl, water, 1.2N HCl, 6.0 and 8.0 buffer pH

- *Thermoanalysis by differential scanning calorimetry*

DSC shows a potential interaction between an API and other substances and offers information about the sample's physical characteristics, such as crystalline or amorphous. An empty cell made of high-purity alpha alumina served as the reference for all calorimetric calculations. It was decided to use high-purity indium metal as a reference for measuring instruments. In a nitrogen environment, at rate of heating at 10°C min^{-1}, dynamic scans were performed at the temperature ranging from 10 to 300°C.[19]

Characterization of fingolimod microspheres

- *Particle size*

The formulated fingolimod polymeric microspheres were evaluated for size of the particles and PDI (polydispersity index) on Master sizer, Malvern instruments, based on laser diffraction principle, and temperature of 25°. The measurments were done for 12 times. The precipitation occurring during the measurments were avoided by dispersing the samples in water at a ratio of 1:10. The measurments were made trice for optimal results.[20]

- *Particle morphology*

Scanning electron microscopy (SEM) was performed by placing few drops of the prepared microsphere suspension on the glued aluminium stub. The stub was air dries at room temperature to remove moisture until complete dryness. The formulated microsphere samples were then coated with gold-palladium for around 90 seconds and further the SEM images were captured under an argon atmosphere using SEM (JEOL, Peabody, Model 6300, NY) insturuemnt at 10kV operating accelerating voltage. The dried microspheres were also subjected for SEM.[21]

Lyophilization cycle development

Freeze drying is a process where the thermo sensitive products are converted from frozen phase to solid phase by skipping the intermediate liquid phase. The processes of removal of water from the frozen product occurs *via* sublimation, where in the product is directly transited from solid phase to gaseous phase due to the application of vacuum. Majority of pharmaceutical lyophilized product and the formulation chosen in this project are aqueous based, hence water was taken as the base solvent and various steps involved in the sublimation process are given below.

- *Freezing*

Freezing is a process where the substance to be lyophilized will be freezed under atmospheric pressure either directly in the lyophilizer or in a deep freezer separetly. The product should always be frozen at 10°C lower than the solidification temperature of the product.

- *Evacuation*

Evacuation is a process where in the vacuum pump is activated when the product to be lyophilized is sufficiently frozen. the pressure within the drying chamber is decreased depending on the data obtained by the ice and water vapour pressuer curve.

- *Sublimation*

The process of sublimation starts when the thermal energy is added to the product; this additional energy enables the aqueus moisture inside the lyophylizing product to form water vapour. The ice condenser vapour pressureis always lower than the product vapour pressuer to be dried. Hence, the ice condenser is cooler than that of the formulation to be dried. As a result, released water vapours from the drying products gets condensed on the condenser coils. The primary drying phase removes maximum amount of free water. The secondary drying removes the final traces of bound water by desorption technique. This will be done at higher temperature and at least possible pressure.[22]

Drug content estimation

- *Preparation of 0.5% Tri-ethylamine solution*[23]

Approximately 5.0 mL of Triethylamine was taken in a beaker containing 1000 mL of water. Further, the pH was adjusted using orthophosphoric acid.

- *Preparation of mobile phase*

About 550 mL of 0.5% tri-ethylamine solution and 450 mL of acetonitrile was mixed uniformly. Sonicate to degas for 5 minutes (Table 1).

- *Preparation of hydrochloric acid (0.01 N)*

About 0.85 mL of conc. hydrochloric acid was placed in volumetric flask (1000 mL) containing 200 mL water. Mixed well and made up to volume with water.

- *Preparation of diluent*

In 0.01 N hydrochloric acid (550 mL) and acetonitrile (450 mL) were mixed and sonicate for 5 minutes to degas.

- *Preparation of standard fingolimod solution*[24]

About 1:7.0 mg of standard fingolimod (70 ppm of Fingolimod) was transferd into volumetric flask (100 mL) while adding 50 mL of diluent and sonicated to solubilize. Uniform solution was obtained by making up the volume with diluent under continuous mixing.

- *Preparation of sample solution*

Whole content of 5 vials were transfered into 100 mL volumetric flask, the vial was rinsed with sufficient amount of diluent and transferd into the same volumetric flask. The rinsing procedure was repeated until complete sample was recovered from the vials. And 50 mL diluent was added into the volumetric flask, and sonicated for 5 minuteswhile makingupthe volume with diluent. The 25 mL of the diluted solution was prepared from the above 5 mL of solution.

Table 1: Chromatographic systems[23]

Instrument used	*HPLC with DAD/PDA detector*
Elution	Isocratic
Column	Agilent C18, 4.6 x 150 mili meter, 5 micro meters
Wavelength of detection	220 nm
Injecting volume	10 micro/liter
Temperature inside the column	40°C
Run time of the experiment	10 Minutes
Temperature of sampler	5°C
Flow rate of the injection	1.0 mL/min

- *Procedure*

Inject blank (1 injection), standard solution - 1 (5 injections), standard solution-2 (1 injections), into HPLC equipped with UV/DAD/PDA detector and check for system suitability.

$$\text{Average Assay in \%} = \frac{(\text{\% Assay of sample-1} + \text{\% Assay of sample-2})}{2}$$

Estimation of drug entrapment[25,26]

- *Preparation of bound sample*

About 0.5 mL methanol was passed through HLB filter repeating the process for 3 more times. The one ml of sampe solution was mixed to the filtrste after equilibrate the filters with 10.0 mL of Milli-Q-water and collecting the same into 10.0 mL volumetric flask, the cartridge was washed with 2.0 mL of water, and diluted and filtered through 0.45 µm PVDF filter, first 2 mL was discarded.

- *Preparation of unbound sample*

With help of filter bound sample was washed with 4.0 mL of diluent and collecting the wased, diluted and filtering through a 0.45 µm PVDF filter discarding initial 2 mL of the filtrate. Analyze bound and unbound samples as per drug content method to estimate amount of drug present in both samples.

Methodology of in-vitro drug release[27,28]

- *IVRT media preparation*

About 2.38 g of HEPES Buffer and about 10.0 g of Tween 80 was poured into 1.0 L of water and sonicated for 5 minutes.

- *Handling of float-A-Lyzer*

The float–A–Lyser membrane was removed by carefuly by pulling out the membrane frompacking by twisting top piece collar and packing tube in opposite direction.

- *10% Ethanol preparation*

A mixture of 2 0 mL ethanol and 180 mL water was prepared and sonicated for 5 minutes.

- *Pre-wetting the membrane*

Complete removal of glycerine is the key factor to obtain most effective membrane wetting for obtaining highest permeable membarane. The float-A-Lyzer G2 gadget was first immersed in 10% alcohol, then completely flushed and submerged in HPLC water before sampling. The cap of the float-A-Lyzer G2 device was removed and filled with 10% ethanol and the cap was fastened again and submerged for 10 minutes in 10% alcohol solution with occasional shaking.

- The alcohol solution was aspirated from the float-A-Lyzer G2 device by inverting and shake out the devuce after unscrewing the cap.

- Drying out of the membrane after pre-wetting was avoided.
- Similarly, procedure was followed for the rest of the float-A-Lyzer G2 device.

Loading of sample solution[29]

- Sample preparation 1: About 50 mL of the media was taken in a 50 mL volumetric flask, and transfered into a 100 mL Duran bottle, the sample bottles were then incubated in the shaking water bath per IVRT conditions to achieve desired temperature (37°C).
- The uniform distribution of fingolimod was ensured by vortexing the sample vials. The rubber stopper was removed slowly and the contents were then transferred carefully into a container stopper (Pool 5 vials of sample solution).
- The sample solution was gently shaken to get homogenous solution, and then accurately 1.0 mL of sample was loaded into activated float-A-Lyzer G2 device. A closed seal environment was created by screwing back the cap in place.
- Similarly, same procedure was followed for the remaining sample preparation.
- Then the sample bottles were incubated in the shaking water bath as per IVRT conditions. The 0.5 mL was collected from the sample bottle by replaceing with 0.5 mL of IVRT media (Pre heated at 37°C).
- Immediately after collecting the test solution from Duran bottle, the contents were filled into HPLC vials and kept in HPLC sample tray which is maintained at about 2-8°C (Table 2).

% Fingolimod Release (Fn):

$$= \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{N} \times \frac{P}{100} \times \frac{100}{LC}$$

Where,
AT = Area response of Fingolimod (sample preparation).
AS = Avg. area response of fingolimod (Standard prepration 1)
WS= Fingolimod standard weight in mg.
N = volume of sample used for sample preparation
DS = Standard solution solution in mL.
DT = Sample solution dilution in mL.
P = Fingolimod standard potency in % w/w (as is basis).
LC = Label claim of Fingolimod in mg/mL.

Stability studies to demonstrate stability of product at various conditions

Stability studies performed as per this guideline by loading the lyophilized samples at different conditions like 25°C ± 2°C/60% RH ± 5% RH for the long term & 40°C ± 2°C/75% RH ± 5% RH accelerated condition.[29] The stability study was designed asper the Ich guidelines for all the four climatic zones (Zone I to IV)

Stability studies performed as per this guideline by loading the lyophilized samples at different conditions like 25°C ± 2°C/60% RH ± 5% RH for the long term & 40°C ± 2°C/75% RH ± 5% RH accelerated condition.[30] The stability study was designed asper the Ich guidelines for all the four climatic zones (Zone I to IV)

Table 2: IVRT Conditions[29]

Instrument	*Julabo water bath (SW 23)*
Speed (in RPM)	120 rpm
Media Volume	50 mL
Time Points (in Hours.)	1, 3, 18, 24, 28, 42, 48, 66, 72 and 115
Sampling Volume	1.0 mL of sample solution
Sample Withdraw Volume	0.5 mL
Media Replacement	0.5 mL
Media Temperature	37°C

Comparative pharmacokinetic study of Fingolimod following oral gavage, intravenous bolus and intramuscular administration test formulations and reference formulation in Sprague-Dawley male rats[31]

This study intended to study and contrast the pharmacokinetic properties of Fingolimod and Fingolimod phosphate after oral gavage, intravenous bolus, and intramuscular injection of test and standard formulations in male Sprague-Dawley rats. A parallel study was design (n = 10/group) and ummarized in the Table 3.

Serial blood sampling design was used for blood collection. Blood samples were collected at 4, hrs interval for about 14 days and post dose for G1 and G3 for about 7 days and post dose for G2. For G1 and G2 group 250 µL of blood was collected. To extract hemolyzed blood, roughly 0.200 mL of blood will be put to a microfuge tube filled with an equivalent volume of milli-Q water. For group 3, 0.500 mL of blood was taken by the retro orbital plexus and placed in a labeled microfuge tube with 200 mM K2EDTA solution (20 µL/mL). The blood samples were centrifuged at 5000 rpm for 5 minutes at a temperature of 4°C. An aliquot of 200 µL plasma was transferred to an Eppendorf tube with 40 µL glycerol. All samples were kept below -60°C until bioanalysis.[32]

The samples were tested for Fingolimod and Fingolimod phosphate using a fit-for purpose LC-MS/MS technique with lower limit of quantification (LLoQ) values of 20 and 50 pg/mL, respectively. The pharmacokinetic properties of Fingolimod and Fingolimod phosphate were estimated using the non-compartmental analysis tool of the verified Phoenix® WinNonlin® program (version 8) Table 4.[33]

Haematological study in sprague dawley rats, to estimate lymphocyte count[34,35]

The formulated Fingolimod microspheres in suspension for Injection (7.0 mg/mL) was administred intramuscular route to Sprague Dawley rats to determining the lymphocytes number. The Sprague Dawley rats were taken for the study. These animals were grouped into 4 groups (G1, G2, G3 and G4) containing 6 animals of either sex totalling to 48 rats. Rats in the group G2, G3 and G4 were injected with 1, 5 and

Table 3: PK study animal group[34]

Treatment	*Group/Route*	*No. of rats*	*Dose (mg/kg)*	*Dose volume (mL/kg)*	*Concentration (mg/mL)*
TF_1	G1/IM	10	0.361	0.103	3.5
Reference formulation	G2/ PO	10	0.051	0.025	0.5
TF_2	G3/IV	10	0.051	0.102	0.5

Vehicle: Ready to use dose formulation was supplied by the sponsor; Food and water was provided ad libitum. a: Represented as 0.5 mg/ capsule

10mg/kg body weight dosage of Fingolimod microsphere injectable suspension (7.0 mg/mL) with a dose volume of 0.142, 0.714 and 1.428 mL/kg body weight respectively. Group G1 was administered with placebo for fingolimod microsphere injectable suspension (7.0 mg/mL) of 1.428 mL/kg. The entire amount volume of placebo and test item was administered through the muscles, having equal amounts gave at two distinct places (left and right thigh muscles). Ready-to-use dose formulations provided by the sponsor were used for administration.

Throughout the studies the rats were thoroughly monitored for clinical signs and symptoms daily and twice a day for mortality/morbidity. Along with that the weight of the animals were recorded. The fasting body weight was measured on day 15.

The site of injection was examined for erythema and oedema on the day of therapy at 1, 4, 6 and 24 hours post dose (Table 5).

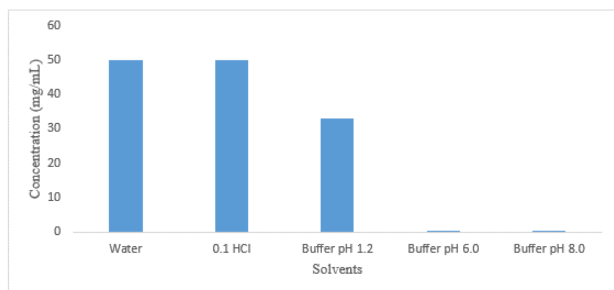

Figure 1: Solubility profile of Fingolimod HCl in different pH

Study Design: The selected animals were assigned to treatment and control groups as shown in Table 6.

RESULTS

Preformulation Studies

Identification test

- *Raw material appearance*

The obtained Fingolimod HCl was in crystalline and white in color.

- *Determination of melting point*

Fingolimod HCl melting point by the capillary method was found in the range of 107 to 111ºC.

- *Solubility*

Fingolimod aqueous solubility, was studied in different pH buffers, and the relevant solubility profile is specified below. The drug was also soluble in water, and 0.1N HCl as specified in the literature and thus confirms the identity of the drug. Table 7. Figure 1 is shown.[36]

- *Thermoanalysis*

Thermoanalysis was done on fingolimod HCl by DSC at 10°C/min. A sharp endothermic melting cutve at of 109.54°, the thermograms obtained is shown in Figure 2 and corresponding temperature values are captured in Table 8

Formulation of microspheres loaded with Fingolimod HCl

As indicated in Table 9, Fingolimod-loaded PLGA microspheres with good polidispersity index were produced in the size range

Table 4: Animal study sample collection details[36]

Anticoagulant	*K2EDTA (200 mL 20 µL/mL) blood assay*
Collection site	Retro orbital plexus
Sample collection	G1 & G2: 4, 8, 12, 16, 24, 48, 72, 120, 168 192, 216, 240, 264, 288, 312 and 336 hours post dose. Approximately, 0.250 mL of blood will be collected from retro orbital plexus from each animal at respective time points. 0.200 mL of blood will be transferred to microfuge tube containing equal volume of milli-Q water to obtain hemolyzed blood.
Sample process	Blood tubes will be placed on ice and shaked at 6000 rpm for five minutes at 4 ± 2ºC within 1-hour of scheduled time. Plasma will be separated and stored at -60°C until bioanalysis. Remaining cellular component will also be stored for analysis.
Data analysis	
Bioanalysis	Hemolyzed blood samples will be evaluated using a fit-for-purpose technique for quantifying total Fingolimod base, phosphate form and total Fingolimod LLoQ 20 pg/mL
Pharmacokinetic analysis	Non-compartment model analysis tool of the validated Phoenix WinNonlin software (version 8) will be used for pharmacokinetic analysis.

Table 5: Test system for haematological study[36]

Animal	*:*	*Rat*
Species/Strain	:	Sprague Dawley
Selection rationale	:	As the dosage is an injectable suspension with a lower volume of injection, Rats were chosen as the suitable species and also considering the regulatory guideline for pre-clinical studies.
Animal source	:	In-house
Body weight	:	141.06–167.26 g (males) 130.32–151.39 g (females)
Grouping	:	Placebo control (G1) small dose(G2) Middle dose (G3) Large dose(G4)
No. of animals/group	:	06 Male + 06 Female / group Total of 29 males and 29 females (nulliparous and non-pregnant) Extra animals were euthanized after initiation of treatment)
Treatment age	:	7 weeks/49 days
Identification of animal	:	The tails of the animals were marked with marker pen and cage cards during acclimatization period while with tumeeric solution during the treatment period

Table 6: Control & treatment group[36]

Group	*Group description*	*Dose* as per body weight*	*Con* (mg/mL*)*	*Dose volume as per body weight*	*No. of Animals/Group*	*Animal No*	
						Male	*Female*
G1	Placebo Control	0	0	1.428	6M + 6F	Rf4651 to Rf4656	Rf4675 to Rf4680
G2	small Dose	1		0.142	6M + 6F	Rf4657 to Rf4662	Rf4681 to Rf4686
G3	Mediu Dose	5	7	0.714	6M + 6F	Rf4663 to Rf4668	Rf4687 to Rf4692
G4	Large Dose	10		1.428	6M + 6F	Rf4669 to Rf4674	Rf4693 to Rf4698

M: Male, F: Female; *: Dosed at two different thigh muscles

Table 7: Solubility analysis of Fingolimod HCl

S. No	*Solvent*	*Solubility (mg/mL)*
1	Water	50
2	0.1 HCl	50
3	Buffer pH 1.2	33
4	Buffer pH 6.0	0.1
5	Buffer pH 8.0	0.1

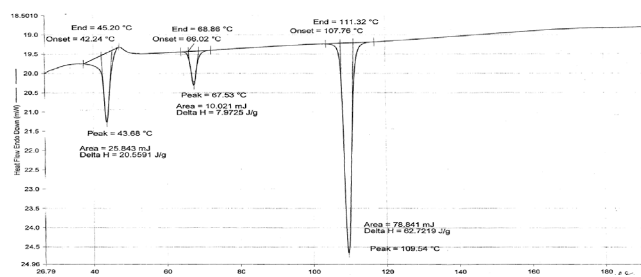

Figure 2: DSC thermograms of Fingolimod HCl

Table 8: DSC data Fingolimod HCl

S. No.	*Formulation*	*To (°C)*	*Tm (°C)*	*Tc (°C)*
1	Fingolimod HCl	107.76	109.54	111.32

of 10 to 15 µm. The presence of an emulsifier often affects the size of PLGA microparticles. In this instance, PVA was utilised to stop the polymer from clinging to the stirring surface causing emulsion droplet agglomeration. Particles may be stabilised with 0.25% PVA even while they were being stored. This is supported by the polydispersity index, which was extremely low due to the absence of aggregation and the persistence of the particles' narrow dispersion, as also demonstrated by the SEM research. To correlate additional parameters like surface area and size distribution while working with micro particulate systems, it is frequently advantageous to visualise particle shapes and surface characteristics. As seen in Figure 3, the solvent evaporation technique produced spherical, extremely smooth, fingolimod-loaded PLGA microspheres. The immunosuppressive agent's loading had no discernible impact on the animals' morphology. The restricted particle size distribution discovered by PSD analysis for each batch was supported by SEM micrographs. As it can be seen, the preparation technique had little effect on the particle morphology.

Characterization of Fingolimod Microspheres

Particle size

The size of the particle and its distribution analysis on the optimised formulation was performed to evaluate the applicability of the prepared microsphere particles for injectable purpose. The polydispersity index (PDI) was found to be 0.32 and the particle size to be 3.16. The values were in good agreement with reference to the particle size required to be qualified as an injectable formulation as specified in the literature (Table 9).

Particle morphology

The restricted particle size distribution discovered by PCS testing for each batch was supported by SEM micrographs. The particles were round, homogenous, and had a smooth surface, according to the results of the exterior morphological analyses

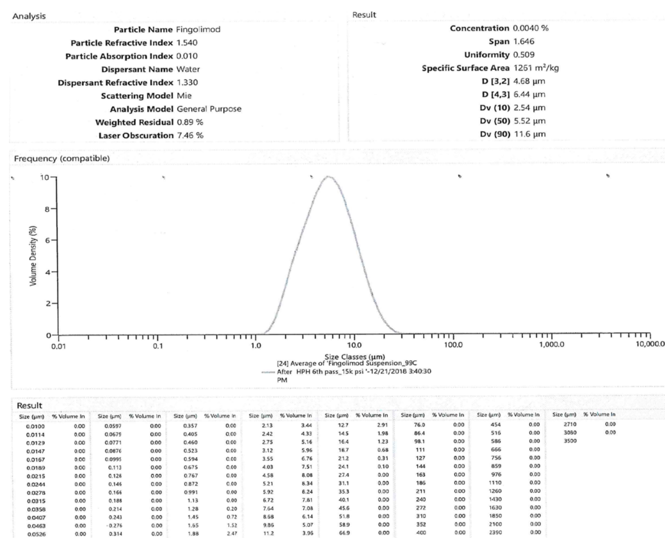

Figure 3: Optimized Fingolimod loaded microspheres particle size distribution

Table 9: Particle size distribution of microsphere formulations

Formulation code	*Particle size (µm)*		
	D10	*D50*	*D90*
FM1	3.7	7.1	15.6
FM2	4.4	8.2	14.8
FM3	3.2	7.1	13.1
FM4	3.1	5.5	12.2
FM5	2.7	7.1	11.7
FM6	3.0	6.2	12.1
FM7	2.5	5.5	11.6
FM8	2.6	6.0	12.9

conducted under the SEM. As demonstrated in Figures 4 and 5, fingolimod-loaded PLGA microspheres created by the solvent evaporation technique had a spherical form and an extremely smooth surface. The immunosuppressive agent's loading had no discernible impact on the morphology. The morphology is same in case of all the formulations but only slight variation is seen in terms of the particle size. Which is the driving force in choosing an optimum formula to produce micro particles with better physical, morphological and drug release characteristics.

Lyophilization Cycle Development and Optimization

Lyophilization cycle development

Freeze drying cycle is developed based on the parameters considered as per Table 10 to have successful, efficient, repeatable & scaleable lyophilization cycle recipe.

The lyophilization cycle recipe typically contains three stages such as freezing, 1^0drying and 2^0 drying. The freezing extent of is determined by freezing the product at various temperature and for specific time duration and further holding the frozen product intact for several minuted to hours to obtain uniform frozen particle throught the sample in the vials. This process is attributed to the phase transition which is described as a function of pressure and temperature in the curve for vapour pressure of ice and water (sublimation pressure curve).

Further the samples will be dried at two stages, the primary drying where in the maximum moisture is removed by applying various temperature condition for a specified period of time. At this stage the vapour pressure rises as the temperature does.

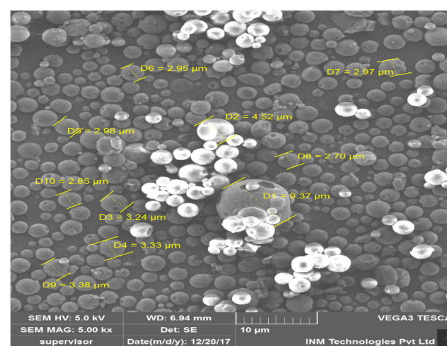

Figure 4: SEM Image of optimized Fingolimod loaded microspheres

Figure 5: Graph of product lyophilization cycle

Figure 6: Final product after lyophilization cycle

And to this when energy is added, the vacuum inhibits the melting of the ice. And during the secondary drying stage the left over moisture will be removed at a lesser pace than that occurs at the primary drying stage and this occurs due to the application of heat energy to a frozen product when it is in a vacuum, the product won't thaw and the water it contains will instead be expelled as water vapour. The complete graphical representation of the lyophilisation cycle recipe is depicted in Figure 5.

The product obtained after lyophilisation is presented in Figure 6, this show that the lyophilised cake formed is amorphous and there is no sign of melting back or evident shrinkage of the cake as shown in Table 10.

Table 10: Optimized lyophilization cycle

S. No.	*Steps*	*Temperature*	*Time*	*Vacuum*	*Ramp-Hold*
1.	Freezing	-5	100	-	$
		-5	200	-	#
		-25	50	-	$
		-25	90	-	#
		-50	60	-	$
		-50	100	-	#
2.	Primary Drying	-50	30	750	#
		-35	120	400	$
		-35	800	400	#
		-25	150	300	$
		-25	400	300	#
		20	150	100	$
		20	300	100	#
		25	30	100	$
3.	Secondary Drying	25	60	100	#

$ Ramp # Hold
*Total freeze-drying cycle was reduced from 67.41 to 44 hours.
Moisture content was reduced from 2.8 to 1.4%
Reconstitution time of the product was reduced from 4 to 1.5 minutes.
Further this will be freezed as final lyophilizatioin cycle as it validated with optimized parameters.

Drug Content Estimation; Method Precision

For the development of the analytical assay method, the following variables were taken into account.

A homogenous batch sample should be examined six times for technique precision. By doing so, you can tell if a procedure consistently produces the same results for every batch. Sample were prepared 6 times and analysed as per method condition. The Table 11 suggest that the data obtained during method precision test meets the acceptance criterion (Table 11).

Table 11: The % assay for Fingolimod for method precision analysis

Trial sample preparation	*Assay (%)*	*Average*	*SD*	*% RSD*
Trial Sample 1	98.3	98.5	0.3800	0.39
Trial Sample 2	98.2			
Trial Sample 3	98.1			
Trial Sample 4	99.1			
Trial Sample 5	98.6			
Trial Sample 6	98.7			

Estimation of Drug Entrapment

The samples were estimated for bound and un-bound drug in the microspheres. An essential factor in determining how well microspheres can load drugs is the drug entrapment efficiency. This parameter is established by the synthesis method, the medication's physical, chemical characteristics, and formulation factors. The drug's capture rate varied from 86 to 88%. Table 12 is shown.

Method Development and *In-vitro* Evaluation of Drug Release

In-vitro release was tested as per validated method and the results in Table 13 shows the percentage of drug release from the microsphere more than 98% of the drug was released in 163 hrs. The data obtained are represented in Tables 13 and 14 and represented graphically figure 7.

In-vitro release results indicate that the release pattern of 7 mg of Fingolimod was released over a period of 163 hours, almost 0.5 mg/day. These results to be simulated

Table 12: Evaluation of drug entrapment

Trial sample set	*Bound percentage*	*Un-bound percentage*
1	87.3 ± 0.12	13.2 ± 1.04
2	87.8 ± 1.67	13.6 ± 0.86
3	86.7 ± 0.89	12.8 ± 0.79
4	87.1 ± 1.35	12.9 ± 0.84
5	87.4 ± 0.93	13.2 ± 0.53
6	88.0 ± 0.57	12.8 ± 1.64

with the pharmacokinetics data of drug release pattern in *in-vivo* conditions. IVRT was tested with 2 units which were operated at same condition and results indicate that the method adopted and the operating conditions like media, temperature, rpm etc. implemented were validated and found to be satisfactory. The drug released was estimated using assay method by HPLC and all condition were same as per assay method development.

Drug entrapment was estimated by preparing samples as described in the methodology using HLB filters, here concentration of free drug and encapsulated drug was analysed and estimated using the same assay method of analysis. As per the results it is evident that about 87% of drug is in an encapsulated form, hence the correlation of encapsulation and drug release pattern were satisfactory to freeze out the formulation and can subjected for further stability studies.

Stability Studies

Study based on stability was conducted and all analytical data depicted in the Tables 14 and 15, fascinates on the stability of product qualifying all the limits specified for each tests. Even at the accelerated condition observation till 3 months do not foresee any abrupt results in any of the physico-chemical parameters. Hence the product is continued to be stability conditions till 6 months.

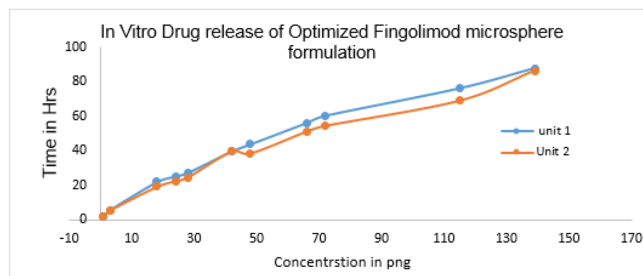

Figure 7: *In-vitro* release of fingolimod injectable suspension

Syringebility Studies

The Fingolimod microspheres formulation is targeted to be delivered through parenteral route *via* intramuscular route where the product forms depot in the deltoid muscles. Removal of the formulated product from the container and further injecting through the dermal tissue is highly dependent on the fluid property. Hence, syringebility plays a crucial role. Based on the obtained data in Table 16 it can be declared that the prepared formulation can be administered *via* parenteral route without any obstruction.

Furthermore, the study was expanded to analyze the influence of syringebility on particle dispersion, and the data is summarized in Table 17.

Syringebility study was performed using 4 types of needles depending on the particle size in the formulation. Needle size selected was selected based on syringebility of the particle size data. Here in assay results we can clearly make out that in 26-gauge needle there was a significant drop in the total API content in the formulation which was injected through 26-gauge needle, and the average results was 93.98, which was on the lower side comparing to assay results of other gauge needles. Even in the particle size distribution measured using lasser diffraction technique implies that there was significantly

Table 13: Percentage drug release from microsphere

Time points	*Unit-1*	*Unit-2*	*Avg % of release*	*Std. Dev.*	*%RSD*	*Min.*	*Max.*
1	2	2	2	0.001	0	2	2
3	6	5	6	0.154	3	6	6
18	22	19	20	1.955	10	22	22
24	25	22	24	1.884	8	25	25
28	27	25	26	1.890	7	27	27
42	40	35	37	3.463	9	40	40
48	44	38	41	3.740	9	44	44
66	56	51	54	3.503	7	56	56
72	60	54	57	4.128	7	60	60
115	76	69	73	5.088	7	76	76
139	88	86	87	1.034	1	88	88
163	98	98	98	0.007	0	98	98
0	5	5	5	0.256	5	5	5

Table 14: SOP for accelerated stability study at 40 ± 2°C/75 ± 5% RH

Parameters		*Initial:*	*1st Month:*	*3rd Month:*
Product subjected stability Fingolimod extended release injectable suspension 7.0 mg/mL in 2 mL flint 13 mm neck vials, 13 mm omniflex flurotec coated rubber stopper, and 13 mm tear off clear lacquered aluminium seals.				
Description: (White to off white injectable suspension in clear tubular glass vial, grey rubber stopper and aluminium seal.)		White opaque homogeneous suspension.	White injectable suspension in clear tubular glass vial, grey rubber stopper and aluminium seal.	
Identification		Complies	Complies	Complies
pH (Between 6.0 and 8.5)		7.48	7.40	7.42
Osmolality (250–350 mOsmol/kg)		311	307	305
Zeta potential (For information only)		-33.5	-33.7	-34.7
Viscosity (Between 18–cps)		20	NP	NP
Assay by HPLC (NLT 90.0% & NMT 110.0%)		103.7	101.4	102.1
Particle Size	D10	2.5	2.8	3.1
Distribution	D50	5.5	5.2	6.2
by Master sizer (in µ)	D90	11.6	11.7	11.4

noticeable reduction in the particle size which was ranging in the range of 29.2 micron where as other gauge needles injection produced around 36 microns. This also clearly evident that there is a resistance to the smooth flow of particles through 26-gauge needle. Hence it is of clear evident that 26 gauge and above bore size needle cannot be recommended for administration to patient. The final syringebility size of the needle ranges from 21 gauge to 25-gauge needles can be effectively used in delivering the drug content to the patients

Comparative Pharmacokinetic Study of Fingolimod Following Oral Gavage, Intravenous Bolus and Intramuscular Administration of Sample Formulations and Standard Formulation in Male Sprague-Dawley Rats

The Fingolimod and Fingolimod phosphates are the active metabolite formed after injecting the drug through intra muscular, oral gavage (Reference Product) and intravenous route. These active metabolites were determined and the pharmacokinetic profiles were tabulated.

Further the haemolysed blood concentration with respect to time was analysed and the amount of Fingolimod and Fingolimod phosphate active merabolites were measured upto 14 days with 12-hour intervals. The detailed results are tabulated in Table 18.

Following single intramuscular administration of test formulation (TF_1) to rats (0.361 mg/kg), the median time to attain peak concentration plasma for fingolimod and fingolimod phosphate was found to be 6.00 hours (4-168 hours) and 8.00 (4.00–216 hours), respectively. The corresponding peak concentrations were 4.57 and 12.7 ng/mL. The exposure (AUC last) ratio of Fingolimod phosphate to Fingolimod was found to be 3.54 indicating comparatively higher exposure of Fingolimod phosphate.

Following administration of refrence formulation via single oral gavage to rats (0.051 mg/kg), the median time to attain peak concentration at plasma for fingolimod and fingolimod phosphate was found to be 12.0 hours (8–12 hours) and 12.0 (8.00-24 hours), respectively. The corresponding peak plasma concentrations were 0.899 and 2.86 ng/mL. The exposure (AUClast) ratio of Fingolimod phosphate to Fingolimod was found to be 4.11 indicating comparatively higher exposure fingolimod phosphate.

By single intravenous bolus of test formulation to rats (0.051 mg/kg), the median time to attain peak cellular components concentration for fingolimod phosphate and fingolimod was found to be 10.00 hours (8-16 hours and 4.00 (4.00-24 hours), respectively. The corresponding peak plasma concentrations were 6.21 and 21.3 ng/mL. The exposure (AUClast) ratio of Fingolimod posphate to fingolimod was found to be 4.22 indicating comparatively higher exposure of fingolimod phosphate.

By single intravenous bolus administration of test formulation to rats (0.051 mg/kg), the median time to attain peak concentration at plasma for fingolimod encapsulated, free fingolimod and fingolimod phosphate was found to be 4.00 h (4–4 hours), 8.00 (4.00–16.0) and 8.00 (4.00–12 hours), respectively. The corresponding peak concentrations at plasma were 0.0547, 0.31 and 1.11 ng/mL. The exposure (AUClast) ratio of Fingolimod Encapsulated to free form was found to be 16.1 indicating comparatively higher exposure of fingolimod encapsulated (Figures 8-11).

Haematological Study in Sprague Dawley Rats to Estimate Lymphocyte Count

The lymphocytic count was determined as there is a significant alteration of lymphocyte count due to multiple sclerosis. After the Fingolimod injectable suspension administration the lymphocytic counts estimated to determine the improvement of the disease condition. The data was generated at various routes such as IM, SC and IV route and tabulated in table.

Table 15: SOP for Real time stability study 25 ± 2°C/60 ± 5% RH

Parameters		*Initial*	*1st Month*	*3rd Month*
Description: (White to off white injectable suspension in clear tubular glass vial, grey rubber stopper and aluminium seal.)		White opaque homogeneous suspension	White injectable suspension in clear tubular glass vial, grey rubber stopper and aluminium seal.	
Identification		Complies	Complies	Complies
pH (Between 6.0 and 8.5)		7.48	7.34	7.34
Osmolality (250–350 mOsmol/kg)		311	308	308
Zeta Potential (For information only)		-33.5	-31.3	-32.9
Viscosity (Between 18–80 cps)		21	NP	NP
Assay by HPLC (NLT 90.0% & NMT 110.0%)		103.7	101.4	102.2
Particle size distribution by Master sizer (in µ)	D10	2.5	2.8	3.1
	D50	5.5	5.2	6.2
	D90	11.6	11.7	11.4
	Total impurity NMT (3.0%)	0.03	0.03	0.05

Table 16: Syringebility data with different gauge needle

S .NO.	*Vial no.*	*Assay %*			
		Gauge 21	*Gauge 23*	*Gauge 25*	*Gauge 26*
01	Vial No. 1	101.1	98.2	98.2	98.2
02	Vial No. 2	100.4	94.2	94.2	94.2
03	Vial No. 3	101.7	100.6	100.6	92.6
04	Vial No. 4	102.4	103.6	103.6	90.6
05	Vial No. 5	98.9	101.4	101.4	94.4
06	Vial No. 6	105	99.9	99.9	93.9
Avg		101.6	99.7	99.7	93.98

Table 17: Syringebility effect on particle size distribution

B. No.	*Stage*	*PSD (µm)*				
		Uniformity	*D10*	*D50*	*D90*	*Span*
(Stirring time: 15 min) (D90 = 15 µ)	Initial	0.52	2.2	6.5	15.3	1.729
	21G needle	0.586	2.8	6.77	14.7	1.887
	23G needle	0.582	1.9	6.38	14.0	1.86
	25G needle	0.582	2.0	6.08	15.2	1.86
	26G needle	0.582	1.5	5.2	13.8	1.86

- There were no clinical indication of mortality or toxicity were seen in either sex groups.
- No indication of treatment associated skin modifications the site of injection was seen.
- In all the groups of either sex, there was no changes in body weight or percent change in day 1 with regard to body weight.

Figure 8: Pharmacokinetic study of Fingolimod following oral gavage in SD rats

Figure 9: Pharmacokinetic study of Fingolimod following IV in SD rats

Figure 10: Pharmacokinetic study of Fingolimod following intramuscular route in SD rats

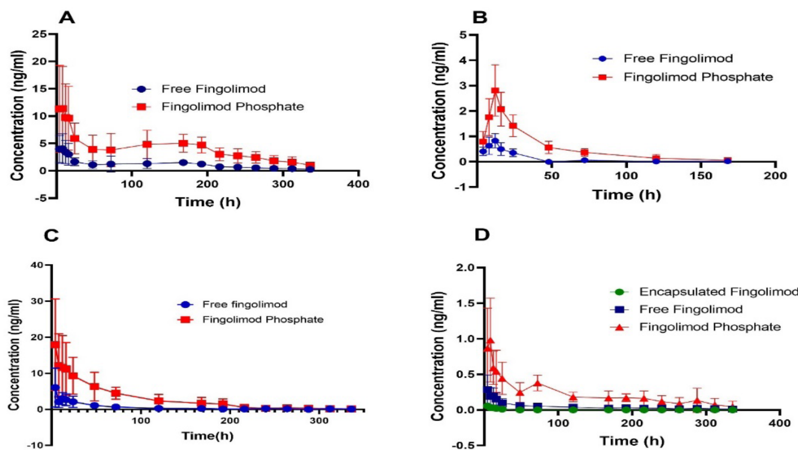

Figure 11: concentration vs time plasma profile of Fingolimod and Fingolimod phosphate A) Intramuscular route, B) oral route reference product and C & D) intravenous bolus administration at cellular and plasma component respectively of TF_1 form

Table 18: Pharmacokinetic parameters of Fingolimod and Fingolimod phosphate

Treatment (Route)	*Matrix*	*Analyte*	*T_{max} (h)*	*C_{max} (ng/mL)*	*AUC (ng.h/mL)*	*MRT (h)*	*AUC ratio*
TF_1 (IM)	Hemolyzed blood	Fingolimod	6.00 (4.00–168)	4.57 ± 2.66	376 ± 131	126 ± 32.1	NA
		Fingolimod phosphate	8.00 (4.00–216)	12.7 ± 7.52	1330 ± 309	134 ± 33.3	3.54
RF_1 (PO)		Fingolimod	12.0 (8.00–12.0)	0.899 ± 0.242	21.4 ± 5.85	30.8 ± 10.2	NA
		Fingolimod phosphate	12.0 (8.00–24)	2.86 ± 0.965	88.0 ± 22.2	36.9 ± 8.35	4.11
TF_2 (IV)	Cellular components	Fingolimod	4.00 (4.00–16.0)	6.21 ± 5.26	234 ± 129	60.7 ± 25.6	NA
		Fingolimod phosphate	10.0 (4.00–24.0)	21.3 ± 12.1	988 ± 248	65.2 ± 31.2	4.22
	Plasma	Fingolimod encapsulated	4.00 (4.00–4.00)	0.0547± 0.0413	0.979 ± 0.627	6.14 ± 2.47	NA
		Fingolimod free	8.00 (4.00–16.0)	0.31 ± 0.193	15.8 ± 4.46	98.7 ± 33.6	16.1
		Fingolimod phosphate	8.00 (4.00–12.0)	1.11 ± 0.555	77.7 ± 20.4	102 ± 28.7	NA

- Absence of ocular changes after treatment.
- Decrease in white blood cells and differential leukocyte count in all tested group was noted which reveals the immunosuppressant nature of the test item fingolimod which correlates with decreased corticomedullary ratio in thymus from mid dose females and at high dose males; and depletion of lymphocytes in marginal zone of white pulp in spleen histopathologically and is considered to be of test item related effects. Changes seen in other variable counts may be related to how the test items were administered and are consistent with how fingolimod was administered.
- No toxicologically significant modifications were noted in coagulation, urinalysis parameters and clinical chemistry.
- Ther were no changes related to organ weight, body weight and their ratios were observed which were associated to the test item treatment. Decrease in thymus weight in low dose males was not associated with changes microscopicaly.
- No observation of gross pathological modifications was made in either of the sex groups including site of injection.
- Changes connected to the test item were seen at the microscopic level in thymus and spleen. In thymus, minimal to mark decreased corticomedullary ratio was observed from mid dose females and at high dose males. This change was characterized by enlarged medullary region with accumulated lymphocytes and relatively thin cortical region. In spleen, white pulp cellularity was decreased at mild to moderate level was observed in group administered with higher dose and further characterized

Table 19: Hematological study in sprague male dawley rats, to estimate to lymphocyte countat various dosage

Haematocrit parameters	*Sprague male Dawley rats*							
	G1		*G2*		*G3*		*G4*	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Leucocyte count (WBC) (10^3 cells/µL)	10.54	4.13	5.29	2.67	3.46*	0.86	6.27	6.26
Total erythrocyte count (RBC) (10^6 cells/µL)	7.73	0.34	7.44	1.18	8.24	0.61	7.09	1.40
Hemoglobin (HGB) (g/dL)	15.55	0.88	15.23	1.72	15.72	0.75	14.42	2.05
Haematocrit (HCT) (%)	47.72	1.52	45.60	4.32	47.77	2.49	44.93	3.56
Mean corpuscular volume (MCV) (fL)	61.78	2.52	61.93	5.66	58.05	1.97	65.43	13.34
Mean corpuscular hemoglobin (MCH) (pg)	20.10	0.62	20.65	1.91	19.12	0.71	20.65	2.17
MCHC (g/dL)	32.57	1.47	33.57	4.08	32.90	0.40	31.95	2.52
Platelet Count (PLT) (10^3 cells/µL)	867.33	151.01	833.17	199.70	877.00	67.27	908.00	259.31
Mean platelet volume (MPV) (fL)	6.23	0.33	6.72	0.80	6.60	0.37	6.98	0.69
Reticulocyte count (Retic) (%)	4.76	3.42	4.69	4.92	4.46	1.06	12.56	18.00
Neutrophils (Neut) (%)	21.27	4.67	33.98	11.27	51.60*	3.57	55.23*	16.34
Lymphocytes (Lymph) (%)	71.98	3.85	56.07*	13.12	35.57*	3.86	32.97*	16.27
Monocytes (Mono) (%)	3.73	1.34	5.58	2.67	8.63*	0.67	7.72*	0.86
Eosinophils (Eos) (%)	0.93	0.48	1.68	0.52	2.20	0.99	1.97	1.35
Basophils (Baso) (%)	0.68	0.17	1.45	1.28	0.93	0.35	0.58	0.20
Absolute reticulocyte count (Retic) (10^9 cells/L)	360.42	239.64	309.72	292.52	364.75	78.12	687.02	730.64
Absolute neutrophils (Neut) (10^3 cells/µL)	2.28	1.21	1.74	0.95	1.78	0.45	3.70	4.14
Absolute lymphocytes (Lymph) (10^3 cells/µL)	7.57	2.86	3.07*	2.07	1.23*	0.35	1.88*	1.65
Absolute monocytes (Mono) (10^3 cells/µL)	0.39	0.18	0.25	0.10	0.30	0.09	0.47	0.42
Absolute eosinophils (Eos) (10^3 cells/µL)	0.09	0.05	0.09	0.06	0.07	0.02	0.12	0.12
Absolute basophils (Baso) (10^3 cells/µL)	0.08	0.04	0.08	0.10	0.03	0.02	0.04	0.03
Prothrombin time (PT) (Seconds)	18.67	3.41	17.52	2.46	17.17	1.47	17.18	1.37
Activated prothrombin time (APTT) (Seconds)	23.88	1.26	25.82	9.20	22.88	4.42	23.32	1.92

by depletion of lymphocytes in marginal zone of white pulp.

- Significant statistical rise in neutrophils percentage in G3 (+142.63%), G4 (+159.72%) and percent monocytes in G3 (+131.25%), G4 (+106.70%); decrease in WBC in G3 (-67.18%) and absolute lymphocytes in G2 (-59.43%), G3 (-83.70%), G4 (-75.15%); and in females, increase in percent neutrophils in G2 (+145.42%), G3 (+203.39%), G4 (+186.08%); percent monocytes in G4 (+143.84%); percent eosinophils in G2 (+468.97%), G3 (+548.28%); and decrease in white blood cells in G2 (-59.54%), G3 (-58.97%), G4 (-46.92%); absolute basophils in G2 (-65.22%), G3 (-78.26%), G4 (-47.83%); percent lymphocytes in G2 (-75.14%), G3 (-80.67%),G4 (-75.29%) were noted. The noted changes in white blood cells and differential leukocyte count in all tested group reveals the immunosuppressant nature of the test item fingolimod which correlates with decreased corticomedullary ratio in thymus from mid dose females and at high dose males; and depletion of lymphocytes in marginal zone of white pulp in spleen histopathologically and is considered to be of test item related effects. Changes seen in other

variable counts may be related to how the test items were administered and are consistent with how Fingolimod was administered (Table 19).

DISCUSSION AND CONCLUSION

Discussion

The current research work has made an attempt to formulate a sustained release depot injection of microspheres containing Fingolimod HCl at active pharmaceutical ingredient, which gives prolonged availability of the drug from the depot site of intramuscular tissues, without need of frequent administration as in case of daily oral intake of Fingolimod capsules. Thus, establishing improved patients' compliance as, patients may skip daily doses if taken oral capsules, thus establishing patient adherence to treatment along with reduced side effects like myocardial infractions. the obtained results from the study are summarized.

- Intramuscular depot forming microspheres which contains Fingolimod was successfully formulated by O/W solvent evaporation technique.
- Initial studies were carried out to optimize the microsphere formulation and process variables that can affect the desired characteristics of the intramuscular injectable microspheres.
- Identification studies viz., solubility determination, melting point analysis, DSC, revealed that the procured samples confirmed their individual identity.
- Preformulation studies revealed optimum concentration of PLGA, PVA and drug to be used for obtaining drug loaded MS with desired inhalable characteristics.
- Compatibility studies viz. DSC, showed that chosen drug and polymer did not had any compatibility issues with each other.
- The pre-formulation studies were performed using central composite design to fabricate desired drug loaded microspheres.
- The fabricated drug loaded MS showed improved drug loading and entrapment efficiency with a desired inhalable particle size.
- Formulation was stabilized by freeze drying technique, where a robust lyophilzation cycle was developed to have uniformly freeze-dried product along with longer stability.
- Drug content estimation and impurity profile estimation method was developed using HPLC and the method was validated.
- SEM microphotographs of the formulation indicated that the produced microspheres were virtually spherical in shape, with little adsorbed free drug on the surface.
- The fabricated MS showed sustained and controlled release when compared to free API alone.
- Syringeability studies were performed to check right needle for injecting the lyophilized product through intramuscular route to pateints.
- *In-vitro* drug release studies were carried out to check the drug release data of the product, and it was found that the drug from the site was releasing for a period of 168 hours.
- The stability study results revealed that the optimize drug loaded MS were stable with respect to their visual appearance, drug loading and entrapment efficiency, drug content, particle size & impurity profile at room temperature than that of the accelerated temperature.
- *In-vivo* studies were performed in Sprague-Dawley rats, a Comparative pharmacokinetic study of Fingolimod following oral gavage, intravenous bolus and intramuscular administration test formulations and reference formulation. Fingolimod and Fingolimod phosphate were evaluated and compared, and the results showed that the intramuscular drug distribution profile complied with our estimated drug release features.
- Hematological study was done in Sprague Dawley rats for estimate of lymphocyte count decrease in white blood cells and differential leukocyte count in all tested group was noted which reveals the immunosuppressant nature of the test item Fingolimod which correlates with decreased corticomedullary ratio in thymus from mid dose females and at high dose males.

CONCLUSION

Our study reveals that the fabricating an intramuscular injection of Fingolimod can release drug in a sustained and controlled manner without any burst release and no increase in impurity levels in the formulation, this can be administered to patients to have prolonged action for about seven days, and in comparison to oral administration of fingolimod capsule which has to administered on a daily basis can be replaced, improving the patient compliance as patients affected by this condition will experience neurological disorder associated with memory loss, which will make patient to skip their daily intake of oral capsules. And the study also reveals that there is no haematological toxicity associated with large dose administration through intramuscular route.

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