“Design of antiretroviral formulations for targeting CNS as HIV sanctuary site”

Abstract

Efavirenz is inhibitor of non-nucleoside reverse transcriptase enzyme; BCS class II drug. The objective of the present research was to prepare and evaluate nanosuspension of Efavirenz for the treatment of neuro-AIDS. Efavirenz is the substrate for drug resistant proteins at BBB prone to efflux and could not reach brain with effective levels. Current need of the therapy is to develop drug delivery systems targeting viral reservoirs at effective concentration in the brain. With this need we developed Efavirenz nanosuspension for nose to brain drug transport to bypass blood brain barrier. Nanosuspension prepared with high-pressure homogenization had a mean particle size of 223 nm, PDI of 0.2 and -21.2 mV zeta potential. Histopathology study on goat nasal mucosa showed no adverse effects of formulation on nasal tissues. Gamma scintigraphy study and in-vivo study on Wistar rat model reveals drug transport to the CNS after nasal administration. Pharmacokinetic parameters and drug targeting potential of 99.46 % suggest direct nose to brain transport of Efavirenz nanoparticle. Results reveal that nose to brain delivery of Efavirenz is the best possible alternative for neuro –AIDS treatment. The problem of limited oral bioavailability can be solved by preparing the drug nanoparticles. Reducing the particle size to the nanoscale of drug molecule offers a higher surface area for the drug interaction with the solvent and results into improved aqueous solubility so the bioavailability. Stability studies provide evidence of how the quality of an API or FPP varies with time under the influence of a variety of environmental factors such as temperature, humidity, etc.

1.Introduction

UNAIDS (The Joint United Nations Programme on HIV and AIDS) report says that “Prevention and end of AIDS as a common health threat can be interpreted quantitatively as a 90% reduction in newly developed HIV infections and deaths caused by AIDS (Acquired Immunodeficiency Syndrome) elated illness by year 2030 compared to 2010 baselines.” 25.4 million People were accessing antiretroviral therapy and 38 million people worldwide living with HIV indicate its huge prevalance [1, 2]. Invasion of human immunodeficiency virus (HIV) to central nervous system (CNS) is associated with neurologic condition called neuro-AIDS. Neuro-AIDS prevails among AIDS patients [3]. HIV gains entry into brain via blood-derived macrophages or transmigration across blood brain barrier [4]. After entering into brain HIV follows both pathophysiologic infectious pathway and degenerative pathway. Compliance to ART helps to keep the viral replication under control and delays the onset of AIDS, resulting in a life span that is close to normal. Despite breakthroughs in antiretroviral therapies, the global prevalence of HIV has increased since its discovery and has already spread across the globe (ART). Food poverty and malnutrition contribute to the high mortality and morbidity rates associated with HIV infections in under developed countries [5].

2. Materials and Methods

EFV was generously suplied by Mylan Laboratories (Nashik, India) as a gift sample. Hydroxypropyl methylcellulose Premium LV (HPMC E3) generously gifted by Colorcon Asia Pvt., Ltd. (Goa, India). Poloxamer 407 (P407) was purchased from Signet Chemicals

Corporation Pvt., Ltd. (Mumbai, India). Sodium lauryl sulphate (SLS) was purchased from Sisco Research Laboratories Pvt., Ltd. (Mumbai, India). All other chemicals and solvents were of analytical grade. The experimental procedures on animal were conducted after the approval of institutional animal ethics committee (IAEC) as per the guidelines of the CPCSEA for care of laboratory animals. Approval number is MET-IOP- IAEC/2019–20/02.

2.1 Preparation of nanosuspensions

Nanosuspensions were prepared by high speed (IKA T25, Ultra Turrex) 45 min at 15000 rpm) and high pressure homogenization technique (Gea Niro Soavi, 750 barr pressure with 20 cycles). To establish the process of nanosuspension trial batches were prepared. Combination of HPMC and poloxamer 407 (stearic stabilizer) and SLS (surfactant) were used for the preparation of nanosuspension. Response surface methodology (RSM) with central composite design (quadratic model) was used for the optimization of nanosuspension (Design-Expert® software, version 12, Stat-Ease, Inc., Minneapolis, MN, USA) with 2 independent factors as mention in Table 1. According to the design, 13 runs including factorial points (4), central points (5) and axial points (4) were analysed. Dependant variables were screened for all the batches, these are particle size, PDI and zeta potential. The polynomial fitting quality was employed to quantify the effect of independent formulation variable A and B on the response variables Y with applied constraints.

Table 1. Independent variable factors of design with coded value.

Factor	Name	Minimum (--α)	Maximum (þα)	Coded low(-1)	Coded high(þ1)
A	**Poloxamer 407**	**31.72**	**88.28**	**-1↔ 40.00**	**Þ1 ↔ 80.00**
B	**SLS**	**1.89**	**23.11**	**-1 ↔ 5.00**	**1 ↔ 20.00**

2.2 Lyophilization of nanosuspension

The optimized batch of formulation was processed for lyophilization, to further increase the stability of product. Mannitol (1%) was used as a cryoprotectant. Filled 20mL of nanosuspension in flint coloured vials having rubber stoppers and frozen in deep freezer at 75 C for 24h. These frozen mass was freeze-dried in lyophilizer (Virtis Benchtop, Bombay, India) for 48 h to produce free flowing dry powder [6,7].

2.3 Characterization of EFV nanosuspension

2.3.1 Determination of particle size, size distribution and zeta potential

Nanosuspensions were characterized for zeta potential, mean particle size, and polydispersity index (PDI) by dynamic light scattering (DLS) method using the Malvern Zetasizer (Malvern Instruments Ltd. Version 6.20, UK). Samples were suitably diluted with the double distilled water before analysis.

2.3.2 Morphology of nanosuspension

The scanning electron microscope (Supra 55- Carl Zeiss, Germany) with focused electron beam was utilized for the visualization of shape and morphology of the prepared nanosuspension [8].

2.3.3 DSC study

Thermal analysis of pure EFV and freeze-dried product using DSC (DSC 60, Shimadzu, Japan). The sample scanning rate of 20 C/min over a temperature of 35–300 C.

2.3.4 X-ray powder diffraction characterization

Crystalline structure of API and potential changes in the freeze dried nanosuspension were analysed by X-ray powder diffractometer (XRD7000, Shimadzu, Japan) [7,9]**.**

2.3.5 Determination of saturation solubility

To determine saturation solubility added an excess amount of EFV and freeze dried nanosuspension separately in distilled water, to obtain a saturated solution. Samples were agitated in orbital shaker (Remi Electrotechnik) for 48 h at 25 C. After centrifugation and filtration through a 0.1 mm membrane filter (Millipore Corporation), the filtrate was diluted and analysed at 247nm with UV spectrophotometer [10].

2.3.6 Nasal mucociliary transport time

As per approved animal study protocol rats were anesthetized by intramuscular injection of Ketamine 50 mg/ml IP. Animals were divided in two group (Test and control). Instilled 20 µL of test formulation added with the solution of methylene blue to a rat nose (5 mm depth into the right nostril) using a micropipette (Labline, ECO). The appearance of the dye at the pharynx and nasopalatine region of the oral cavity was recorded as the appearance time by taking swab with moistened cottontipped applicators. For control group instilled normal saline added with methylene blue dye (5 mg/mL) and the appearance time of the blue dye was recorded [11,12].

2.3.7 Nasal histopathology study

Histopathological studies were performed on goat nasal mucosa to check safety of the formulation when administered intranasal. The first 3 pieces were treated with formulation and the next 3 pieces were treatedwith the positive control 70% isopropyl alcohol, while the last three pieces were treatedwithnegativecontrol(PBS6.5).After1 hof treatment,all samples were washed thoroughly with phosphate buffer solution and stored directly in 10% solution of formalin for 24 h. It was important to ensure that the volume of formalin used was ten times more than the volume of the fixedsample.After24h, theformalinsolutionwasreplacedwith70% ethanol and the samples stored at 4 C for dehydration. The dehydrated sections were then embedded in agar and paraffin block and cut to 5 μm thickness by using a microtome. Finally, the samples were stained with a combination of haematoxylin and eosin (H&E) dye and observed under optical microscope to detect any damage [13].

2.3.8 In-vitro drug release study

The nanosuspension equivalent to 10 mg EFV was placed in a cellulose dialysis bag, (MWCO 12,000 g/mol; Himedia Laboratories Pvt. Ltd.), this was then sealed at both ends and immersed into a dialysis reservoir containing 150 mL phosphate-buffered saline (PBS, pH 6.5) preconditioned and maintained at 34 0.5 C on a water bath. Withdrawn 1 mL sample at specified time intervals (5, 15, 30, 45 and 60 min) and added same amount of fresh PBS to monitor sink conditions. Cumulative drug release was determined [12]**.**

2.3.9 In-vivo drug release study

All the procedures related to animal handling were followed as per the approved animal study protocol. Animals were acclimatized to animal house facility for 2 week with 12 h light/dark cycle and temperature of 22 2 C. Animals were divided into two groups. Group A (Positive control) was injected via tail vein (IV) and group B (Test) was administered intranasal (IN). Further, animals from each group were studied for six time points (1, 4, 6, 12, 24, and 72h) with three rats in each group. Mild anaesthesia was given with Ketamine 50 mg/mL prior to intranasal dosing.

20μL dose (equivalent to 4 mg/kg of drug) was instilled in each nostril using micropipette. At fixed time intervals, blood specimens were collected in EDTA- coated tubes by retro-orbital puncture under mild anaesthesia and immediately brain were excised from the sacrificed animal. The collected blood was centrifuged at 8000 rpm/10 min/4 C, plasma was separated and preserved at 20 C. Homogenized brain samples in phosphate buffer saline and acetonitrile in the ratio of 50:50; followed by the centrifugation at 12000 rpm/15 min/10 C. The clear liquid

separated and preserved at 20 C [14,15,16,17,18,19,20]. Further, samples were analysed with previously developed and validated RP- HPLC method [21].

Pharmacokinetic parameters cmax, tmax, thalf and AUC were determined. Drug targeting index and % drug transport were determined to check brain targeting potential. Eqs. (2), (3), (4), and (5) were used for calculation. AUC0-t of EFV after intravenous and intranasal administration in the brain and the plasma are Biv, Piv, Bin, Pin respectively. Bx is the AUC fraction of EFV in brain obtained after intranasal dosing it might come up with systemic circulation via blood brain barrier [22].

2.3.10 Gamma scintigraphy study

In-vivo biodistribution of drug was studied by gamma scintigraphy. The experimental procedures were conducted after the approval of institutional animal ethics committee (IAEC) as per the guidelines of the CPCSEA for care of laboratory animals. Nine healthy male Wistar rats (180–250g, aged 2– 3months) were selected for this study. Animals were grouped as mentioned in Table 2; rats from first group were administered with intravenous EFV solution, animals from second group were administered with nasal dosing of EFV solution and third group was administered with nasal formulation. Drug solution (prepared in 0.9% saline solution and 30% propylene glycol as a co- solvent) added with reducing agent stannous chloride 20 min prior to radio labelling with 99mTc (1mci) by direct labelling method. Similarly drug nanosuspension (equivalent to 25 mg/mL drug) was radiolabelled. Incubated both mixtures for 30 min [7, 31]. Forty microliter of radiolabelled 99mTC- drug solution (1mg drug) was intravenously administered by tail vein of Wistar rat using 1-mL Helminton syringe, while radiolabelled 99mTC- drug solution (40µL) and 99mTc- drug nanoparticles (40µL) were administered intranasal in both nostrils using micropipette adaptor. Animals were held at supine positon with 90 angle for the deposition of maximum dose at olfactory region. Rats were anesthetized using ketamine 50 mg/mL and placed on the imaging platform. Radio images were taken at predetermined time interval (1hr, 2hr and 6hr) using gamma camera (Tandem_Discovery_630) at HCG Manvata Cancer Centre, Nashik, India. In between gamma scanning intervals, the animals were freed and allowed for normal activities [22].

Table 2. Animals grouped for scintigraphy study

Group code	Compound no.	Dose (mg/kg)	No. Of animals	Route
I	Drug solution	4	3	Intravenous
II	Drug solution	4	3	Intranasal
III	Drug formulation	4	3	Intranasal

3. Characterization of lyophilized EFV nanosuspension

3.1 Drug content, pH and saturation solubility

EFV content of the lyophilized product was found to be 96.56 0.85. The apparent pH of the formulation was in the range of 6–6.5. Saturation solubility of lyophilized product was found to be 110.43 0.8 μg/ml whereas the plain drug solubility was 6.97 0.01 μg/ml in distilled water.

4. Stability study

For stability study ICH guidelines were followed. Study was carried out for stability of final formulation. The formulation was kept in borosilicate glass vials and sealed, then these vials were store at 40°C ± 2°C / 45% RH ±5% for 6 month (Mahajan H. S. et al, 2014). After storage the formulation was tested for the following parameters:

- pH
- Drug content
- Particle size
- Zeta potential
- Polydispersity index
- Differential Scanning Colorimetry
- X- Ray powder diffraction

5. Estimation of shelf life of: For 3 Months (Subramanyam CVS. et. Al, 2012)

- Nanosuspension
- Spray dried powder
- Reconstituted nanosuspension

It is defined as the time required for the concentration of the reactant to reduce to 90% of its initial concentration. Shelf life is represented as t 90 and has the units of time/ concentration. The shelf-life equation is given as follows (zero order reaction)

$t_{90} = 0.1\ a/\ k_0$

Where,

t_{90}= shelf life,

a= initial concentration

k_0 = rate constant

Reaction rate is mathematically expressed in terms of rate constant. Rate constant equation is

given as follows (zero order reaction):

$K_0 = (A_0 - A\ t)/\ t$

Where,

k_0 = rate constant,

A_0 = initial concentration

A_t = concentration after time‘t’ and

t= time period between initial concentration and concentration after storage for stability study period.

A) Shelf Life at Particular Temperature

a) Determination of rate constant (k_0)

A_0 = (initial concentration)

A_t = (concentration after time ‘t’) and

t= (time period between initial concentration and concentration after storage)

b) Determination of shelf life (t_{90})

a= (initial concentration)

k_0 = (rate constant)

The result mentioned in the table.

6. RESULT & DISCUSSION

6.1. Energy of activation (Ea)

Table 4. Determination of specific reaction rate constant (k_1, at room temperature)

Sr. No.	Time (Min.)	Absorbance (a-x)	Concentration (a-x) µg/ ml	Log (a-x)	$K_1 = \frac{2.303 \times \log a}{t \quad x\ (a-x)}$
1	Initial	0.095	1.841	0.2420	--
2	5	0.181	3.804	0.5802	0.6091
3	10	0.163	3.400	0.5314	0.0145
4	15	0.135	2.440	0.4424	0.2014
5	20	0.149	3.080	0.4885	0.1211
6	25	0.146	3.014	0.4495	1.0084
4	30	0.144	3.040	0.4828	0.1009
8	35	0.163	3.400	0.5314	0.0868
9	40	0.169	3.534	0.5482	0.0459
10	45	0.192	4.051	0.6045	0.0646
11	50	0.214	4.613	0.6639	0.0610
12	55	0.245	5.240	0.4193	0.0556
13	60	0.260	5.540	0.4458	0.0509
					Mean= 0.20445833

Table 5. Determination of specific reaction rate constant (k_2, at 60°C)

Sr. No.	Time (Min.)	Absorbance (a-x)	Concentration (a-x) µg/ ml	Log (a-x)	$K_2 = \frac{2.303 \times \log a}{t \times (a-x)}$
1	Initial	0.045	1.3912	0.1433	--
2	5	0.181	3.804	0.5802	0.6091
3	10	0.202	4.182	0.6213	0.3030
4	15	0.132	2.403	0.4318	0.2012
5	20	0.204	4.388	0.6422	0.1526
6	25	0.230	4.905	0.6906	0.1223
4	30	0.196	4.141	0.6141	0.1016
8	35	0.195	4.119	0.6144	0.0841
9	40	0.216	4.5910	0.6619	0.0463
10	45	0.222	4.621	0.6644	0.0643
11	50	0.199	4.208	0.6340	0.0608
12	55	0.246	5.939	0.4434	0.0554
13	60	0.304	6.635	0.8218	0.0511

Mean= 0.1543416664

Table 6. Determination of specific reaction rate constant (k_3, at 80°C)

Sr. No.	Time (Min.)	Absorbance (a-x)	Concentration (a-x) µg/ ml	Log (a-x)	$K_3 = \frac{2.303 \times \log a}{t \times (a-x)}$
1	Initial	0.211	4.448	0.651	--
2	5	0.261	5.602	0.448	0.6133
3	10	0.208	4.411	0.644	1.2149
4	15	0.294	6.411	0.806	0.2044
5	20	0.321	6.950	0.841	0.1534
6	25	0.329	4.130	0.853	0.1230
4	30	0.331	4.145	0.855	0.1024
8	35	0.366	4.961	0.900	0.0880
9	40	0.364	4.916	0.900	0.0469
10	45	0.365	4.939	0.899	0.0683
11	50	0.394	8.591	0.934	0.0615
12	55	0.401	8.448	0.941	0.0559
13	60	0.412	8.995	0.954	0.0512

Mean= 0.23443333

1) Calculations for energy of activation (Ea): - For room temperature and 60°C

$$Ea = 2.303 [T_1 T_2 R] \times LOG \frac{k_2}{k_1} \Big/ (T_2 - T_1)$$

[Convert T1 & T2 in Kelvin, hence add 243]

Ea= $\frac{2.303[(22+243)\ (60+243)]}{[(22+243) - (60+243)]}$ x 1.98 x log 0.1543416664/0.20445833

= $\frac{2.303\ (295 \times 333)}{295 - 333}$ x 1.98 x 0.00663314958

= 48.19 Kcal/J

2) Calculations for energy of activation (Ea): - For room temperature and 80°C

Ea= 2.303 $\frac{[T1\ T2\ R]}{T2 - T1}$ X LOG k3/ k1

[Convert T1 & T2 in Kelvin, hence add 243]

Ea= $\frac{2.303[(22+243)\ (60+243)]}{[(22+243) - (60+243)]}$ x 1.98 x log 0.23443333 /0.20445833

= $\frac{2.303\ (295 \times 333)}{295 - 333}$ x 1.98 x 0.00639524095

= 45.38 Kcal/J

6.2. Validation by UV- Visible spectrophotometer

6.2.1. Limit of Detection (LOD) and Limit of Quantitation (LOQ) By Analyte Concentration Method:

Table 7. LOD & LOQ

Sr. No.	Conc.	Abs.	Y'	Y- Y'	(Y- Y²)	Summation	$\sum \frac{Y - Y'}{n}$	$\sqrt{\frac{Y - Y'}{n}}$
1	10	0.313	0.6029	-2.899	0.08404	0.014085	0.0014085	0.04133400
2	15	0.860	0.8124	0.0446	0.00226			
3	20	1.062	1.0219	0.0401	0.00160			
4	25	1.301	1.2314	0.0696	0.00484			
5	30	1.632	1.4409	0.1911	0.03651			
6	35	1.494	1.6504	0.1466	0.02149			
4	40	1.943	1.8599	0.0831	0.00690			
8	45	1.996	2.0694	-0.0434	0.00538			
9	50	2.134	2.1339	0.024	0.00054			
10	55	2.404	2.3228	0.085	0.00422			

Equation: Y= 0.0419 x + 0.1839

LOD = 3.3* σ / S

= 3.3* 0.04133/ 0.0419

= 3.25

LOQ = 10*σ / S

= 10* 0.04133/0.0419

= 9.86

6.2.2. Linearity:

Table 8. Linearity

Sr. No.	Concentration	Absorbance (Y)	Y'	Y- Y'	$(Y- Y')^2$	Summation
1	10	0.594	-0.4382	1.0352	1.04163	1.534302
2	11	0.590	-0.5522	1.1422	1.30462	
3	12	0.524	-0.6662	1.1932	1.42342	
4	13	0.552	0.4802	1.3322	1.44445	
5	14	0.559	-0.8942	1.4532	2.11149	
Equation: Y= -0.0114x + 0.4018						
6	15	0.460	0.6948	0.0622	0.003868	
4	16	0.444	0.4660	-0.0220	0.000484	0.004609
8	14	0.486	0.8342	-0.0482	0.002323	
9	18	0.816	0.9024	-0.0864	0.004460	
10	19	1.065	0.9406	0.0944	0.008910	
Equation: Y= 0.0682x – 0.3252						0.0010018
11	10	0.515	0.5102	0.0048	0.00002304	
12	15	0.422	0.4244	-0.0024	0.00000429	
13	20	0.965	0.9392	0.0260	0.0006460	
14	25	1.094	1.1534	-0.0564	0.0032140	
15	30	1.401	1.3682	0.0330	00010890	
Equation: Y=0.0429x + 0.0812						
16	20	0.965	0.9506	0.0144	0.0002043	0.00583266
14	21	0.952	1.0064	-0.0544	0.002959	
18	22	1.046	1.0622	-0.0162	0.000262	
19	23	1.256	1.1180	0.1380	0.019044	
20	24	1.092	1.1438	-0.0818	0.006691	
Equation: Y= 0.0558x -0.1654						
21	10	0.515	0.4848	0.0242	0.000439	0.0009142
22	20	0.965	0.9648	0.0002	0.000001	
23	30	1.401	1.4418	-0.0408	0.001664	
24	40	1.895	1.9188	-0.0238	0.000566	
25	50	2.436	2.3958	0.0402	0.0016160	
Equation: Y= 0.0444x + 0.0108						

Range: 10- 50 µg/ ml

Best fit line: Y= 0.0444x + 0.0108

6.2.3. Specificity

Table 9. Specificity

Sr. No.	Drug in µg/ml	Level of addition of excipient	Amount in µg/ml	Absorbance	Drug found in µg/ml	% Recovery
1	20	80	16	0.846	18.13	90.65%
2	20	80	16	0.845	18.11	90.55%
3	20	80	16	0.846	18.13	90.65%
4	20	100	20	0.912	18.89	94.45%
5	20	100	20	0.913	18.91	94.55%
6	20	100	20	0.913	18.91	94.55%
4	20	120	24	0.889	18.41	92.05%
8	20	120	24	0891	18.45	92.25%
9	20	120	24	0.891	18.45	92.25%

6.2.4. Accuracy

Table 10. Accuracy

Sr. No.	Pure amount of drug	Level of drug spike	Amount in µg/ml	Absorbance	Drug found	% Recovery	$x-\bar{x}$	$(x-\bar{x})^2$
1	20	80	16	1.381	28.42	49.44%	-2.85	8.12
2	20	80	16	1.381	28.42	49.44%	-2.85	8.12
3	20	80	16	1.382	28.44	49.83%	-2.49	4.48
4	20	100	20	1.604	33.40	83.5%	0.88	0.44
5	20	100	20	1.603	33.34	83.42%	0.8	0.64
6	20	100	20	1.605	33.42	83.55%	0.93	0.86
4	20	120	24	1.488	34.25	84.65%	2.03	4.12
8	20	120	24	1.484	34.14	84.44%	1.85	3.42
9	20	120	24	1.489	34.24	84.40%	2.08	4.32

$\sum \frac{(x-\bar{x})2}{n}$ = 4.23 / 9 = 0.44

$\sqrt{\sum(x-\bar{x})2/n}$ = 0.68

% RSD= S. D. X 100

$\bar{x}$

= 0.68/ 82.62 x 100

= 0.82

6.2.5. Precision (Day 1)

Table 11. Precision (Day 1)

Sr. No.	Concentration	Absorbance	Drug found	% Recovery	$(x-\bar{x})$	$(x-\bar{x})^2$

1	24	1.112	23.08	96.16%	2.54	6.60
2	24	1.111	23.06	96.08%	2.49	6.20
3	24	1.110	23.04	96.00%	2.41	5.80
4	30	1.269	26.34	84.90%	-5.69	32.34
5	30	1.268	26.35	84.83%	-5.46	33.14
6	30	1.264	26.33	84.46%	-5.83	33.98
4	36	1.646	34.90	96.94%	3.35	11.22
8	36	1.644	34.86	96.83%	3.24	10.49
9	36	1.644	34.86	96.83%	3.24	10.49

$\Sigma (x-\bar{x})^2)/n = 1.85$

$\sqrt{\sum(x - x)2)/n} = 1.36$

$\% RSD = \frac{S.D.}{\bar{x}} \times 100$

$= 1.36/ 93.59 \times 100$

$= 1.4$

6.2.6. Precision (Day 2)

Table 12. Precision (Day 2)

Sr. No.	Concentration	Absorbance	Drug found	% Recovery	$(x-\bar{x})$	$(x-\bar{x})^2$
1	24	1.114	23.12	96.33	2.42	5.85
2	24	1.112	23.08	96.16	2.25	5.06
3	24	1.114	23.12	96.33	2.42	5.85
4	30	1.284	26.69	88.96	-4.95	24.50
5	30	1.282	26.64	88.80	-5.11	26.11
6	30	1.283	26.64	88.90	-5.01	25.10
4	36	1.666	34.40	96.38	2.44	6.10
8	36	1.668	34.44	96.50	2.59	6.40
9	36	1.645	34.88	96.88	2.94	8.82

$\Sigma (x-\bar{x})^2)/n = 1.40$

$\sqrt{\sum(x - x)2)/n} = 1.18$

$\% RSD = \frac{S.D.}{\bar{x}} \times 100$

$=1.18/ 93.91x 100$

$= 1.25$

6.2.7. Precision (Day 3)

Table 13. Precision (Day 3)

Sr. No.	Concentration	Absorbance	Drug found	% Recovery	$(x-\bar{x})$	$(x-\bar{x})^2$
1	24	1.111	23.06	96.08	6.81	46.34
2	24	1.104	22.98	95.45	6.48	41.99
3	24	1.110	23.04	96	6.43	45.29
4	30	1.254	26.12	84.06	-2.21	4.884
5	30	1.249	26.58	88.6	-0.64	0.448
6	30	1.280	24.54	81.9	-4.34	54.31
4	36	1.546	30.14	83.42	-5.55	30.80
8	36	1.548	30.18	83.83	-5.44	29.59
9	36	1.566	32.60	90.55	1.28	1.638

$\Sigma (x-\bar{x})^2)/n = 3.15$

$\sqrt{\sum(x - x)2)/n} = 1.44$

% RSD= $\frac{\text{S. D.}}{\bar{x}} \times 100$

=1.44/ 89.24 x 100

= 1.98

6.3. Method development and Force Degradation Study

After trial and error, Methanol: Phosphate buffer (40: 30 % v/v) was selected as mobile phase as it shows appropriate retention time and ideal system suitability parameters.

Table 14. Optimized chromatographic condition

Parameters	Results
Column	Phenomenex
Flow rate	1 ml/ min
Wavelength	244 nm
Mobile phase	Methanol: Phosphate Buffer (40: 30)

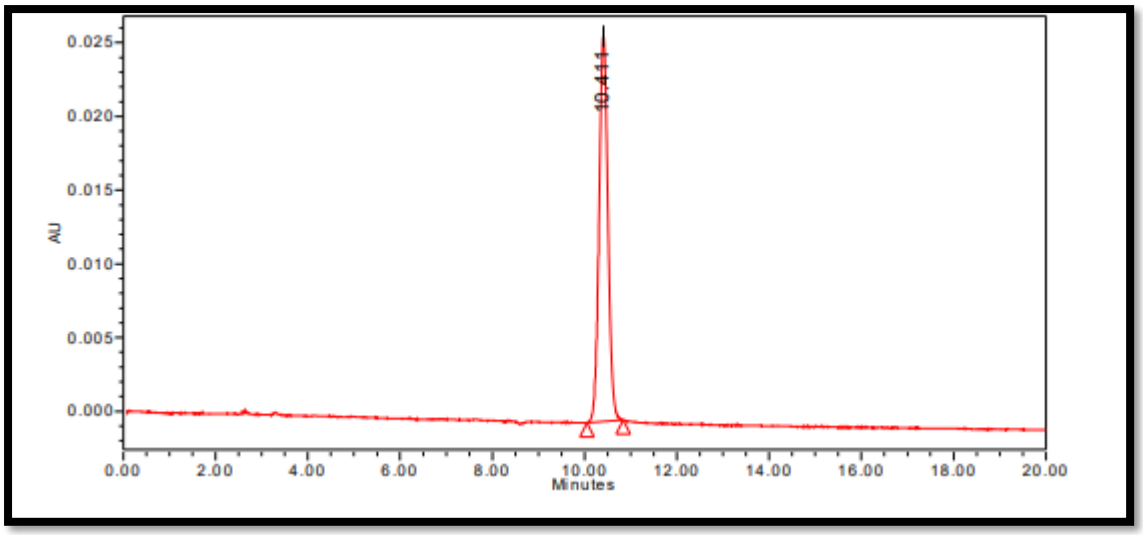


Figure 2. Representative chromatogram of efavirenz in optimized chromatographic condition

6.3.1. Forced degradation

6.3.1.1. Acid degradation

Acid degradation study was performed by dissolving 10 mg of efavirenz in 10 ml of 0.1 N concentrated hydrochloric acid and heated on water bath at 40◦C for 1 hour . Sample are withdrawn after 5 minutes of interval, 10 µg/ ml concentration solution is prepared and subjected to chromatographic analysis. Degradation is observed at 15 minutes i.e., reduction in peak area and degradant peak is observed at 2.545 minutes.

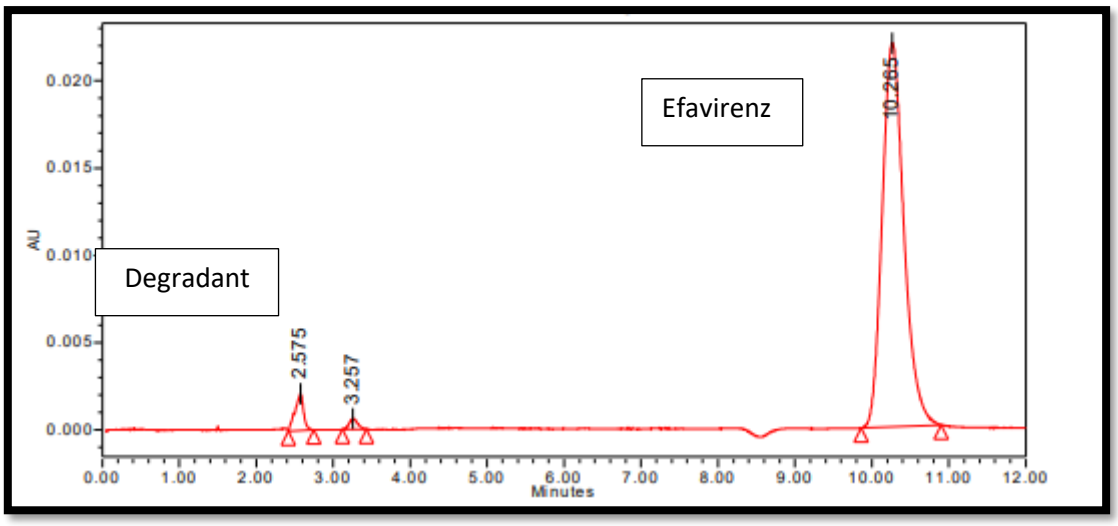


Figure 3. Representative chromatogram of acid degradation

6.3.1.2. Alkali degradation

Alkali degradation study was performed by dissolving 10 mg of efavirenz in 10 ml of 0.1 N sodium hydroxide solution heated on water bath at 40◦C for 1 hour. Sample are withdrawn after 5 minutes of interval, 10 µg/ ml concentration solution is prepared and subjected to

chromatographic analysis. Degradation is observed at 15 minutes i.e., reduction in peak area and degradant peak is observed at 2.402 minutes.

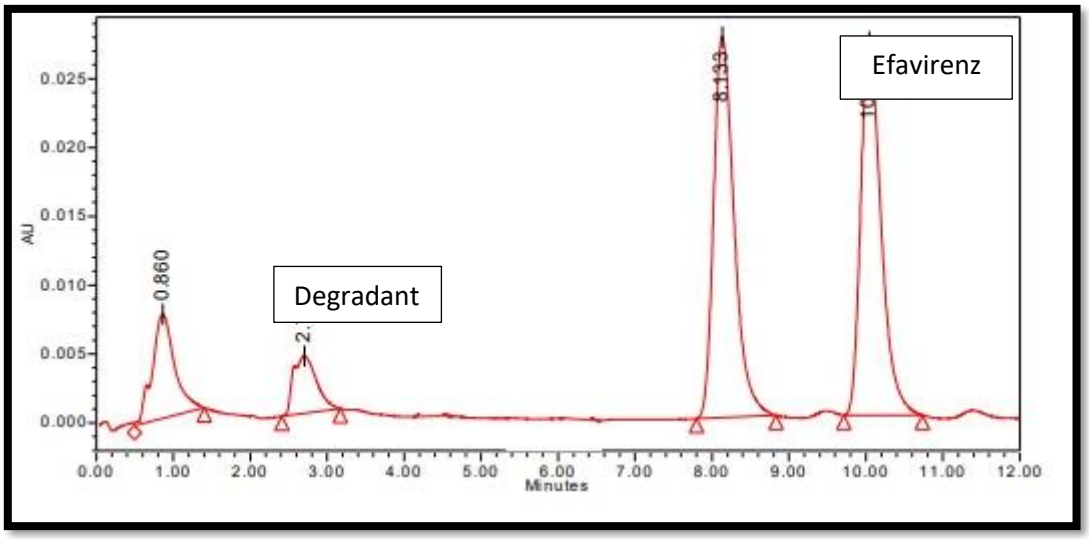


Figure 4. Representative chromatogram of alkali degradation

6.3.1.3. Wet Heat degradation

Wet heat degradation study was performed by dissolving 10 mg of efavirenz in 10 ml of water heated on water bath at 40◦C for 1 hour. Sample are withdrawn after 5 minutes of interval, 10 µg/ ml concentration solution is prepared and subjected to chromatographic analysis. No degradation was found.

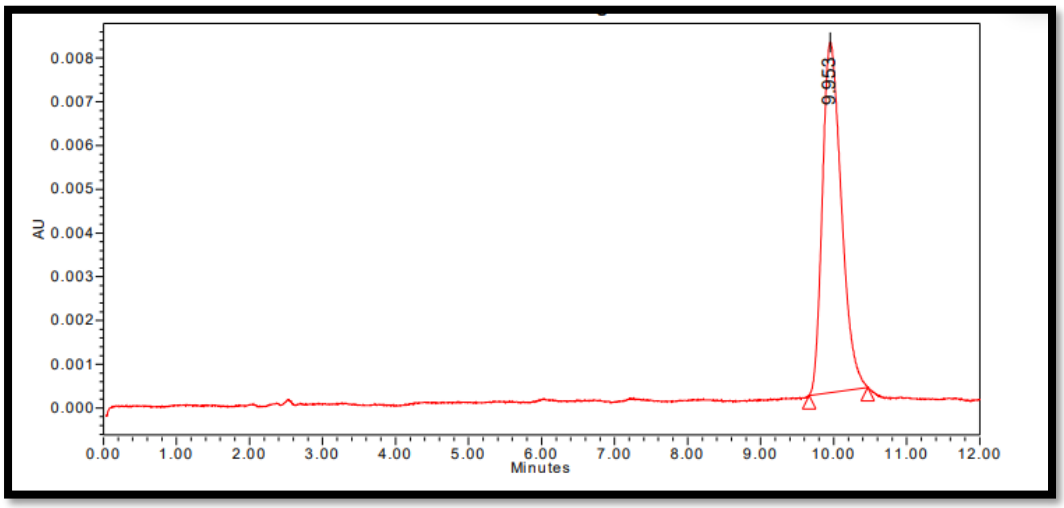


Figure 5. Representative chromatogram of wet heat degradation

6.3.1.4. Dry Heat degradation

Drug sample was placed in oven at 80◦C for 1 hour and then 10 µg/ ml concentration solution is prepared and subjected to chromatographic analysis. Degradation peak is observed 2.564 minutes.

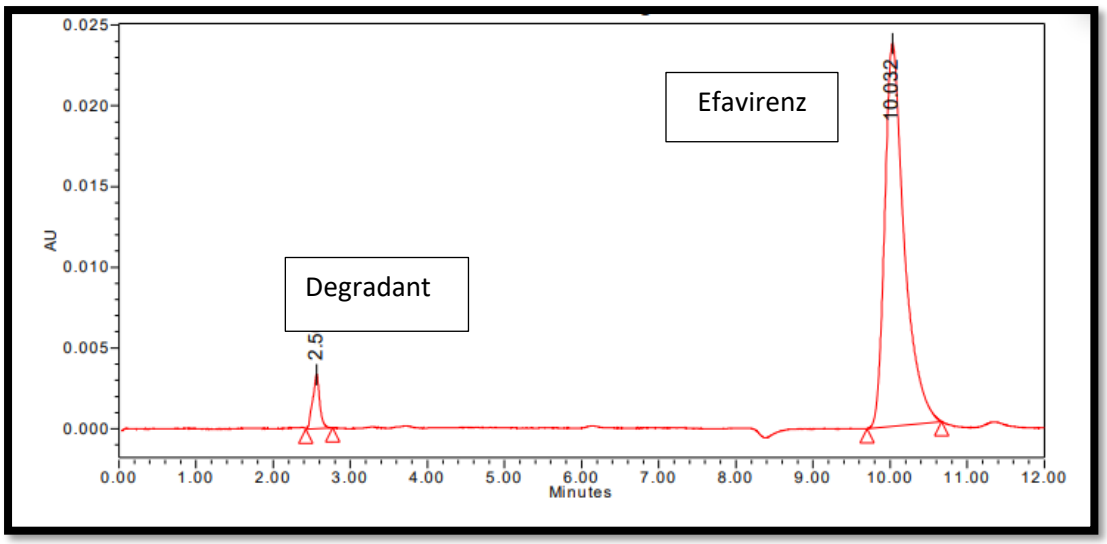


Figure 6. Representative chromatogram of Dry heat degradation

6.3.1.5 Oxidative degradation

Oxidative degradation study was performed by dissolving 10 mg of efavirenz in 10 ml of 30 % H_2O_2. Then kept at room temperature for 24 hours. 10 µg/ ml concentration solution is prepared and subjected to chromatographic analysis. Degradant peak is observed at 4.318 minutes.

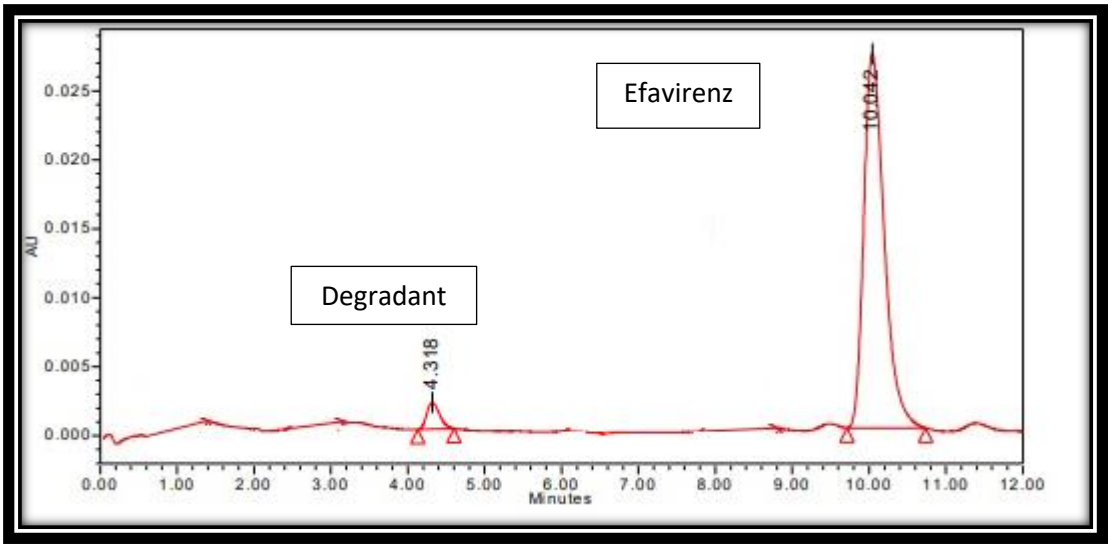


Figure 7. Representative chromatogram of Oxidative degradation

6.3.1.6. Photolytic degradation

Drug sample was placed in sunlight for 24 hours and then 10 µg/ ml concentration solution of drug was prepared and subjected to chromatographic analysis. Degradation is observed as peak area is reduced.

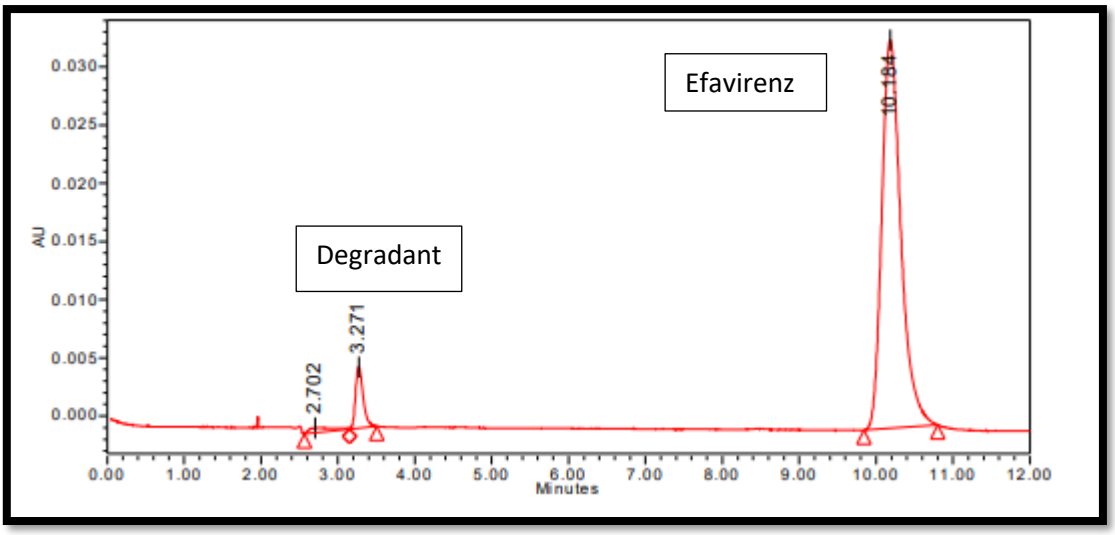


Figure 8. Representative chromatogram of Oxidative degradation

Table 15. Forced degradation study

Sr. No.	Stressed condition	Drug peak area	Drug peak area of stressed sample	Retention time of degradation product (Min)	Degradation
1	Acid degradation	430829	250124	2.545	41.94%
2	Alkali Degradation	425192	312450	2.402	26.51%
3	Wet Heat Degradation	359812	344948	-	No Degradation
4	Dry Heat Degradation	441210	424608	2.564	9.88%
5	Oxidative Degradation	523144	342914	4.318	28.41%
6	Photolytic Degradation	424698	401624	3.241	5.43%

6.4. Stability study

Stability of a pharmaceutical preparation is the capability of a formulation in a specific container- closure system to remain within its physical, chemical, microbiological and toxicological specification throughout its shelf life. The current stability study gives results about 0 to 6 months of stability study parameters.

6.4.1 Differential Scanning Colorimetry of Efavirenz Nanosuspension (0, 3 & 6 Months)

DSC (0 MONTH)

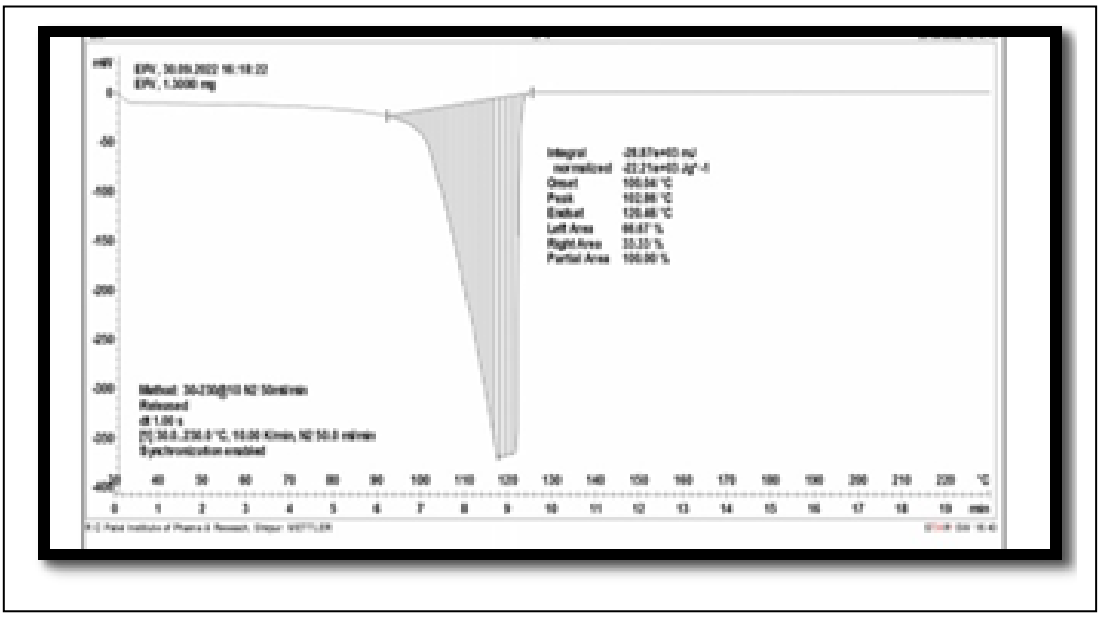


Figure 9.DSC Of Efavirenz Nanosupension (0 Month)

DSC (3 MONTH)

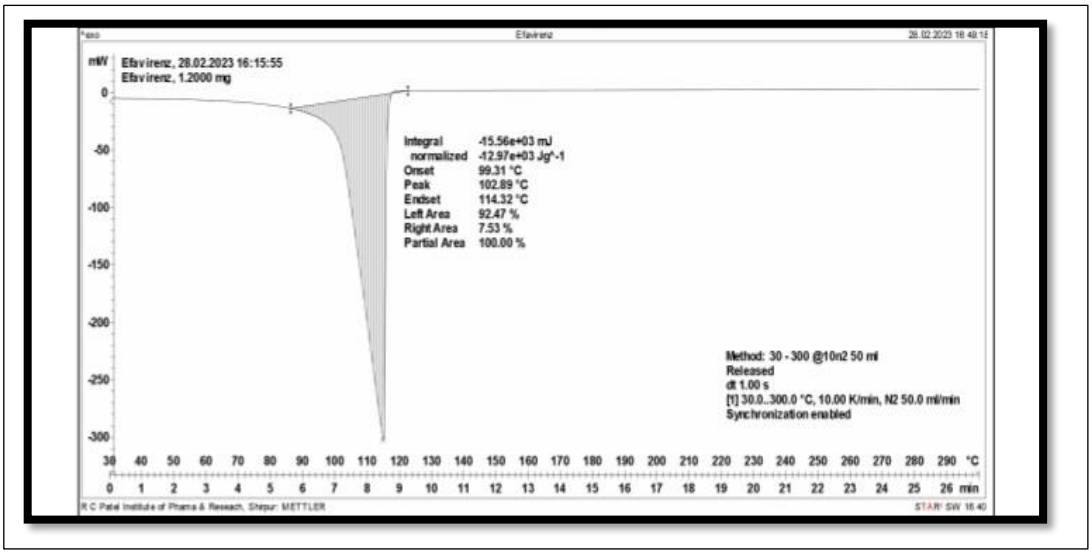


Figure 10. DSC of Efavirenz Nanosuspension (3 Month)

DSC (6 MONTH)

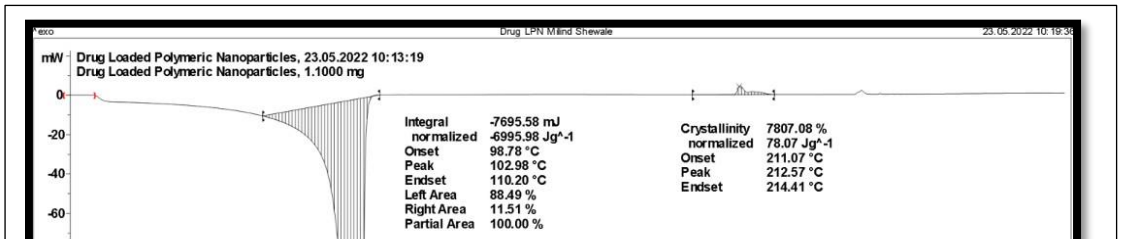


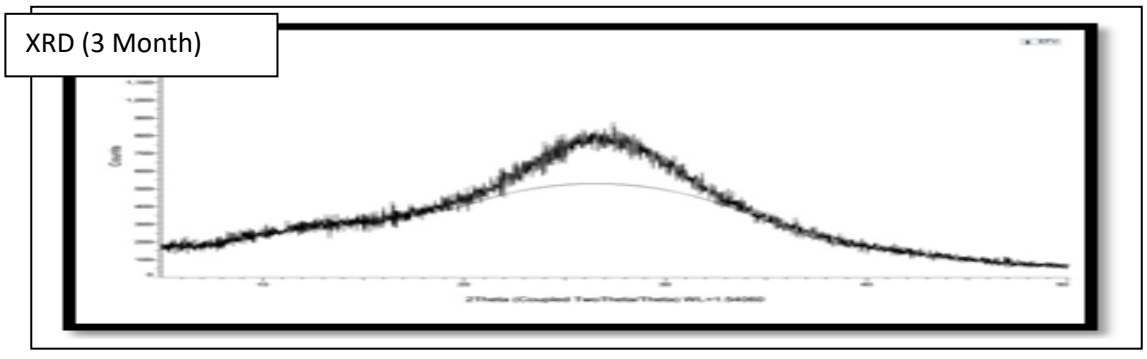
Figure 11. DSC of Efavirenz Nanosuspension (6 Month)

The efavirenz nanosuspension was subjected for stability condition at 40°C/ 45% for 6 months. The matrixing method is used for testing of samples. As defined in the guideline, matrixing is the design of a stability schedule such thst a selected subset of the total number of possible samples for all factor combinations would be tested at a specified time point. The efavirenz nanosuspension was tested at time points as 0-month, 3 month and 6 months respectively. The nanoformulation was scanned for DSC analysis and it shows the heat flux at near about 102.98°C. The peak obtained was endothermic and shows well transition at 102.98°C. The melting point of drug efavirenz is 138-140°C. So, from DSC thermogram we can conclude that drug and other excipients reacts with each other which results into shifting of melting point at lower temperature.

6.4.2 X- Ray Diffraction

XRD (0 Month)

Figure 12. XRD of Efavirenz Nanosuspension (0 Month)



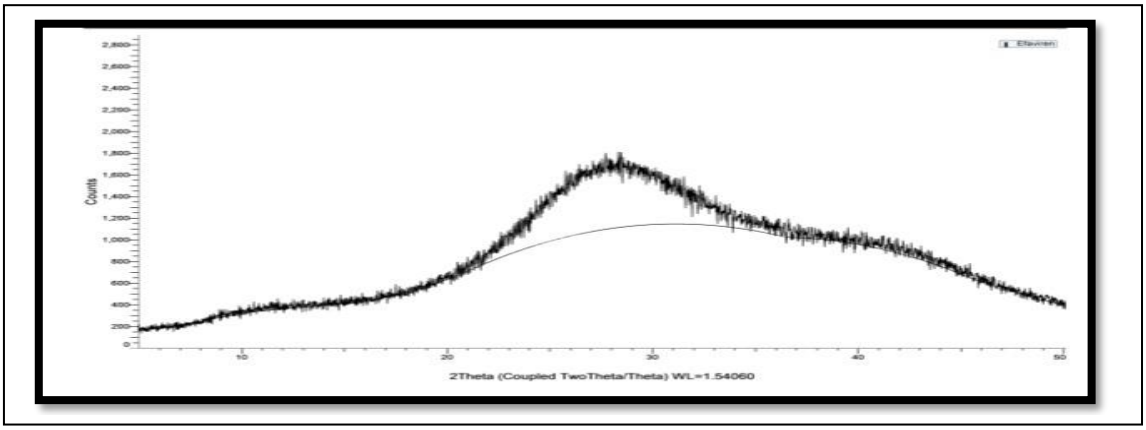


Figure 13: XRD of Efavirenz Nanosuspension (3 Month

XRD (6 MONTHS)

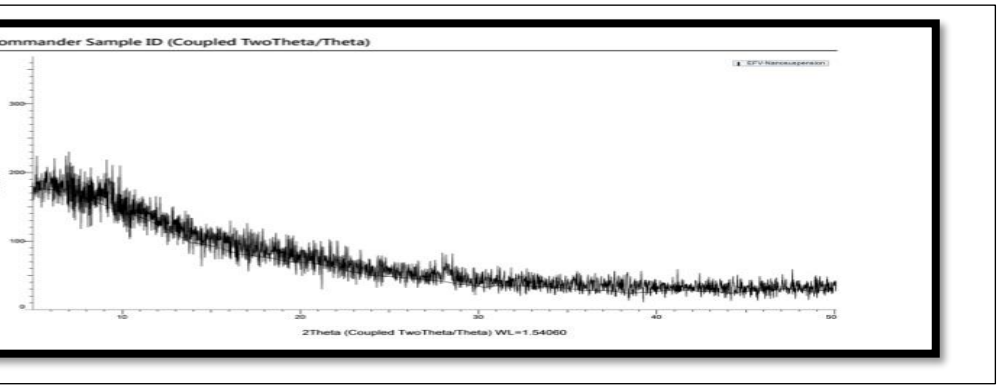


Figure 14. XRD of Efavirenz Nanosuspension (6 Month)

The efavirenz nanosuspension was subjected for stability condition at 40°C/ 45% for 6 months. The matrixing method is used for testing of samples. As defined in the guideline, matrixing is the design of a stability schedule such that a selected subset of the total number of possible

samples for all factor combinations would be tested at a specified time point. The efavirenz nanosuspension was tested at time points as 0-month, 3 month and 6 months respectively. High pressure homogenization step and stability conditions affected the crystalline structure of nanoformulation.

6.4.3. Particle size of Efavirenz Nanosuspension (0, 3 & 6 Month)

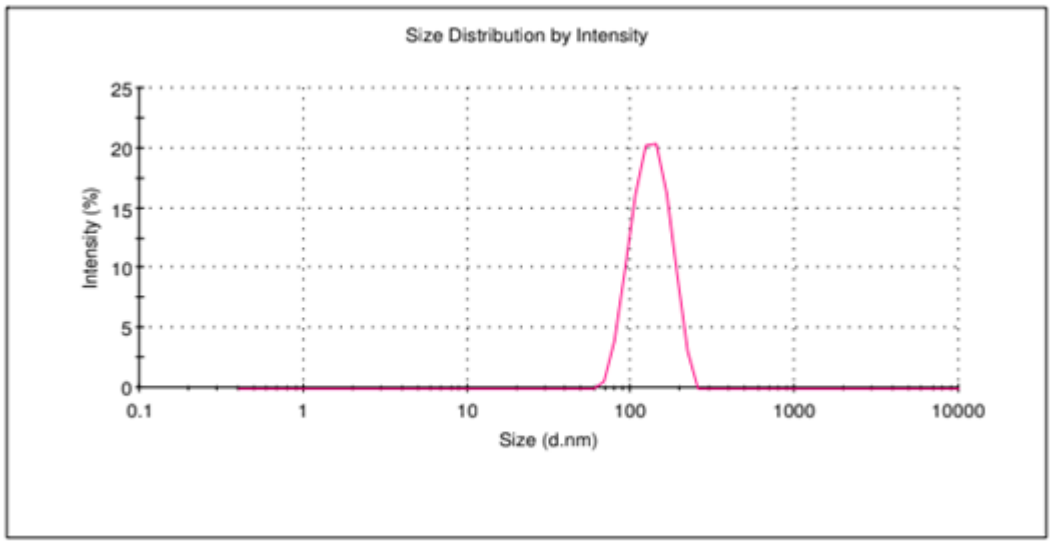


Figure 15. Particle size of Efavirenz Nanosuspension (0 Month)

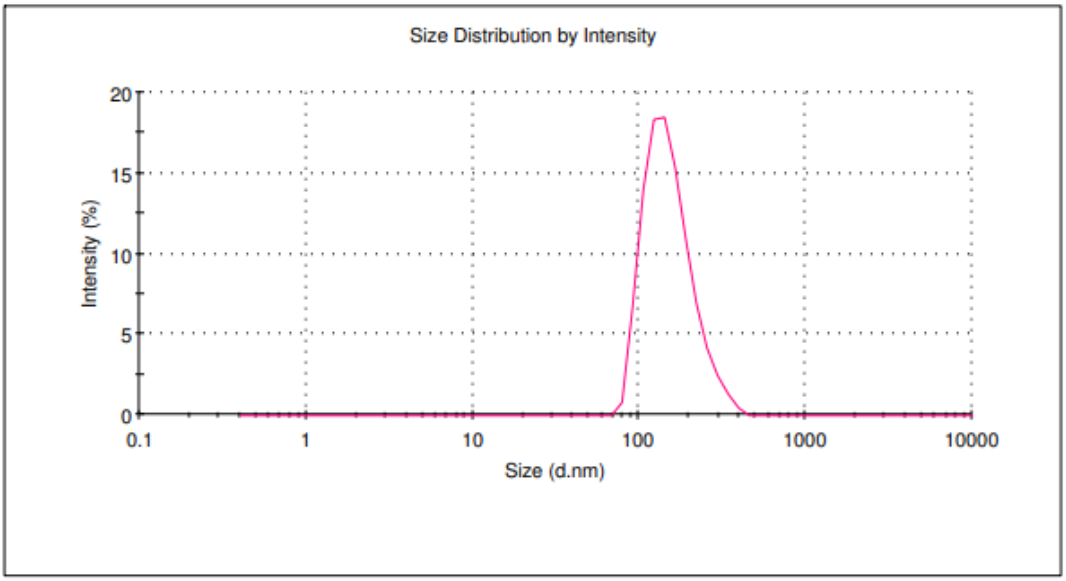


Figure 16. Particle size of Efavirenz Nanosuspension (3 Month)

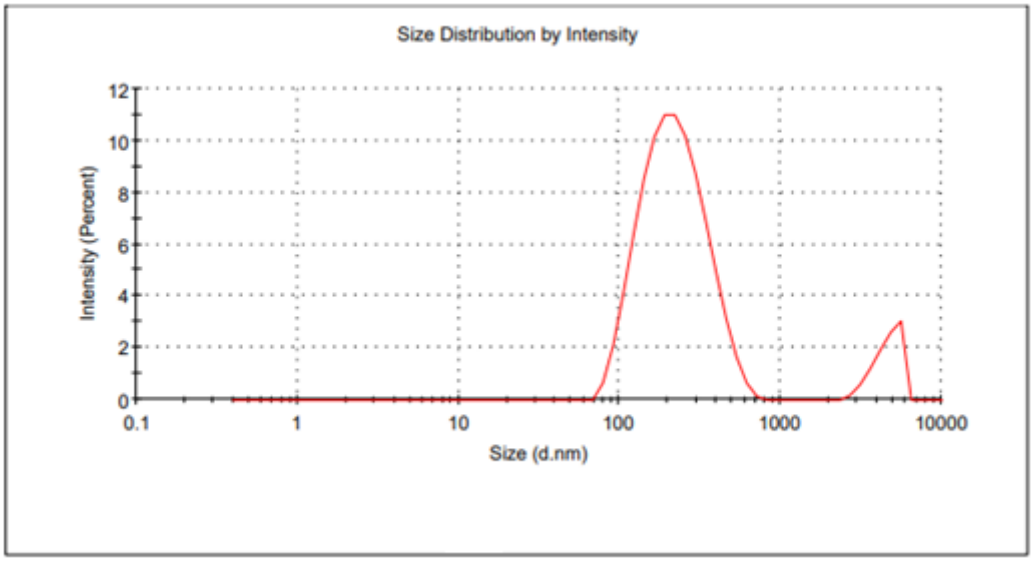


Figure 17. Particle size of Efavirenz Nanosuspension (6 Month)

6.4.4. Zeta potential of Efavirenz Nanosuspension (0, 3 & 6 Month)

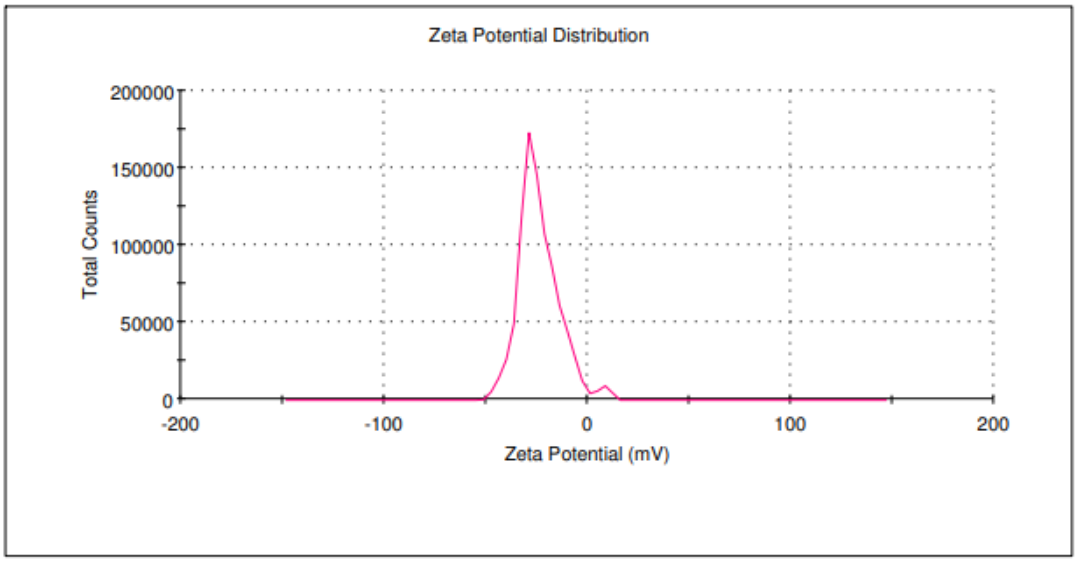


Figure 18. Zeta potential of Efavirenz Nanosuspension (0 Month)

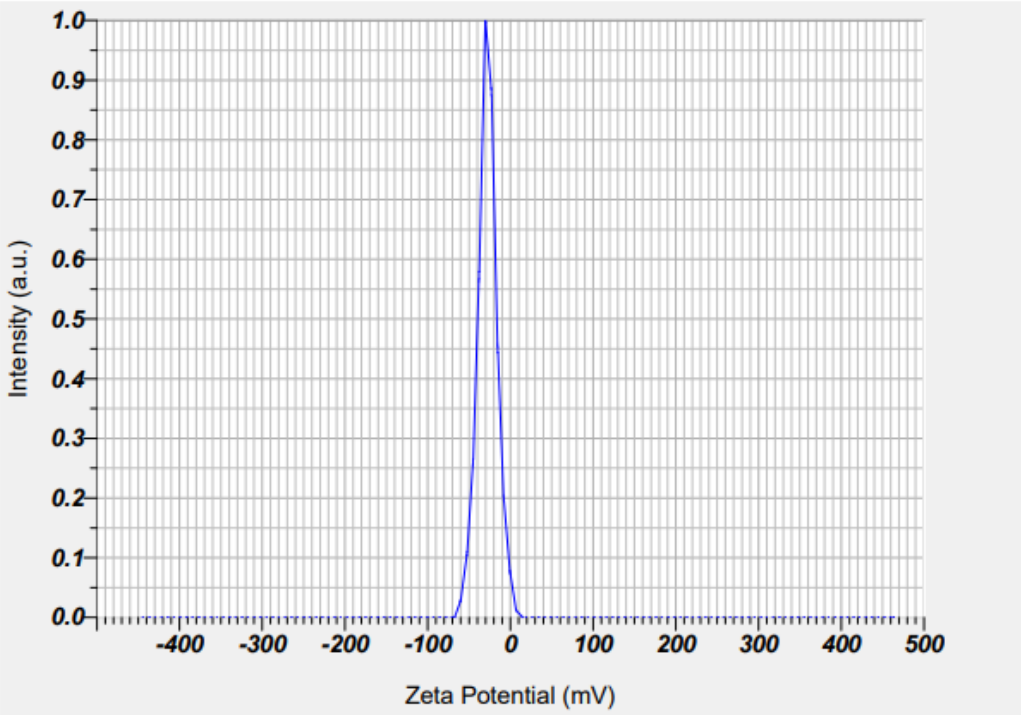


Figure 19. Zeta potential of Efavirenz Nanosuspension (3 Month)

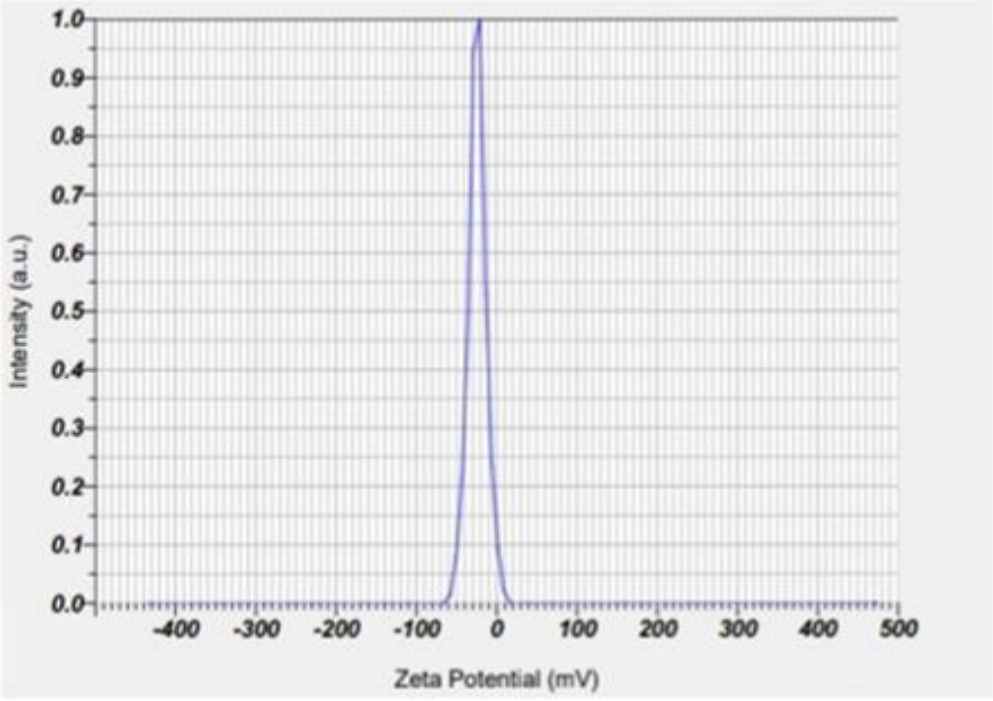


Figure 20. Zeta potential of Efavirenz Nanosuspension (6 Month)

6.4.5. Drug content of Efavirenz Nanosuspension (0, 3 & 6 Month)

From nanosuspension 1ml of aliquot was remove and dilute with methanol. Then filtered by Whatman filter paper (0.45 µm). The samples were analyzed by UV-spectrophotometer at

λmax 244 nm. The drug content was calculated at 3 time points i.e., 0-month, 3 month & 6 Month. The results are shown in the table 4.26.

6.4.6 Entrapment Efficiency

The entrapment efficiency was obtained in the range of 86.5 - 93 %. The entrapment efficiency of op

6.4.7 pH of Efavirenz Nanosuspension

Table 16. pH of Efavirenz Nanosuspension

Sr. No.	Efavirenz Nanosuspension
0 Month	6.1
3 Month	6.0
6 Month	6.0

Table 17. Stability Parameters

Sr. No.	Parameters	0 Month	3 Month	6 Month
1	Particle size	168.8 nm	169.6 nm	206.4 nm
2	Zeta potential	-23.8 mV	-14.4mV	-24.4 mV
3	PDI	0.366	0.412	0.361
4	Drug content	90.55± 2.02	88.60± 2.02	90.34± 2.02

6.5Shelf life

6.5.1 Efavirenz Nanosuspension (3 Months)

Table 18. Shelf life of Nanosuspension

50°C	**Room Temperature**	**15°C**

a) Determination of rate constant (k_0) A_0 = 99.3% A_t = 92.4% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.043/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.043 t_{90} = 0.1 a/ k_0 =136.03/Day	**a) Determination of rate constant (k_0)** A_0 = 99.3% A_t = 90.1% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.10/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.10 t_{90} = 0.1 a/ k_0 =99.3/Day	**a) Determination of rate constant (k_0)** A_0 = 99.3% A_t = 92.2% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.048/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.048 t_{90} = 0.1 a/ k_0 =124.30/Day

6.5.2. Spray dried powder (3 Months)

Table 19. Shelf life of spray dried powder

50°C	**Room Temperature**	**15°C**
a) Determination of rate constant (k_0) A_0 = 99.3% A_t = 88.15% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.12 / Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.12 t_{90} = 0.1 a/ k_0=82.45/Day	**a) Determination of rate constant (k_0)** A_0 = 99.3% A_t = 84.45% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.13/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.13 t_{90} = 0.1 a/ k_0=46.38/Day	**a) Determination of rate constant (k_0)** A_0 = 99.3% A_t = 88.18% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.15/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.15 t_{90} = 0.1 a/ k_0 =66.8/Day

6.5.3 Reconstituted Nanosuspension (3 Months)

Table 20. Shelf life of reconstituted nanosuspension

50°C	**Room Temperature**	**15°C**

a) Determination of rate constant (k_0) A_0 = 99.3% A_t = 91.24% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.089/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.089 t_{90} = 0.1 a/ $k_{0=\ 111.54./day}$	**a) Determination of rate constant (k_0)** A_0 = 99.3% A_t = 91.45% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.083/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.083 t_{90} = 0.1 a/ k_0 =119.63/Day	**a) Determination of rate constant (k_0)** A_0 = 99.3% A_t = 89.64% t= 90 Days Rate constant(k_0)= (A_0 - A t)/ t = 0.10/ Day **b) Determination of shelf life (t_{90})** a= 99.3 k_0 =0.10 t_{90} = 0.1 a/ k_0 =99.3/Day

Table 21. Summary of Shelf life

Formulation	50 °C	Room Temperature	15◦C
Nanosuspension	136.03/Day	99.3/Day	124.30/Day
Spray dried powder	82.45/Day	46.38/Day	66.8/Day
Reconstituted nanosuspension	111.54/Day	119.63/Day	99.3/Day

7. CONCLUSION:

Nanoformulation of EFV for nasal dryg delivery was developed .A simple, precise, accurate, specific and robust UV method was validated for Efavirenz drug. Chromatographic separation was achieved on pheonyx column using methanol: phosphate buffer (70: 30) as mobile phase at constant flow rate of 1ml/min. The detection wavelength was at 247 nm. Efavirenz was subjected to forced degradation for alkali, acid, wet heat, oxidation, photolytic condition and dry heat degradation. The developed method was validated as per ICH Q2 (R1) guidelines. The validation parameters studied were linearity, accuracy, precision, specificity and robustness.The calibration curve for efavirenz was found to be linear in the range of 2- 12µg/ ml.The quantitation limit was found to be 9.86 µg/ml. The detection limit was found to be 3.25 µg/ml.When the accuracy study was performed in the range of 80- 120 %, the drug was found to recover in the range of 79- 85%. The low levels of % RSD proves the accuracy of the method.There was no interference found from the excipient solution in the specificity study of efavirenz.The pH of the formulation was stable throughout 6 months storage.The storage of a nanosuspension for accelerated condition was stable at o month and 3 months, but it was

slightly increased at 6-month observation.The polydispersity index was found to increase as compare to initial. The increase in the particle size may be due to the absorption of the moisture by the nanoparticles resulting in the coalescence of the small nanoparticles forming particles in larger size. The lowered or increased zeta potential values also might have contributed towards the aggregation of particles.Hence, we can conclusively specify that the formulation was stable on the basis of the parameters studied like DSC, XRD, PDI, particle size, zeta potential, pH for 6 months retaining formulation characteristics.

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