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The use of experimental design for the development and validation of an HPLC-ECD method for the quantitation of efavirenz

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A high performance liquid chromatography with electrochemical detection (HPLC-ECD) method for the quantitation of efavirenz (EFV) was developed, since traditional HPLC-UV methods may be inappropriate, given that EFV undergoes photolytic degradation following exposure to UV light. This work describes the use of response surface methodology (RSM) based on a central composite design (CCD) to develop a stability-indicating HPLC method with pulsed ECD in direct current (DC) mode at an applied potential difference and current of +1400 mV and 1.0 µA for the analysis of EFV. Separation of EFV and imipramine was achieved using a Nova-Pak® C_{18} cartridge column and a mobile phase of phosphate buffer (pH 4.5): acetonitrile (ACN) (55:45 v/v). Mobile phase pH, buffer molarity, ACN concentration and applied potential difference were investigated. The optimized method produced sharp well resolved peaks for imipramine and EFV with retention times of 3.70 and 8.89 minutes. The calibration curve was linear (R^2 = 0.9979) over the range 5-70 µg/mL. Repeatability and intermediate precision ranged between 3.37 and 4.34 % RSD and 1.31 and 4.29 % RSD and accuracy between -0.80 and 4.71 % bias. The LOQ and LOD were 5.0 and 1.5 µg/mL. The method was specific for EFV and was used to analyse EFV in commercially available tablets. The HPLC-ECD method is more suitable for quantitative analysis of EFV than HPLC-UV.

1. Introduction

Efavirenz (EFV) (Fig. 1), is a non-nucleoside reverse transcriptase inhibitor used for the treatment of HIV-1-infected individuals (Marzolini et al. 2001; Veldkamp et al. 1999; Vrouenraets et al. 2007). EFV selectively binds to HIV-1 reverse transcriptase (RT) causing an allosteric change in RT thus leading to non-productive binding of an incoming nucleotide during DNA polymerization (Wang and Hutchins 1999). EFV is chemically known as (4S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one. The drug is weakly acidic with a pKa of 10.2 and is largely unionised at physiological pH (Rabel et al. 1996). It is a white to slightly pink crystalline powder practically insoluble in water with an intrinsic aqueous solubility of 9.2 µg/mL at pH 8.7 at 25 °C (WHO 2005). EFV possesses a chiral carbon at position 4 and the 4S enantiomer is used in commercially available formulations (Rabel et al. 1996).

Quantitative analysis of EFV has been achieved in biological samples using analytical methods including gas chromatography-mass spectroscopy (GC-MS) (Lemmer et al. 2005), HPLC (Veldkamp et al. 1999; Jiaping et al. 2008; Langmann et al. 2001; Matthews et al. 2002; Sarasa-Nacenta et al. 2001) and capillary zone electrophoresis (CZE) (Jiaping et al. 2008). A limited number of researchers have reported the use of HPLC-UV for quantitative analysis of EFV in dosage forms either alone (Montgomery et al. 2001; Hamrapurka et al. 2010; Rao and Nikalje 2009) or in combination with other antiretroviral drugs (Rebiere 2007). HPLC is a powerful and reliable analytical tool that not only permits separation and provides quantitative data but can eliminate almost all interference challenges (Garcia et al. 2002). However, the use of HPLC with UV detection for the quantitaive analysis of EFV may be inappropriate as the drug is photolabile and has been reported to undergo extensive photolytic degradation following exposure to light at wavelengths of 254 nm (Gadkari et al. 2010) and 320-400 nm (Hamrapurka et al. 2010).

The analysis of EFV in pharmaceutical formulations with HPLC has been previously accomplished using UV detection at a wavelength ranging between 220 and 390 nm (Montgomery et al. 2001; Rao and Nikalje 2009; Gadkari et al. 2010). These conditions may be unsuitable and there is a need to explore the use of an alternative detection technique for the quantitative analysis of EFV with HPLC. EFV has a nitrogen atom on the benzoxazine ring, which can undergo electrochemical oxidation (Dogan-Topal et al. 2009). Consequently, electrochemical detection (ECD) may be a better tool that can be used to ensure accurate determination of EFV in dosage forms. Dogan-Topal et al. (2009) further reported the use of a HPLC-ECD method for the determination of EFV in formulations using a pencil graphite electrode. However, this method is non-stability-indicating and involves the use of an electrochemical biosensor with the primary aim of studying DNA–EFV interactions, and therefore may not be suitable for routine analysis of the drug in dosage forms. This manuscript reports the use of a simple, stability-indicating HPLC method with an amperometric ECD for the accurate quantitation of EFV in pharmaceutical formulations and was developed using a design of experiments (DoE) approach.

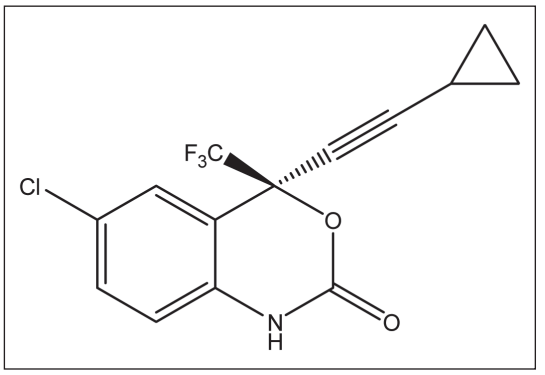

Fig. 1: Chemical structure of EFV ($C_{14}H_9ClF_3NO_2$, MW = 315.7) (Cristofoletti et al. 2013)

DoE was used to identify optimum conditions during analytical method development. The conventional approach to establish optimum operating conditions is by changing a single parameter whilst keeping all others constant and this approach does not permit the study of interactive effects of different variables and consequently does not depict the complete effects of the operational parameters on a process (Bas and Boyaci 2007). Furthermore this approach is time-consuming and tedious as it requires the analyst to perform a vast number of experiments and may lead to excessive use of reagents and materials (Bas and Boyaci 2007; Khamanga and Walker 2011). DoE is a valuable statistical tool that could be used to overcome these limitations during optimization studies. In these studies, a central composite design (CCD) approach for evaluating quadratic response surfaces (Bas and Boyaci 2007) was used to describe the interactions between the various experimental variables that were evaluated.

The CCD approach uses a second order design that consists of a complete or fractional 2^k factorial design with the factor levels coded as either +1 or -1 and axial portions consisting of $2k$ points at a distance viz., á from the design centre point with n_o centre points included (Andre and Mukhopadhyay 2010). Different authors have applied a DoE approach to the development of HPLC methods for the analysis of active pharmaceutical ingredients (API) in dosage forms and for the determination of the purity of compounds including natural products (Srinubabu et al. 2006, 2007; Barmpalexis et al. 2009; Fauzee and Walker 2013). The mathematical model that is associated with optimization of factors using a CCD approach is depicted in Eq. (1).

$$Y = (\beta_0 + \varepsilon) + \sum_{i=1}^{n} \beta iXi + \sum_{i=1}^{n} \beta iiXi2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta ijXiXj \qquad (1)$$

where,

Y = the measured experimental response,
n = the number of factors,
X = the factor under to be examined, and
βo, βi, βj = coefficients for the main or interactive effects (Khamanga and Walker 2011; Barmapalexis et al. 2009).

The objective of these studies was to develop a stability-indicating HPLC-ECD method for the quantitative analysis of EFV in pharmaceutical formulations and to monitor EFV release during *in vitro* dissolution studies. The method was developed and then validated according to International Conference on Harmonisation (ICH) guidelines (ICH 2006). To our knowledge this is the first report of the use of experimental design for the development of a simple, rapid, sensitive and reliable method for the quantitation of EFV in pharmaceutical dosage forms using HPLC with an amperometric ECD. Furthermore, to our knowledge this is the first study to use CCD to optimize the method using ACN content, buffer molarity, buffer pH and voltage.

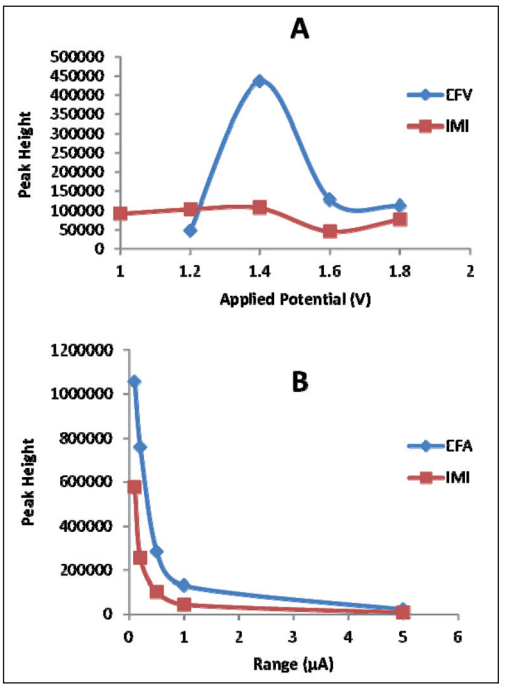

Fig. 2: (A) HDV for EFV and IMI generated in DC mode and (B) scan for background current

2. Investigations, results and discussion

2.1. Method development

It was essential to conduct preliminary hydrodynamic voltammetric (HDV) studies to establish an appropriate applied oxidising potential and analytical current suitable for the detection of EFV in solution. The HDV of EFV and imipramine (IMI) generated in direct current (DC) mode at potentials ranging between +1000 mV and +1800 mV (A) at a scan background current ranging between 0.5-5.0 µA (B) is depicted in Fig. 2.

These data reveal that the maximum oxidising potential for EFV was achieved at +1400 mV that corresponds to oxidation of the secondary amine and electro-active site (Dogan-Topal et al. 2009) as shown in Fig. 1. The limiting current for EFV and IMI plateaued at 1.0 µA. The optimal detector potential is selected following optimization of the chromatographic conditions due to the fact that the electrochemical behaviour of an analyte is also dependent on conditions such as the composition and pH of the mobile phase (ESA 2012). Consequently, an analytical potential ranging between +1300 mV and +1500 mV was included in the CCD design in order to establish the optimum chromatographic conditions for this analysis. However the plateau background current of 1.0 µA observed in these studies was selected and used in all subsequent experiments for the separation and detection of EFV (70 µg/mL) and IMI (5 µg/mL). An additional consideration for aqueous solutions of EFV is that the reported pH of maximum stability is between 4-5 (Maurin et al. 2002). Consequently, a buffer of pH 4 was selected as the appropriate pH for commencing these studies.

2.2. Method optimization

All experiments for optimization studies were performed randomly as generated by Design Expert® statistical software in order to eliminate possible experimental bias. The selected design summary is described by four quadratic models and the mathematical models for the four independent factors were postulated. Optimization of the significant model variables was then undertaken to generate the best response. The influence of four factors *viz*., buffer molarity, buffer pH, ACN content and detector voltage in addition to any interaction with respect to retention time, peak symmetry and peak resolution were described using one factor graphs and two dimensional contour plots.

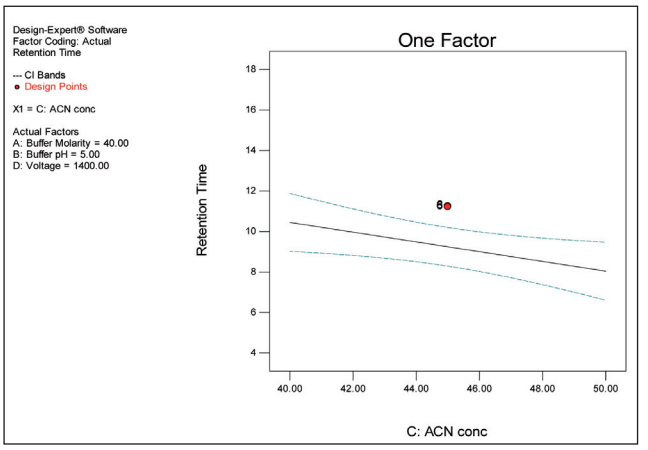

Fig. 3: One factor plot depicting the impact of ACN content on retention time

2.2.1. *Effect on retention time*

The retention time (Y_1) was considered the singular critical response in terms of establishing an appropriate analytical run time for all future experiments. CCD analysis revealed that the content of ACN in the mobile phase was the most significant input variable to affect the retention of EFV. The influence of ACN content on the retention time of EFV is depicted in Fig. 3.

These data reveal that a decrease in the retention time of EFV occurs when the content of ACN is increased from 40-50% v/v. ACN has an intermediate dielectric constant and an increase in the ACN content in a mobile phase decreases the polarity of the mobile phase (Moreau and Douheret 1976) that leads to preferential partitioning of EFV into the mobile phase due to the hydrophobic nature of EFV thereby facilitating rapid elution and short retention times. ANOVA was used to determine if the quadratic model for retention time was significant and the Fisher F-ratio was calculated. This ratio was used to determine the significant terms with an error estimate of p = 0.05. Values of "Prob > F" < 0.0500 indicate that the model terms are significant and conversely values > 0.1000 indicate the model terms are not significant. The overall contribution of the model factors to retention time was not statistically significant since Prob > F is > 0.1 and the data is summarized in Table 1.

The "Model F-value" of 0.89 implies the model is not significant relative to the noise. There is a 58.25% chance that this "Model F-value" could be due to noise. The "Lack of Fit F-value" of 27764.19 implies that the lack of fit is significant. The significant lack of fit is due to many insignificant model terms and is usually undesirable. Therefore, it was essential to perform model reduction through a background elimination procedure with the primary aim of improving the model fit. This meant that insignificant model terms were reduced resulting in a significant model as shown in Table 2.

The data summarised in Table 2 reveal that the Model F-value is 5.34 implying that the model is significant with only a 2.85% probability that the "Model F-Value" could be a consequence of noise. The backward method is considered a robust choice for algorithmic model reduction since all model terms are considered for inclusion in the model (NIST/SEMATECH 2012). Forward elimination and stepwise selection procedures commence with a minimal core model and therefore some terms are never included (NIST/SEMATECH 2012). The "Adeq Precision" measures the signal to noise ratio, and a ratio > 4 is desirable. Therefore although, the lack of fit was significant following model reduction (Table 2) the ratio of 7.306 indicates an adequate signal for these data. Consequently, this model was used to navigate the design space and the method that was developed was used to predict the retention time of EFV within the limits of the design space. The equation for Y_1 (retention time) is shown in Eq. (2).

$$Y_1 = 9.25 - 1.20C_3 \quad (2)$$

The normal probability plot of residuals for retention time is depicted in Fig. 4 and reveals that the points generally fall on a

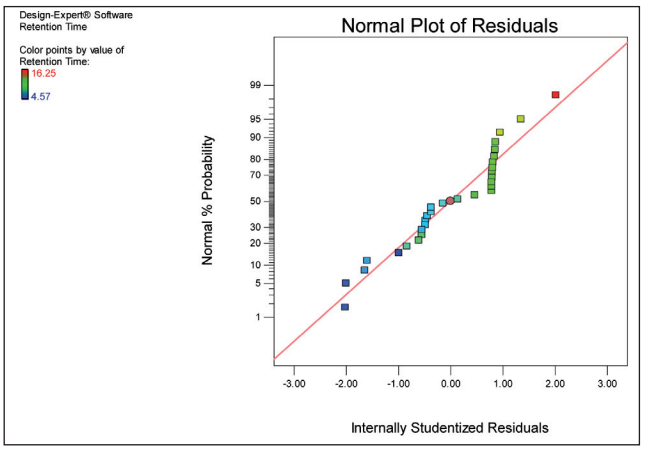

Fig. 4: Normal plot of residuals for retention time of EFV

Table 1: ANOVA table for response surface quadratic model for retention time

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	98.59	14	7.04	0.89	0.5825	**Not significant**
A-Buffer Molarity	1.23	1	1.23	0.16	0.6983	
B-Buffer pH	7.59	1	7.59	0.96	0.3424	
C-ACN conc.	34.75	1	34.75	4.40	0.0533	
D-Voltage	0.066	1	0.066	8.325E-003	0.9283	
AB	0.30	1	0.30	0.038	0.8488	
AC	5.50	1	5.50	0.69	0.4171	
AD	2.28	1	2.28	0.29	0.5989	
BC	12.78	1	12.78	1.61	0.2227	
BD	0.40	1	0.40	0.050	0.8256	
CD	1.66	1	1.66	0.21	0.6528	
A^2	12.87	1	12.87	1.63	0.2212	
B^2	4.87	1	4.87	0.62	0.4446	
C^2	17.77	1	17.77	2.25	0.1543	
D^2	8.76	1	8.76	1.11	0.3090	
Residual	118.46	15	7.90			
Lack of fit	118.46	10	11.85	27764.19	< 0.0001	Significant
Pure Error	2.133E-003	5	4.267E-004			
Cor Total	217.05	29				
Std. Dev.	2.81	R^2	0.4542			
Mean	9.25	Adj R^2	-0.0552			
C.V. %	30.44	Pred R^2	-2.1437			
Press	682.34	Adeq. Precision	3.859			

Table 2: ANOVA table for response surface reduced linear model for retention time

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	34.75	1	34.75	5.34	0.0285	Significant
C-ACN conc	34.75	1	34.75	5.34	0.0285	
Residual	182.30	28	6.51			
Lack of fit	182.30	23	7.93	18576.27	< 0.0001	Significant
Pure error	2.133E-003	5	4.267E-004			
Cor Total	217.05	29				
Std.Dev.	2.55	R2	0.1601			
Mean	9.25	Adj R^2	0.1301			
C.V. %	27.60	Pred R^2	-0.0048			
PRESS	218.09	Adeq. Precision	7.306			

straight line suggesting a more or less normal distribution of errors thereby confirming an adequate fit of the data to the model.
The Box-Cox plot for power transformations is shown in Fig. 5. This plot is used when data transformation is required to increase the applicability and usefulness of an applied statistical test. The blue line points the current transformation and in this case, the observed value is 1 indicating that no transformation is required. The red lines indicate the 95% confidence interval and in this case the 95% confidence interval includes 1 implying that the data are approximately in the best possible and optimum region of parabola. This ultimately confirms that no transformation of the model data is necessary and implies the normality of distribution of these data.

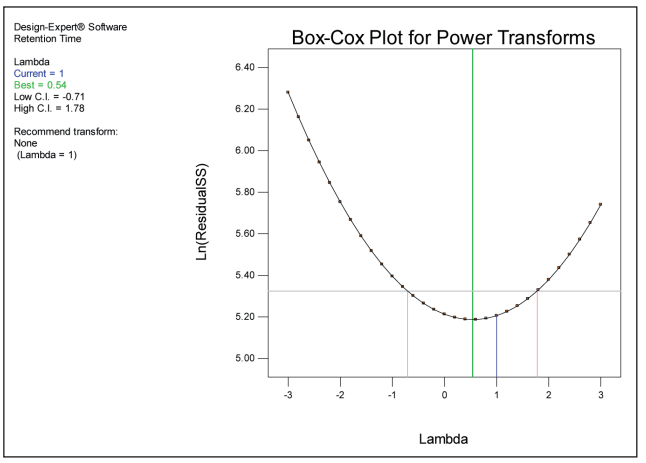

Fig. 5: Box–Cox plot for power transformation for retention time of EFV

2.2.2. *Peak symmetry*

The peak symmetry response (Y_2) was evaluated using the peak asymmetry factor (As). Excellent columns produce an As value of between 0.95 and 1.1, albeit values of < 1.5 are also acceptable (Snyder et al.1997). The peak symmetry or the peak asymmetry factor was established using Eq. (3).

$$A_s = \frac{b}{a} \tag{3}$$

where,

As = peak asymmetry factor,
b = distance from the point at peak midpoint to the tailing edge (measured at 10% of peak height), and
a = distance from the leading edge of peak to the midpoint (measured at 10% of peak height)

ANOVA analysis reveals that the model for peak symmetry was not significant (p=0.0852) and consequently model reduction was performed using background elimination. The significant model terms established in the significant model (p = 0.0005) were ACN content (p= 0.0256) and analytical potential (p= 0.0256) and affected peak symmetry for EFV. The combination of ACN content and voltage is therefore influential in the response of peak symmetry (p=0.0032). These factors can be manipulated to improve peak symmetry for EFV in these analyses. The synergistic effect of ACN content and voltage on peak symmetry is depicted in Fig. 6.

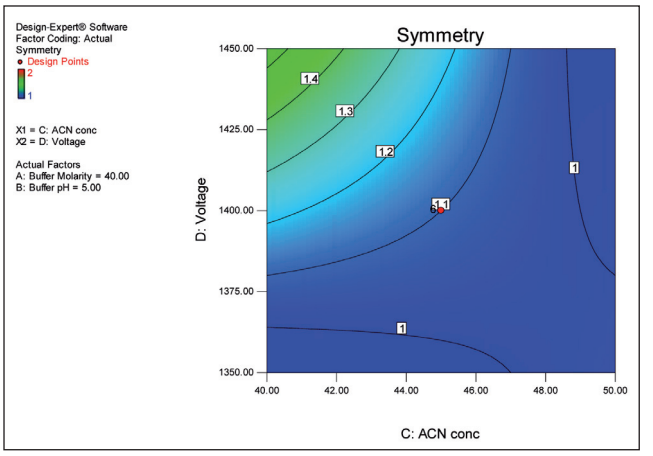

Fig. 6: Contour plot depicting the impact of ACN content and applied voltage

It can be seen from the contour plot that peak symmetry can be improved to achieve acceptable peak symmetry by manipulating ACN content and analytical potential. An approximate combination of 40-47 % v/v ACN content and an analytical potential of between +1375 mV and +1450 mV results in a region of acceptable values of peak symmetry. Peak symmetry of 1.1 with a % RSD of 0.18 was observed for the experimental run using the optimized chromatographic conditions and this was considered adequate for peak symmetry for this analytical method for EFV. The symmetry was therefore considered appropriate for the method that was developed and the equation for Y_2 peak symmetry response is shown in Eq. (4).

$$Y_2 = +1.00 - 0.042A + 0.042B - 0.13C + 0.12D + 0.063AB + 0.063AC - 0.062AD - 0.062BC + 0.063BD - 0.19CD + 0.031A^2 + 0.031B^2 + 0.031C^2 + 0.031D^2 \tag{4}$$

2.2.3. *Effect on peak resolution*

The peak resolution was also monitored as an experimental response in these studies since the resolution between the analyte of interest and internal standard is vital in order to ensure that accurate quantitation of the two compounds of interest is

achieved. The quadratic model was not significant (p=0.4265) and an F-value of 1.10 was generated. The "Model F-value" of 1.10 implies that the model is not significant relative to the noise associated with the analysis. Consequently there is a 42.65% chance that the "Model F-value" could be due to noise. The ratio of the maximum to the minimum resolution values was 23.67 and a ratio > 10 indicates that transformation of the data is required so as to meet the assumptions that make the ANOVA valid. In this case inverse transformation was used to transform the data and a significant (p=0.0412) two factor interaction (2FI) model was observed. Buffer pH was a significant model term with a p-value of 0.0233 and combination factors of buffer pH and ACN content in addition to ACN content and voltage were established as significant model terms with p-values of 0.0179 and 0.0371, respectively. Two dimensional contour plots of the combination factors influencing peak resolution are depicted in Figs. 7 and 8.

The data depicted in Fig. 7 reveal that adequate peak resolution is obtained with a mobile phase containing a buffer pH < 5 and any amount of ACN content in mobile phase. The chemical stability of EFV is influenced by pH, the consequence of which is that a change in pH may lead to a change in the migration mobility of EFV and hence resolution of the analysis. Analyte resolution that was considered acceptable in these studies were generated by a combination of ACN content of approximately 44-50% v/v and a detector potential ranging between +1380 mV and +1450 mV as depicted in Fig. 8. Peak resolution for the experimental run for the optimized chromatographic conditions was calculated using Eq. (5).

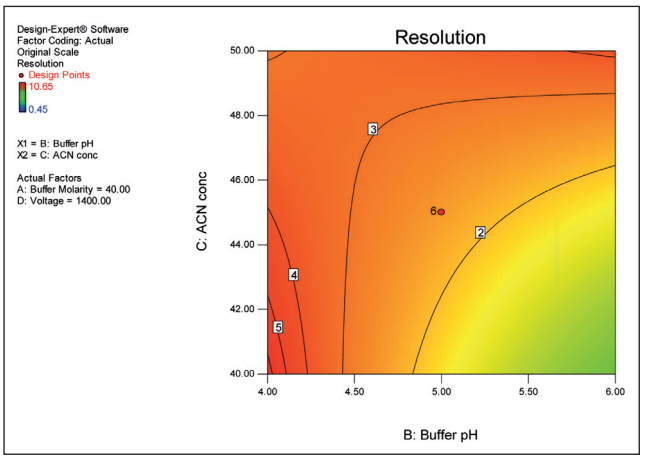

Fig. 7: Contour plot depicting the impact of buffer pH and ACN content on peak resolution

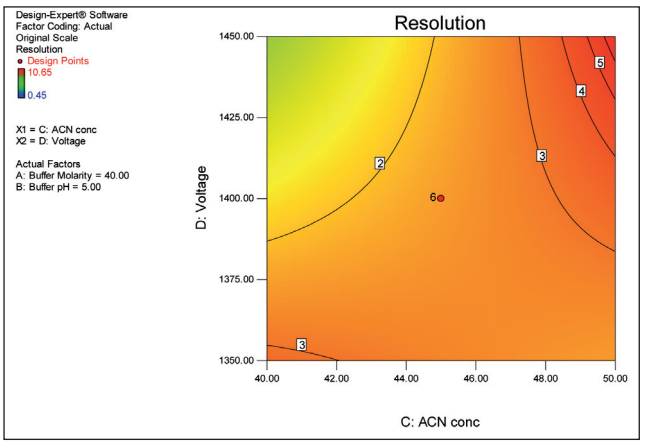

Fig. 8: Contour plot depicting the impact ACN content and applied voltage on peak resolution

$$R_S = \frac{t_2 - t_1}{0.5\,(W_1 + W_2)} \quad (5)$$

where,

R_s = peak resolution,
t_2 = Retention time for second eluting peak,
t_1 = Retention time for first eluting peak,
W_1 = Width of first eluting peak at the base, and
W_2 = Width of second eluting peak at the base.

FDA recommends a resolution factor of > 2 for the separation of two compounds (Dong et al. 2012; FDA 2011). The resolution between EFV and IMI was 3.52 with a % RSD of 0.59 suggesting a more than adequate resolution between these two compounds suggesting the separation was suitable for these and future studies. The equation for Y_3 (peak resolution) response is shown in Eq. (6).

$$\frac{1}{Y_S} = +0.43 + 5.086E - 003A + 0.18B - 0.14C + 0.059D + 0.021AB - 0.11AC + 0.084BC + 0.063BD - 0.20CD \quad (6)$$

2.2.4. *Optimized chromatographic conditions*

The overall solutions for the chromatographic analysis of EFV with IMI were distilled through optimization of the aforementioned models using Design Expert statistical software. The optimized conditions for the overall response, Y are listed in Table 3.

Table 3: Optimized chromatographic conditions for the overall separation of EFV and IMI

Parameter	Results
Buffer molarity	30 mM
Buffer pH	4.5
Acetonitrile content	45 % v/v
Applied voltage	+1400 nV

The optimized chromatographic conditions that were generated were applied to the quantitative analysis of EFV and produced sharp and well resolved peaks for IMI and EFV with retention times (Rt) of 3.70 and 8.89 min, respectively. The Rt were considered suitable

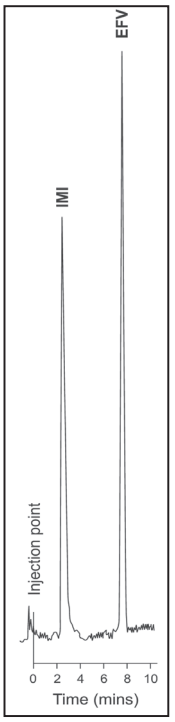

Fig. 9: Typical chromatogram depicting the separation of an EFV (8.9 min) and IMI (3.70 min) solution

for regular analyses since the total run time for sample analysis was limited to 10 min suggesting that the development of a rapid method for EFV analysis had been achieved. The % RSD for the retention time of EFV using the optimized conditions in relation to the predicted retention time was 2.54% and was considered adequate as it was less than the laboratory set % RSD of 5%. A typical chromatogram of the separation of EFV and IMI achieved using the optimized chromatographic conditions is depicted in Fig. 9.

2.3. Method validation

2.3.1. Linearity and range

The linearity of the method was evaluated over the concentration range of 5-70 µg/mL and least squares linear regression analysis of the peak height ratio *versus* concentration data was used to test the linearity of the method. The calibration curve was found to be linear with a $R^2 = 0.9979$, a slope of 0.0151 and a y-intercept of 0.0163, yielding a regression equation of
$y = 0.0151x + 0.0163$. The correlation coefficient is normally used to assess linearity of a method and a value of ≥ 0.99 is usually considered sufficient to deem linearity of the data across the concentration range (Causey et al. 1990). Consequently, the HPLC-ECD method was found to be linear over a relatively wide concentration range for EFV.

2.3.2. Precision

The precision of the method was evaluated at two different levels *viz.,* repeatability (intra-day precision) and intermediate precision (inter-day precision). The intra-day precision and inter-day precision data for the analysis of EFV at three different concentrations (n = 6) are summarized in Table 4. These data reveal that in all cases the % RSD values were < 5%, indicating that the HPLC-ECD analytical method is precise and can be used as intended.

2.3.3. Accuracy

The accuracy of the HPLC-ECD method was determined by replicate analysis of samples containing known amounts of EFV. A summary of the data generated in these studies is listed in Table 5. The largest value generated for % Bias was + 4.11%, which indicates that no value deviated by more than 5% from the expected value. In addition, the resultant % RSD values for all analyses were < 5%, indicating that the HPLC-ECD analytical method is accurate and suitable for its intended purpose.

2.3.4. LOQ and LOD

The LOQ of the method was determined by evaluating the lowest concentration of EFV that resulted in a precision of < 5% RSD, and the LOD was taken as 0.3 x LOQ value (ICH 2006). Consequently, the LOQ in these studies was 5.0 µg/mL (% RSD = 4.29) and by convention, the LOD value was taken as 1.5 µg/mL, which, when injected onto the HPLC system results in a detectable but not quantifiable peak.

2.3.5. Specificity

The specificity of the HPLC-ECD method was evaluated by subjecting EFV to different stress conditions. The forced degradation studies were also intended to ensure that the analytical method was stability-indicating. Data obtained from these studies are summarized in Table 6.

Table 4: Intra- and inter-day precision data for EFV analysis

Intra-day precission									
Concentration (µg/mL)				**Calculated concentration Mean ± SD**			**% RSD**		
10.00				9.86 ± 0.26			4.34		
30.00				29.67 ± 0.89			3.37		
50.00				48.54 ± 1.01			3.99		
Inter-day precision									
Quality control									
	Day 1			**Day 2**			**Day 3**		
Theoretical concentration (µg/mL)	10.00	30.00	50.00	10.00	30.00	50.00	10.00	30.00	50.00
Actual concentration (µg/mL)	10.23	29.87	48.95	9.88	30.12	50.04	9.96	29.97	51.03
% RSD	4.24	2.79	1.86	4.26	1.67	1.31	3.58	2.29	1.35

Acceptable degradation levels of EFV for degradation studies was set at ≤ 5 % degradation of EFV following 8 h exposure to each stress condition. It is clearly evident that when exposed to basic and oxidative stress conditions, EFV undergoes extensive degradation. Base hydrolysis has been reported to be the major degradation pathway of EFV solutions due to the presence of a cyclic carbamate functional group (Gadkari et al. 2010). Hydrogen peroxide is also thought to oxidise EFV, the consequence of which is the impairment of the ability of the drug to undergo electrochemical oxidation during HPLC-ECD analysis resulting in no detection of the analyte of interest. EFV exhibited limited degradation in solution at 80 °C and was considered stable in dry heat. The extent of degradation was close to the limit set but was considered acceptable since EFV

Table 5: Accuracy results for blinded EFV samples

Theoretical concentration (µg/mL)	Actual concentration (µg/mL)	% RSD	% Bias
7.50	7.80 ± 0.039	3.08	+4.11
33.50	32.78 ± 0.339	2.91	-2.15
65.50	64.94 ± 0.464	2.32	-0.85

Table 6: Forced degradation data of EFV following exposure to various stress conditions

STRESS CONDITION	% RECOVERED	REMARKS
Acid hydrolysis	99.58	No degradation
Base hydrolysis	0.00	Degradation
Neutral hydrolysis	99.50	No degradation
Thermal degradation (80°C)	94.39	Degradation
UV	64.78	Degradation
Dry Heat	98.64	No degradation
Oxidation	0.00	Degradation

is unlikely to be exposed to the harsh conditions used for this study during routine analysis. Exposure of EFV to UV light resulted in approximately 35% degradation of EFV, which confirms data that have been published previously (Hamrapurka et al. 2010). However UV-induced degradation does not result in the loss of oxidising potential for EFV implying that the compound may be detected in the presence of degradation products. EFV is stable when exposed to acidic and neutral conditions and is thermostable. The analysis of EFV in commercially available tablets produced clear and sharp peaks without interference from excipients used to manufacture the product. These studies indicate that the HPLC-ECD method is specific for EFV and is stability-indicating.

2.3.6. Stability of the analyte

The stability of the analyte in mobile phase was tested over a 3-day period, following storage at 4 °C, 22 °C and in an autosampler. These studies reveal that the concentration of EFV remained constant over the three days of the study and analysis under the stated conditions with % RSD value of < 0.17 %. Consequently, EFV was considered stable in the mobile phase and the HPLC-ECD was suitable for quantitative analysis of EFV. Samples for analysis could be stored under the stated conditions and analysis could take place over at least three consecutive days.

2.4. Assay

The HPLC-ECD method was applied to the analysis of EFV in commercially available dosage forms, viz., Stocrin® 600 mg tablets. These studies revealed that the tablets contained on average 95.4±3.24 % of the label claim with a precision of 3.40 % RSD. These data fall within the limits of 92-110 % as specified in the USP (USP 2009). Therefore, the HPLC-ECD method is suitable for the quantitative analysis of EFV in pharmaceutical formulations.

A simple and rapid stability-indicating HPLC-ECD has been developed using a DoE approach. The method has been validated for quantitative determination of EFV in pharmaceutical products. The method is linear over a relatively wide range of EFV concentration and is sensitive for the intended purpose. Furthermore the method is selective, precise and accurate. The major advantage of the HPLC-ECD method over previously reported stability-indicating HPLC-UV methods is that the former allows for the quantitative analysis of EFV in pharmaceutical formulations without running the risk of exposing the photolabile molecule to UV radiation that is more than likely to cause the decomposition of the drug during routine analysis. The HPLC-ECD method is also specific to degradation products therefore not quantitating EFV that has lost the oxidative functionality that would be detected using HPLC-UV.

3. Experimental

3.1. Instrumentation

The modular HPLC system consisted of a Model 510 Waters solvent delivery module (Waters Associates, Milford, MA, USA), a Waters WISP 710 B autosampler (Waters Associates, Milford, MA, USA) and a Model 464 pulsed amperometric electrochemical detector (Waters Associates, Milford, MA, USA) operated in the DC mode. The operating potential and current were set at +1400 mV and 1 µA, respectively. The initial separation of IMI and EFV was achieved using isocratic conditions with a Nova-Pak C_{18}, 4 µm (150 mm x 3.9 mm i.d.) cartridge column (Waters Corporation, Milford, MA, USA) and 30 mM phosphate buffer (pH 4.5) and ACN in a 55:45 v/v ratio. The flow rate and the injection volume were 1.0 mL/min and 20 µL, respectively. The analytical column was maintained at 30 °C using a Model LC-22 temperature controller (Bioanalytical Systems, West Lafayette, IN, USA) and data acquisition was achieved using a Model SP Thermo Separations Data Jet Integrator (Spectra Physics, San Jose, CA, USA) set at an attenuation of 128, an offset of 0 and a chart speed of 0.25 cm/min.

3.2. Chemicals and reagents

All chemicals and reagents that were used were at least of analytical reagent grade. EFV was donated by Adcock Ingram Limited (Johannesburg, Gauteng, South Africa) and the internal standard (IS), imipramine (IMI) was donated by Aspen Pharmacare (Port Elizabeth, Eastern Cape, South Africa). HPLC far UV-grade acetonitrile (ACN) was purchased from Microsep® (Pty) Ltd (Port Elizabeth, Eastern Cape, South Africa). A Milli-Q Academic A-10 water purification system (Millipore, Bedford, MA, USA) consisting of an Ionex® Ion-Exchange cartridge and a Quantum Ex-Ultrapore Organex cartridge fitted with a 0.22 µm Millipak 40 sterile filters (Millipore, Bedford, MA, USA) was used to produce HPLC-grade water. Potassium dihydrogen orthophosphate, *o*-phosphoric acid (85% v/v) and sodium hydroxide pellets were purchased from Merck Laboratories (Wadeville, Gauteng, South Africa).

Table 7: Randomised coded experimental runs for CCD

		Factor 1	Factor 2	Factor 3	Factor 4
Std	Run	A:Buffer Molarity (mM)	B:Buffer pH	C:ACN conc (% v/v)	D:Voltage (mV)
6	1	1	-1	1	-1
9	2	-1	-1	-1	1
11	3	-1	1	-1	1
29	4	0	0	0	0
7	5	-1	1	1	-1
25	6	0	0	0	0
16	7	1	1	1	1
19	8	0	-2	0	0
21	9	0	0	-2	0
22	10	0	0	2	0
3	11	-1	1	-1	-1
2	12	1	-1	-1	-1
12	13	1	1	-1	1
30	14	0	0	0	0
24	15	0	0	0	2
14	16	1	-1	1	1
13	17	-1	-1	1	1
20	18	0	2	0	0
4	19	1	1	-1	-1
15	20	-1	1	1	1
1	21	-1	-1	-1	-1
8	22	1	1	1	-1
23	23	0	0	0	-2
27	24	0	0	0	0
26	25	0	0	0	0
18	26	2	0	0	0
28	27	0	0	0	0
5	28	-1	-1	1	-1
17	29	-2	0	0	0
10	30	1	-1	-1	1

3.3. Preparation of stock solutions and calibration standards

Standard stock solutions of EFV (140 µg/mL) and IMI (100 µg/mL) were prepared by accurately weighing approximately 14 mg and 10 mg of each compound and transferring into a 100 mL A-grade volumetric flask and making up to volume with mobile phase. The calibration standards for EFV over the concentration ranging between 5 and 70 µg/mL were prepared by serial dilution of the standard stock solution on the day of analysis using mobile phase as the solvent. An aliquot of the IMI stock solution equivalent to a concentration of 5 µg/mL was added to each of the calibration standards. The stock solutions were protected from light using aluminium foil and were stored in a refrigerator at 4 °C prior to use. All solutions were used within three days of preparation.

3.4. Preparation of buffers and mobile phase

A 30 mM phosphate buffer solution of was prepared by accurately weighing 4.08 g of potassium dihydrogen orthophosphate into a 1000 mL A-grade volumetric flask and making up to volume with HPLC grade water. The pH of the buffer was adjusted to 4.5 using *o*-phosphoric acid and was measured using a Model GLP 21 Crison pH-meter (Crison Instruments, Barcelona, Spain) at 25 °C. The mobile phase was prepared by mixing the appropriate volumes of buffer and HPLC-grade acetonitrile that were then added together and degassed by passing through a 0.45 µm Durapore HVLP membrane filters (Millipore Corporation, Bedford, MA, USA) under vacuum using an Eyela Aspirator A-2S degasser (Rikakikai Co.,LTD, Tokyo, Japan) prior to use.

3.5. Method development

A rotatable CCD design consisting of 30 experiments with 6 centre points and 24 non-centre points was generated and is depicted in Table 7 with the corresponding experimental levels that were investigated listed in Table 8. Briefly, the minimum and maximum values of the buffer molarity were maintained at 30 mM and 50 mM, respectively with the lower and upper axial points kept at 20 mM and 60mM, respectively. Similarly, pH 4 and pH 6 were the minimum and maximum value of the buffer pH, respectively with the lower and upper axial values maintained at a pH of 3 and 7, respectively. The concentration of ACN in the mobile phase was kept at either a minimum and maximum value of 40 % v/v or 50 % v/v, respectively with the lower and upper axial levels maintained at 35 % v/v and 55 % v/v, respectively. The voltage of the detector was set at a minimum of +1350 mV or a maximum of +1450 mV with the lower and upper axial levels set at either +1300 mV or +1500 mV, respectively. The independent input variables and ranges were selected on the basis of preliminary studies of the chromatographic separation for EFV and IMI. The retention time of the last peak to elute, peak symmetry and peak resolution were monitored as the dependent or output responses.

Table 8: Values for input variables for the experimental design.

Variable	Level			
	-α	**-1**	**1**	+α
Buffer molarity (mM)	20	30	50	60
Mobile phase pH	3	4	6	7
Organic solvent (%)	35	40	50	55
Applied voltage (mV)	1300	1350	1450	1500

3.6. Data analysis

The data generated from the experiments and the responses that were monitored were analysed using Design Expert Version 8.0.2 statistical software (Stat-Ease Inc., Minneapolis, MN, USA). The Fisher's test for Analysis of Variance (ANOVA) was used to establish whether significant differences existed between the mean values for the different factors that were investigated.

3.7. Method validation

3.7.1. Calibration, linearity and range

The calibration curve for EFV was constructed by plotting the peak height ratio of EFV to IMI *vs.* EFV concentration over the concentration range 5-70 µg/mL. The linearity of the method was subsequently evaluated by performing the least squares linear regression analysis of the constructed calibration curve and reported in terms of the correlation coefficient (R^2) and the equation for the linear regression curve (Green 1996; Shabir 2003).

3.7.2. Precision

The repeatability and intermediate precision (Hokanson 1994) of the method was established by calculating the coefficient of variation (% RSD) of the peak height ratio of EFV to IMI following repeated measurement (n =6) of three calibration standards of concentrations that represented low, medium and high concentrations of EFV. The analysis was performed on a single day to evaluate the repeatability of the method and on three consecutive days to establish the intermediate precision of the method (Green 1996; Shabir 2003). The acceptance criterion for repeatability and intermediate precision was set at ≤ 5 % RSD for each of the concentrations investigated.

3.7.3. Accuracy

The accuracy (Hokanson 1994) of the method was established following replicate analysis (n =6) of three known concentrations of EFV representing low (7.50 µg/mL), medium (33.5 µg/mL) and high (65.50 µg/mL) concentrations that were prepared in mobile phase. The accuracy of the method was reported as the % recovery, % RSD and % bias. The acceptance criteria for accuracy were a mean % recovery and % RSD of 100 ± 5% and ≤ 5 % RSD, respectively at each concentration level investigated. The percent bias was required to be < 5% at each level. Adherence to each criterion was necessary to infer the accuracy of the method.

3.7.4. Limits of quantitation (LOQ) and detection (LOD)

The LOQ and LOD (Green 1996; Hokanson 1994) were established using a statistical coefficient of variation or % RSD approach. The LOQ was determined by evaluating the lowest concentration of EFV that resulted in a precision that did not exceed 5% and the LOD was then established by convention and reported as a concentration that was 30% lower than the value for LOQ.

3.7.5. Specificity and forced degradation or stress studies

Forced degradation or stress studies (Ngwa 2010; Bakshi and Singh 2002) were undertaken in order to establish whether the analytical method that was developed was stability-indicating and specific (Hokanson 1994). All degradation studies with the exception of studies using dry heat were performed in solution with an initial EFV concentration of 100 µg/mL. It has been suggested that stress testing studies are only valid when a drug substance is forced to degrade to a level that is between 5 - 20% of the initial concentration of the analyte under investigation (Ngwa 2010). Therefore a tolerance level of 5% was used to determine whether EFV had degraded under conditions studied.

3.7.5.1. Heat-induced hydrolysis

A 100 µg/mL solution of EFV was heated in increments of 10 °C in the range 50-80 °C as suggested in the ICH guidelines (ICH 2005). The solutions were heated in a Model NB-34980 Colora Ultra-Thermostat water bath (Colora, Lorch, Germany) and the solution was maintained at each temperature for 8 h, allowed to cool down to room temperature (22 °C) and then analysed by HPLC.

3.7.5.2. Acid and alkali-induced hydrolysis

Acid-induced stress degradation was carried out by adding 50 mL of 0.1 M hydrochloric acid to 50 mL of 100 µg/mL EFV solution and refluxing the mixture at 70 °C for 8 h (Bakshi and Singh 2002). The mixture was left to reach room temperature (22 °C) prior to analysis by HPLC. Base-induced stress degradation was performed by adding 50 mL of 0.1 M sodium hydroxide to 50 mL of 100 µg/mL EFV solution and refluxing the mixture at 70 °C for 8 h (Bakshi and Singh 2002). The mixture was allowed to reach room temperature (22 °C) prior to analysis by HPLC.

3.7.5.3. Neutral-induced hydrolysis

In these studies, 50 mL of HPLC-grade water was added to 50 mL of 100 µg/mL EFV solution. The mixture was refluxed at 70 °C under reflux for 8 hours (Bakshi and Singh 2002) with subsequent cooling to ambient temperature (22 °C) prior to analysis.

3.7.5.4. Oxidative degradation

To study the effect of oxidation on EFV, 50 mL solution of 3 % hydrogen peroxide was added to 50 mL of 100 µg/mL EFV and the mixture was refluxed at 70 °C for 8 hours (Bakshi and Singh 2002). The solution was then allowed to reach room temperature (22 °C) prior to analysis.

3.7.5.5. Photolytic degradation

Photolytic degradation studies were performed by exposing a 100 µg/mL solution of EFV to 500 W/m^2 for 8 hours in a Suntest CPS+ photostability chamber (ATLAS® Material Testing Solutions, Chicago, IL, USA) (Bakshi and Singh 2002) prior to analysis by HPLC.

3.7.5.6. Dry heat studies

The influence of dry heat on EFV was investigated by exposing EFV powder to a temperature of 97 °C for 8 h in a Gallenkamp drying cabinet (Weiss Technik, Loughborough, LE, UK). The heated drug powder was allowed to cool to ambient (22 °C) temperature and used to prepare a 100 µg/mL solution of EFV solution prior to analysis to establish the true concentration of EFV using HPLC.

3.7.6. Stability of EFV

The stability of EFV in solution alone or in combination with IMI was evaluated in mobile phase by analysing solutions that were maintained at 4 °C, ambient temperature (22 °C) and in the auto sampler for a period of 3 days. A tolerance level of 1% RSD of the initial concentration of EFV was considered acceptable in order to infer stability of EFV.

3.8. Assay of Stocrin® 600 mg tablets

The method that was developed and validation was applied to the analysis of EFV in commercially available tablets, viz., Stocrin® 600 mg tablets. Twenty tablets were crushed in a mortar using a pestle and an aliquot of powder equivalent to the weight of one tablet was then quantitatively transferred to a 100 mL A-grade volumetric flask. Approximately 80 mL of the mobile phase was then added to the volumetric flask and the mixture was sonicated with regular shaking until complete dissolution of the powders had been observed (USP 2009). The solution was allowed to cool to room temperature (22 °C) prior to making up to volume with mobile phase. An aliquot of the resultant mixture was centrifuged at 500 rpm for 10 minutes (USP 2009), filtered through a 0.45 µm Millipore Millex-HV Hydrophilic PVDF filter membrane (Millipore Co., Bedford, MA, USA) and analysis using HPLC.

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References

Andre IK, Mukhopadhyay S (2010) Response surface methodology. WIREs Comp Stat 2:128-149.

Bakshi M, Singh S (2002) Development of validated stability-indicating assay methods-critical review. J Pharm Biomed Anal 28:1011-1040.

Barmpalexis P, Kanaze FI, Georgarakis E (2009) Developing and optimizing a validated isocratic reversed-phase high-performance liquid chromatography separation of nimodipine and impurities in tablets using experimental design methodology. J Pharm Biomed Anal 49: 1192-1202.

Bas D, Boyaci H (2007) Modeling and optimization I: Usability of response surface methodology. J Food Eng 78: 836-845.

Causey AG, Hill HM, Phillips LJJ (1990) Evaluation of criteria for the acceptance of bioanalytical dataPharmaceut. Biomed Anal 8: 625-628.

Cristofoletti R, Nair A, Abrahamsson B, Groot DW, Kopp S, Langguth P, Polli JE, Shah VP, Dressman JB (2013) Biowaiver monographs for immediate release solid oral dosage forms: efavirenz. J Pharm Sci 102:318-329.

Dogan-Topal B, Uslu B, Ozkan SA (2009) Voltammetric studies on the HIV-1 inhibitory drug Efavirenz: the interaction between dsDNA and drug using electrochemical DNA biosensor and adsorptive stripping voltammetric determination on disposable pencil graphite electrode. Biosens Bioelec 24: 2358-2364.

Dong, M., Paul, R., Gershanov, L., Getting the peaks perfect: System suitability for HPLC. Today's Chemist At Work, 2012.

ESA (2012) Hydrodynamic Voltammograms:Generation, Explanation, and Optimization of Applied Potentials, ESA.Inc, 2012.

Fauzee AFB, Walker RB (2013) Forced degradation studies of clobetasol 17-propionate in methanol, propylene glycol, as bulk drug and cream formulations by RP-HPLC. J Sep Sci 36: 849-856.

FDA (2011) Guidance for Industry: Bioanalytical Method Validationl, Food and Drug Administration.

Gadkari T, Chandrachood P, Ruikar A, Tele S, Deshpande N, Salvekar J, Sonawane S (2010) Validated stability indicating LC-PDA-MS method to investigate pH rate profile and degradation kinetics of efavirenz and identification of hydrolysis product by LCMS. Int J Pharm Pharmaceut Sci 2:169-176.

Garcia CV, Breier AR, Steppe M, Schapoval EES, Oppe TP (2002) Determination of dexamethasone acetate in cream by HPLC. J Pharmaceut Biomed Anal 31: 597-600.

Green JM (1996) A practical guide to analytical method validation. Anal Chem 68: 305A-309A.

Hamrapurka PD, Patil PS, Phale M D, Shah N, Pawar SB (2010) Optimization and validation of RP-HPLC stability-indicating method for determination of efavirenz and its degradation products. Int J App Sci Eng 8:155-165.

Hokanson GC (1994) A life cycle approach to the. validation of analytical methods during. pharmaceutical product development. part II: Changes and the need for additional validation. Pharm Tech 18: 92-100.

ICH Q2 (R1) (2006) Harmonised tripartite guideline. Validation of analytical procedures, texts and methodology.

Jiaping L, Jun W, Zongwei C (2008) Nucleoside reverse transcriptase inhibitors and their phosphorylated metabolites in human immuno deficiency virus-infected human matrices J Chromatogr B 868: 1-12.

Khamanga SM, Walker RB (2011) The use of experimental design in the development of an HPLC-ECD method for the analysis of captopril. Talanta 83: 1037-1049.

Langmann P, Schirmer D, Vath T, Zilly M, Klinker H (2001) High-performance liquid chromatographic method for the determination of HIV-1 non-nucleoside reverse transcriptase inhibitor efavirenz in plasma of patients during highly active antiretroviral therapy. J Chromatogr B 755:151-156.

Lemmer P, Schneider S, Schuman M, Omes C, Arendt V, Tayari JC, Fundira L, Wennig R, (2005) Determination of Nevirapine and Efavirenz in Plasma Using GC/MS in Selected Ion Monitoring Mode. Ther Drug Monit 4: 521-525.

Marzolini C, Telenti A, Decosterd LA, Greub G, Biollaz J, Buclin T (2001) Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. AIDS 15: 71-75.

Matthews CZ, Woolf E J, Mazenko RS, Haddix-Wiener H, Chavez-Eng C M, Constanzer M L, Doss G A, Matuszewski B K (2002) Determination of efavirenz, a selective non-nucleoside reverse transcriptase inhibitor, in human plasma using HPLC with post-column photochemical derivatization and fluorescence detection. J Pharm Biomed Anal 28: 925-934.

Maurin MB1, Rowe SM, Blom K, Pierce ME (2002) Kinetics and mechanism of hydrolysis of efavirenz. Pharm Res 19:517-21.

Montgomery ER, Edmanson AL, Cook SC, Hovsepian PK (2001) Development and validation of a reverse-phase HPLC method for analysis of efavirenz and its related substances in the drug substance and in a capsule formulation. J Pharm Biomed Anal 25: 267-284.

Moreau C, Douhéret G (1976) Thermodynamic and physical behaviour of water+acetonitrile mixtures. Dielectric properties. J Chem Thermody 8: 403-410.

Ngwa G (2010) Forced degradation as an integral part of HPLC stability-indicating method development. Drug Del Tech 10: 1-4.

NIST/SEMATECH (2012) e-Handbook of Statistical Methods, Engineering Statistics Handbook, NIST SEMATECH.

Rabel SR, Maurin MB, Rowe SM, Hussain M (1996) Determination of the pKa and pH-solubility behavior of an ionizable cyclic carbamate, (S)-6-chloro-4-(cyclopropylethynyl)-1,4dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one (DMP 266). Pharm Dev Technol 1:91-95.

Rao BU, Nikalje AP (2009) Stability-indicating HPLC method for the determination of Efavirenz in bulk drug and in pharmaceutical dosage form. Afr J Pharm Pharmacol 3: 643-650.

Rebiere H, Mazel B, Civade C, Bonnet PA (2007) Determination of 19 antiretroviral agents in pharmaceuticals or suspected products with two methods using high-performance liquid chromatography. J Chromatogr B 850: 376-383.

Sarasa-Nacenta M, Lopez-Pua Y, Lopez-Cortes LF, Mallolas, J, Gatell JM, Carne X (2001) Determination of efavirenz in human plasma by high-performace liquid chromatography with ultraviolet detection. J Chromatogr B 763: 53-59.

Shabir GA (2003) Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. J Chromatogr A 987: 57-66.

Srinubabu G, Jaganbabu K, Sudharani B, Venugopal K, Girizasankar G, Rao JVLNS (2006) Development and validation of a LC method for the determination of pramipexole using an experimental design. Chromatographia 64: 95-100.

Srinubabu G, Raju C, Sarath N, Kumar PK, Rao JV (2007) Development and validation of a HPLC method for the determination of voriconazole in pharmaceutical formulation using an experimental design. Talanta 71: 1424-1429.

Snyder LR, Kirkland J J, Glajch J L (1997) Practical HPLC Method Development, John Wiley and Sons, Inc, New York.

USP (2009) Efavirenz Monograph, The United States Pharmacopeial Convention.

Veldkamp AI, Van Heeswijk RPG, Meenhorst PL, Mulder JW, Lange JMA, Beijnen JH, Hoetelmans RMW (1999) Quantitative determination of efavirenz (DMP 266), a novel non-nucleoside reverse transcriptase inhibitor, in human plasma using isocratic reversed phase high-performance liquid chromatography with ultraviolet detection. J Chromatogr B 734: 55-61.

Vrouenraets SM, Wit FW, van Tongeren J, Lange JM (2007). Efavirenz: A review. Expert Opin Pharmacother 8:851– 871.

Wang J, Hutchins LD (1995) Thin-layer electrochemical detector with carbon electrode coated with a base-hydrolysed cellulosic film. Anal Chem 57: 1536-1541.

WHO (2005) International Pharmacopoeia Monograph on Efavirenz, World Health Organization. World Health Organization , Geneva, Switzerland.