

Research Article

A Simple RP-HPLC Method to Simultaneously Assay the Contents of Lamivudine, Tenofovir, and Nevirapine in Fixed Dose Combined Oral Antiviral Medicines

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Received 3 July 2020; Accepted 10 August 2020; Published 8 September 2020

Academic Editor: Patricia E. Allegretti

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An accurate and rapid reverse HPLC method has been developed and validated for the simultaneous quantification of lamivudine, nevirapine, and tenofovir disoproxil fumarate. Suitable separation was achieved on Phenomenex Synergi C18 (250 × 4.6 mm, 4 μm) using mobile phase, methanol (50%): ammonium acetate buffer (adjusted to pH 2.80) (40%): acetonitrile (10%) in an isocratic mode. The drugs were detected at 270 nm with a flow rate of 1.0 ml/min, and the retention times were found to be 3.26, 5.42, and 7.55 minutes for lamivudine, nevirapine, and tenofovir disoproxil fumarate, respectively. The developed method was validated per ICH guidelines. Good linearity was obtained within the concentration ranges of 10–59 μg/ml, 7–42 μg/ml, and 15–90 μg/ml with a correlation coefficient of not less than 0.990. The % RSD values for precision (intraday and interday) and accuracy studies were found to be less than 2%. The results obtained from quantitative analysis conform to USP content requirements for marketed tablet dosage forms, RICOVIR-LN, and tenofovir disoproxil fumarate/lamivudine tablets. The method is therefore useful for routine quality control of antiretroviral tablet dosage forms containing tenofovir disoproxil fumarate, lamivudine, and nevirapine.

1. Introduction

HIV/AIDS is a major public health issue and as such forms a significant part of the Sustainable Development Goals, with the aim of ensuring healthy lives and promoting well-being for all at all ages [1]. The introduction of highly active antiretroviral therapy (HAART), a treatment regimen comprising of the combination of three or more antiretroviral drugs [2], has revolutionized the management of the disease condition, with the resultant dramatic reduction in mortality rates and the incidences of opportunistic infections [3]. In Ghana and other parts of the world, three of the widely used antiretrovirals (ARVs) in HAART include lamivudine (3TC), nevirapine (NVP), and tenofovir

disoproxil fumarate (TDF). It may be argued that a lasting impact on morbidity and mortality on people living with HIV/AIDS (PLWHA) could be assured if the quality of ARVs was adequately monitored. With recent reports of substandard medicines available in Ghanaian health facilities [4], it has become particularly important to increase surveillance on the quality of all medicines procured for public health facilities, especially for antiretrovirals, used for a significant public health intervention programme. This therefore calls for the development and validation of reliable analytical methods to achieve such quality control purposes.

In the past, most of the methods developed had focused on the detection and quantitation of ARVs in biological samples [5, 6]; just a handful had concentrated on methods

for the oral dosage formulations [7–9]. In as much as these methods have provided useful tools to assess the qualities of some ARVs, it is necessary that very efficient alternative methods with the target scope of ARVs in the HAART programme be developed to achieve similar quality control outputs. The aim of the current study therefore was to develop a simple, affordable, and reliable method for the quality assessment of ARVs used in the HAART programme in Ghana and other parts of the world, where applicable. Thus, the method when developed would be used for the analyses of 3TC, NVP, and TDF in monotherapies and fixed-dose combination products as well.

3TC is chemically known as 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1H)-one [10]. It is a nucleoside reverse transcriptase inhibitor and is available for the treatment of HIV-I and HIV-II infections, as well as hepatitis B virus [11]. TDF is a derivative of adenosine *S'*-monophosphate and is a prodrug [11]. Chemically, it is known as [[[(1R)-2(6-amino-9H-purin-9-yl)-methylethoxy] methyl] phosphonate, bis (isopropoxyloxycarbonyloxymethyl ester), fumarate (1:1). It inhibits both HIV-I and HIV-II. It is a nucleotide analogue that is available for use in antiretroviral therapy [11]. NVP, on the other hand, belongs to the non-nucleoside reverse transcriptase inhibitor class of ARVs. Its chemical name is 11-cyclopropyl-4-methyl-5, 11-dihydro [3, 2b: 2', 3'-e] [1,4] diazepin-6-one hemihydrate. It is approved for the treatment of HIV infections in adults and children in combination with other antiretroviral agents [11].

2. Materials and Methods

2.1. Standards and Samples. The working standards used for the study included confirmed 3TC (assay: 102.0%), NVP (assay: 100.1%), and TDF (assay: 100.2%) (Table 1), which were donated by Danadams Pharmaceutical Industry Limited, Spintex, Ghana. The drug products, samples A1 (containing TDF 300 mg/3TC 300 mg tablets + NVP 200 mg) and A2 (containing TDF 300 mg/ 3TC 300 mg) both claimed to be manufactured by Mylan Laboratories Limited, India, were used.

2.2. Chemicals and Reagents. The reagents and solvents used to prepare the samples and carry out the analyses were of analytical and HPLC grades, respectively. Solvents including acetonitrile and methanol were procured from Fisher Scientific, United Kingdom. Ammonium acetate and glacial acetic acid purchased from VWR International Limited and Merck House, respectively, were also used for the analysis. Purified water was freshly produced in-house, terminally sterilized with ultraviolet radiation, and filtered through a 0.45 μm membrane filter before being used to prepare all solutions and buffers.

2.3. Equipment and Instrument. The HPLC system used comprised of Shimadzu prominence UFLC series system, consisting of LC-20A quaternary pump (part G1311 A), DGU-20A₅ in-line vacuum degasser (part no. G1322A), and

SPD-20A ultraviolet detector. Data acquisition was performed by LC solutions software (version A.10.02 Build 1757). The chromatographic separation was carried out using on a C18 Phenomenex Synergi column (250 \times 4.6 mm; 4 μm). An electronic analytical balance (Mettler Toledo, AB204-S/FACT), a digital pH meter (Mettler Toledo Seven Compact pH/Ion S220), and a sonicator were also used.

2.4. Preparation of Solutions

2.4.1. Buffer Preparation. A 500 ml of 0.02 M ammonium acetate buffer was prepared by weighing a determined quantity of ammonium acetate powder, dissolving with some amount of distilled water, and transferred into a 500 ml volumetric flask. The pH was adjusted to 2.8 with acetic acid, and with continuous stirring, the solution was topped up with distilled water to the required volume. It was then filtered with a 0.45 μm membrane filter.

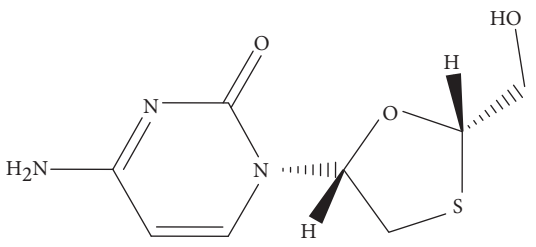
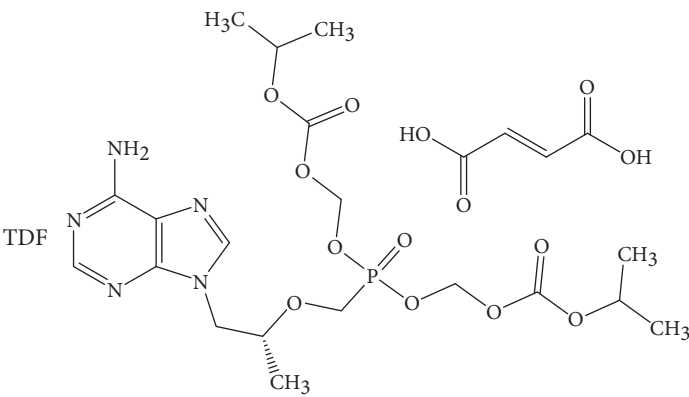
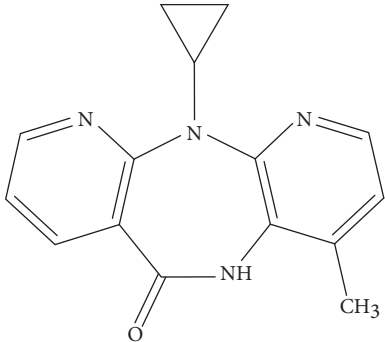
2.4.2. Mobile Phase/Diluent Preparation. The solvent system used was a mixture of buffer (pH = 2.8), methanol, and acetonitrile in the ratio 40:50:10 (v/v), respectively. This solution was also used as the diluent to prepare solutions of the standards and samples.

2.4.3. Preparation of Standard Solutions. A stock standard solution containing 75 mg of 3TC, 35 mg of NVP, and 50 mg of TDF was prepared in a 100 ml volumetric flask. The content in the flask was sonicated at 37°C for 10 minutes before topping up to the required volume with the diluent, after cooling. The resulting solution was filtered with a 0.45 μm membrane filter. 3 ml of the stock solution was pipetted to prepare 50 ml of working standard solution containing 45 $\mu\text{g/ml}$ of 3TC, 21 $\mu\text{g/ml}$ of NVP, and 30 $\mu\text{g/ml}$ of TDF.

2.4.4. Preparation of Sample Solutions. Solutions of powdered product samples A (containing TDF and 3TC) and B (containing two formulations, with B₁ containing TDF and 3TC and B₂ containing NVP) were prepared for analysis. For samples A and B₁, a weight of each containing an equivalent of 75 mg of 3TC was weighed and transferred into a 100 ml volumetric flask. About 50 ml of methanol was added, sonicated for 10 minutes at 37°C, and made up to volume with the diluent. The resulting solution was then filtered through a Whatman No. 1 filter paper, discarding the first 5 ml of the filtrate. 3 ml of the resulting filtrate was pipetted and transferred into a 50 ml volumetric flask and made up to volume using the diluent. For sample B₂, a weight containing an equivalent of 35 mg of NVP was transferred into a 100 ml volumetric flask and a similar procedure as described above was employed to prepare the sample B solution.

2.5. Development and Validation of Method. The method for detection, separation, and quantitation of the three antiretroviral drug substances in the product was developed empirically (S1), and validated in accordance with the

TABLE 1: Chemical data on drug substances considered in the study.

Drug substance	Code	Chemical structure	Mol wt. (g/mol)	cLog P	pKa	Solubility
Lamivudine	3TC	 <chem>C8H11N3O3S</chem>	229.3	-1.46	13.90	Soluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96%)
Tenofovir disoproxil fumarate	TDF	 <chem>C23H34N5O14P</chem>	635.5	2.65	3.75	Slightly soluble in water, soluble in methanol, very slightly soluble in dichloromethane
Nevirapine	NVP	 <chem>C15H14N4O</chem>	266.3	2.65	2.8	Practically insoluble in water, sparingly soluble or slightly soluble in methylene chloride, slightly soluble in methanol

International Council for Harmonisation (ICH) Q2(R) guidelines [12]. The validation parameters investigated included linearity and its range, limits of detection and quantitation, specificity, stability of test solution, robustness, accuracy, and precision.

2.5.1. Specificity/Selectivity. Specificity and selectivity were assessed by comparing the chromatograms from a matrix without expected analytes (blank sample) with that of matrices containing the expected analytes (that is, 3TC, NVP, and TDF) [13, 14]. The selectivity/specificity was

confirmed by comparing mean \pm SD of the retention times for the analytes using one-way ANOVA followed by Tukey's *post hoc* test [15].

2.5.2. Accuracy. Accuracy was investigated by determining recoveries of the analytes at three concentration levels (80%, 100%, and 120%) [16, 17].

2.5.3. Precision. Precision was demonstrated by determining repeatability (intraday precision) and intermediate precision of the method. Intraday precision was confirmed

from the peak areas of the analytes, from triplicate injections of three dilute concentrations of the standard solution within the same day (that is, 15 $\mu\text{g/ml}$, 45 $\mu\text{g/ml}$, and 90 $\mu\text{g/ml}$ for 3TC; 7 $\mu\text{g/ml}$, 21 $\mu\text{g/ml}$, and 42 $\mu\text{g/ml}$ for NVP; and 10 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, and 59 $\mu\text{g/ml}$ for TDF). Intermediate precision, on the other hand, involved studying the variation in response at 100% concentration of the working standard on three different days. The results obtained were statistically analysed by determining the relative standard deviations and carrying out ANOVA, where applicable [12].

2.5.4. Linearity and Range. The linearity of the developed method was investigated by injecting six concentrations prepared from the stock standard solution, to obtain concentrations for 3TC, NVP, and TDF within the ranges of 15 $\mu\text{g/ml}$ –90 $\mu\text{g/ml}$, 7 $\mu\text{g/ml}$ –42 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$ –59 $\mu\text{g/ml}$, respectively [12]. Triplicate determinations were carried out for each test concentration and the peak areas, reported as mean \pm SD were plotted against test concentrations. Statistical analysis was performed by the least-squares method [13]. Linearity was predicted by estimating the regression coefficient (R^2) and the linear regression y -intercept of the response versus concentration plot. The regression model was also tested for fitness by determining the level of significance of the F -value of the model in an ANOVA at 5% risk level [13]. Additionally, the residual plots for the sets of test data were generated.

2.5.5. Limit of Detection (LOD) and Limit of Quantitation (LOQ). The limits of detection and quantification were determined from the intercept on the y -axis slope from the linear regression model derived from the linearity test [12]. The formulae below were used for calculating the LOD and LOQ. The results are shown in Table 2.

$$\text{LOD} = \frac{3.3 \times \text{standard deviation of the response}}{\text{slope of the calibration curve}}, \quad (1)$$

$$\text{LOQ} = \frac{10 \times \text{standard deviation of the response}}{\text{slope of the calibration curve}}.$$

2.5.6. Robustness. The robustness of the developed method was tested by monitoring the effects of deliberate changes in the flow rate (± 0.1 ml/min) and the wavelength of detection (± 2 nm) on the general performance of the developed method [13, 18].

2.5.7. Stability of Test Solution. The stability of the working standard solution was assessed over a 6-hour period at room temperature by monitoring the peak areas of the drug substances with time [13].

2.6. Analysis of Commercial Products. The contents of 3TC, NVP, and TDF in samples A, B₁, and B₂ as prepared above were assessed from peak areas from triplicate injections of

the samples and applying the linear regression models obtained to the recorded peak areas.

3. Results and Discussion

3.1. Method Development. The chromatographic method was developed empirically, guided by the physicochemical properties like acid dissociation constant (pKa) and partition coefficient (cLogP) (Table 1). The conditions comprising suitable column, mobile phase composition, wavelength for analyte detection, flow rate, column temperature, and injection volume were determined empirically by monitoring the resolution, peak symmetry, and run time for the analytes (S1). The investigations resulted in the choice of a mobile phase system consisting of methanol (50%), ammonium acetate buffer pH of 2.8 (40%) and acetonitrile (10%), and a flow rate of 1 ml/min, among others (Table 3), which were then used to develop the chromatographic method (Figure 1).

3.2. Method Validation. The results from the validation are summarized in Table 2. In establishing the specificity and selectivity of the developed method, the chromatogram generated from the blank sample showed only noises, with no apparent peak observed within 0–20 minutes (S2). Upon injection of the working standard solutions (containing 3TC, NVP, and TDF), three resolved peaks were observed. It was further shown that the retention times for each of the analytes were different from each other ($p < 0.0001$; Table 2). The outcome showed that the method was capable of independently detecting the three analytes and distinguishing one from the other, thus specific and selective. In order to evaluate the quantifying power of the method, its accuracy was also determined. It was shown that the percentage recoveries obtained, complied with the acceptance criteria of 98%–102% [13, 14] (with minimum deviations) over the concentration range, 80%–120% of test concentration for the three analytes (Tables 2 and S3).

The responses for 3TC, NVP, and TDF were shown to be linear within the ranges 15 $\mu\text{g/ml}$ –90 $\mu\text{g/ml}$, 7 $\mu\text{g/ml}$ –42 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$ –59 $\mu\text{g/ml}$, respectively (Table 2 and Figure 2). The regression coefficient of correlation (R^2) obtained for the abovementioned analytes was 0.9959, 0.9948, and 0.9971, respectively. F -values from the linear regression models were also shown to be significant, further demonstrating strong correlations between the concentration of the analytes and peak area responses (Table 2). The method was shown to detect at least 5.5032 $\mu\text{g/ml}$, 3.1496 $\mu\text{g/ml}$, and 3.9267 $\mu\text{g/ml}$ of 3TC, NVP, and TDF, respectively. However, quantitation could only be carried out with the method when their concentrations were at least 16.6762 $\mu\text{g/ml}$, 9.5443 $\mu\text{g/ml}$, and 10.0433 $\mu\text{g/ml}$, respectively (Table 2).

Method precision was demonstrated with repeatability and intermediate precision (Tables 2 and S4). It was observed in both evaluations that the RSDs for the method responses (peak areas) were less than 2%, proving their consistency and precision [12, 13]. In addition, it was

TABLE 2: Results from validation carried out on the developed method.

Validation parameter	3TC	NVP	TDF	Remarks
Specificity/ selectivity (S2)	3.269 ± 0.0074 min (N = 5)	5.423 ± 0.0015 min (N = 5)	7.555 ± 0.0024 min (N = 5)	Retention times are significantly different from each other ($F_{(2,12)} = 1.102e + 006$; $p < 0.0001$). Method selectively and specifically identifies the three analytes.
Accuracy (S3)	36 µg/ml = 99.30% ± 0.34 (N = 3) 45 µg/ml = 100.80% ± 0.32 (N = 3) 54 µg/ml = 97.90% ± 0.46* (N = 3)	16.8 µg/ml = 102.99% ± 0.45* (N = 3) 21 µg/ml = 99.71% ± 0.72 (N = 3) 25.2 µg/ml = 98.22% ± 0.51 (N = 3)	24 µg/ml = 101.75% ± 0.28 (N = 3) 30 µg/ml = 98.58% ± 0.71 (N = 3) 36 µg/ml = 98.18% ± 0.26 (N = 3)	Developed method accurately estimates the content of the analytes. Acceptance criteria: [98.00%–102.00%].
Linearity and range	Regression equation $Y = 14487x + 38521$ $R^2 = 0.9959$ $Sy.x = 25214$ F -value, p value 3900, $p < 0.0001$ Range 15 µg/ml–90 µg/ml	$Y = 9363x + 11417$ 0.9948 8543 3090, $p < 0.0001$ 7 µg/ml–42 µg/ml	$Y = 5283x + 8162$ 0.9971 5062 5429, $p < 0.0001$ 10 µg/ml–59 µg/ml 3.9267 µg/ml	Acceptance criteria: [$R^2 > 0.9900$] linearity of responses established for the specified analytes concentration ranges.
LOD	5.5032 µg/ml	3.1496 µg/ml	3.9267 µg/ml	
LOQ	16.6762 µg/ml	9.5443 µg/ml	10.0433 µg/ml	
Precision (S4)	Repeatability (3 conc terms) RSD = 0.4000% ± 0.2291, N = 3 Intermediate precision (3 analysts for 3 days) RSD = 0.52%–1.70%, N = 9; $F_{(4,18)} = 1.786$, $p = 0.1756$	RSD = 0.7967% ± 0.342, N = 3 RSD = 0.41%–1.94%, N = 9; $F_{(4,18)} = 2.890$, $p = 0.0520$.	RSD = 0.9467% ± 0.6704, N = 3 RSD = 0.29%–1.63%, N = 9; $F_{(4,18)} = 0.08900$, $p = 0.9847$	Acceptance criteria: [RSD < 2.0%]. Two-way ANOVA showed that the differences in the responses produced from the different concentrations on different days not were significant ($p \geq 0.05$). Developed method responses were precise.
Robustness (S5)	(i) Retention time = 3.416 ± 0.019 min and 3.131 ± 0.131 min at 0.90 ml/min and 1.1 ml/min, respectively. (ii) % deviation of peak area < ±5%	(i) Retention time = 8.375 ± 0.033 min and 3.417 ± 0.022 min at 0.90 ml/min and 1.1 ml/min, respectively. (ii) % deviation of peak area < ±5%	(i) Retention time = 9.573 ± 0.011 min and 6.783 ± 0.036 min at 0.90 ml/min and 1.1 ml/min, respectively. (ii) % deviation of peak area < ±5%	Deliberate changes in flow rate resulted in retention times of the analytes maintaining their distinction from each other. Hence, peaks maintained resolutions at 0.90 ml/min [$F_{(2,6)} = 60322$; $p < 0.0001$] and 1.10 ml/min [$F_{(2,6)} = 1949$; $p < 0.0001$]. Deliberate changes in the wavelength detection did not show significant differences in peak areas (RSD < 2%; % deviation < 5%). In addition, proportionate detection was maintained ($p > 0.005$). Method is fairly robust.

*Minimal deviations of results from acceptance criteria.

TABLE 3: Optimised chromatographic conditions adopted for the validation.

Condition	Description
Column	Phenomenex Synergi C18 (250 × 4.6 mm, 4 μm)
Mobile phase	Acetonitrile (10%): ammonium acetate buffer (adjusted to pH 2.8) (40%): methanol (50%)
Diluent	Acetonitrile (10%): ammonium acetate buffer (adjusted to pH 2.8) (40%): methanol (50%)
Flow rate	1 ml/min
Column temperature	25°C
Injection volume	10 μL
Wavelength	270 nm

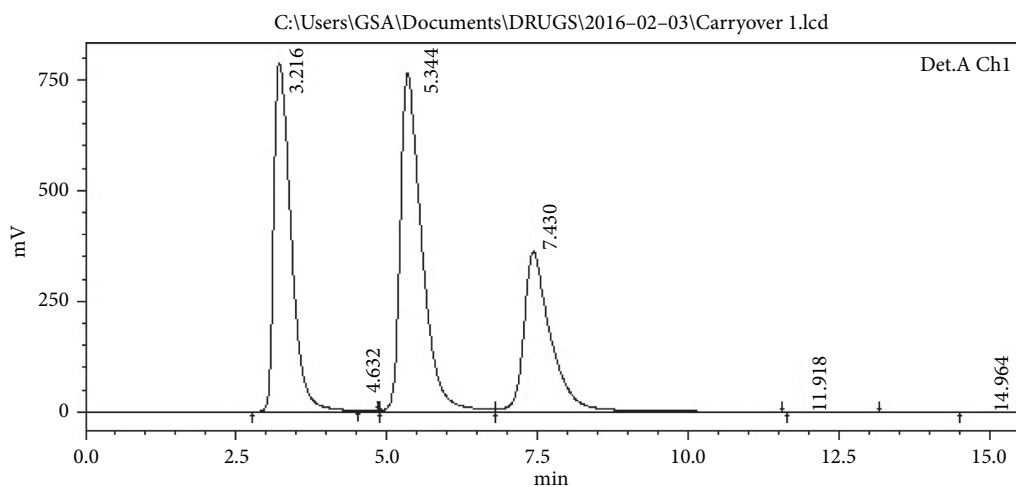
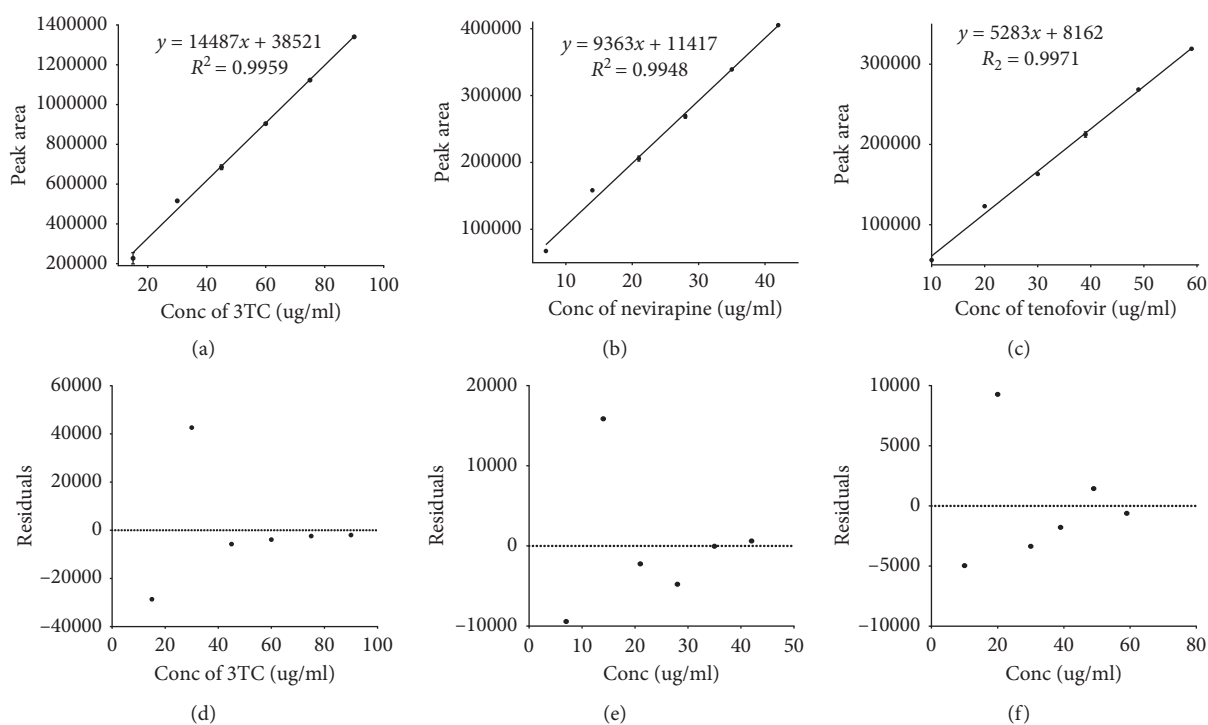


FIGURE 1: Chromatogram showing optimised separation of lamivudine (3.216 min), nevirapine (5.344 min), and tenofovir disoproxil fumarate standard (7.430 min) (from left to right) using the developed method.

FIGURE 2: Proof of linearity of the developed method. (a) A₁, (b) B₁, (c) C₁, (d) A₂, (e) B₂, and (f) C₂.

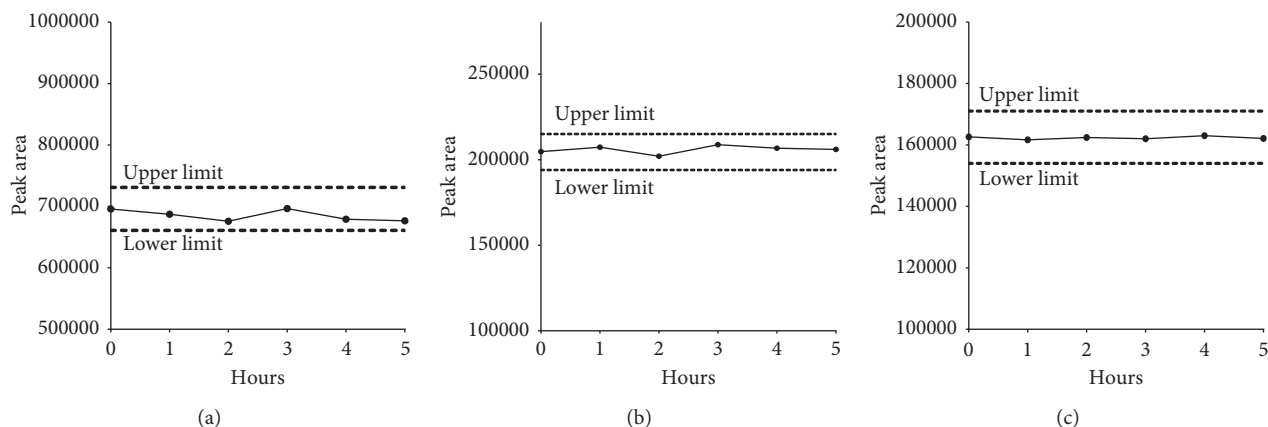


FIGURE 3: Stability of analytes in test solution within 6-hour period. (a) 3TC, (b) NVP, and (c) TDF.

TABLE 4: Percentage content obtained for sampled products.

	Analyte	Content (%)	Acceptance criteria (%)	Inference
Sample A	TDF	97.50 ± 0.75	90–110	Passed
	3TC	97.72 ± 0.09		Passed
Sample B ₁	TDF	98.55 ± 0.17	90–110	Passed
	3TC	105.33 ± 0.85		Passed
Sample B ₂	NVP	99.20 ± 1.17		Passed

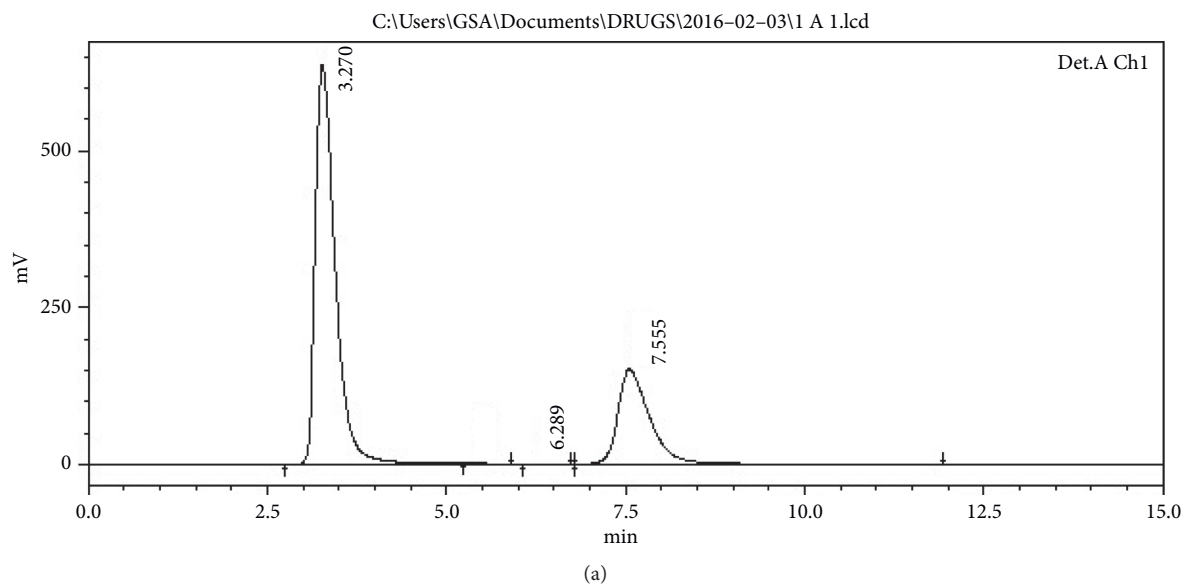


FIGURE 4: Continued.

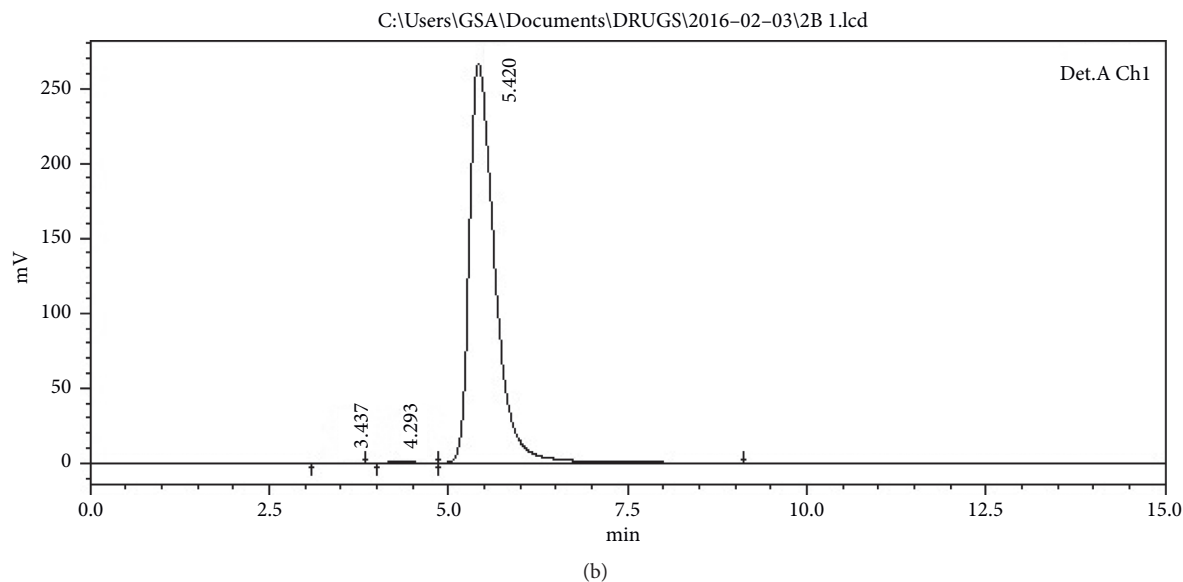


FIGURE 4: Chromatogram showing the peaks of lamivudine and tenofovir in a sample B₁ and nevirapine in sample B₂. Retention times are 3.27 and 7.55 minutes, and 5.42 minutes, respectively.

observed from the intermediate precision determinations that the peak areas recorded on different days for the different concentrations adopted were not significantly different from each other ($F_{(4,18)} = 0.08900$, $p = 0.9847$) (Tables 2 and S4).

In testing for the robustness of the method, it was observed that deliberate changes in the flow rate did not significantly affect the resolution of the peaks, as they remained well separated from each other ($F_{(2,6)} = 60322$; $p < 0.0001$ and $F_{(2,6)} = 1949$; $p < 0.0001$). Upon changes effected to the wavelength of detection, the deviations observed were also not significant (that is, % deviations $< \pm 5\%$) (Tables 2 and S5). Thus, the method was considered robust. The analytes in the test solution were also found to be stable within the period of analysis, which in the current study was estimated to be 6 hours (Figure 3).

3.3. Assay of Sampled Products. The validated method demonstrated its usability in the analysis of commercial products. The outcome of such investigation showed that the products complied with content assay specifications as indicated in the United States Pharmacopeia [19] (Table 4 and Figure 4).

4. Conclusion

An accurate, precise, and selective reverse HPLC method for the simultaneous estimation of lamivudine, nevirapine, and tenofovir disoproxil fumarate has been developed and validated. The method was validated per the ICH guidelines and passed all tests for the various validation parameters. The developed method is specific and selective as well as robust, accurate, and precise. The HPLC method was successfully applied to quantitatively estimate the active pharmaceutical ingredients in

commercial products A₁ (containing TDF 300 mg/ 3TC 300 mg tablets + NVP 200 mg) and A₂ (containing TDF 300 mg/ 3TC 300 mg).

Data Availability

Processed data used to support the findings of this study are included within the article. Some of the data related to the validation of the developed method are also included within the article while others are included as Supplementary Data (S1–S5).

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

The authors are grateful to Danadams Pharmaceutical Industry Limited, Spintex, Accra, for the donation of the pure antiretroviral APIs used in this research. The authors are also thankful to Mr. Brown of Ghana Standards Authority for providing HPLC instrument and all reagents used.

Supplementary Materials

S1: investigations carried out to determine suitable chromatographic conditions in method development. S2: specificity/selectivity. S3: results for accuracy studies involving analytes. S4: precision repeatability—triplicate injections of three different concentrations of the analytes, to investigate the effect of concentration change on the precision of the method. S5: robustness examining effects from change in flow rate. (*Supplementary Materials*)

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