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METHODOLOGY

# Development and Validation of Bioanalytical Method for Efavirenz in Dried Blood Spot Using High Performance Liquid Chromatography–Photodiode Array

Dysaprita Nabila Putri[1], Yahdiana Harahap[1,2], Febrina Amelia Saputri[1]

[1]Faculty of Pharmacy, Universitas Indonesia, Depok, Indonesia; [2]Faculty of Military Pharmacy, Republic of Indonesia Defense University, Bogor, Indonesia

Correspondence: Yahdiana Harahap, Email yahdiana03@yahoo.com

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**Abstract:** Efavirenz requires adequate serum concentrations (1–4 µg/mL) to ensure therapeutic efficacy and avoid central nervous system toxicity. Due to significant interindividual pharmacokinetic variability, therapeutic drug monitoring (TDM) is often necessary. Reliable bioanalytical methods for quantifying efavirenz in biological matrices are therefore essential. Previous dried blood spot (DBS) methods using High-Performance Liquid Chromatography–Photodiode Array (HPLC–PDA) showed suboptimal performance and lacked compliance with current bioanalytical guidelines. This study aimed to develop and validate an improved HPLC–PDA method for efavirenz quantification in DBS using an internal standard. The optimized sample preparation employed protein precipitation and methanol extraction, followed by chromatographic separation under isocratic conditions with UV detection at 245 nm. The method demonstrated excellent linearity ($r^2$ = 0.9995) within 0.1–30 µg/mL, accuracy within ±9.3%, and precision (CV < 4.6%). Recovery ranged from 91.4–95.7%, and no significant carryover or matrix effect was observed. Validation results met the acceptance criteria specified by the US FDA (2018) and EMA (2022) guidelines. The proposed method offers enhanced sensitivity, accuracy, and precision, supporting its suitability for routine TDM of efavirenz to improve treatment safety and effectiveness.

**Keywords:** efavirenz, dried blood spot, high performance liquid chromatography, HPLC, HIV, therapeutic drug monitoring

## Introduction

Since the first diagnosis of acquired immunodeficiency syndrome (AIDS) in 1981 and the discovery of the human immunodeficiency virus (HIV)-1 virus in 1983, HIV and AIDS have become major global public health issues. Up to the present, the disease caused by this virus has claimed 40.4 million lives, with ongoing transmission in all countries. Among the many combinations of antiretroviral therapy (ART), one frequently used regimen includes efavirenz as the component. Efavirenz has been part of first-line antiretroviral therapy for over 15 years.[1]

Efavirenz is one type of ART belonging to the non-nucleoside reverse transcriptase inhibitor (NNRTI) class. For optimal therapeutic outcomes, efavirenz must achieve serum concentrations between 1 and 4 µg/mL.[2] Concentrations below this range can lead to virologic failure, while excessive concentrations may result in central nervous system toxicity.[3] Clinical studies have highlighted the interindividual variability in efavirenz absorption, metabolism, and clearance, contributing to significant pharmacokinetic variability and diverse treatment responses.[4] This variability underscores the critical need for drug monitoring, particularly to ensure that patients achieve therapeutic concentrations while avoiding toxicity.

Historically, efavirenz quantification has relied on plasma-based assays, primarily using liquid chromatography-tandem mass spectrometry (LC–MS/MS), which are highly sensitive but costly and technically demanding. In contrast, high-performance liquid chromatography with photodiode array detection (HPLC–PDA) offers a more accessible

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alternative. Although it is less sensitive and robust than LC–MS/MS, HPLC–PDA can be optimized for clinical use, making it a promising option for efavirenz monitoring.[5–7] Earlier studies utilizing LC–MS/MS and plasma-based assays have demonstrated high sensitivity but are limited by instrument cost and sample preparation complexity. The HPLC–PDA/DBS approach developed in this study offers a simpler, accessible, and guideline-compliant alternative for routine therapeutic drug monitoring.

Blood is recognized as the primary and most potent source of HIV transmission. It is higher risk in occupational settings, particularly among healthcare workers exposed to needless-stick injuries and other percutaneous incidents.[8] This can be addressed by using dried blood spot (DBS) matrices, as it requires only a small amount of blood sample, minimizing exposure and patient discomfort. Furthermore, the finger prick sampling technique allows patients to collect their own samples under supervision. HIV concentrations in DBS are typically low, and the virus is susceptible to degradation, reducing the risk of transmission.[9]

The previously published method for determining efavirenz concentration in DBS using HPLC-PDA did not include any internal standard (IS).[10] Meanwhile, internal standards help compensate for variability introduced during sample preparation and analysis. Therefore, this study is conducted to develop a validated analytical method that provides accurate, sensitive, and selective results using accessible instrumentation and materials. To the best of our knowledge, no validated HPLC–PDA method for efavirenz in DBS using an internal standard has been reported in accordance with the updated EMA (2022) and FDA (2018) guidelines. This study therefore provides a novel contribution by improving sensitivity, reproducibility, and compliance with current bioanalytical standards. Therapeutic drug monitoring (TDM) of efavirenz is clinically recommended in specific patient groups, such as those with virological failure, central nervous system adverse effects, or potential drug–drug interactions. Genetic polymorphisms in CYP2B6 can also contribute to significant interindividual variability in efavirenz plasma concentrations. Therefore, developing a reliable, validated, and accessible analytical method is crucial to support effective TDM implementation in these populations.

# Materials and Methods

## Material

Efavirenz and warfarin sodium as internal standard were purchased from Indonesian Food and Drug Authority (Jakarta, Indonesia); acetonitrile, methanol, ethanol, potassium dihydrogen phosphate (Darmstadt, Germany); ultrapure water (Jakarta, Indonesia); dried blood spot paper Perkin Elmer 226 (USA); six whole blood bags from different donor sources were acquired from the Indonesian Red Cross (Jakarta, Indonesia), spiked with efavirenz, and sent straight to the DBS.

## Preparation of Stock Solution, Calibration Samples, and Quality Control Samples

Efavirenz and warfarin were eluted using methanol. Standard solutions were at 1,000 µg/mL and stored in the freezer at −20°C. The calibration ranges of 0.1, 0.5, 1; 5; 10; 15; and 30 µg/mL for efavirenz were obtained by diluting working solutions in the blood at a concentration of 1:10. Quality control (QC) solutions were prepared by diluting working solutions in the blood with a 1:10 concentration of blood at 0.3 µg/mL (QCL), 9.0 µg/mL (QCM), and 22.5 µg/mL (QCH).

## Instrument and Chromatographic Condition for Analysis

The HPLC system used was the Shimadzu HPLC with LC-20AD pump, autosampler SIL-20a (Japan), photodiode array detector (Waters 2996), and an integrator using Lab Solutions as the system controller. The analytical column was a reverse-phase C-C18 column (SunfireTM; 250 × 4.6 mm; 5 µm).

Efavirenz standards, controls, and samples were autosampled at an injection volume of 20 µL, separated isocratically. The mobile phase was selected from mixtures including acetonitrile-water (60:40 v/v); acetonitrile-phosphate buffer (70:30 v/v); 63:37 v/v; and methanol-acetonitrile-phosphate buffer (10:55:35 v/v). Flows of 0.8, 1.0, and 1.2 mL/min were tested for optimization. Column temperatures of 30°C, 40°C, and 50°C were also evaluated. The optimum analytical condition was assessed with a system suitability test. The system suitability test was considered acceptable if the percent coefficient of variation (%CV) of the generated responses was below 2%.[11]

## Preparation of DBS Samples

In the development of the method, DBS sample preparation was assessed using the protein precipitation method with various spotting volumes of 20 and 30 µL; blood spots drying time of 1, 2, and 3 hours; composition extraction solvent of methanol, ethanol, and acetonitrile and the volume of 300, 400, and 500 µL; vortexing time at 10, 15, and 30 seconds; sonication time at 1, 3, and 5 minutes; also centrifugation speed and time of 5,000 rpm and 10,000 rpm for 3 minutes and 5 minutes. Aliquots of 200 µL of supernatant were evaporated using nitrogen (N2) gas flow. The residue was reconstituted with 100 µL of mobile phase, then vortexed for 15 seconds and sonicated for 10 minutes. Subsequently, 20 µL of the aliquot was injected into the chromatography system. Optimal conditions were chosen based on the highest response and the effectiveness of sample preparation time.

## Method Validation

Validation of the analytical method was conducted in accordance with the requirement of full validation from the European Medicine Agency 2022 and the US Food and Drug Administration 2018. Parameters of full validation assessed were lower limit of quantification (LLOQ), calibration curve and the linearity of calibration, selectivity, accuracy, precision, recovery, carryover, dilution integrity, stability, and reinjection reproducibility.[11,12] Each validation parameter was tested in at least five replicates unless otherwise specified. Data were analyzed using Microsoft Excel 2016 with calculation of mean, %CV, and %diff according to FDA (2018) and EMA (2022) criteria.

### LLOQ

Efavirenz standard solution was diluted in blood to achieve a concentration of approximately 0.1 µg/mL. LLOQ was tested with a minimum of five replicates. The acceptance criteria required that the %diff and %CV values did not exceed ±20%. If the results met the criteria, the analysis was repeated at 0.5 times the initial concentration. If the criteria were not met, the LLOQ concentration was either maintained or increased.[11,12]

### Calibration Curve and Linearity

A calibration curve was constructed using six efavirenz concentrations (0.1, 0.5, 1, 5, 10, 15, 20, 30 µg/mL), along with zero and blank samples. Three replicates were analyzed for each concentration. The calibration curve was accepted if % diff and %CV were ≤±15%, except for LLOQ which was ≤±20%.[11,12]

### Selectivity

Selectivity was evaluated using 6 blank matrices from different blood sources, with 6 replicates of efavirenz standard solutions diluted to LLOQ concentrations. The results were accepted if the analyte response at LLOQ was less than 20% and the internal standard response in the blank samples was less than 5%.[11,12]

### Accuracy and Precision

Accuracy and precision were assessed by analyzing 5 replicates at LLOQ, QCL, QCM, and QCH concentrations in blood, both intraday and interday (three independent tests on at least two different days). Accuracy was considered acceptable if %diff was within ±15% for QC concentrations and ±20% for LLOQ, while precision was acceptable if % CV was ≤15% for QC and ≤20% for LLOQ.[11,12]

### Recovery

Recovery testing was performed by comparing the peak areas of extracted blood samples with those of blank samples spiked with efavirenz at QCL, QCM, and QCH concentrations, including 30 µL of warfarin as the internal standard. Tough 100% recovery was not required; recovery was considered consistent and reproducible if the %CV was ≤15%.[11,12]

### Carry Over

Carry over was tested by measuring the analyte response in a blank sample after analyzing a sample with ULOQ concentration. Five replicates were performed for each test. The results were accepted if the carry over was ≤20% of the LLOQ for the analyte and ≤5% for the internal standard.[11,12]

### Dilution Integrity

Dilution integrity validation was performed by spiking blood with concentrations 1.5 to 2 times higher than QCH (45 µg/mL), followed by dilution to QCH (22.5 µg/mL) and ½ QCH (11.25 µg/mL). The analysis was conducted with at least five replicates for each concentration. The results were accepted if the average accuracy was within ±15% of the nominal concentration and the precision (%CV) was ≤15%.[11,12]

### Stability

Stability tests evaluation include stock solution stability, short-term analyte stability, long-term analyte stability, and autosampler stability. Stock solution stability was evaluated at 25°C for short-term stability and −20°C for long-term stability. The stock solution is considered stable if the concentration difference and %CV obtained are not more than 10%. Short-term analyte stability in DBS was tested at 25°C for short-term stability and −20°C for long-term stability. Autosampler stability was tested according to autosampler conditions. Analyte stability in DBS and autosampler stability were considered acceptable if the average concentration difference at each level fell within the ±15% of the true concentration.

### Reinjection Reproducibility

The reinjection reproducibility test was conducted by injecting samples after storage at room temperature for 24 hours. Reproducibility of reinjection was assessed by re-injecting the process, which included calibration curve concentrations and at least five replicates of QCL, QCM, and QCH concentrations. The suitability of the reinjection process was determined by accuracy and precision values, with %CV and %diff not exceeding 15%.

# Result and Discussion

## Optimization of Efavirenz Analytical Condition

Optimization was performed for efavirenz and warfarin at a concentration of 10 ppm. The maximum absorbance wavelength for efavirenz was 245 nm, producing an area of 445.125 µV/s, while warfarin's maximum wavelength was 281 nm, yielding 423.695 µV/s. Based on these results, 245 nm was selected for analysis to maximize absorbance, particularly at the LLOQ, ensuring acceptable accuracy and precision. Although warfarin showed good absorbance in the 201–250 nm range, the use of 245 nm still effectively detected warfarin, yielding an area of 136.720 µV/s at 10 ppm. To improve warfarin's response, its concentration was increased to 200 ppm, resulting in an area of 1.183.139 µV/s at 245 nm. The wavelength optimization results are shown in Figure 1.

The detection method was performed using isocratic elution with a C18 column (Sunfire™ 5 µm; 250 × 4.6 mm) maintained at 40°C, with a mobile phase of acetonitrile:10 mM phosphate buffer pH 3 (70:30), a flow rate of 0.8 mL/min, and UV detection at 245 nm. This condition was selected as it provided the largest chromatogram area, with 657,316 µV/s for efavirenz and 161,323 µV/s for warfarin (IS), compared to other analytical conditions. Parameters such as retention time, resolution, HETP, theoretical plates, and tailing factor were also evaluated during method development, with the optimal values selected based on each parameter to ensure adequate analytical conditions. Detailed results are not shown in this section.

## System Suitability Test

The System Suitability Test (SST) is a critical pre-analysis procedure used to confirm that the analytical system is functioning properly and can produce accurate and precise data. In this study, SST was conducted to verify that the HPLC–PDA system could accurately and precisely quantify efavirenz and the internal standard (warfarin) under the validated conditions. According to both the US Food and Drug Administration (FDA, 2018) and the European Medicines Agency (EMA, 2022) guidelines, system suitability parameters such as coefficient of variation (CV) for peak area and retention time should generally not exceed 2% to indicate stable system performance. The SST was performed using isocratic elution with a C18 column (Sunfire™ 5 µm; 250 × 4.6 mm) maintained at 40°C, with a mobile phase of acetonitrile:10 mM phosphate buffer pH 3 (70:30), a flow rate of 0.8 mL/min, and UV detection at 245 nm. Under these conditions, all SST acceptance criteria were met. Representative chromatograms of efavirenz and warfarin are presented in Figure 2. Efavirenz showed the mean peak area is 655,648 ± 2.509 µV/s with CV of 0.38%, and retention time is 8.814

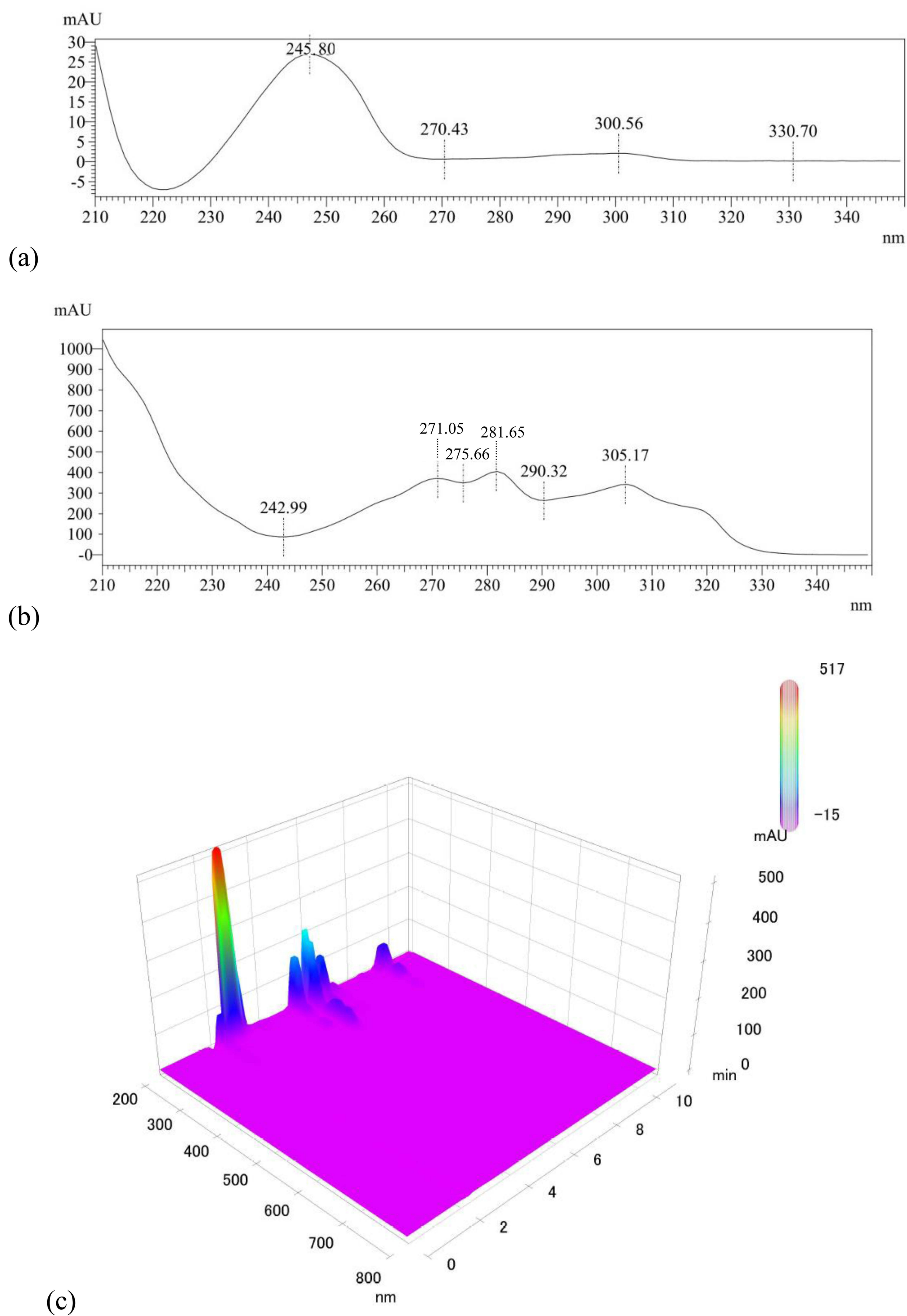


**Figure 1** Spectrum of the Wavelength Optimization Results. (**a**) Efavirenz in multiwavelength spectrum, (**b**) Warfarin in multiwavelength spectrum, (**c**) Plot 3D *Wavelength* Efavirenz-Warfarin in optimum condition.

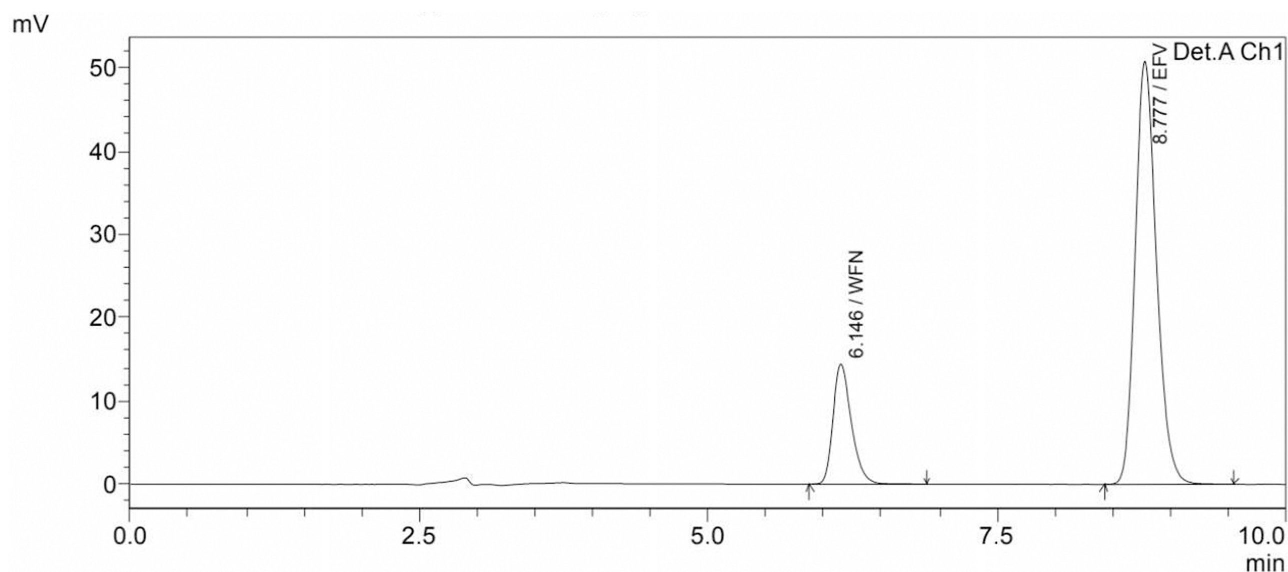


**Figure 2** Representative chromatograms from system suitability test.
**Abbreviations**: WFN, warfarin (6.146 minutes); EFV, efavirenz (8.777 minutes).

± 0.353 min with CV of 0.40%. Warfarin yielded a mean peak area of 163,430 ± 1.654 µV/s with CV of 1.01% and retention time of 6.151 ± 0.181 min with CV of 0.19% (Table 1).

## Optimization of Efavirenz Sample Preparation

Sample preparation optimizations were conducted by comparing the peak area ratio responses of various parameters. The optimization of the sample preparation for efavirenz with various treatments and their results are shown in Table 2. The process began with optimizing the blood spotting volume, which was found to be 30 µL. One critical factor in using DBS for bioanalysis is the effect of hematocrit and blood viscosity from individual samples. Variations in hematocrit significantly affect the peak area ratios in the analysis. Hematocrit represents the relative volume of red blood cells and influences blood viscosity, leading to differences in blood distribution and spread on the card. This correlates with the diameter and volume of the blood spot analyzed.[13] Therefore, optimizing the blood spotting volume is essential during method development when using DBS to

**Table 1** System Suitability Test Results

| No | Area (µV/s) | | Retention Time (min) | |
|---|---|---|---|---|
| | Efavirenz | Warfarin | Efavirenz | Warfarin |
| 1 | 653,361 | 160,149 | 8.777 | 6.146 |
| 2 | 651,731 | 164,310 | 8.798 | 6.159 |
| 3 | 657,341 | 164,626 | 8.804 | 6.159 |
| 4 | 657,820 | 163,846 | 8.812 | 6.163 |
| 5 | 656,281 | 163,490 | 8.881 | 6.116 |
| 6 | 657,357 | 164,160 | 8.809 | 6.162 |
| Mean | 655,648 | 163,430 | 8.814 | 6.151 |
| SD | 2.509 | 1.654 | 0.353 | 0.181 |
| KV (%) | 0.38 | 1.01 | 0.40 | 0.29 |

**Table 2** Optimization of Efavirenz Sample Preparation

| **Blood Volume (µL)** | | **Area (µV/s)** | |
|---|---|---|---|
| | | **Efavirenz** | **Warfarin (IS)** |
| 20 | | 95507 | 21261 |
| 30* | | 136002 | 19619 |
| **Drying time (hour)** | | **Area (µV/s)** | |
| | | **Efavirenz** | **Warfarin (IS)** |
| 1 | | 98885 | 17093 |
| 2* | | 136002 | 19619 |
| 3 | | 121270 | 20094 |
| **Extracting Solution** | | **Area (µV/s)** | |
| | | **Efavirenz** | **Warfarin (IS)** |
| Methanol* | | 90414 | 25075 |
| Acetonitril | | 50215 | 13298 |
| Ethanol | | 73057 | 23521 |
| **Extraction Solution Volume (µL)** | | **Area (µV/s)** | |
| | | **Efavirenz** | **Warfarin (IS)** |
| 300* | | 91797 | 501349 |
| 400 | | 67637 | 247688 |
| 500 | | 49443 | 190121 |
| **Vortexing Time (seconds)** | | **Area (µV/s)** | |
| | | **Efavirenz** | **Warfarin (IS)** |
| 10* | | 98712 | 347892 |
| 15 | | 93210 | 323043 |
| 30 | | 89076 | 343866 |
| **Sonication Time (minutes)** | | **Area (µV/s)** | |
| | | **Efavirenz** | **Warfarin (IS)** |
| 1* | | 100321 | 454921 |
| 3 | | 99012 | 434609 |
| 5 | | 98092 | 409825 |
| **Centrifugation Time (Minutes)** | **Centrifugation Speed (rpm)** | **Area (µV/s)** | |
| | | **Efavirenz** | **Warfarin (IS)** |
| 3 | 5.000 | 90132 | 327520 |
| | 10.000 | 92543 | 324390 |
| 5* | 5.000* | 100684 | 349388 |
| | 10.000 | 92367 | 359429 |

**Note**: *Selected as optimum sample preparation.

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ensure consistent and reliable results. The optimization of DBS drying time was conducted with variations of 1, 2, and 3 hours. The results indicate that a 1-hour drying time gives a lower analyte value compared to a 2-hour drying time. At a 3-hour drying time, there is a decrease in the analyte area response compared to the 2-hour drying time. This may occur due to the decreased stability of the analyte as it is exposed to air for longer periods, affecting the area response and analyte recovery. Based on these results, the optimal drying time selected is 2 hours. Extraction of efavirenz was performed using a protein precipitation method, and it was observed that 300 µL of methanol extracted more efavirenz compared to other extracting solutions. This method offers a simplified sample preparation procedure compared to the previously reported HPLC–PDA method for efavirenz quantification in DBS, which employed 10 mM potassium phosphate buffer with 75% acetonitrile as the extraction solvent.[10] In contrast, the current method utilizes 100% methanol for protein precipitation, eliminating the need for buffer preparation and simplifying solvent handling. Additionally, the optimal conditions for vortexing (10 seconds), sonication (1 minute), and centrifugation (5 minutes at 5000 rpm) were systematically determined to maximize the extraction efficiency of efavirenz from DBS. These parameters were selected based on the highest peak areas obtained for both the analyte and internal standard, along with well-defined chromatographic peak shapes. This streamlined procedure reduces sample preparation time and complexity, thereby increasing its suitability for routine therapeutic drug monitoring applications.

## Method Validation

### LLOQ

The LLOQ determination was validated starting with a concentration of 0.1 µg/mL, which is below 0.2 µg/mL, representing 1/20 of the maximum therapeutic concentration of efavirenz in blood (4 µg/mL). The actual concentration at 0.1 µg/mL was calculated using the calibration curve. The obtained actual concentration at 0.1 µg/mL resulted in a % diff ranging from −12.53% to 9.43%, with a %CV of 10.29%.

### Calibration Curve and Linearity

The calibration curve parameter validation was performed using 8 concentrations: 0.1, 0.5, 1, 5, 10, 15, 20, and 30 µg/mL, along with blank and zero samples. The data from the first replicate of the eight calibration points resulted in a %diff of −2.19% for the LLOQ concentration, and %diff ranging from −3.81% to 8.48% for the other concentrations. These results demonstrate that the calibration curve meets the accuracy and linearity criteria set.[11,12] Additionally, a between-run calibration curve test was conducted to assess the precision of the analysis, and the results are presented in Table 3.

### Selectivity

The interference response at the retention time of efavirenz was 0%, indicating no interference, and the response at the retention time of warfarin, the internal standard, ranged from 0% to 2.13%. These results demonstrate that the analytical method is selective, capable of distinguishing the analyte and internal standard from other components present in the matrix.[11,12]

### Accuracy and Precision

The intraday accuracy %diff ranged from −3.92% to 8.43% with %CV ≤ 4.62% (QCL, QCM, and QCH concentrations) and ranged from 2.58% to 6.28% with a %CV of 2.28% (LLOQ concentration). The interday accuracy %diff ranged from −6.20% to 9.26% with % CV ≤ 3.89% (QCL, QCM, and QCH concentrations) and ranged from −1.23% to 6.28% with %CV of 2.28% (LLOQ concentration). Figure 3 displays chromatograms obtained during the analysis for blank, LLOQ, QCL, QCM, and QCH samples.

**Table 3** Data of Interday Calibration Curve of Efavirenx

| Interday Replicate | Slope | Intercept | R |
|---|---|---|---|
| 1 | 0.0227 | 0.0120 | 0.9991 |
| 2 | 0.0296 | 0.0016 | 0.9998 |
| 3 | 0.0297 | −0.0022 | 0.9996 |

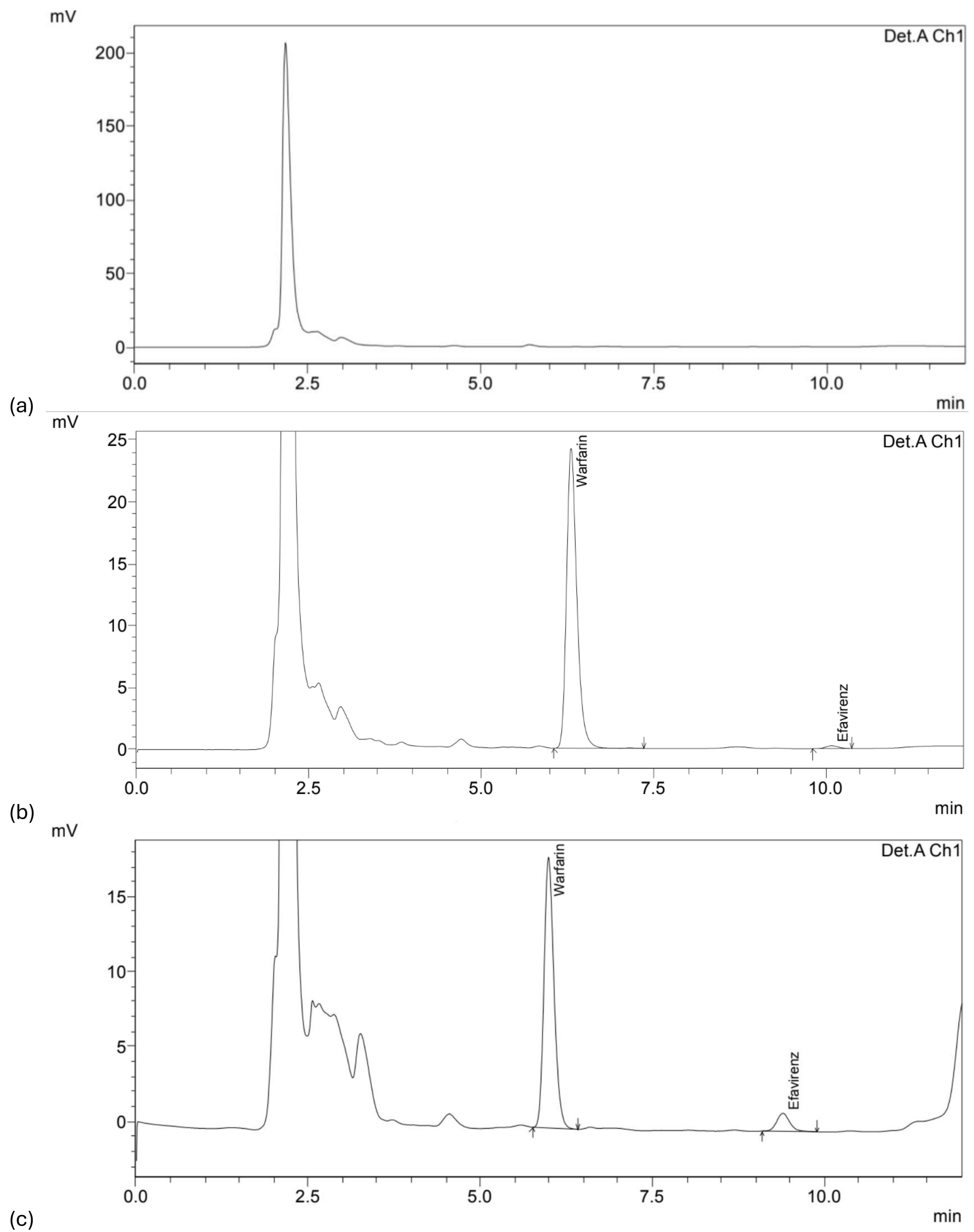


**Figure 3** Continued.

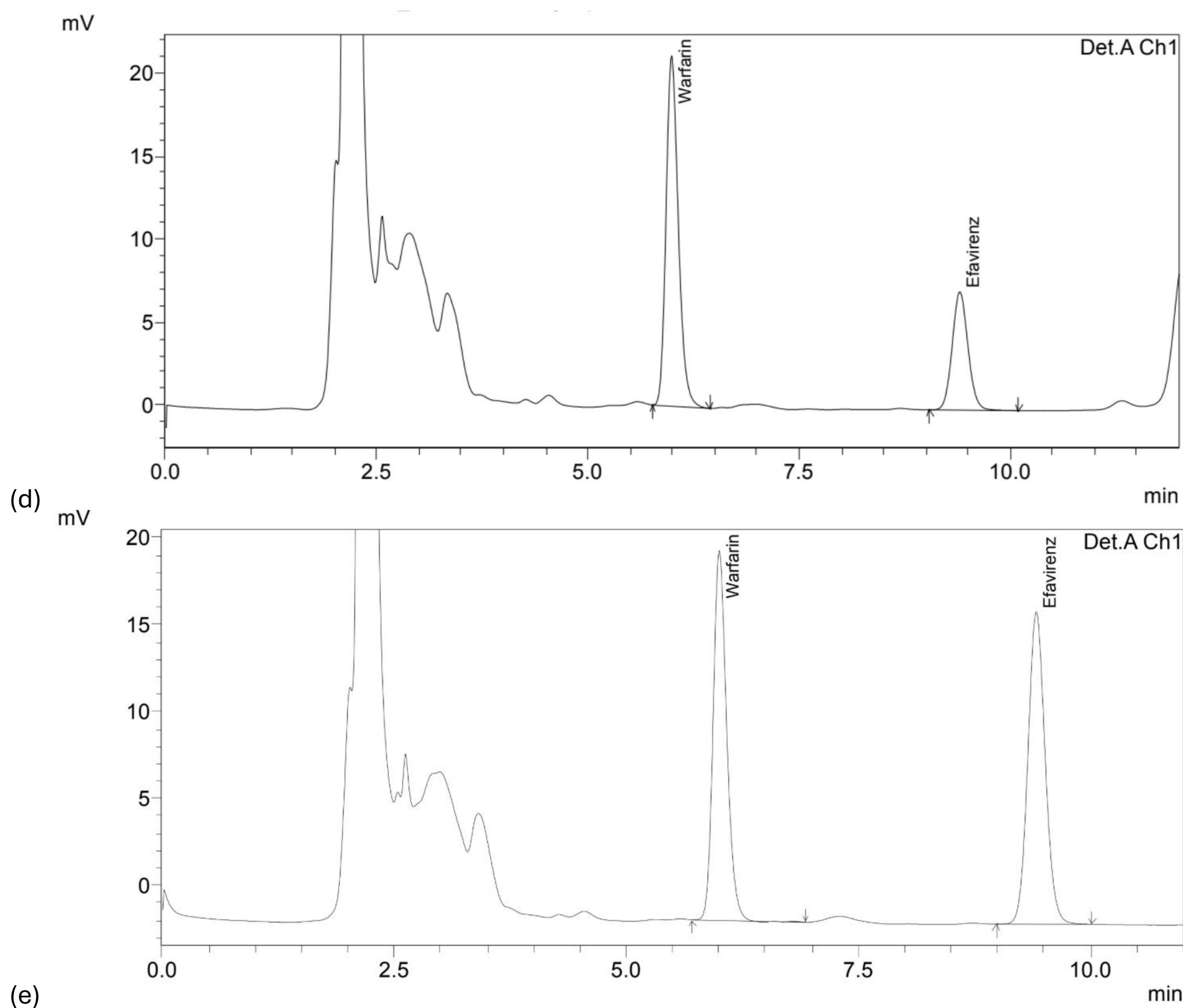


**Figure 3** Representative chromatogram in (**a**) blank, (**b**) LLOQ, (**c**) QCL, (**d**) QCM, and (**e**) QCH.

The within-run and between-run accuracy and precision obtained from this method were overall better than those of the efavirenz analysis method developed previously which did not use an internal standard. In their method, the %diff for within-run accuracy during three days at LLOQ (0.3125 μg/mL) ranged from −1.7% to 9.1%, and for low (QCL), medium (QCM), and high (QCH) validation concentrations, %diff ranged from 3.4% to 9.2%, 2.1% to 11.2%, and −4.3% to 1.5%, respectively. The %diff for the measured concentrations in the between-run test for LLOQ, QCL, QCM, and QCH were 3.64%, 4.76%, 5.12%, and 1.18%, respectively. The between-run precision at LLOQ, QCL, QCM, and QCH ranged from 8.72% to 8.9%.[10] These results suggest that the use of an internal standard improves accuracy and precision, and the method developed using warfarin as an internal standard demonstrated better accuracy and precision than those without it.

## Recovery

In the validation of this method, the average recovery of efavirenz at QCL, QCM, and QCH concentrations were 91.37% ± 6.55%, 95.65% ± 9.08%, and 92.45% ± 4.28%, with %CV values of 7.17%, 9.49%, and 4.36%, respectively. The average recovery of the internal standard warfarin was 88.77% ± 6.26%, with a %CV of 7.05%. These results demonstrate that the developed method meets recovery requirements, and the %CV values indicate that the extraction

and analysis methods are reproducible.[11,12] The overall average recovery of efavirenz for the developed method was 93.16% ± 2.23%. This recovery is higher compared to the previous method, which achieved an average recovery of 91.5%.[10]

## Carry Over

The carryover test results for this method ranged from 0.00% to 11.43% for the efavirenz analyte, and from 0.00% to 0.91% for the internal standard warfarin. Carryover may result from residual analyte remaining on the column or autosampler. However, these results still meet the acceptable carryover criteria.[11,12]

## Dilution Integrity

In the twofold dilution, a %CV of 9.89% and %diff within the range of −9.34% to 9.79% were obtained. In the fourfold dilution, a %CV of 4.83% and %diff within the range of 3.30% to 12.67% were obtained. The %diff and %CV values indicate that dilution does not affect accuracy and precision.[11,12]

## Stability

The results of the stability test are shown in Table 4. The results indicate that both efavirenz and warfarin stock solutions are stable at room temperature for up to 24 hours. However, a decrease in response is observed over time, suggesting that stock solutions should be refrigerated when not in use. Storage at −20°C maintains stability for up to 30 days, despite a slight decrease in response by day 30, which still meets stability criteria. Therefore, during analysis, stock solutions should not be left at room temperature for extended periods and should be refrigerated to maintain stability. All obtained data met stability requirements, indicating that the analyte in DBS samples remained stable at room temperature for 24 hours. Therefore, DBS sample preparation can be performed at room temperature. The results also indicate that DBS samples stored at room temperature or in a refrigerator at −20°C remain stable for up to 30 days. If this method is used to analyze drug levels in the blood of patients taking efavirenz, blood samples collected using DBS can be stored and analyzed up to 30 days after collection. The stability of the autosampler was assessed at 0, 6, and 24 hours to determine the maximum time samples could be analyzed and stored in the autosampler. The results indicate that samples remain stable during autosampling for up to 24 hours.

**Table 4** Stability Test Results

| **Stability Test** | | **Stable to-** | **Accuracy (%diff)** | **Precision (%CV)** |
|---|---|---|---|---|
| Short-term stock solution stability | Efavirenz | 24 hours | −3.78 to −2.97 | 0.81 |
| | Warfarin | | −3.37 to −1.75 | 0.83 |
| Long-term stock solution stability | Efavirenz | 30 days | −9.61 to −9.06 | 0.30 |
| | Warfarin | | −1.42 to −1.29 | 0.35 |
| Short-term EFV in DBS sample stability | | 24 hours | −14.09 to −11.84 (QCL) | 1.32 (QCL) |
| | | | 6.62 to 8.61 (QCH) | 1.02 (QCH) |
| Bench-top EFV in DBS sample stability | | 30 days | −14.34 to −0.87 (QCL) | 8.58 (QCL) |
| | | | −14.28 to −9.34 (QCH) | 3.19 (QCH) |
| Long-term EFV in DBS sample stability (freezer at −20°C) | | 30 days | −3.49 to 2.22 (QCL) | 2.83 (QCL) |
| | | | 5.09 to 9.89 (QCH) | 2.37 (QCH) |
| Autosampler stability | | 24 hours | −0.87 to 3.28 (QCL) | 2.06% (QCL) |
| | | | 6.93 to 8.82, (QCH) | 0.99 (QCH) |


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### Reinjection Reproducibility

Accuracy was achieved from the reinjection of the calibration curve, with a %diff of −10.47% for the LLOQ and ranging from −7.74% to 10.80% for other calibration points. Testing on QCL, QCM, and QCH samples resulted in %diff values ranging from 1.35% to 12.87%, with a %CV of ≤4.58%. These results demonstrate that the method meets the reproducibility requirements for injection repeatability, allowing for recalibration and reinjection of samples if any issues arise during analysis.[11,12] Compared to LC–MS/MS-based assays, this HPLC–PDA method provides a cost-effective alternative with sufficient sensitivity (LLOQ = 0.1 µg/mL vs 0.05 µg/mL in LC–MS/MS). While less sensitive, it demonstrates excellent reproducibility and accessibility for routine monitoring in low-resource settings.

## Discussion

Based on the validation results, the developed method for analyzing efavirenz in DBS using warfarin as an internal standard meets all the validation requirements established by the European Medicines Agency (2022) and the US Food and Drug Administration (2018). Full validation was conducted, covering parameters such as sensitivity, calibration curve linearity, selectivity, accuracy, precision, recovery, carryover, dilution integrity, stability, and reproducibility of repeated injections. Compared to the study by previous method, which reported a recovery rate of 91.5% for efavirenz, the developed method demonstrates superior extraction efficiency, as indicated by the higher recovery rate achieved.[10] This method also has a LLOQ of 0.1 µg/mL and shows linearity across the concentration range of 0.1–30 µg/mL, with lower %diff and %CV values. These results reflect a more sensitive approach compared to previous methods which had an LLOQ of 0.3 µg/mL.[10] A detailed comparison of the %diff and %CV values is provided in Accuracy and Precision.

However, the method was validated exclusively using efavirenz-spiked whole blood, without evaluating its performance using real patient-derived samples. Efavirenz undergoes extensive hepatic metabolism and exhibits high plasma protein binding, and it may undergo other changes in vivo that could influence extraction efficiency and chromatographic behavior. Although these alterations are unlikely to significantly affect the results, they cannot be ruled out. Therefore, the method's performance in real clinical conditions remains uncertain. For context, the previous method has validated theirs using whole blood and plasma from HIV-positive patients undergoing efavirenz therapy, which may better reflect the true clinical environment. Further research using patient-derived samples is necessary to confirm the method's applicability in clinical practice.

This study was limited to in-vitro validation using spiked blood samples; therefore, clinical validation using patient-derived DBS is needed to confirm its applicability for therapeutic drug monitoring. Future studies should also assess matrix effects from different hematocrit levels and storage conditions.

## Conclusion

The method developed for analyzing efavirenz in DBS using warfarin as an internal standard meets all the validation requirements set by EMA 2022 and showed linearity within the concentration range of 0.1–30 ug/mL. This method can be applied for therapeutic drug monitoring in the future.

## Data Sharing Statement

Data included in article/referenced in article.

## Ethical Approval

The study was based on anonymized secondary data and did not involve any direct human participation. Therefore, it is exempt from ethical approval in accordance with the institutional review policy.

## Disclosure

The authors declare no conflicts of interest in this work.

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