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

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Abstract

A stability-indicating high performance liquid chromatographic (HPLC) method was developed for the assay of efavirenz, a non-nucleoside reverse transcriptase inhibitor used in the treatment of AIDS. The HPLC method, which is used to determine potency in efavirenz capsules and related substances in efavirenz drug substance and capsules, was validated per ICH guidelines. This method, which uses a cyano column, is capable of separating efavirenz from its *trans*-alkene reduction product. This paper will discuss development and validation of this method, which, to the best of our knowledge, is the first known separation of homologs containing double and triple bonds using reverse-phase HPLC. © 2001 Dupont Pharmaceutical Company. Published by Elsevier Science B.V. All rights reserved.

Keywords: Efavirenz; Capsule formulation; Reverse-phase HPLC; Cyano column; Separation of double/triple bond homologs

1. Introduction

Reversed phase HPLC is the separation method of choice for most pharmaceutical compounds, both hydrophilic and hydrophobic, due to the stable, reproducible nature of the HPLC columns, the largely aqueous composition of the mobile phase, and the relative ease of reproducing the methods in a variety of laboratories. However, some separations have not been achievable on reversed phase systems. Among these are the sep-

aration of compounds whose structure differs by the presence of a double bond vs. a triple bond. Evershed et al. [1] and van Beek and Subrtova [2] reported on the separation of *cis* and *trans* alkenes by making silver complexes and effecting the separation on silica. Andersson, Demirbuker and Blomberg [3,4] achieved separation of alkanes and alkenes using a CN column by supercritical fluid chromatography (SFC) and using silver-complexed alkanes and alkenes by SFC on silica columns.

Careri et al. [5] achieved separation of iron complexes of alkynes using normal phase and

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reversed phase HPLC. However, no corresponding alkene complexes were tested. Careri et al. [6] achieved separation of alkynes by reversed phase HPLC using ruthenium complexes. Mangia et al. [7] reported separation of iron complexes of geometrical isomers via reversed-phase HPLC. Phelan and Miller [8] separated silver complexes of alkene isomers on octadecylsilane columns.

Described herein is separation via reversed-phase HPLC of an alkene–alkyne pair. Design and development of the method, as well as validation, including evaluation of the robustness of the separation, are also discussed.

Efavirenz is a non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV-1 reverse transcriptase. Efavirenz is used in combination with other anti-retroviral agents for the treatment of HIV-1 infection in children and adults. Currently, efavirenz is marketed in 50, 100 and 200 mg strength capsules. The structure of efavirenz is shown in Fig. 1.

2. Experimental

2.1. Method development

2.1.1. Method requirements

A reversed phase method for separation of efavirenz from its known related substances, potential degradation products, and excipients was needed for analysis of up to five strengths of capsules, varying from 50 to 200 mg, as well as for two series of in-process tests during capsule manufacture. The same method also needed to be able to analyze efavirenz drug substance impuri-

Fig. 1. Structure of efavirenz.

ties and purity and drug product potency and degradation products. The method would also be used at drug substance contract manufacturing, drug product manufacturing, and contract testing sites. Therefore, robustness requirements had to be identified during validation so that these method transfers would be successful. To expedite formulation development for this needed therapy, a capsule formulation was selected early in development as the commercial dosage form. While only limited validation to establish potency and safety would be done at phase I, efavirenz was submitted for accelerated approval. Therefore, drug substance and drug product methods had to be finalized and fully validated during phase II.

2.2. Method design

While many of the efavirenz drug substance impurities and degradation products could be separated from efavirenz and each other by reversed phase HPLC, one of the impurities, SR695, differs from efavirenz in that it contains a *trans*-double bond while efavirenz contains a triple bond (Fig. 2). Preliminary separation of this peak pair was achieved on a Zorbax SB-CN column in reversed phase, using a methanol/water gradient. The *cis*-double bond analog of SR695 eluted well before SR695 and was thus not as critical a separation. This separation could not be achieved in an acetonitrile/water gradient and was optimized using methanol/water and trifluoroacetic acid. On-column loading and sample solvent composition were chosen to allow reliable quantitation of efavirenz and all known impurities and degradation products in both the drug substance and in the presence of the excipients in the drug product and in-process samples. In this method, the concentration of any related substance (synthetic impurity or degradation product) was determined by comparison of its peak area with the peak area of efavirenz in the standard solution, and correcting for differences in absorptivity with relative response factors for the known related substances. Unknown related substances were assumed to be equal in absorptivity to efavirenz. In the drug product, only the known degradation products SD573 and SM097 and any unknown degradation
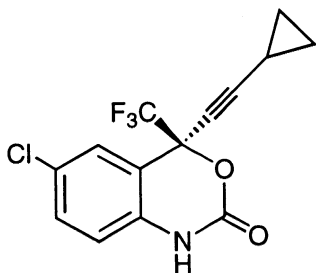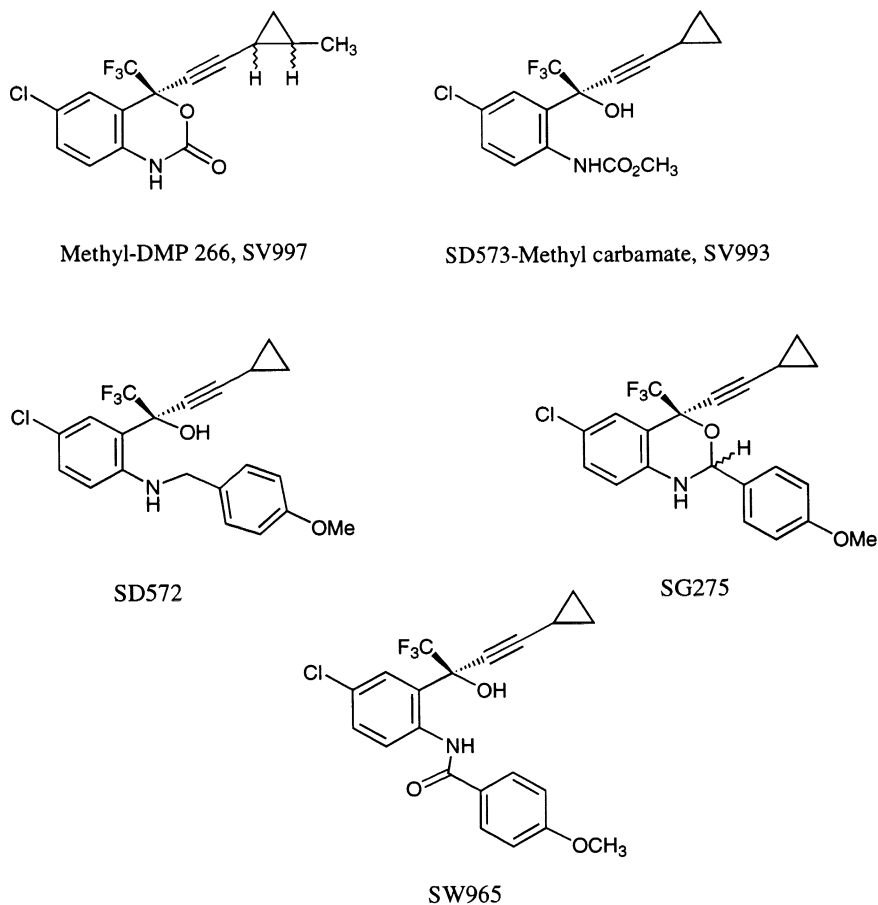
Fig. 2. Chemical structures of efavirenz potential synthetic impurities and degradation products.

Table 1
Chromatographic conditions for efavirenz drug substance and drug product assay and related substances or degradation products analysis

Column	Zorbax® SB-CN, 15 cm × 4.6 mm i.d.
Mobile phases	(A) 90% water, with 0.05% trifluoroacetic acid/ 10% methanol
	(B) 90% methanol/10% water, with 0.05% trifluoroacetic acid
Gradient profile	A linear gradient from 60:40 (A:B) to 50:50 over 16 min, then to 35:65 over 7 min, then to 30:70 over 5 min, then to 20:80 over 1 min. Hold for 2 min and return in 1 min to 60:40 (A:B) and equilibrate for at least 8 min before the next injection.
Flow rate	1.5 ml/min
Injection volume	35 µl
Column temperature	40°C
Detector	UV at 250 nm
Run time	40 min

products were monitored and quantitated as appropriate. The method parameters as listed in Table 1 were then validated per ICH and internal guidelines.

2.2.1. Linearity

Linearity was evaluated for the assay of efavirenz by preparing six standard concentrations of efavirenz ranging from 120 to 360 μg/ml (48–144% of target) and assaying in duplicate. Linearity was evaluated for the known related substances by preparing five or more standard concentrations, in duplicate, of efavirenz, SP234, SR695, SM097, SW965, and SE563 ranging from 0.025 to 2.0 μg/ml (0.01–0.8%), and for SD573 ranging from 0.04 to 2.0 μg/ml (0.02–0.8%). Structures of these related substances are shown in Fig. 2.

2.2.2. Detection and quantitation limit

The detection limit was determined by diluting known concentrations of each related substance and efavirenz drug substance until the average responses were approximately three times the standard deviation of the responses for three replicate determinations. The quantitation limit was determined for all methods by diluting known concentrations of each related substance and efavirenz drug substance until the average responses were approximately ten times the standard deviation of the responses for three replicate determinations. Concentrations at which the signal-to-noise ratio was three-to-one and ten-to-one were also measured.

2.2.3. Accuracy and precision

For the assay method for drug substance, drug product, and in-process testing, the accuracy and precision were evaluated using a 3×3 matrix.

For the drug substance validation, these studies were performed by preparing triplicate samples from lot 013 at 80, 100 and 120% of target. Accuracy was determined by evaluating the recovery of analyte at these levels.

The accuracy and precision for the drug product assay were evaluated using a 3×3 matrix. All capsule strengths have an initial efavirenz concentration of 5 mg/ml and a final concentration of 0.25 mg/ml. In addition, the excipient to drug ratio is the same for all strengths. Therefore, the recovery was done only on the 200 mg (gold) and 100 mg (white) capsules.

For the 100 mg and 200 mg capsules, accuracy and precision studies were performed by fortifying efavirenz drug substance lot 15M at 70, 100 and 130% of label, in triplicate, into placebo lot DMP 5683-122 and adding white or gold capsule shells. The placebo contained sodium lauryl sulfate, lactose, magnesium stearate, and sodium starch glycolate.

For the in-process blend, accuracy and precision studies for the samples were performed by fortifying efavirenz drug substance lot 13 at 70 and 100% of one capsule content in triplicate, and 130% of three capsule contents, in triplicate, into either placebo lot DMP 5683-122 or into 80 mg of sodium starch glycolate. In this manner, the in-process recovery was determined for both pre- and final blend samples.

Intermediate precision was demonstrated using the data in the recovery and precision studies, each of which was performed by two analysts using different instruments over 2 days in the same laboratory.

Accuracy and precision of the method for all related substances except SW965 was determined by fortifying placebo lot DMP 5683-122 with efavirenz drug substance lot 013 and each related substance in triplicate and adding white capsule shells. Accuracy and precision for SW965 was determined by fortifying placebo lot DMP 5683-122 with efavirenz drug substance lot 015M, and SW965 in triplicate and adding white capsule shells.

2.3. Method robustness

The robustness of the assay/related substance method was evaluated throughout the development and validation of the method. A filter comparison study was done to compare PVDF with nylon filters for filtering stock sample solutions. HPLC systems with both high pressure and low pressure mixing (identified in the equipment section) were evaluated using a pre-run system equilibration time of 30 min. Several lots of columns were used during method validation. The column temperature was varied from 25 to 40°C. The mobile phase was evaluated with and without the 0.05% trifluoroacetic acid (TFA).

Also as part of the evaluation of robustness, solution stability for efavirenz drug substance and drug product in the assay and related substance methods was evaluated by monitoring the peak area response of the assay standard, the related substances standard, and a sample over a total of 9 days. Finally, the solution stability of SD573, a known degradation product of efavirenz, was measured by spiking both an efavirenz drug substance and drug product sample with 0.5% SD573 and monitoring SD573 and SM097 levels over a period of 6 days.

To evaluate the effect of sample solvent composition, a final sample solvent concentration of 47.5/52.5 (H_2O/ACN), was compared with sample solvent containing 50/50 (H_2O/ACN) by analyzing three preparations of three lots with each sample solvent.

To show that the sample solvent composition of 47.5/52.5 (H_2O/ACN) has no impact on the levels of the degradation products SD573 and SM097, a recovery study was done by fortifying placebo lot DMP 5683-122 with drug substance lot SB706-15M and 0.10% each of SD573 and SM097, in triplicate, and adding capsule shells. These results were compared to the recovery results obtained at a sample solvent composition of 50/50 (H_2O/ACN). The sample solvent strength was also varied from 25/75 (H_2O/ACN) to 50/50 (H_2O/ACN).

2.3.1. Specificity

Three types of specificity experiments were performed. In the first experiment, placebos were prepared as 100 and 200 mg capsule samples and examined for possible excipient interferences. In a second specificity experiment, efavirenz was spiked with its known potential synthetic impurities and degradation products (Fig. 2). The third specificity test involved forced degradation of efavirenz, to determine whether the degradation products are resolved from the efavirenz peak. This was done by subjecting efavirenz drug substance, capsules and placebo to conditions that would cause up to 5% degradation of efavirenz, thereby forming degradation products that would resemble those seen by the drug substance or capsules under longer term room temperature storage. These conditions are described below.

2.3.2. Acid stress

Efavirenz drug substance (lot 013; 250 mg) and 100 and 200 mg efavirenz capsules and placebo capsules were dissolved in 500 ml of methanol and the pH adjusted with 0.1 N hydrochloric acid until the solution was acidic (based on apparent pH). This sample was refluxed for 1 hour and diluted to nominal assay concentration with sample solvent. The sample was analyzed for degradation products and the efavirenz peak purity determined using a Hewlett–Packard 1090 series II diode-array detector and a purity threshold score of 999 of a possible 1000.

2.3.3. Base stress

Efavirenz drug substance (lot 013; 250 mg) and 100 and 200 mg capsules were dissolved in 500 ml of 0.1 N sodium hydroxide. This solution was sampled after 4 h and diluted to nominal assay concentration with sample solvent. The sample was analyzed for degradation products and the efavirenz peak purity determined using a Hewlett–Packard 1090 series II diode-array detector and a purity threshold score of 999 of a possible 1000.

A similar experiment was conducted with 100 and 200 mg placebo capsules, except that no diode array spectrum was taken. The placebo was assayed after 1 h of reflux.

2.3.4. Peroxide stress

Efavirenz drug substance (lot 013; 50 mg) was dissolved in 50 ml of 3% hydrogen peroxide and stored at ambient temperature. This solution was sampled after 2 h and diluted to nominal assay concentration with sample solvent. The sample was analyzed for degradation products and the efavirenz peak purity determined using a Hewlett–Packard 1090 series II diode-array detector and a purity threshold score of 999 of a possible 1000.

2.3.5. Photolytic stress

A drug substance sample (lot 019) and 100 and 200 mg capsules were exposed to ICH photostability conditions (6 days, 1.2 million lux hours overall illumination with near UV energy ≥ 200 W h/m²). The sample was analyzed for degrada-

tion products and the efavirenz peak purity determined using a Hewlett–Packard 1090 series II diode-array detector and a purity threshold score of 999 of a possible 1000.

2.3.6. Response factors

The response factors for potential synthetic impurities were generated versus the 0.50% efavirenz related substance standard. The response factors are the ratios of the peak areas from injections of known concentrations of each related substance to the peak areas of injections of known concentrations of efavirenz drug substance. The response factors were determined on two different HPLC systems with different detectors.

3. Equipment

The following systems were used for the validation of the assay/related substances method.

- System 1: Hewlett Packard 1050 series pump (low pressure mixing), autosampler, column oven, and detector.
- System 2: Hewlett Packard 1100 series pump (low pressure mixing), autosampler, column oven, and detector.
- System 3: Waters 717 autosampler, gradient controller, 510 pumps (high pressure mixing), and column oven, with an Applied Biosystems 783A detector.
- System 4: Waters 717 autosampler, gradient controller, and column oven, with an Applied Biosystems 759A detector and Spectraphysics SP8800 pumps (high pressure mixing).
- System 5: Waters 717 autosampler, 600E controller, 60C pumps (high pressure mixing), and column oven, with an Applied Biosystems 759A detector.

- System 6: Waters 717 autosampler, gradient controller, and column oven, with an Applied Biosystems 759A detector and Spectraphysics SP8800 pumps (high pressure mixing).
- System 7: Waters 715 autosampler, gradient controller column oven and 510 pumps (high pressure mixing), with an Applied Biosystems 759A detector.
- System 8: Waters 717 autosampler, gradient controller, column oven and 510 pumps (high pressure mixing), with an Applied Biosystems 783 detector.
- System 9: Waters 717 autosampler, 600 controller, column oven and 60F pumps (high pressure mixing), with an Applied Biosystems 759A detector.
- System 10: Hewlett Packard 1100 series pump (low pressure mixing), and autosampler, with an Applied Biosystems 759A detector and Waters column oven.
- System 11: Waters 717 autosampler, gradient controller, and 600 pumps (high pressure mixing), with an Applied Biosystems 783 detector and DuPont column oven.

4. Results and discussion

4.1. Linearity

The linearity of the HPLC method used for efavirenz assay, content uniformity, and in-process blend samples was evaluated by injecting standard concentrations of efavirenz drug substance ranging from 120 to 360 µg/ml (48–144% of target). A summary of the data showing the slopes, y-intercept values, and P-values for the y-intercept values for assay and related substances is given in Table 2.

Table 2
Linearity data for efavirenz drug substance and drug product assay and related substances or degradation products analyses

Component (range µg/ml)	Analysis	Slope [V s/(mg/mL)]	y-Intercept (V s)	Correlation coefficient	P-value for intercept
Efavirenz (120–360)	Assay	26.25	0.0584	0.99991	0.26724
Efavirenz (0.02–2.0)	Related substances	25.76	0.0000806	0.99999	0.33987

Table 3
Linearity data for drug substance and drug product related substances or degradation products

Component (range 0.025–2.08 µg/ml)	Slope (ml µv-s/µg)	y-Intercept (µv-s)	Correlation coefficient	P-value for intercept
SR695	25389.8	−43.23117	1.00000	0.33928
SE563	19409.7	−89.9189	1.00000	0.01321
SM097	50854.7	−196.8502	1.00000	0.01699
SP234	26658.6	−108.217	1.00000	0.04651
SW965	14182.4	171.173	0.99996	0.08293
SD573	9060.12	−41.15861	0.99999	0.02150

Linearity of the HPLC method for SR695, SE563, SM097, SP234, and SW965, was evaluated by injecting standard concentrations of each of the impurities at concentrations ranging from 0.025 to 2.08 µg/ml (0.01–0.8%). Linearity of the method for SD573 was evaluated from concentrations of 0.04–2.0 mg/ml (0.02–0.8%). A summary of the data showing the slopes and y-intercept values and P-values for the y-intercept value is given in Table 3.

The correlation coefficients for the assay of efavirenz and analysis of related substances were all greater than 0.999. In addition, the analysis of residuals for the assay and the related substance standard show that the values are randomly scattered around zero which shows a good fit with the linear model. To evaluate whether the y-intercepts were significantly different than zero, the P-value was determined for each line. If the P-value was > 0.05 then the intercept was considered statistically equal to zero. For SR695, SW965, and the efavirenz assay and related substances standards, the P-value was > 0.05; therefore, the intercept was statistically equal to zero, as shown in Tables 2 and 3. For the other related substances the intercept is statistically different from zero and is shown in Table 3; however, the value of the y-intercept was $< 5\%$ of the response at the quantitation limit and therefore unimportant. Based on these results, a single point calibration was established for the assay and related substances/degradation products method.

4.2. Detection limit and quantitation limit

The detection limit for efavirenz drug substance, and for all related substances (determined relative to efavirenz), determined using the standard deviation of the response or the signal-to-noise method were less than or equal to 0.01%. Similarly, the quantitation limit for efavirenz drug substance and all its related substances determined using either measurement method was less than or equal to 0.05%. A suitable range was established from 0.05 to 0.60% since precision and accuracy were equivalent over this range for all related substances.

4.3. Accuracy, precision and range

The accuracy and precision for the determination of drug substance, dosage form and in-process assay were evaluated using a 3×3 matrix. The accuracy of the HPLC method for the assay analysis of efavirenz was determined by preparing drug substance samples (lot 013) at 80, 100 and 120% of target (0.20–0.30 mg/ml). Recovery of efavirenz drug substance ranged from 99.3 to 100.5%. The data are presented in Table 4. No difference in precision between the upper and lower limits of the method range (80.0–120.0%) was found using an analysis of variance (ANOVA) with a 95% confidence limit as evidenced by the f-ratio. This indicated that the assay method gave a value that accurately represented the efavirenz content in prepared samples over the method range of 80–120% of

target. Intermediate precision was demonstrated by showing that equivalent results were obtained by different analysts on different days in the same laboratory.

The accuracy and precision for the drug product were evaluated using a 3×3 matrix. Nine assay determinations for the 100 and 200 mg efavirenz capsules were performed by fortifying placebo with known amounts of efavirenz drug substance at 70, 100 and 130% of label strength (0.175–0.325 mg/ml). Overall recovery of efavirenz from 100 mg spiked placebo ranged from 99.0 to 100.3% with a mean recovery of 99.7 and 95% confidence interval of ± 0.19 (Table 5). The recovery from 200 mg spiked placebo ranged from 99.1 to 101.2% with a mean recovery of 99.9 and a 95% confidence interval of ± 0.29 (Table 6). No difference in precision between the upper and lower limits of the method range (70.0–130.0%)

was found using an ANOVA at the 95% confidence level as evidenced by the f-ratio. This indicates that the assay value obtained accurately represented the true drug content in the formulation over the method range of 70–130% of label. Intermediate precision was demonstrated by showing that equivalent results were obtained by different analysts on different days in the same laboratory.

Similarly, nine assay determinations for the pre-blend and final blend were determined by fortifying placebo with known amounts of efavirenz at 70, 100 and 130% of one capsule content and 130% of three capsule contents. Overall recovery of efavirenz from pre-blend ranged from 99.1 to 101.3%, with a mean recovery of 100.3% and a 95% confidence interval of ± 0.20 (Table 7). The recovery of efavirenz from final blend ranged from 99.2 to 101.9%, with a mean recovery of

Table 4
Assay recovery of efavirenz drug substance

% of target		Efavirenz % recovery	
		Analyst 1, day 1, system 6	Analyst 2, day 2, system 4
80		99.9	100.2
		99.9	100.3
		100.1	100.5
	Mean	100.0	100.3
	% RSD	0.1	0.2
100		100.0	99.7
		99.9	100.3
		99.7	100.3
	Mean	99.9	100.1
	% RSD	0.2	0.3
120		99.6	99.8
		99.3	99.7
		99.3	99.7
	Mean	99.4	99.7
	% RSD	0.2	0.1
	Daily mean	99.7	100.1
	% RSD	0.3	0.3
	Grand mean	99.9	
	% RSD	0.3	
	95% Confidence interval	± 0.17	

Table 5
Assay recovery of efavirenz from placebo fortification at 70–130% of 100 mg efavirenz capsules

% of target		Efavirenz % recovery	
		Analyst 1, day 1, system 1	Analyst 2, day 2, system 4
70		100.2	100.0
		99.7	100.3
		100.0	100.3
	Mean	100.0	100.2
	% RSD	0.3	0.2
100		99.3	99.5
		99.9	99.6
		99.3	99.8
	Mean	99.5	99.6
	% RSD	0.3	0.2
130		99.7	99.5
		99.0	99.4
		99.4	99.4
	Mean	99.4	99.4
	% RSD	0.4	0.1
	Daily mean	99.6	99.8
	% RSD	0.4	0.4
	Grand mean	99.7	
	%RSD	0.4	
	95% Confidence interval	±0.19	

100.5% and a 95% confidence interval of ±0.32 (Table 8). No difference in precision between the upper and lower limits of the method range (70.0–130.0%) was found using an ANOVA at the 95% confidence level as evidenced by the *f*-ratio. This indicates that the assay value obtained accurately represented the true drug content in the formulation over the method range of 70–130% of label. Intermediate precision was demonstrated by showing that equivalent results were obtained by different analysts on different days in the same laboratory.

The accuracy of the HPLC method for the analysis of efavirenz related substances was determined by fortifying placebo and efavirenz drug substance with known amounts of the related substances at concentrations from 0.05 to 0.6%. The overall recovery of the related substances (Table 9) were as follows: SR695 ranged from 92.6 to 117.3%, with a mean recovery of 99.7%, SP234 ranged from 84.4 to 101.7%, with a mean recovery of 95.7%, SM097 ranged from 78.6 to 103.6%, with a mean recovery of 91.7%, SE563 ranged from 95.5 to 114.9%, with a mean recovery of 104.7%, SW965 from 85.8 to 119.3%, with a mean recovery of 98.4%, and SD573 ranged from 88.1 to 134.3%, with a mean recovery of 111.2%. Intermediate precision was demonstrated by showing that essentially equivalent results were obtained by different analysts on different days in the same laboratory. As expected, the greatest variability in recovery for most related substances is observed at the low (0.05%) spiking level; however, all recovery values are acceptable over the method range from 0.05 to 0.60%. The difference in the recovery of SM097 between analyst 1 and analyst 2 is due to instrument differences. An SM097 recovery sample prepared by analyst 2, when analyzed on system 1, gave 100% recovery. Because 250 nm is not the optimum wavelength

for SM097, and the response factor for SM097 is 1.983, small differences in detector performance can cause variability in the recovery. The difference in recovery for SD573 is also due to response factor differences between instruments. SD573 is not typically observed in efavirenz capsules. The method provides for determination of SD573 quantitation factor at time of use if amounts are to be reported.

4.4. Method robustness

To evaluate method robustness a variety of parameters were deliberately varied. As seen by the recovery data (Tables 4–6), the method, including the separation of SR695 and efavirenz, is robust with respect to different instruments and column lots. As long as the mobile phase was either continuously sparged or continuously degassed, the type of mixing (high or low pressure) had no effect on the reliability of the method. As a result of the filter study, two suitable sample filters, PVDF and nylon, were identified.

However, other parameters were found to effect either the chromatography or the performance of the method. For example, the temperature of the column was very important to reliability. Operating temperatures less than 40°C slowed the elution of efavirenz and its related substances such that peaks eluted on the steep part of the gradient on some HPLC systems, leading to problems with the integration. However, in all cases acceptable resolution between SR695 and efavirenz was obtained. In addition, resolution of SR695 and efavirenz decreased with increasing temperature. This behavior indicates that the resolution is not mass transfer-limited. This effect is being further studied and will be reported on in a forthcoming publication. The addition of trifluoroacetic acid to the mobile phase sharpened the peak shape of some related substances, which in turn improved integration.

Table 6
Assay recovery of efavirenz from placebo fortification at 70–130% of 200 mg efavirenz capsules

% of target		Efavirenz % recovery	
		Analyst 1, day 1, system 4	Analyst 2, day 2, system 1
70		100.1	99.6
		99.8	99.6
		100.9	99.6
	Mean	100.3	99.6
	% RSD	0.6	0.0
100		100.1	99.7
		100.6	100.1
		100.5	99.3
	Mean	100.4	99.7
	% RSD	0.3	0.4
130		101.2	99.4
		99.6	99.4
		100.3	99.1
	Mean	100.4	99.3
	% RSD	0.8	0.2
	Daily mean	100.3	99.5
	% RSD	0.5	0.3
	Grand mean	99.9	
	%RSD	0.6	
	95% Confidence interval	±0.29	

Table 7
Assay recovery of efavirenz in process pre-blend

% of target		Efavirenz % recovery		
		Analyst 1, day 1, systems 3 and 5	Analyst 2, day 2, systems 8 and 9	Analyst 2, day 3, system 7
70		101.2	100.4	100.4
		101.2	100.2	100.4
		101.3	100.1	100.8
	Mean	101.2	100.2	100.5
	% RSD	0.06	0.2	0.2
100		100.5	99.9	99.9
		100.3	100.2	100.3
		100.0	100.0	99.9
	Mean	100.3	100.0	100.0
	% RSD	0.3	0.2	0.2
130		100.0	100.0	99.1
		100.0	100.0	99.5
		99.9	99.6	99.2
	Mean	100.0	99.9	99.3
	%RSD	0.06	0.2	0.2
130% of three capsule contents		100.3	101.1	
		100.2	100.6	
		100.4	101.6	
	Mean	100.3	101.1	
	%RSD	0.1	0.5	
	Daily mean	100.4	100.3	99.9
	% RSD	0.5	0.6	0.6
	Grand mean		100.3	
	% RSD		0.6	
	95% Confidence interval		±0.20	

Solution stability was evaluated for efavirenz standards and samples at assay and related substances concentrations. For the assay method, efavirenz standards were stable for up to 216 h (∼ 9 days), the change in efavirenz peak area response for the assay standards over 216 h being 2.0%. The change in efavirenz peak area response for the dosage form assay samples over 216 h was 1.4%. The efavirenz peak areas in both standard and sample increased over the 9 days that solution stability was measured. This increase is most likely due to instrument drift; however, the ratio of the sample response to the standard response did not change. The percent change from time zero for all standards and samples was within acceptable variability. Therefore, assay samples and standards were stable for at least 216 h.

There was no change in peak area response for the related substance standard over 216 h. There was no change in the number of related substances observed in efavirenz drug substance nor in their size, or retention time from a comparison of the chromatograms over 216 h.

Solution stability was also evaluated for samples prepared from 100 to 200 mg efavirenz capsules. The change in the efavirenz peak area

response over 216 h was 1.4% for both strengths. There was no change in the number of related substances observed in efavirenz drug product samples nor in their size or retention time from a comparison of the chromatograms over 216 h.

Although there was no change in the related substances profile of the samples, there was reason to believe that SD573 might be unstable in solution. SM097 forms from degradation of SD573, so SM097 was also monitored. The solution stability of SD573 was measured over 7 days by spiking it into both drug substance and drug product. At time zero there was 0.52% SD573 spiked into the drug substance and 0.58% SD573 spiked into the drug product. By the 6th day,

there was 0.53% SD573 and 0.04% SM097 in the drug product sample, and 0.49% SD573 and 0.04% SM097 in the drug substance sample. While this level of degradation is within the method precision, by day 7 the concentration of SD573 in the drug product spiked sample had degraded to less than 90% of that present at time zero. Therefore, for the degradation products analysis, solution stability of 6 days has been established for drug substance and drug product, because the percent change from time zero for the degradation product SD573 over this time period was within acceptable variability.

To show that there is no difference in the assay results due to small changes in sample solvent

Table 8
Assay recovery of efavirenz in process final blend

% of target		Efavirenz % recovery	
		Analyst 1, day 1, system 5	Analyst 2, day 2, system 6
70		99.7	100.8
		100.2	101.1
		100.2	101.1
	Mean	100.0	101.0
	% RSD	0.3	0.2
100		99.1	101.2
		100.0	100.9
		99.9	101.2
	Mean	99.7	101.1
	% RSD	0.5	0.2
130		99.2	100.1
		99.5	100.5
		99.6	100.6
	Mean	99.4	100.4
	% RSD	0.2	0.3
130% of three capsule contents		100.9	100.7
		100.8	101.8
		101.0	101.9
	Mean	100.9	101.5
	% RSD	0.1	0.7
	Daily mean	100.0	101.0
	% RSD	0.6	0.5
	Grand mean	100.5	
	% RSD	0.8	
	95% Confidence interval	±0.32	

Table 9
Recovery[a] of efavirenz impurities

% Spiked		% Recovery			
		SR695		SP234	
		Analyst 1, day 1, systems 1 and 2	Analyst 2, day 2, system 6	Analyst 1, day 1, systems 1 and 2	Analyst 2, day 2, system 6
0.05		106.1	109.1	98.6	87.5
		103.2	113.6	99.6	84.4
		98.7	117.3	95.8	89.1
	Mean	102.7	113.3	98.0	87.0
	% RSD	3.6	3.6	2.0	2.7
0.1		97.8	96.5	98.7	101.7
		96.1	92.6	96.8	96.3
		97.1	95.5	95.4	98.4
	Mean	97.0	94.9	97.0	98.8
	% RSD	0.9	2.1	1.7	2.8
0.6		95.8	93.2	97.4	95.4
		96.9	94.3	98.1	96.5
		94.9	95.0	96.0	97.5
	Mean	95.9	94.2	97.2	96.5
	% RSD	1.0	1.0	1.1	1.1
	Daily mean	98.5	100.8	97.4	94.1
	% RSD	3.8	9.6	1.5	6.1
	Grand mean		99.7		95.7
	% RSD		7.2		4.6
		SM097		SE563	
0.05		97.3	86.6	114.8	109.4
		97.1	82.9	104.3	111.0
		96.7	84.7	109.7	114.9
	Mean	97.0	84.7	109.6	111.8
	% RSD	0.3	2.2	4.8	2.5
0.1		101.3	78.6	109.9	99.8
		103.6	86.3	106.5	95.6
		102.0	86.2	108.0	97.4
	Mean	102.3	83.7	108.1	97.6
	% RSD	1.2	5.3	1.6	2.2
0.6		98.1	86.0	104.5	95.5
		95.6	85.7	105.4	96.8
		97.5	85.0	103.2	97.5
	Mean	97.1	85.6	104.4	96.6
	% RSD	1.3	0.6	1.1	1.1
	Daily mean	98.8	84.7	107.4	102.0
	% RSD	2.8	3.0	3.4	7.4
	Grand mean		91.7		104.7
	% RSD		8.4		6.1

Table 9 (*Continued*)

% Spiked		% Recovery			
		SD573		SW965	
		Analyst 1, day 1, systems 1 and 2	Analyst 2, day 2, systems 1 and 6	Analyst 1, day 1, system 2	Analyst 2, day 2, system 1
0.05		101.2	115.1	104.7	93.9
		99.7	108.2	119.3	98.2
		88.1	111.1	105.1	90.1
	Mean	96.3	111.5	109.7	94.1
	% RSD	7.4	3.1	7.6	4.3
0.1		122.8	110.6	108.7	93.0
		127.4	106.6	106.7	86.0
		134.3	104.1	104.7	89.2
	Mean	128.2	107.1	106.7	89.4
	% RSD	4.5	3.1	1.9	3.9
0.6		118.9	106.8	102.9	85.8
		119.8	105.0	102.8	89.9
		117.5	104.5	102.1	88.9
	Mean	118.7	105.4	102.6	88.2
	% RSD	1.0	1.1	0.4	2.4
	Daily mean	114.4	108.0	106.3	90.6
	% RSD	13.0	3.4	5.0	4.3
	Grand Mean	111.2		98.4	
	% RSD	9.9		9.4	

[a] Recovery of each impurity was corrected for the amount of that impurity present in the drug substance.

composition, four lots of efavirenz capsules were analyzed with a sample solvent composition of 47.5/52.5 ACN/H_2O and 50/50 (ACN/H_2O). The assay results are shown in Table 10.

An additional method robustness experiment, which looked at small changes in sample solvent, was done using the degradation products SD573 and SM097. Three recovery samples were prepared by fortifying placebo lot DMP 5683-122 with drug substance lot 15M and 0.10% each of SD573 and SM097. The recovery in this modified sample solvent (Table 11) was compared to the recovery in the original sample solvent (see Table 9).

Varying the final sample solvent composition from 50/50 ACN/H_2O to 52.5/47.5 ACN/H_2O had no effect on either assay or related substances results (Tables 10 and 11). Likewise, varying the sample solvent from 5050 (H_2O/ACN) to 25/75 (H_2O/ACN) did not effect the assay or related substances results. However, the resolution between SR695 and efavirenz decreased with increasing solvent strength, but in all cases the resolution was 1.1 or better. A system suitability criterion for resolution of not less than 1.2 has been established. This has been shown to be achievable in a number of laboratories as demonstrated by interlaboratory qualification. In addition, resolution of 1.2 allows acceptable precision and accuracy in quantitating both efavirenz and SR695. This result is being further investigated and will be reported on in a forthcoming paper.

Table 10
Comparison of the efavirenz capsules assay results as a function of final sample solvent composition

Lot	Sample solvent 50/50 (ACN/H$_2$O) System 2, % of label	Sample solvent 52.5/47.5 (ACN/H$_2$O) System 1, % of label	Difference
DMP722-003, 50 mg	99.3	99.9	
	101.1	101.3	
	97.7	99.3	
Mean	99.4	100.2	+0.8
RSD	1.7	1.0	
LE207, 100 mg	100.7	101.3	
	101.7	100.6	
	100.6	101.8	
Mean	101.1	101.2	0.1
RSD	0.6	0.6	
LE208, 150 mg	99.7	98.5	
	99.3	99.8	
	98.8	99.0	
Mean	99.3	99.1	−0.2
RSD	0.5	0.7	
LE209, 200 mg	99.1	99.6	
	100.4	99.7	
	99.1	99.6	
Mean	99.5	99.6	+0.1
RSD	0.8	0.1	

Analysis of the results of recovery studies showed no significant column-to-column variability. Likewise, column lot had no effect on separation of SR695 and efavirenz. Recovery samples were prepared by multiple analysts at different concentrations with no operator bias observed. In addition, comparable results for drug substance and drug product were obtained between sites during interlaboratory qualifications. Instrument to instrument differences were not observed for assay results or for resolution and/or quantitation of SR695, SP234, SW965, or SE563. However, they were noted during validation and interlaboratory qualification for the potential degradation products SD573 and SM097. These impurities have quantitation factors which differ by 100% or more from efavirenz. To accurately measure these potential degradation products on a routine basis, the quantitation factor should be measured on the same instrument or authentic substances used.

When the critical factors are taken into account this method is robust with respect to column, instrument, analyst, and site, as evidenced by the comparable results observed among analysts for the recovery studies, the Interlaboratory Qualifications (ILQ) which were successfully performed at eight sites worldwide to date, and during routine analysis. This indicates that the separation of SR695 and efavirenz is achievable under a variety

Table 11
Recovery of efavirenz degradation products in modified sample solvent

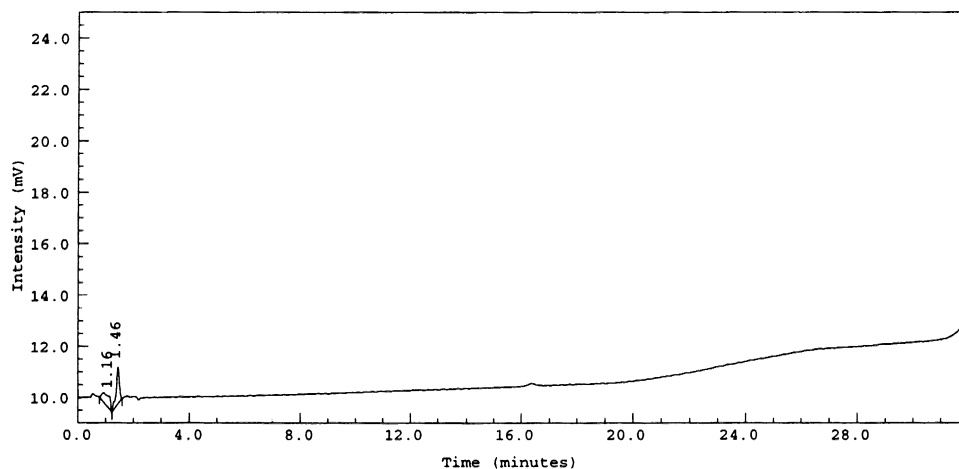| Amount spiked	SD573, system 3	SM097, system 1
0.10%	125.1	102.7
	127.4	97.9
	109.5	98.5
Mean	120.7	99.7
RSD	8.1	2.6

Fig. 3. Representative (100 mg capsules) placebo chromatogram under efavirenz drug product assay and degradation products analysis conditions.

of conditions. Therefore, this column/mobile phase combination should be applicable to similar peak pairs.

4.4.1. Specificity

A number of experiments were done to show specificity of the method for determination of assay of both drug substance and efavirenz capsules. To prove noninterference from excipients and capsule shells, placebos which matched 100 and 200 mg efavirenz capsules were prepared and analyzed as samples. The efavirenz drug product assay method was shown to be free from interference from the placebo and from both gold and white hard gelatin capsules with purple and black printing (Fig. 3).

To show method specificity for known potential related substances and degradation products, efavirenz drug substance was spiked with available samples of its known potential synthetic impurities and degradation products (shown in Fig. 2). A second spiking experiment was also done when small amounts of additional impurities were identified after the method was initially validated. The chromatograms in Figs. 4 and 5 show that efavirenz and its known potential synthetic impurities and degradation products are resolved by the assay and related substances method.

4.4.2. Forced degradation

The third specificity test was to determine whether new degradation products were produced from the forced degradation of efavirenz drug substance, whether these degradation products are resolved from the efavirenz chromatographic peak, and to verify the peak purity of the efavirenz peak. Therefore, efavirenz drug substance and placebo capsules were stressed under the conditions most likely to cause degradation based on the known chemistry of efavirenz.

4.4.3. Acid stress

Total degradation of $\sim 0.1\%$ was observed in efavirenz drug substance stressed in 0.1 N hydrochloric acid. These degradants were observed at relative retention time (RRT) of 0.17, 0.32, and 0.83, at amounts $< 0.05\%$ each. No degradation ($< 0.1\%$) of efavirenz was observed for efavirenz 100 and 200 mg capsules stressed in 0.1 N hydrochloric acid. Peak purity showed that the efavirenz peak was pure.

No degradation of placebo capsules which would interfere with measuring efavirenz in efavirenz capsules was observed after refluxing for 1 h in 0.1 N hydrochloric acid.

4.4.4. Base stress

Degradation of 3.6% was observed in efavirenz drug substance stressed in 0.1 N sodium hydroxide for 4 h at ambient temperature. This degradation appeared as formation of SD573, which is known to be the primary degradation product of efavirenz. Efavirenz 100 and 200 mg capsules stressed in 0.1 N sodium hydroxide produced both SD573 and SM097. Peak purity showed that the efavirenz peak was pure.

No degradation of placebo capsules which would interfere with measuring efavirenz in efavirenz capsules was observed after refluxing for 1 h in 0.1 N sodium hydroxide.

4.4.5. Peroxide stress

Degradation of less than 0.1% was observed in efavirenz drug substance stressed in 3% hydrogen peroxide for 2 h. Degradation products appeared at RRT 0.24 and 0.36 at < 0.05% each. Peak purity showed that the efavirenz peak was pure.
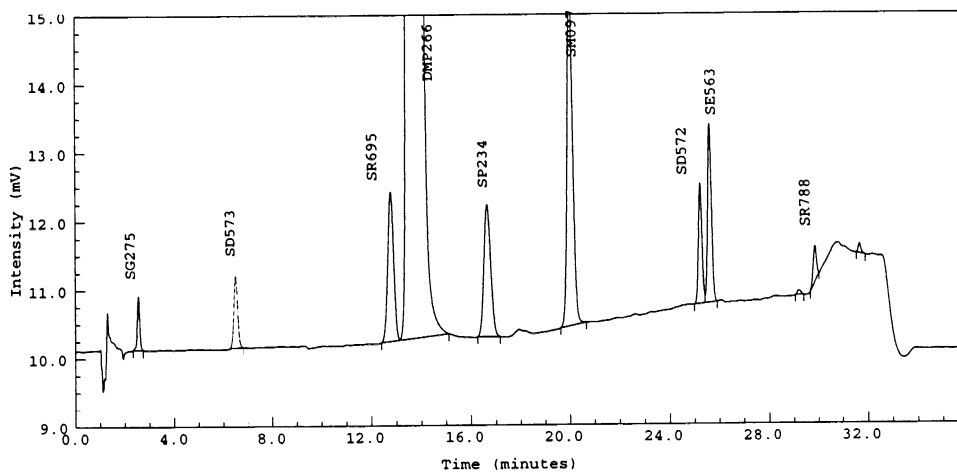

Fig. 4. Chromatogram of efavirenz drug substance spiked with its known potential synthetic impurities (at 0.5%) under assay and related substances conditions.
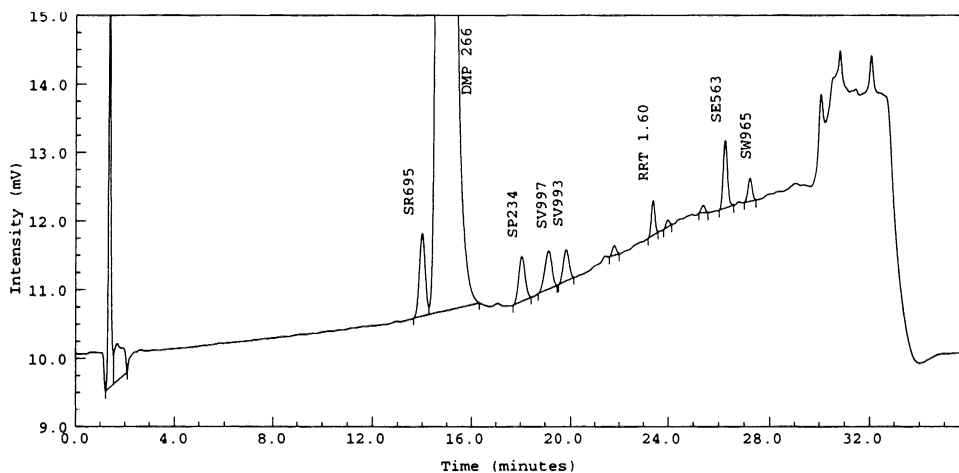

Fig. 5. Chromatogram of efavirenz drug substance containing additional related substances (SR695 is present at 0.13%).

Table 12
Efavirenz drug substance and drug product related substances quantitation factors and response factors

Related substance	Response factor[a] (RF)	Quantitation factor[b] (QF)
SD573	0.2649	3.8
SP234	0.9865	1.0
SM097	1.983	0.51
SE563	0.7143	1.4
SW965	0.5493	1.8
SR695	0.9434	1.1

[a] RF, related substance peak area/efavirenz peak area at related substances standard concentration.
[b] QF = 1/RF.

4.4.6. Photolytic stress

An efavirenz drug substance sample exposed to ICH photostability conditions was analyzed. Only drug substance was analyzed since efavirenz capsules stored under ICH stability conditions show no degradation. Degradation of 0.12% was observed as SM097, a known degradation product of efavirenz. Peak purity showed that the efavirenz peak was pure.

Because the results of spiking experiments and forced degradation showed no interference with the efavirenz peak, the method is both specific and stability-indicating.

4.5. Response factors

The response factors for potential synthetic impurities were generated versus the efavirenz related substance standard. They are the ratios of the peak areas from injections of known concentrations of each related substance to the peak areas of injections of known concentrations of efavirenz drug substance. The quantitation factors used in the test method are the reciprocals of the response factors. These factors are shown in Table 12. Results on the two detectors were in good agreement except for SD573, which is known to have relatively poor molar absorptivity.

Response factors for the related substances of efavirenz were in good agreement with the slope of the linearity curves for each related substance (Table 3). These factors will allow quantitation of these related substances using a 0.5% efavirenz standard. Results for the two detectors were in good agreement except for SD573, which is known to have relatively poor molar absorptivity. Variability in the QF of SD573 was also observed during Interlaboratory Qualification.

5. Conclusion

Linearity, precision, and recovery data are excellent for both the drug substance and drug product assay and related substances methods over their intended ranges. These methods are accurate, robust, specific, and stability-indicating. The quantitation and detection limits and accuracy are acceptable. The methods have been successfully transferred to eight sites worldwide for assay efavirenz drug substance, drug product, in-process samples, and synthetic impurities or degradation products. This method has been applied to analysis of solution, oral liquid, and tablet dosage forms.

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