Chapter 2

Analytical Method Development and Validation for Quantification of Efavirenz and Enfuvirtide from Efavirenz-Enfuvirtide Co-Loaded Polymer-Lipid Hybrid Nanoparticles

1. Introduction

Long-acting (LA) nanoformulations have been reckoned as an alternative for frequently administered drugs with low patient adherence and fluctuating plasma drug delivery. Since anti-retroviral therapy needs to be continued for a prolonged duration; the development of LA antiretroviral nanoformulation was one objective of current research work. To this end, drugs with differential physicochemical properties namely; Efavirenz (Efa) and Enfuvirtide (Enf) were co-loaded into polymer-lipid hybrid nanoparticles (PLN) and further evaluated for their LA potential. A suitable analytical method was required for the estimation of each drug from PLN and release media. Therefore, quantification of Efa was done by developed and validated RP-HPLC method in presence of Enf, other formulation excipients, and release media. Whereas, Enf was analyzed in the presence of Efa, formulation excipients, and release media by developed and validated spectrofluorophotometric method. The current chapter describes each method in succinct.

Efa (Figure 2.1 (a)) (DB00625, (S)-6-Chloro-4-cyclopropylethynyl-4-trifluoromethyl-1,4-dihydro-benzo[d] [1,3]-oxazin-2-one) [1] is a non-nucleoside reverse transcriptase inhibitor approved by USFDA under accelerated review process [2]. As per WHO guidelines, Efa is a part of combination antiretroviral therapy (cART) for the treatment of HIV infection [3]. Few analytical methods have been reported for quantification of Efa in plasma [4], human hair [5], formulation [6], and bulk drug [7]. However, they have limitations of longer retention time, lack of reproducibility, tedious sample preparation technique, and lack of method applicability for analysis of Efa from combination drug nanoformulations. Further, to optimize the nanoparticles with respect to critical process and formulation parameters which would affect % entrapment efficiency (% EE), drug content, and % drug release, a simple and specific HPLC method is desirable.

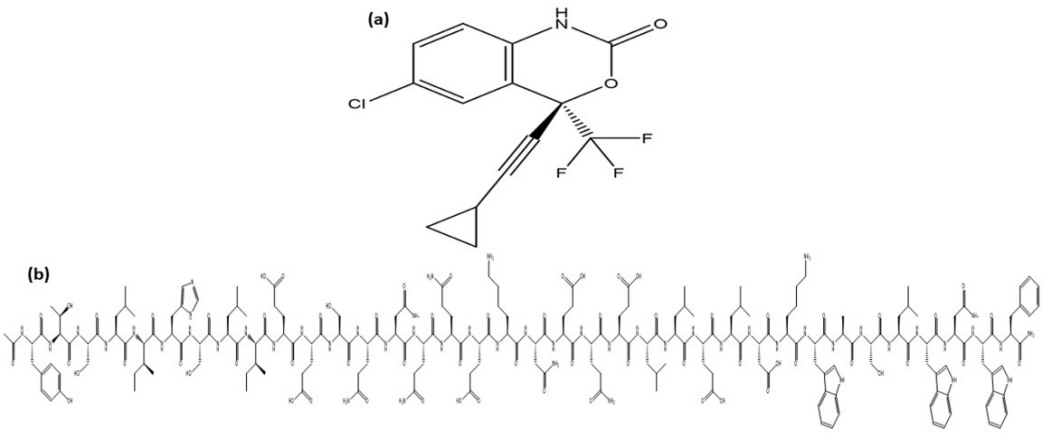

Figure 2.1: Chemical structure of (a) Efavirenz and (b) Enfuvirtide.

Whereas, Enf (Figure 2.1 (b)) is a fusion inhibitor peptide approved by USFDA in 2003 to be administered in combination with other antiretroviral drugs [8]. It elicits increased solubility in aqueous buffers (pH 7.5) of 85-142 g/ 100 ml [9] and log P of -17.2 [10]. It has 3-tryptophan residues amongst the 36-amino acids which encompass the ability to fluoresce [11]. Also, Enf was quantified by the spectrofluorimetric method from polymeric nanoparticles previously [12]. Therefore, spectrofluorophotometric method was developed and validated for the estimation of Enf from Efa-Enf PLN.

2. Materials and methods

2.1 Materials

Efa was a kind gift sample from Ranbaxy Laboratories Ltd. (Gurgaon, India). Enf was purchased from Prospec Protein Specialists (Rehovot, Israel). HPLC grade acetonitrile, potassium dihydrogen orthophosphate, sodium hydroxide, stearic acid, and dichloromethane were procured from S D Fine-Chem Limited (Mumbai, India). PLGA (lactide:glycolide-50:50, mol.wt. 30000-60000 Da) was procured from Sigma-Aldrich Chemicals Company (Missouri, United States), Cremophor HS-15 was a kind gift sample from BASF Chemicals Company (Navi-Mumbai, India) and soy lecithin was procured from HiMedia Laboratories

Pvt. Ltd. (Mumbai, India). All other chemicals, solvents, and reagents utilized were either HPLC or analytical grade. HPLC grade water was obtained from the Milli-Q system (Millipore GmbH, Germany). The solvents and buffers prepared were suitably filtered through 0.22 µ Millipore™ membrane filter (Merck, Darmstadt, Germany) and suitably degassed in an ultrasonic bath for 30 minutes.

2.2 RP-HPLC method development and validation for quantification of Efavirenz

2.2.1 Instrument

HPLC method for quantification of Efa was developed using HPLC (Shimadzu, Kyoto, Japan) equipped with a binary pump (LC-20AD), photo-diode array (PDA) detector (SPD M20A), and autosampler (SIL-HTC, Shimadzu, Japan). Efa was separated using Waters Spherisorb® 5 µm ODS (C18) column (4.6 x 250 mm) with a sample injection volume of 20 µl after pre-equilibrating the column for 40 min. The hardware control and data processing were done using LCsolution software version 1.2 SP1.

2.2.2 Chromatographic conditions

Separation of Efa was done in isocratic mode at room temperature. The mobile phase comprised A-acetonitrile and B-phosphate buffer (10 mM, pH 6.8) with a composition of 70:30 (%A:%B) at a flow rate of 1 ml/min. The run time and detection wavelength determined by spectrum scan were 7 min and 246 nm respectively. Peak area was utilized to quantify Efa.

2.2.3 Preparation of standard solutions

A stock standard solution of 1 mg/ml was prepared by dissolving accurately weighed 100 mg of Efa in 100 ml of acetonitrile. The working standard solutions of 100 µg/ml and 10 µg/ml were prepared by further suitably diluting stock standard solution using acetonitrile:

phosphate buffer (10mM, pH 6.8) in 70:30 %v/v. The secondary standard solutions (500, 750, 1000, 2000, 4000, 8000, 12000, 16000 and 20000 ng/ml) were prepared from working standards (100 µg/ml) by diluting an aliquote of 0.025, 0.0375, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 ml in 5 ml volumetric flask; respectively, using acetonitrile: phosphate buffer (10 mM, pH 6.8) in 70:30 %v/v. Further, 10 µg/ml was utilized to prepare 160, 320, 485, 640, and 800 ng/ml by suitably diluting aliquots of 160, 320,485, 640, and 800 µl up to 10 ml using acetonitrile: phosphate buffer (10 mM, pH 6.8) in 70:30 %v/v to determine Limit of detection (LOD) and limit of quantitation (LOQ).

2.2.4 Preparation of sample solution

When analyzing Efa nanodispersion, aliquote was diluted 10-20 times using acetonitrile, bath sonicated for 3 min and further centrifuged at 10000 rpm for 10 min. The supernatant was withdrawn and subjected to HPLC analysis. Other aqueous samples containing free Efa alone were directly analyzed by HPLC with/without dilution with acetonitrile.

2.2.5 Method validation

The analytical method for quantification of Efa was validated as per ICH guideline Q2R(1) with respect to specificity, linearity, range, accuracy, precision, and robustness [13].

Specificity: To determine the method specificity, a standard solution of Efa (100 µg/ml) was spiked in a solution of nanoparticle excipients including stearic acid, PLGA, solutol HS-15, soy lecithin, Enf, and dissolution medium. Chromatogram was assessed for the presence of interfering peaks corresponding to Efa. Further, the specificity of Efa was also determined after degradation of Efa under different stress conditions including acidic or basic pH, UV, and oxidation. Briefly, 12.5 mg of Efa was suitably dissolved in 25 ml methanol and pH was adjusted to 2.3 with 0.1 N hydrochloric acid and refluxed for 8 h. For degradation of Efa

under basic pH, 12.5 mg of Efa was dissolved in 25 ml of 0.1 N NaOH and refluxed for 1 h. For degradation of Efa under oxidative stress, 50 mg of Efa was dissolved in 50 ml of 3%v/v hydrogen peroxide in methanol and kept under stirring at room temperature for 8 h [14,15]. While, for solid-state degradation studies under ultraviolet (UV) radiation, 10 mg Efa was kept in UV light up to 48 h, appropriate sample was withdrawn at 24 h and 48 h and analyzed by RP-HPLC after dissolving 1 mg Efa in 1 ml acetonitrile and further appropriately diluting the stock solution with the mobile phase.

Linearity and range: To determine the linearity and range, solutions of different concentrations of Efa including 500, 750, 1000, 2000, 4000, 8000, 12000, 16000, and 20000 ng/ml were injected six times per concentration. The peak area of each concentration was recorded and a plot of Efa concentration versus peak area was constructed. Since the ordinary least square (OLS) can lead to statistically erroneous results for heteroscedastic data, both OLS and weighted least square (WLS) were tested for heteroscedasiticity by F-test. Based on the correlation obtained between $F_{observed}$ and F_{table} ($F_{observed}>F_{table}$), WLS regression analysis was performed on different weights (w_i) including $1/\sqrt{x}$, $1/x$, $1/x^2$, $1/\sqrt{y}$, $1/y$ and $1/y^2$. % relative error (% RE) and total % relative error ($\sum$%RE) were determined for each model of different weights and the model with the least $\sum$%RE was selected [16–18].

The range of developed analytical method was determined based on the plot obtained for peak area against concentration and response factor against concentration for every calibration standard. Further, the % RSD of the obtained response factor was determined to establish an appropriate range.

Accuracy: accuracy was determined by estimating the % recovery of known concentration of Efa spiked in three different working standards (500 ng/ml, 2000 ng/ml, and 16000 ng/ml).

The analysis was performed in triplicate for each concentration and %recovery and %RSD were calculated.

Precision: Precision was determined by calculating %RSD of peak area of three different concentrations (500 ng/ml, 2000 ng/ml, and 16000 ng/ml) each in triplicate within a day over the entire calibration curve range. While, intermediate precision was established by determining %RSD of nine different determinants (three concentrations of each in triplicate) for consecutive 3 days.

Limit of detection (LOD) and limit of quantitation (LOQ): LOD and LOQ were determined based on calibration curve using equation (2.1) and (2.2) respectively[13]

$$LOD = \frac{3.3\sigma}{s} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.1)$$

$$LOQ = \frac{10\sigma}{s} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.2)$$

Wherein, σ is the standard deviation of the y-intercept of the calibration curve and s is the slope of the calibration curve.

Robustness: Robustness of the method was evaluated by deliberate variation in chromatographic conditions including analytical instrument from Shimadzu HPLC 1 (Shimadzu, Kyoto, Japan) to HPLC 2 (ThermoScientific, Waltham, United States), pH of buffer from 6.8 to 7, column oven temperature to 30°C and mobile phase ratio to 72:28. % recovery was evaluated as a response for each condition with respect to the standard solution at 750, 4000, 8000, and 16000 ng/ml.

System suitability: To verify the suitability of the chromatographic system for intended analysis, a system suitability test was performed by six replicate injections of a standard solution of Efa (2 µg/ml) and determining %RSD between obtained retention time, tailing

factor (10%), number of theoretical plates (N) and height equivalent to a theoretical plate (HETP).

2.3 Spectrofluorophotometric method development and validation for Enfuvirtide

2.3.1 Instrument and spectrofluorophotometric conditions

Spectrofluorophotometeric method of Enf was developed using spectrofluorophotometer RF-5301 PC (Shimadzu, Japan) equipped with a recorder, 1x1 cm^2 quartz cell, xenon arc lamp, and RFPC fluorescence spectroscopy software for RF-5301PC (Version 2.04) for data interpretation. Relative fluorescence intensity was utilized to analyze Enf at excitation (λ_{ex}) and emission wavelength (λ_{em}) of 287 nm and 353 nm respectively as determined through spectral scan. The samples were prepared in phosphate buffer (10 nM, pH 6.8) at 4°C and analyzed in spectral and quantitative mode to yield emission spectra and fluorescence intensity respectively.

2.3.2 Preparation of standard solution

A stock solution of 500 µg/ml was prepared by dissolving 10 mg of Enf in a 5 ml volumetric flask using phosphate buffer (10 mM, pH 6.8). The standard solution was utilized to prepare calibration standard solution of 2.5, 5, 10, 20 and 40 µg/ml by diluting aliquots of 0.025, 0.050, 0.100, 0.200 and 0.4 ml respectively in 5 ml volumetric flask using phosphate buffer (10 mM, pH 6.8).

2.3.3 Preparation of sample solution

An aliquot (1 ml) of the sample containing Enf was diluted 2 times using phosphate buffer (10 mM, pH 6.8) and bath sonicated up to 15 min at 25°C. Thereafter, the sample was centrifuged at 15000 rpm for 10 min and supernatant (2 ml) was utilized for quantitative

analysis of Enf. Release media (2 ml) containing Enf was directly analyzed after nullifying the background fluorescence from release media at the initial time-point.

2.3.4 Method validation

The developed spectrofluorophotometric method for quantification of Enf was validated with respect to linearity, range, specificity, limit of detection, limit of quantification, accuracy, and precision as per ICH Q2(R1) guideline [13].

To determine the specificity of the method, Enf (33.34 µg/ml) was spiked in nanoparticle dispersion which included stearic acid, PLGA (50:50), soy lecithin, solutol HS-15, Efa, and release media consisting of phosphate buffer (10 mM, pH 7.4 and 1% tween 80). An aliquot of sample (1 ml) was diluted twice in phosphate buffer (10 mM, pH 6.8) and analyzed in quantitative mode. A spectral scan of the sample was obtained to determine any interference of formulation excipient, co-loaded Efa, or release media components [19]. The linearity of the developed spectrofluorophotometric method for Enf was determined by the ordinary least square regression analysis. Enf standard solution of 2.5,5,10,20 and 40 µg/ml were prepared as described above and the fluorescence intensity of each was determined in triplicate. Thereafter, the calibration curve was obtained from the fluorescence intensity versus concentration graph. The linearity of the method was determined by the correlation coefficient. The range of present analytical methods was determined based on calibration standards obtained from the calibration curve. LOD and LOQ were determined based on the calibration curve and linear regression equation using previously mentioned equations 2.1 and 2.2 respectively.

The method was also validated for accuracy, intra-and, inter-day precision. The ability of the method to estimate the analyte to its true value was determined by % recovery representing the accuracy of the method. Whereas, the ability of the developed method to repeatably and

reproducibly measure the analyte concentration was determined by 3 assays in a day for each QC level (LQC, MQC and HQC), each concentration run in triplicate per assay for intra-day precision. Whereas, the inter-day precision was determined by three assays on three consecutive days at LQC, MQC and HQC with each value determined in triplicate. Precision was represented as % RSD.

2.4 Statistical analysis

Statistical data analysis was performed using a student t-test with $p< 0.05$ as a minimal level of significance. The results were expressed as mean ± standard deviation (SD) obtained from three separate experiments (n=3). Mathematical fit functions were performed by ANOVA analysis.

3. Results and Discussion

3.1 RP-HPLC method development and validation for quantification of Efavirenz

3.1.1 Method development

The RP-HPLC method development for quantification of Efa was initiated with phosphate buffer (10 mM, pH 6.8) as an aqueous phase, and acetonitrile (ACN) as an organic phase to prepare the mobile phase. Efa was not eluted at 50:50 ratio of ACN: phosphate buffer (10 mM, pH 6.8) and 1 ml/min flow rate until 20 min. Thereafter, mobile phase comprising of various ratios of ACN: phosphate buffer (10 mM, pH 6.8) including 60:40, 70:30, and 80:20 were used for separation of Efa and evaluated for several chromatographic parameters including retention factor (K'), HETP, N and retention time (Table 2.1). As the amount of ACN in the mobile phase was increased from 60% to 80% in the mobile phase, the retention time was found to decrease from 9.6±0.03 min to 4.1±0.002 min. However, it also led to an undesirable decrease in retention factor, N, HETP, and an increase in tailing factor (>1.2) at

246 nm (λ_{max}). For basic molecules, weakly acidic to neutral aqueous phase may lead to a decrease in irreversible retention and tailing in chromatogram unlike at acidic conditions; therefore, with an increase in the aqueous phase in the mobile phase, a decrease in tailing factor for Efa peak was observed [20]. Although the mobile phase comprising of ACN and phosphate buffer (10 mM, pH 6.8) in the ratio of 60:40 showed higher retention time than 70:30, an insignificant difference in tailing factor was observed ($p<0.1$). Therefore, ACN: phosphate buffer (10 mM, pH 6.8) in 70:30 ratio at 1 ml/min flow rate was selected as the mobile phase for Efa analysis.

Table 2.1 Optimization of Mobile phase for quantification of Efavirenz by RP-HPLC method

Chromatographic parameters	**Acceptance criteria**	**Results***		
A: Acetonitrile: B: Phosphate buffer (10 mM, pH 6.8) (%A:%B)		60:40	70:30	80:20
Efa retention time (min)	--	9.6±0.03	5.6±0.04	4.1±0.002
Retention factor, κ'	$2<\kappa'<10$	3.5±0.02	2.2±0.02	0.8±0.01
Tailing factor	0.8-1.5	1.12±0.007	1.18±0.01	1.26±0.02
Number of theoretical plates, N	>2000	12149±149	9602±887	7230±110
HETP	--	34.5±0.52	26.1±2.41	20.5±0.25

*Data represents mean±S.D., $p<0.001$ by two-way ANOVA followed by Tukey's multiple comparison test

3.1.2 Method validation

3.1.2.1 Linearity and range

Initially, linearity was determined by the least-square linear regression analysis; wherein, all the nine calibration curve levels were found to be linear in the concentration range of 500 ng/ml-20000 ng/ml (R^2-0.9995, $p<0.0001$ by F-test) (Figure 2.2(a)). The linear regression equation for the calibration curve was as follows:

$$y = 65.221x - 3833.8 \ldots\ldots\ldots (2.3)$$

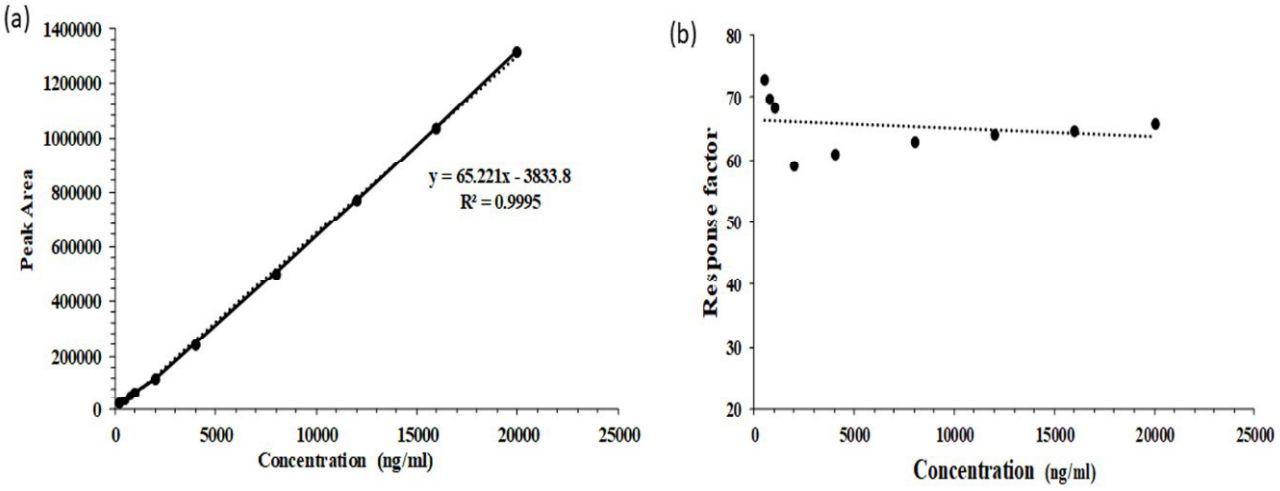

Figure 2.2 Linearity of Efavirenz RP-HPLC method (a) Calibration curve. (b) Response factor versus Efavirenz concentration.

Where, y corresponds to the peak area for corresponding *x* concentration (ng/ml) [21]. The obtained regression equation was utilized to calculate the % bias between calculated and theoretical concentration (Table 2.2) [21,22].

Table 2.2 % bias of calibrated concentration range of Efavirenz

Concentration (ng/ml)	% bias
500	22.79±1.49
750	14.06±7.69
1000	10.52±3.40
2000	6.96±1.27
4000	4.78±2.15
8000	2.78±1.14
12000	1.05±0.88
16000	3.90±1.85
20000	1.21±0.10

To further confirm the linearity of the developed calibration curve, response factors were plotted against each concentration (Figure 2.2(b)). Near zero slope (-0.0001) and %RSD of 6.5 of the average response factor, confirmed the linearity of the method. %RSD of <10 was used to establish the concentration range of the method [23]. However, this OLS regression

analysis is well suited only for homoscedastic data. Therefore, obtained responses were tested for heteroscedasticity by F-test. Wherein, it was assumed that the obtained responses were homoscedastic, if $F_{observed} > F_{table}$ (0.99 confidence interval, n-1) or vice versa. Since $F_{observed}$ was higher than F_{table} (Table 2.3), therefore the responses were heteroscedastic with the highest %RE at lower concentration and vice versa (Figure 2.3).

Table 2.3 Test for homogeneity of variance, F-test for calibrated concentration range of Efavirenz

Concentration (ng/ml)	**Responses**	**Variance (S^2)**
500	45567	S_1^2:27097756.8
	32204	
	31382	
	36468	
	33489	
	34328	
20000	1304305	S_2^2:2197760851
	1405479	
	1342766	
	1298812	
	1269451	
	1324334	
$F_{observed}$ (S_2^2/ S_1^2) =81.1		
F_{table} (0.99,5,5) =10.9		

Heteroscedasticity could be minimized by either narrowing the calibration range or applying WLS regression analysis. Since the developed RP-HPLC method has to be utilized for estimation of Efa from various nanoformulations as well as release media a wide calibration range was desirable. Therefore, the WLS regression equation, regression coefficient (r), and ∑% RE for each weighted factor were obtained (Table 2.4). Model 4 ($w_{i-1/x}^2$) led to the least ∑%RE as determined by the plot of %RE versus concentration (Figure 2.3) and appropriately defined the correlation between RP-HPLC responses and concentration of Efa.

Table 2.4 Regression equation, correlation coefficient and sum of the relative errors (∑%RE) for each weighted and unweighted factor of RP-HPLC method of Efavirenz

Model no	w_i	WLS/LS regression equation	Correlation coefficient (r)	∑%RE
1	Unweighted	y=65.5276x-6287.0742	0.9996	71.36
2	$1/\sqrt{x}$	y=64.9875x-1330.4149	0.9996	47.38
3	1/x	y=64.4588x+1342.6371	0.9994	34.16
4	$1/x^2$	y=62.8493x+4022.9739	0.9985	25.64
5	$1/\sqrt{y}$	y=64.9926x-1556.4484	0.9996	48.43
6	1/y	y=64.4456x+1071.2767	0.9994	35.48
7	$1/y^2$	y=62.6495x+4005.1601	0.9984	27.17

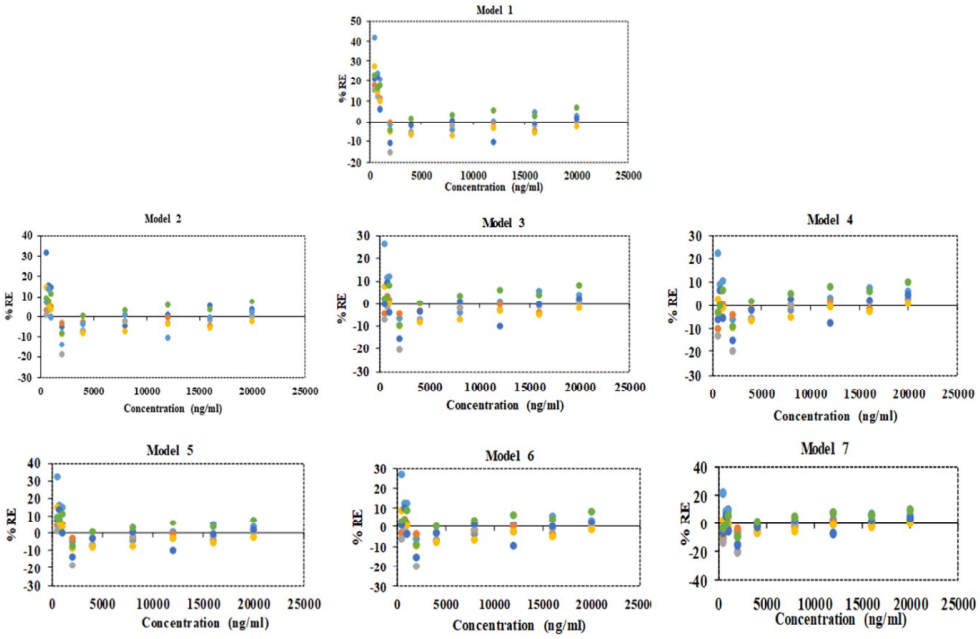

Figure 2.3 % relative error distribution based on concentration of Efavirenz for unweighted and weighted factors. (model 1: unweighted factor, model 2: $1/\sqrt{x}$, model 3: 1/x, model 4: $1/x^2$, model 5: $1/\sqrt{y}$, model 6: 1/y and model 7: $1/y^2$).

3.1.2.2 Specificity

The chromatogram of Efa using ACN: phosphate buffer (10mM, pH 6.8) in 70:30 ratios and 1 ml/min flow rate demonstrated the absence of interference of any other peak corresponding to formulation excipients at 246 nm compared to standard Efa solution (Figure 2.4(a-b)). Further, no interference from the component of the release medium was observed during the analysis of Efa content in *in-vitro* release studies (Figure 2.4(c)). The resolution between Efa and release media peak was found to be 3.59±0.26 which confirmed the specificity of the

developed RP-HPLC method for Efa. Similarly, no interference of degradants was observed during forced degradation studies (Figure 2.4 (d-f)). It was observed that Efa was stable in acidic pH with a retention time of 5.76±0.07 min; while, HPLC chromatogram of Efa when subjected to basic conditions led to elution of Efa degradant at 5.92±0.19 min (R=2.39±0.07). The presence of degradation products could be due to amide or ester hydrolysis which further resulted in cyclization [7]. Further, Efa depicted a high degree of stability upon UV light exposure for 48 h (Figure 2.4(f)). Therefore, the developed HPLC method was specific for the separation and quantification of Efa. Differences in retention time of Efa could be attributed to fluctuation in the total pressure of the system between 800-1000 psi during analysis[24]. %recovery and retention time of Efa during stress degradation study is presented in Table 2.5.

Table 2.5 Assay of 500 µg/ml Efavirenz solution under different stress conditions

Stress condition	**Sample treatment**	**Unexpected peaks, (R)**	**Efa retention times (min)**	**% recovery (±SD)**
Reference	--	--	5.6±0.04	100.4±2.86
pH	0.1 N HCL, 8 h	No	5.7±0.07	102.3±11.6
	0.1 N NaOH, 8h	Yes, (2.39±0.08)	5.9±0.19	66.1±8.62
Oxidation	3% (v/v) H_2O_2, 8h	No	5.8±0.07	91.9±4.56
UV light	24h	No	5.7±0.002	102.6±2.76
	48h	No	5.7±0.01	104.3±6.54

R-resolution, Data represents mean±SD, n=3

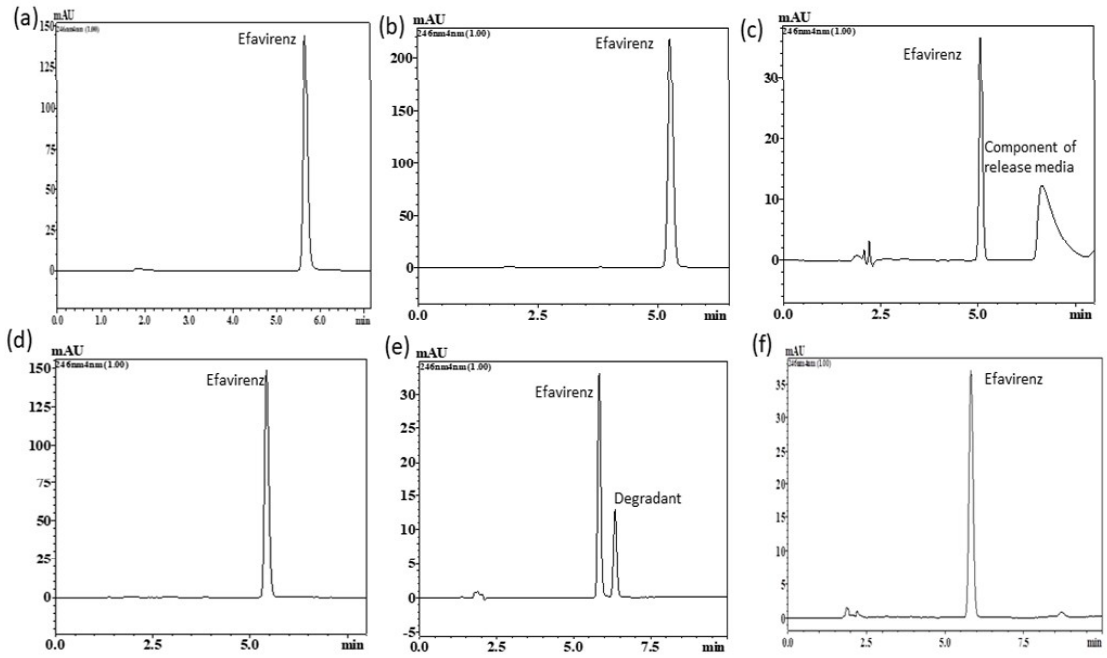

Figure 2.4: Chromatogram of Efavirenz in presence of (a) standard Efavirenz solution (16 µg/ml) (b) formulation excipients (20 µg/ml) (c) release media (4.2 µg/ml), (d) acidic pH (16 µg/ml), (e) basic pH (3.6 µg/ml) and (f) UV exposure for 48 h (4 µg/ml) using Waters Spherisorb® 5 µm ODS (C18) column (4.6 x 250 mm), flow rate: 1.0 ml min^{-1}; injection volume: 20 µl; UV detection: 246 nm; mobile phase A-acetonitrile and B-phosphate buffer (10 mM, pH 6.8) with composition of 70:30 (%A:%B), run time: 7 min.

3.1.2.3 Accuracy and precision

Accuracy of the analytical procedure determines the degree of closeness between the obtained values to the true values which are expressed as %recovery. The overall %recovery for three different concentrations at LQC, MQC, and HQC levels was observed to be 99.9±9.99% which is well within the acceptance criteria of 80-120% [25]. The precision determines the effect of random errors on the repeatability of the method which is expressed as % RSD. The overall % RSD was found to be <2% (Table 2.6).

Table 2.6 Accuracy and precision at different levels of Efavirenz in standard solution

Accuracy (n=3)			
Target %	**% Recovery (mean)**	**SD**	**% RSD**
80	100.1	0.02	1.21
100	90.3	0.01	0.39
120	109.1	0.20	1.03
Inter-day precision (n=3)			
Concentration (ng/ml)	**Measured concentration (ng/ml)**	**SD**	**% RSD**
500	598.3	12.3	2.05
2000	1879.8	25.3	1.34
16000	15785.9	272.6	1.72
Intra-day precision (n=3)			
500	543.2	6.3	1.16
2000	2069.2	7.6	0.36
16000	15383.1	193.7	1.26

3.1.2.4 LOD and LOQ

The LOD and LOQ of the developed RP-HPLC method for Efa, when determined from the standard deviation of the y-intercept and slope of the calibration curve using equation (2.1) and (2.2) respectively, were found to be 160 ng/ml and 480 ng/ml respectively. To further validate the obtained LOD and LOQ, a calibration curve was plotted with a lower concentration from 160-800 ng/ml. Higher %RSD was observed at lower concentrations (Table 2.7). Similarly, 17.15% RSD of response factor at LOD and poor near-zero linear slope (3.8804) depicted a low degree of precision at lower concentration levels of Efa.

Table 2.7 % RSD at LOD and LOQ of Efavirenz

Calibration level (ng/ml)	**Actual average concentration (ng/ml)**	**SD**	**% RSD**
160	163	32.3	19.84
320	316	14.8	4.71
485	492	1.60	0.33
640	645	6.64	1.03
800	803	9.31	1.16

Data represented for n=3

3.1.2.5 Robustness

Change in the analytical instrument from HPLC 1 (Shimadzu, Kyoto, Japan) system to HPLC 2 (ThermoScientific, Waltham, United States) system to determine robustness although led to a change in retention time to 4.81±0.04 min for Efa, the coefficient of regression was found to be 0.9991 with <2% RSD for every calibration standard concentration. The % recovery of Efa was found to be 94.7±4.21% to 113.1±2.74% after changing several chromatographic parameters namely analytical instrument, column temperature from 25°C to 30°C, ACN: phosphate buffer (10 mM, pH 6.8) ratio from 70:30 to 72: 28 and change in pH of phosphate buffer from 6.8 to 7.0 (Table 2.8).

Table 2.8 Robustness results of Efavirenz RP-HPLC method at different conditions

Condition	**Recovery % (±SD)***			
	750 ng/ml	**4000 ng/ml**	**8000 ng/ml**	**16000 ng/ml**
HPLC 2	113.1±2.74	95.5±10.86	95.7±1.69	98.1±1.01
Column temperature (30°C)	106.8±7.76	104.9±1.18	98.5±3.06	94.7±4.21
Mobile phase ratio (72:28)	107.7±3.33	105.2±2.42	99.4±3.39	95.8±5.72
Buffer pH 7	107.2±2.48	104.5±1.05	98.2±3.40	96.3±0.59

*Data represented as mean±SD, n=3

3.1.2.6 System suitability

The retention time of 2 µg/ml Efa standard solution was found to be 5.64±0.002 min. The %RSD chromatographic parameters including retention time, tailing factor, N, and HETP of six replicate injections of a standard solution of Efa (2µg/ml) was found to be <2%. Further, the tailing factor and N were in accordance with the United States Pharmacopeia limit for system suitability (<1.2 and >2000 respectively) (Table 2.9).

Table 2.9 System suitability parameters for RP-HPLC method of Efavirenz

Parameter	Result*
Retention time (min)	5.6±0.002
HETP	20.4±0.18
N	12264.0±105.0
Tailing factor	1.19±0.005

*Data represented as mean±SD, n=6

3.2 Spectrofluorophotometric method development and validation for quantification of Enfuvirtide

3.2.1 Method development

Enfuvirtide (Fuzeon; Roche) is an HIV-1 fusion inhibitor peptide consisting of 36-amino acid [8,26]. Furthermore, it consists of 3-tryptophan residues amongst 36-amino acids which possess intrinsic fluorescence ($\lambda_{ex}/\lambda_{em}$ -280/350 nm) and could be utilized for analysis of Enf [11]. It belongs to BCS class III, possessing high solubility in aqueous buffers up to 85-142 g/100 ml [9]. Thus, the addition of Enf (10 mg) in 5 ml of phosphate buffer (10 mM, pH 6.8) led to its rapid solubilization. Method development was initiated after nullifying the background absorption and emission using phosphate buffer (10 mM, pH 6.8). Thereafter, Enf working standard was scanned for determination of λ_{ex} (287 nm) and λ_{em} (353 nm) (Figure 2.5 (a)). Further, Calibration curve was derived from fluorescence intensity of Enf obtained at predetermined concentration (2.5, 5, 10, 20 and 40 µg/ml) in quantitative mode (Figure 2.5 (b))

3.2.2 Method validation

3.2.2.1 Linearity and range

Linearity was evaluated by the ordinary least square method. All 5 calibration levels depicted linearity in the range of 2.5 µg/ml-40 µg/ml. The linearity was further confirmed by the

regression coefficient (R^2-0.9994). Equation 2.4 represents linear regression equation obtained from the calibration curve

y=18.07x+17.201 ……..(2.4)

where, y corresponds to fluorescence intensity for corresponding x concentration (µg/ml) [27].

3.2.2.2 Specificity

The specificity of the developed analytical method was determined by observation of Enf spectra after extraction from Efa-Enf nanodispersion and release media. The emission spectra of Enf in presence of formulation excipients, Efa, and release media was a replica of standard Enf with the absence of any interference (Figure 2.5 (c))

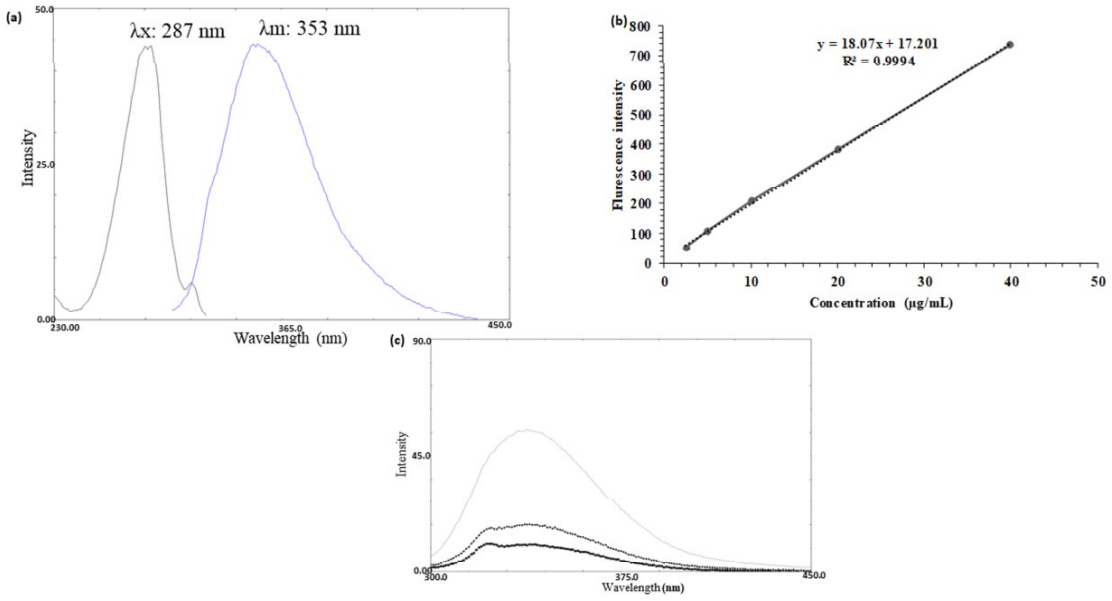

Figure 2.5 Method development and validation of Enfuvirtide (a) Excitation and emission spectra for Enfuvirtide (b) Calibration curve for Enfuvirtide (c) Specificity of developed spectrofluorophotometric method for determination of Enfuvirtide depicting Enfuvirtide standard solution (10 µg/ml), Enfuvirtide from release media (5.1 µg/ml) and Enfuvirtide spectra in presence of formulation components (2.5 µg/ml) from top to bottom respectively.

3.2.2.3 LOD and LOQ

The standard deviation amongst y-intercept of three replicate calibration curves and slope mean was calculated for the determination of LOD and LOQ. The LOD and LOQ for the developed analytical method was found to be 0.443 µg/ml and 1.32 µg/ml using equations 2.1 and 2.2 respectively.

3.2.2.4 Accuracy and precision

The developed spectrofluorophotometric method was found to be accurate with an average % recovery of 104.1±11.50% which was well within limits (80-120%). Furthermore, the method was found to be precise for the determination of Enf with % RSD <2% (Table 2.10).

Table 2.10 Accuracy and precision at different levels of Enfuvirtide in standard solution

Accuracy (n=3)			
Target %	**Nominal concentration (µg/ml)**	**Measured concentration (µg/ml) (mean± S.D)**	**% Recovery (mean± S.D)**
80	12.5	12.9±0.78	103.9±6.28
100	20	18.5±2.19	92.7±10.98
120	50	57.8±5.89	115.7±11.79
Inter-day precision (n=3)			
Concentration (µg/ml)	**Measured concentration (µg/ml)**	**SD**	**% RSD**
2.5	1.5	0.02	1.26
10	8.2	0.05	0.67
40	41.4	0.19	0.47
Intra-day precision (n=3)			
2.5	1.6	0.02	1.67
10	8.3	0.09	1.19
40	42.6	0.83	1.96

Data represented as mean±SD, n=3

4. Conclusion

The RP-HPLC and spectrofluorophotometric method for estimation of Efa and Enf, respectively was validated as per ICH guideline Q2(R1) and could be suitably utilized for analysis of %EE, drug content, %DL and *in vitro* drug release of Efa and Enf from Efa-Enf PLN. The obtained calibration curve for Efa showed heteroscedasticity which was minimized using a weighted least square regression method with $1/x^2$ being the most appropriate weighting with respect to least ∑%RE. The run time of 7 min allowed analysis of a large number of samples in a shorter time duration. Whereas, the intrinsic fluorescence of Enf was useful in the development of an analytical method for the rapid quantification of Enf. Specificity of developed analytical methods enabled its use in the estimation of Efa and Enf from nano-formulations in the presence of different excipients and release media without interference.

4. References

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