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Development and validation of HPLC methods for simultaneous analysis of 6 antiretrovirals in pharmaceutical formulations

Abstract

Background: HIV/AIDS stills one of the world's most significant public health problems, affecting particularly Africa. Hence, there is a need to dedicate more attention to fight against HIV by promoting the use of appropriate medicines for its treatment.

Introduction: Unfortunately, the quality of these medicines is currently a deep concern in the public health sector due to the issue of drugs counterfeiting. Therefore, developing a screening and quantitative high-performance liquid chromatography method to simultaneously analyze 6 antiretrovirals (ARVs) from the same matrix (excipients) can be considered as a commendable breakthrough towards improved quality control of ARV.

Methods: Many specific methods dedicated to ARV analysis in different dosage forms have been previously developed; some of them were predicted and optimized. In this study, we have developed and validated a rapid and affordable method using short column and low cost organic solvent (Methanol) in gradient mode, which seems to be very practical in the Africa context. In addition, we verified our method ability for analysis of various fixed-dose combinations made of FTC/TDF/EFV (two different batches); 3TC/ZDV (one batch) and ZDV/NVP (one batch) in tablets formulations. The validation results satisfied the fixed specifications, then the method was applied to test unknown four(4) samples (N°1;N°2; N°3 and N°4) that were claimed to contain the targeted ARV.

Results: The results obtained with all the samples were encouraging and suggested that samples N°2 and N°4 contained the same ARV as predicted at the beginning of experiment. The developed analytical method appears to be promising for routine analysis of ARV formulations, allow tackling the issue of HIV product counterfeiting.

Keywords: development, validation, quality control, high performance liquid chromatography, anti-retrovirals, muscle relaxant, methanol and trifluoroacetic acid

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Abbreviations: HPLC, high performance liquid chromatography; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; DNA, deoxyribonucleic acid; ARC, antiretroviral

Introduction

HIV/AIDS is one the world's most serious public health problems and affects an important number of populations in Africa; particularly in the Democratic Republic of the Congo (DRC) where the access to a good antiretrovirals (ARVs) treatment remains a big issue. To increase the life quality of people living with HIV/AIDS, it is urgent to secure medicines of good quality and in good quantity for everyone in need. The drugs efficiency and safety depend first on their quality, and secondly on their use.[1] In the particular case of DRC, we estimated that among one million people living with HIV/AIDS[2] only 350,000 were having ARV treatment in 2011-2015. This number, decreased only in 44 000 patients who were effectively treated with ARVs,[3] which represented a coverage of 14 % ARV treatment. This was the lowest rate in the world and shows that more than 300 000 people were in need but were not served.[4] Nowadays, one can tell that the situation remains unchanged and there is an emergency for the HIV/AIDS living people. In addition to poor accessibility to ARV treatment, another important question may arise from the ARV products, and this concerns their quality! Antiretrovirals are medicines used to treat the HIV infection. They act by reducing the viral DNA replication speed, stopping at the same time its propagation in the body[5]. Since the launch of Monetary Found and Medicines Access Program projects, the ARV treatment is given to the public for free, but their access stills a problem in some countries. This is unfortunately the case of the DRC, where the national guide of HIV treatment was elaborated in 2003 and reviewed in 2005, 2008 and 2010, following the new recommendations of WHO.[6,7] For importer countries, it is very important to certify the ARV quality according to the national guidelines and the manufacturer data submitted to the public health authorities before their launch in the market for use.[8]

In DRC for example, six out of ten medicines sold, are not homologated. This is the same for Morocco, with 60 % of counterfeited medicines in the market; which means Africa is seriously affected by the poor quality drugs phenomenon. A part from the disease, the presence of poor quality and counterfeited drugs in the market represents also a real and permanent problem for the public health sector and needs to be fought urgently. It is also important to ensure better access to good quality medicines for AIDS treatment to avoid its aggravation and the resistance phenomenon[9]. In all healthcare system, it is fundamental to guarantee the drugs quality either for locally manufactured or imported products because poor quality drugs can lead to death. Hence, we cannot talk about good healthcare system without the better quality drugs.[10]

Antiretrovirals medicines are not spared from counterfeiting, especially for their substantial high unit costs, long term medical

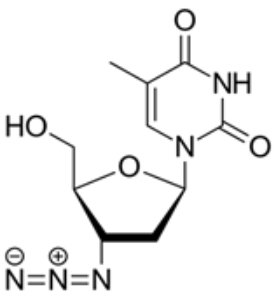
treatment and sustained demand. For example in 2003 the WHO issued an alert that a product called Ginovir 3D , marketed in Ivory coast as a combination of triple ARV, was counterfeited; containing only one of the active ingredients and another non-declared ARV agent.[11] In 2004, Médecins Sans Frontières discovered counterfeited ARVs in the DRC's market, a pharmaceutical composition containing antidepressant and muscle relaxant agents.[12] In 2011, the government of Kenya removed thousands of batches of ARVs from circulation after patients and health workers reported irregularities in the appearance and texture of a widely used antiretroviral product, Zidolam-N.[13,14]

The quality assurance should be found at every level of pharmaceutical manufacturing, including all steps of production from the raw materials (active ingredients and excipients) control up to the final presentation, putting together all the good practices rules and packaging.[15] Before claiming the quality for a batch of products (pharmaceutical dosage forms), it is compulsory to establish the identity, purity, dosage and other characteristics with a reference; and this should be acceptable to the quality specifications.[16] In this case, the main objective of quality control is to determine the product specifications, to evaluate the results according to the specifications and to decide on acceptance or reject of the products based on the fitting acceptability conditions. In this point of view, talking about quality of a product is the same as talking about its improvement. This means, that the search for quality of a product is not the same as its perfection but its improvement, just because of the continuity of this process we must ensure. The quality assessment is based on specifications fixed in different quality referential documents (ISO 9001, ISO 17025, ISO 15189, GLP, GMP, ICH, etc.).[17]The present research aimed to contribute to the better healthcare handling of HIV/AIDS living people by developing and validating an effective, rapid and affordable high-performance liquid chromatography method for simultaneous determination of six ARV in pharmaceutical formulations (used in quality control studies and HIV/AIDS medicines survey).

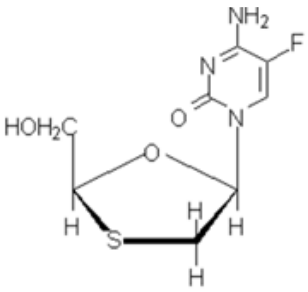
Materials and methods

Chemicals: Tenofovir disoproxil fumarate (>99.1%) and Zidovudine (>99.0%) were purchased from Rockville in USA. Efavirenz (>99.0%), Emtricitabine (>99.0%) and Nevirapine (>99.0%) were sourced from Alsachim (Strasbourg, France). Lamivudine (>99.7%) was bought from European Direction of Drugs Quality (Strasbourg, France). HPLC grade Methanol and trifluoroacetic acid (TFA>99.8%) were from the Merck laboratory (Darmstadt, Germany). Ultrapure water produced by Milli-Q Plus 185 Apparatus (Massachusetts-MA, USA) was sourced from Kim Pharma Laboratory (Kinshasa, DR Congo). Excipients (starch, lactose, magnesium stearate) were all from the Kim Pharma Laboratory (Kinshasa, DR Congo).

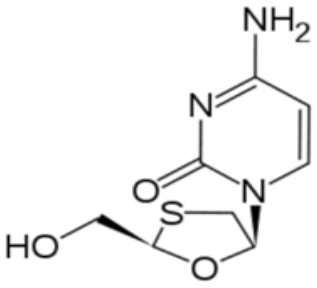
Compounds structures



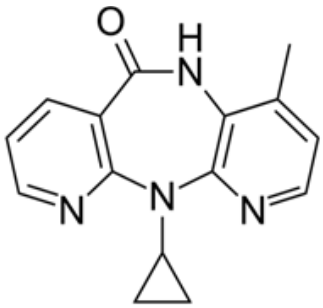
Zidovudine



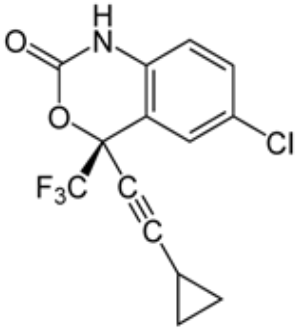
Emtricitabine



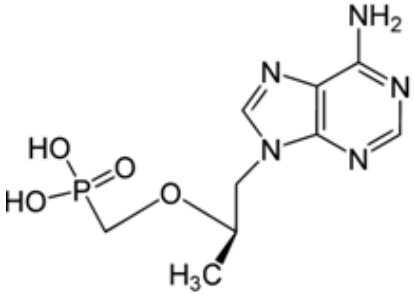
Lamivudine



Nevirapine



Efavirenz



Tenofovir

Methods

Sample preparation

A) Sample solutions for method development

Five solutions have been prepared for method development follows:

Preparation of trifluoroacetic acid 0.05% (TFA): In a 250mL volumetric flask, 50mL of HPLC water was poured and 125mL of Trifluoroacetic acid (98.8%) was added. The total volume was coped to the gauge line with fresh HPLC water.

Individual sample solutions for preliminary data information: Each of the six compounds solution was prepared in accordance with the same operative protocol: In a 10mL volumetric flask, an exactly weighted quantity approximately equal to 10 mg of the active ingredient was transferred, diluted and coped to the gauge line with

Methanol. The same operation was repeated to obtain the appropriate concentration for the study.

ARVs mixture solution (raw materials): In the 10 mL volumetric flask, about 10 mg of each active ingredient (3TC; ZDV, TDF, FTC, NVP and EFV) was weighed. Methanol was added for dissolution and adjustment of the volume to the gauge mark. The resultant solution was diluted ten times with methanol-water mixture (50:50).

B) Sample solutions for calibration and validation

Two groups of solutions were prepared, one for calibration (CS) and another for validation (VS). For every cases, standards were made by dissolving approximately 50 mg of each ARV in some methanol in 50 mL volumetric flask and the total volume was coped to the mark with the same solvent to obtain stock solutions at the concentration of 1 $mg.ml^{-1}$ for each of the anti retrovirals studied. Thereafter, appropriate dilutions were performed using methanol in order to obtain six final calibration standards (CS) in three levels of concentration and six validation standards (VS) at five different concentration levels.

Calibration standards

The (Table 1) shows the corresponding concentration levels for each compound in the CS.

Table 1 Calibration standards

Levels	Percentage (%)	Concentration ($\mu g.ml^{-1}$)
1	40	40
3	80	80
5	120	120

Validation standards

The validation standards (VS) were prepared using the ordinary matrix used in tablets formulations in order to simulate drugs formulations and evaluate the matrix effect on active ingredients analysis. Stock solutions prepared by combination of active ingredients and matrix were subject to appropriate dilutions with methanol in order to produce various concentration levels for each compound (Table 2).

Table 2 Validation standards

Levels	Percentage (%)	Concentration ($\mu g.ml^{-1}$)
1	40	40
2	60	60
3	80	80
4	100	100
5	120	120

C) Analytical solutions

Two solutions were prepared to conduct the analysis:

Sample solution

In a 10 mL volumetric flask, different ARV drugs equivalent to 10mg of each active compound were weighed. Methanol was added for dissolution and dilution to the mark. The resultant solution was subject to 10 dilutions successively using the mixture of methanol and water (50:50) as solvent. Finally, three solutions of individual compound were prepared separately and filtered with 0, 45 µm syringe filters.

D) Reference solutions

In a 10 mL volumetric flask, ARV active ingredients (10mg) was weighed and dissolved with methanol up to the gauge mark. The resultant solution was diluted ten times with the mixture of methanol and water (50:50) as solvent. The solutions of each reference compound taken separately were further prepared and filtered using syringe filters of 0.45 µm pore sizes.

Apparatus and software

Experiments were conducted on a HPLC branded VWR Hitachi instrument coupled to the UV-DAD 5430 (Anvers, Belgium) Detector. Chromaster VWR Hitachi software was used to control the HPLC System together with a DellTM computer (Hangzhou, China) to record the signal and to interpret the generated chromatogram.

We used a GRAM FV-220C electronic balance (IPESAGE S.A.S., France), the XBridget C18, 250x4,6mm, 5µm from Waters (Massachusetts-MA, USA) and an IKA® C-MAG MS4 (Grosseron SAS, France). The obtained data were treated with ENOVA software Excel free version to establish graphs and statistical data analysis; also to treat data during the method validation.

Statistical analysis

All the data were collected trice and presented as mean of results, expressed in percentages ± standard deviation (%±std). The software Chromaster of VWR Hitachi was used to deliver results. When possible, numerical data were treated using ANOVA Excel free version and the significant level considered was $p < 0.05$.

Results

All the development steps and drugs analysis were conducted under the following chromatographic conditions:

Under selected chromatographic conditions, the packs separation observed is presented in (Figure 1). According to the chromatogram given above, the Table 3&4 presents the retention times relative to each antiretroviral.

After the method development, we assessed different validation criteria as presented in the following Tables and Figures.

Selectivity/specificity: it helped to assess the packs purity using the DAD between 200 and 400nm.

Precision: based on the random error, it allowed us to evaluate using both the repeatability and intermediate precision.

Trueness: the result according to the method trueness is given in the Table 5.

It was assessed as it is the closeness of agreement between a conventionally accepted value or reference value that correspond to the introduced concentrations of the 5 ARVs and a mean experimental one.

Linearity: was studied to evaluate the relationship between the

calculated results of the validation standards against the introduced concentrations of six ARVs in the samples. (Figure 2) shows the linearity profile corresponding to each of compounds.

The (Table 6) shows the model of linear regression for all the considered compounds including their related determination coefficient (R^2). The method accuracy was also assessed taking into account the total error (systematic error and the random error) strategy. The accuracy profiles were investigated as shown in the (Figure 3). After development and validation, the method was applied for drugs analysis (ARVs samples from the ministry of the Public Health) and results are presented in (Tables 6–9).

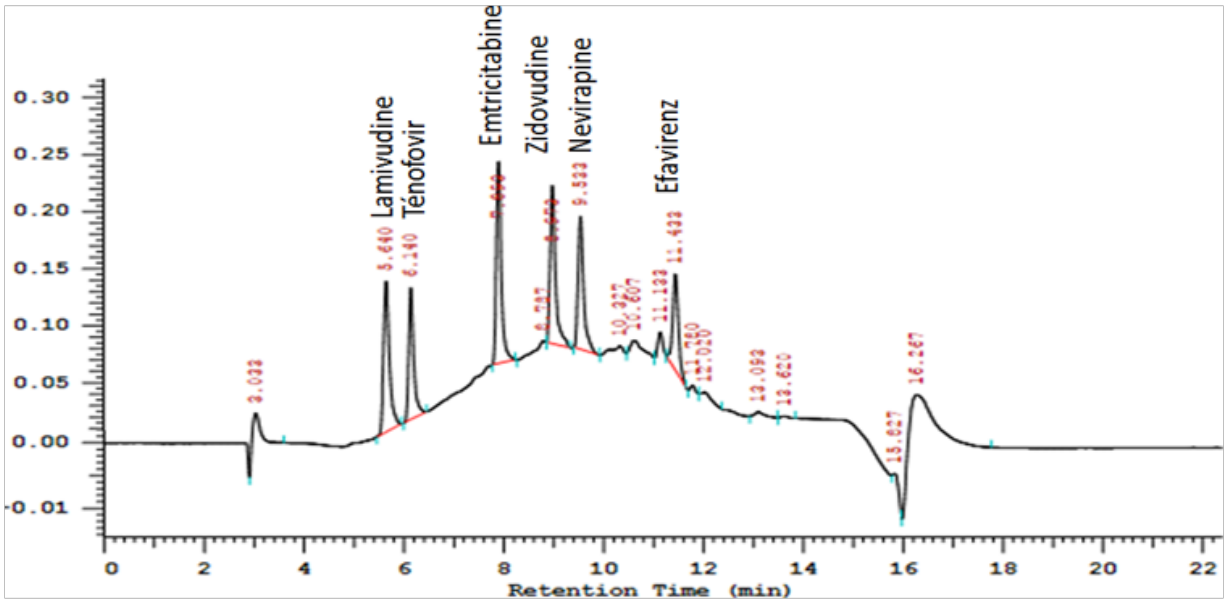


Figure 1 Typical experimental chromatogram of ARVs separation.

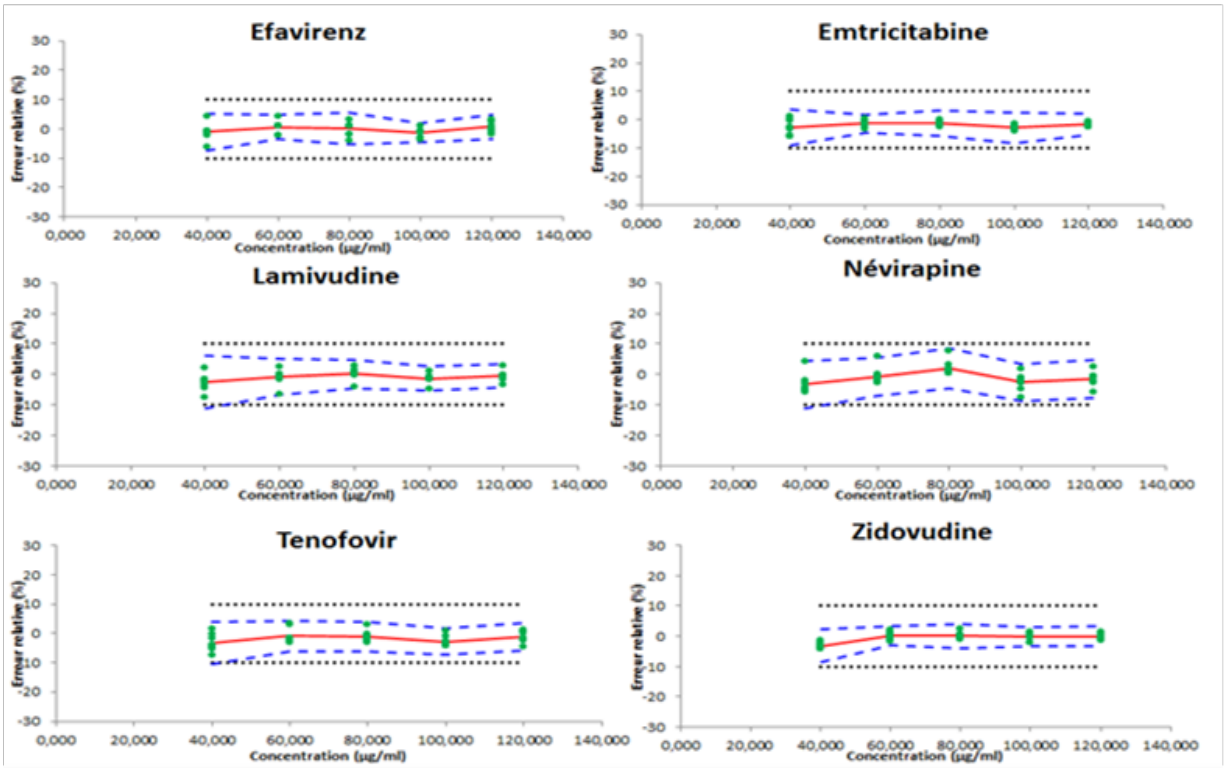


Figure 2 Linearity profile.

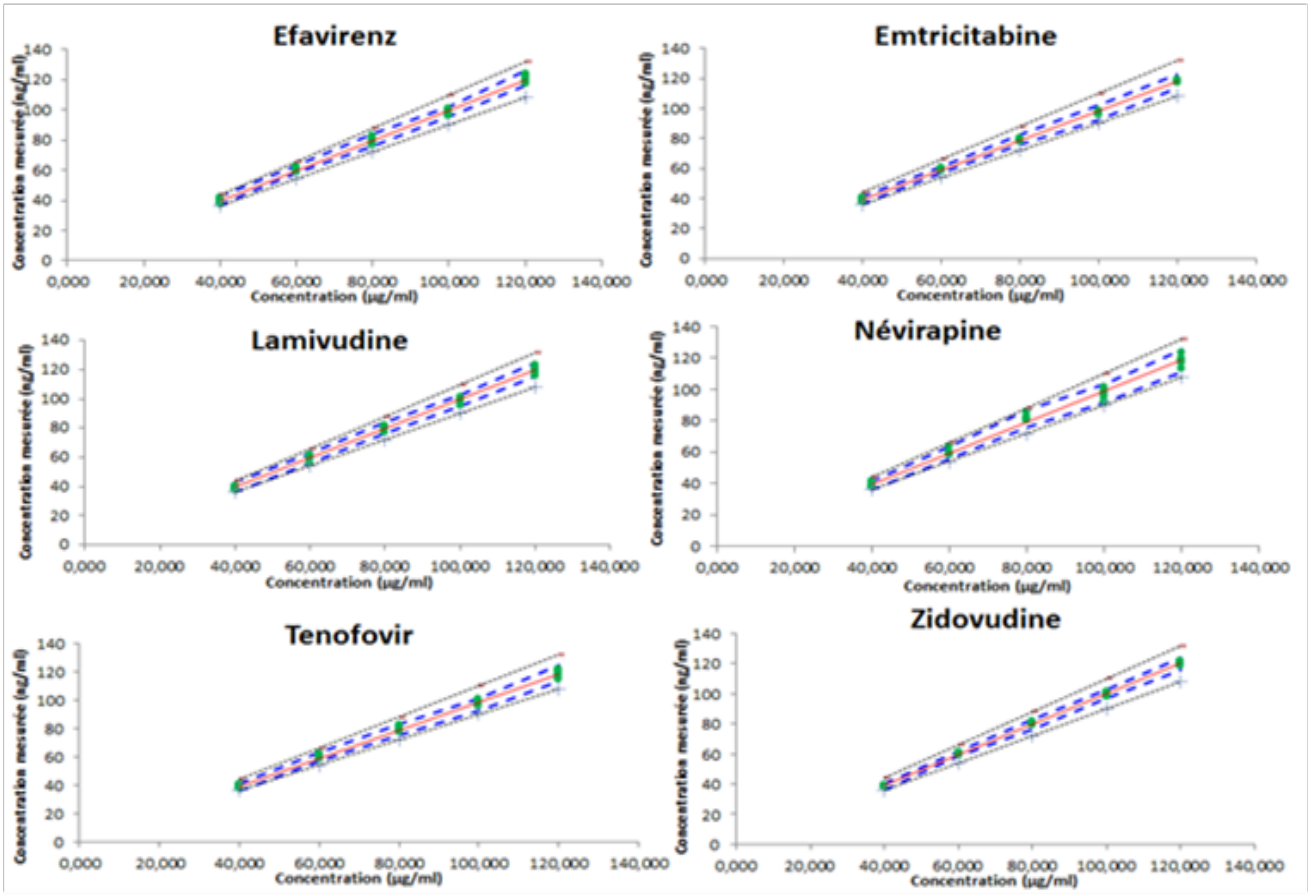


Figure 3 Accuracy profile.

Table 3 Chromatographic conditions

Column	**X Bridge C18, 250x4, 6mm, 5µm**		
Mobile phase A	Methanol		
Mobile phase B	Trifluoroacetic acid 0,05%		
Gradient	Temps (minute)	A (%)	B (%)
	0,0	8	92
	2,5	95	5
	5,0	95	5
	7,5	8	92
	10,5	8	92
Flow rate	1ml/min		
Detection UV	λ = 260 nm		
Injection volume	10µl		
Column temperature	30°C		
Dilution solvent	Eau Milli-Q/Méthanol (50:50		
Concentration	10µg/ml		

Citation: Jocelyn MK, Augustin MM, Adelard MP, et al. Development and validation of HPLC methods for simultaneous analysis of 6 antiretrovirals in pharmaceutical formulations. *J Anal Pharm Res.* 2019;8(6):200–207. DOI: 10.15406/japlr.2019.08.00339

Table 4 The compounds retention times

N°	Compound	Retention time (min)	Relative retention time (min)
1	3TC	5,64	0,49
2	TDF	6,14	0,54
3	FTC	7,89	0,69
4	ZDV	8,97	0,78
5	NVP	9,53	0,83
6	EFV	11,43	1,00

Table 5 Trueness of the method

Levels of conc.	Method developed											
	3TC		ZDV		FTC		NVP		EFV		TDF	
	B.Aµg/ml*	B.R%*	B.Aµg/ml	B.R%	B.Aµg/ml	B.R%	B.Aµg/ml	B.R%	B.Aµg/ml	B.R%	B.Aµg/ml	B.R%
1	-0,98	-2,46	-1,29	-3,23	-1,09	-2,73	-1,32	-3,29	-0,41	-1,03	-1,33	-3,34
2	-0,53	-0,88	0,15	0,25	-0,81	-1,35	-0,45	-0,76	0,38	0,63	-0,51	-0,86
3	0,20	0,25	-0,01	-0,02	-0,98	-1,23	1,68	2,10	0,08	0,10	-0,92	-1,15
4	-1,31	-1,30	-0,26	-0,26	-2,85	-2,85	-2,59	-2,59	-1,25	-1,25	-2,88	-2,88
5	-0,48	-0,39	-0,07	-0,06	-1,92	-1,60	-1,74	-1,45	0,82	0,68	-1,15	-0,96

Table 6 Sample 1 identification

N°	Compound	Reference retention time	Sample retention time
1	3TC	5,64	5,71
2	ZDV	8,97	8,99

Table 7 Sample 1 quantification (results in %±std)

Sample	Results (in %±std, n=3)	
1	ZDV	3TC
	97,0± 0,60	93,3± 0,27

Table 8 Samples 2,3 and 4 identification

N°	Compounds	Reference retention time	ARVs in samples retention time
1	3TC	5,64	5.71
2	TDF	6,14	6,11
3	FTC	7,89	7,56
5	NVP	9,53	9,82
6	EFV	11,43	11,79

Table 9 Samples 2,3 and 4 quantification (results in %±std)

Sample	Analysis results (%±CV, n=3)				
	NVP	FTC	EFV	TDF	3TC
2		98,5±0,98	97,8±1,01	99,6±0,91	
3	101,4±0,67				97,4±0,72
4		99,0±0,84	101,7±1,12	99,0±0,91	

Citation: Jocelyn MK, Augustin MM, Adelard MP, et al. Development and validation of HPLC methods for simultaneous analysis of 6 antiretrovirals in pharmaceutical formulations. *J Anal Pharm Res.* 2019;8(6):200–207. DOI: 10.15406/japlr.2019.08.00339

Table 10 Method precision

Level of conc. µg/ml	Method developed											
	3TC		ZDV		FTC		NVP		EFV		TDF	
	Rép*	F.I*	Rép	F.I	Rép	F.I	Rép	F.I	Rép	F.I	Rép	F.I
40	1,95	2,88	0,36	1,12	2,98	2,87	3,08	3,33	2,64	2,79	2,75	3,07
60	2,35	2,56	0,95	1,22	0,84	1,15	2,92	2,76	2,12	1,95	2,61	2,35
80	1,73	1,94	0,71	1,20	0,41	0,97	1,81	2,41	2,06	2,33	1,56	1,95
100	1,52	1,68	1,03	1,27	0,55	1,15	2,78	2,75	1,31	1,40	1,63	1,88
120	1,59	1,70	0,76	1,13	0,35	0,82	2,10	2,50	2,04	1,82	1,81	2,01

Table 11 Linearity of the method

Parameters	Developed method					
	ZDV	NVP	FTC	EFV	TDF	3TC
Slope	1,010	0,985	0,982	0,986	0,990	1,001
Interception	1,113	0,307	0,052	0,683	0,560	0,716
R^2	0,998	0,993	0,998	0,996	0,996	0,997

Discussion

The method was developed according to the one variable strategy; which means by changing one parameter of study and observing the analytical response (Antiretrovirals medicines packs separation on chromatogram) under selected chromatographic conditions. Inspired from database found in the software, we observed successful results as shown in (Figure 1). According to the outcome from chromatographic separation, we can see distinctive peak separation (six peaks). The chromatographic profile can be insightful for identification of compounds in the mixture based on the retention time and DAD spectra. (Table 4) presented the retention times relative to each antiretroviral for identification by comparison with reference retention times under the same analytical conditions.

The validation approach is nowadays based on the total error strategy which uses the accuracy as the main tool to estimate the analytical specifications of the method. Our method was also studied using the same approach, with a ±10% acceptation limit. A direct validation for all ARVs was undertaken. This is commonly called global validation; since it involves all the validation criteria for all the compounds under the same conditions. This allowed us to conduct analysis without changing chromatographic conditions for a particular compound. The separation and quantification of the ARVs from the mixture were achieved in the same analytical conditions with no modification of analytical parameters as in traditional methods where variations in experimental conditions were necessary for analysis of complex mixtures. The experimental conditions used were deliberately selected to gain in terms of time and have a general method for analyzing each of the compounds involved. A part from gaining time, this method appears to be economical since there was no need for thorough screening of particular conditions for successful individual analysis of all the six molecules in the mixture. The present study helped to finally have a general protocol to study six ARVs in same matrix and under the same analytical conditions for the method validation.

The common validation criteria found in the document Q2 (R1) of the international conference on harmonization (ICH),[18] were considered namely as: Selectivity/Specificity, trueness, precision (repeatability and intermediate precision), accuracy, linearity, limit of detection (LOD)/limit of quantification (LOQ), and the dosing range. First of all, we confirmed method's selectivity by purity assessment of the peaks using DAD between 200 and 400nm. The spectra obtained were compared to the literature data and revealed the similarity between the obtained results and the reference recorded. Secondly, we assessed the method precision, which allowed us to be informed on the uncertainty (random error). The random error was evaluated using both the repeatability and intermediate precision. This parameter expresses the tightness between a measurement series from multiple essays obtained using the same and homogenous sample in same conditions.[19]

In this study, the observed values for different standard deviations of repeatability and intermediate precision for all ARVs in every level of concentrations were within the acceptable range, which indicates good precision for the method developed. Only NVP in the lowest level of concentration hawed relatively large values for repeatability and intermediate precision but not exceeding respectively 3.08 % and 3.33 % (Table 10,11). For the obtained values with all other ARVs, no issue was observed under the analytical conditions used. Noteworthy, all the deviations observed for compounds under study are negligible, confirming the developed method meets well the precision criterion (closeness of agreement among measurements) and can be declared safe to use. To demonstrate the developed method linearity, we evaluated the relationship between the calculated results of the validation standards against the introduced concentrations of six ARVs in the samples. The model of linear regression was determined, and fitted for all the considered compounds, which was demonstrated by the determination coefficient (R^2) (Table 5) values in each case where it is close to 1.[20,21]

The method accuracy was assessed taking into account the total error (systematic error and the random error) strategy. The accuracy profiles were investigated (Figure 3). The analysis of individual graphs shows their accuracy profile, allowing us to conclude that the

developed method is acceptable for screening and quantification of these compounds in same matrix under the same chromatographic conditions. This observation can be confirmed by different tolerance interval, which is included in the acceptation limit fixed at ±10% for the studied molecules, and was valid at all the levels. Notably, the accuracy profiles of four molecules (FTC, 3TC, NVP and TDF) stretched to approach the lowest limit of concentration fixed to 40µg/ml. Nevertheless, this could not make a barrier to the developed method, since in general this method has demonstrated good analytical responses at all the concentration levels in accordance with the limits of acceptation.

In addition, it is important to note that the choice of these limits was done based on the previous experiments and used in accordance with the literature. It is also worth mentioning that the exactitude profile obtained, allowed to decide on our method's capacity to produce good results during routine experiments. The final phase of the development for an analytical method is its application for dosing pharmaceutical dosage forms. The developed method was applied for the routine control of tablet samples using four different pharmaceutical brands made of ARVs drugs. There were samples from the MOH (Minister of public Health) of Democratic Republic of Congo claimed to contain respectively 3TC and ZDV (sample 1); FTC, EFV and TDF (sample 2); NVP and 3TC (sample 3) and FTC, EFV and TDF (sample 4). As one can observe, two samples were claimed to have the same content (samples 2 and 4). All branded samples were tested using our analytical method, following the same protocol and analytical conditions; the Tables 4.a, 4.b, 4.c and 4.d present the results in the mean percentages of the claimed nominal contents in each medicine and the relative standard deviations calculated for all the six independent samples. The developed method was also used to identify all ingredients contained in each formulation as individual compound using the retention time as the basis of comparison with the reference chemical substances.

The obtained results revealed that the investigated products contain the molecules claimed in the labels of each branded medicines. Similarly, the results from quantification demonstrated that the quantitative composition of the studied products was good, with acceptable content limits (90 % - 100 %).

Conclusion

Several studied were conducted for ARVs simultaneous determination in Pharmaceutical forms but most of them were limited according to the analysis time which was too long (more than 12 minutes), and the analytical conditions which should be changed before passing from one compound analysis to another. This work aimed to develop and validate (main validation) an HPLC-UV analytical method for screening and quantification of six ARVs (EFV, FTC, ZDV, 3TC, NVP and TDF) in the same matrix under the same analytical conditions. After developing our generic method, we achieved the main method validation differently from classic validation, because all molecules studied were subject to identical analytical conditions and the validation criteria for all the compounds were studied as it was only one entity. The developed method satisfied to all the validations criteria explored in the present study. Finally, the method was applied for analysis of Pharmaceutical forms within a good time of analysis which was not exceeding 12 minutes.

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Conflicts of interest

The author declares that there are no conflicts of interest.

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