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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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 anti-retroviral 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]. Chemoically, it is known as [(1R)-2-(6-amino-9H-purin-9-yl)-methyl] phosphonate, bis (isopropylxyloxy)carbonyloxymethyl 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-14-methyl-5,11-dihydroxanthan [3, 2b: 2, 3'-e] [1,4] diazein-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 300mg/3TC 300mg 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 x 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 µg/ml of 3TC, 21 µg/ml of NVP, and 30 µg/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
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 ± 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 µg/ml, 45 µg/ml, and 90 µg/ml for 3TC; 7 µg/ml, 21 µg/ml, and 42 µg/ml for NVP; and 10 µg/ml, 30 µg/ml, and 59 µg/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 µg/ml–90 µg/ml, 7 µg/ml–42 µg/ml, and 10 µg/ml–59 µg/ml, respectively [12]. Triplicate determinations were carried out for each test concentration and the peak areas, reported as mean ± SD were plotted against test concentrations. Statistical analysis was performed by the least-squares method [13]. Linearity was predicted by estimating the regression coefficient (R²) 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.

\[
\text{LOD} = \frac{3.3 \times \text{standard deviation of the response}}{\text{slope of the calibration curve}},
\]

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

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 showed to be linear within the ranges 15 µg/ml–90 µg/ml, 7 µg/ml–42 µg/ml, and 10 µg/ml–59 µg/ml, respectively (Table 2 and Figure 2). The regression coefficient of correlation (R²) 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 µg/ml, 3.1496 µg/ml, and 3.9267 µg/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 µg/ml, 9.5443 µg/ml, and 10.0433 µg/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) | Retention time (mean ± SD) | 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 \( (F_{3,12} = 1.102e + 0.06; \ p < 0.0001) \). Method selectively and specifically identifies the three analytes. |
| Accuracy (S3) | % recovery (mean ± SD) | 36 µg/ml = 99.30% ± 0.34 (N = 3) | 16.8 µg/ml = 102.99% ± 0.45* (N = 3) | 24 µg/ml = 101.75% ± 0.28 (N = 3) | Developed method accurately estimates the content of the analytes. Acceptance criteria: [98.00%–102.00%]. |
| Linearity and range | Regression equation | \( Y = 14487x + 38521 \) | \( Y = 9363x + 11417 \) | \( Y = 5283x + 8162 \) | Acceptance criteria: \([R^2 > 0.9900]\) linearity of responses established for the specified analytes concentration ranges. |
| | R\(^2\) | 0.9959 | 0.9948 | 0.9971 | |
| | Sy.x | 25214 | 8543 | 5062 | |
| | F-value, p value | 3900, \( p < 0.0001 \) | 3090, \( p < 0.0001 \) | 5429, \( p < 0.0001 \) | |
| | Range | 15 µg/ml–90 µg/ml | 7 µg/ml–42 µg/ml | 10 µg/ml–59 µg/ml | |
| 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, \( N = 3 \) | RSD = 0.7967% ± 0.342, \( N = 3 \) | RSD = 0.9467% ± 0.6704, \( N = 3 \) | 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. |
| | Intermediate precision (3 analysts for 3 days) | RSD = 0.52%–1.70%, \( N = 9; \ F_{(4,18)} = 1.786, p = 0.1756 \) | RSD = 0.41%–1.94%, \( N = 9; \ F_{(4,18)} = 2.890, p = 0.0520. \) | RSD = 0.29%–1.63%, \( N = 9; \ F_{(4,18)} = 0.08900, p = 0.9847 \) | |
| Robustness (S5) | (i) Retention time \( = 3.416 \pm 0.019 \) 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 \( = 8.375 \pm 0.033 \) 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 \( = 9.573 \pm 0.011 \) 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    |                         | Passed    |
|                        | 3TC     | 97.72 ± 0.09    |                         | Passed    |
| Sample B₁              | TDF     | 98.55 ± 0.17    | 90–110                  | Passed    |
|                        | 3TC     | 105.33 ± 0.85   |                         | Passed    |
| Sample B₂              | NVP     | 99.20 ± 1.17    |                         | Passed    |

Figure 4: Continued.
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 ob-
erved 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, nevir-
apine, and tenofovir disoproxil fumarate has been de-
veloped and validated. The method was validated per the 
ICH guidelines and passed all tests for the various vali-
dation 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 $A_1$ (containing TDF 300 mg/ 3TC 
300 mg tablets + NVP 200 mg) and $A_2$ (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.

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Supplementary Materials
S1: investigations carried out to determine suitable chro-
matographic conditions in method development. S2: spec-
ificity/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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