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Application of design space optimization strategy to the development of LC methods for simultaneous analysis of 18 antiretroviral medicines and 4 major excipients used in various pharmaceutical formulations

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ABSTRACT

As one of the world's most significant public health challenges in low- and middle-income countries, HIV/AIDS deserves to be treated with appropriate medicines, however which are not spared from counterfeiting. For that, we developed screening and specific HPLC methods that can analyze 18 antiretroviral medicines (ARV) and 4 major excipients. Design of experiments and design space methodology were initially applied for 15 ARV and the 4 excipients with prediction thanks to Monte Carlo simulations and focusing on rapidity and affordability thus using short column and low cost organic solvent (methanol) in gradient mode with 10 mM buffer solutions of ammonium hydrogen carbonate. Two other specific methods dedicated to ARV in liquid and in solid dosage formulations were also predicted and optimized. We checked the ability of one method for the analysis of a fixed-dose combination composed by emtricitabine/tenofovir/efavirenz in tablet formulations. Satisfying validation results were obtained by applying the total error approach taking into account the accuracy profile as decision tool. Then, the validated method was applied to test two samples coded A and B, and claimed to contain the tested ARV. Assay results were satisfying only for sample B.

1. Introduction

HIV/AIDS remains one of the world's most significant public health challenges. The World Health Organization (WHO) reports that at the end of 2015, there were approximately 36.7 million people living with HIV, with 2.1 million people becoming newly infected in the same year globally. Low- and middle-income countries and particularly in Sub-Saharan Africa are the most affected with 25.6 million people (i.e. around 70% worldwide) living with HIV [1,2].

On the other hand, antiretroviral (ARVs) 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 counterfeit containing only one of the active ingredients and another non-declared ARV agent [3]. In 2004 "Médecins Sans Frontières" discovered counterfeit ARVs on the market in the Democratic Republic of Congo that contained an antidepressant and a muscle relaxant [4]. In 2011 the Government of Kenya removed thousands of batches of ARVs from circulation after

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patients and health workers reported irregularities in the appearance and texture of a widely used antiretroviral Zidolam – N [5,6].

Furthermore, as the antiretroviral therapy (ART) constitutes a growing pharmaceutical research field that necessitates combining several ARV molecules to maximally suppress HIV and stop the progression of HIV disease, there is need of disposing rapid and efficient analytical methods that can serve in different stages of drug development lifecycle, to ensure quality and guarantee safety and efficacy. There are also few monographs dedicated to the analysis of multiple ARV combinations already on the market, for example there is no monograph yet for the analysis of efavirenz/lamivudine/tenofovir in the three most popular pharmacopoeias i.e. International Pharmacopoeia (Ph. Int.), British Pharmacopoeia (BP) and United States Pharmacopoeia and the National Formulary (USP-NF).

In the frame of fighting against poor quality antiretroviral medicines especially counterfeit/falsified ones, we aimed to develop effective, rapid and affordable methods. In this context HPLC methods were preferred considering classical and short analytical columns and low cost mobile phase such as methanol with classic buffer solutions. Three types of methods were targeted depending on the situation that will be faced: (*i*) the screening methods for which the interest will be the analysis of suspected counterfeit ARV medicines or complex samples that contain several ARV molecules, (*ii*) the generic method(s) that are expected for the analysis of specific cases for example of different fixed-dose combinations (FDC) in liquid dosage forms containing generally preservatives and/or anti-oxidants, and (*iii*) the generic method(s) applicable to different FDCs in solid dosage forms not generally containing preservatives or anti-oxidants.

This study focused on 18 antiretroviral medicines widely used in HIV/AIDS treatment, and 4 major preservatives and anti-oxidants generally used in liquid dosage formulations. Their chemical structures are given in Fig. 1.

A strategic approach based on design of experiments (DoE) and design space (DS) methodology was followed during the development of methods. It presents advantages of allowing the exploration of different chromatographic factors effects and the interactions between them. On the other hand, DS defined as "multidimensional combination and interaction of input variables that have been demonstrated to provide assurance of quality" is suitable to predict optimal analytical conditions within the experimental domain [7–10]. Then in order to ensure its fit-for-purpose, one of the candidate methods was submitted to validation prior to its use in routine analysis and its proposal to sustain monographs. We privileged the concept of total error strategy using accuracy profiles as decision tool while considering the ICH Q2 (R1) guidelines about the validation of analytical procedures [11–15].

2. Experimentation

2.1. Materials and methods

2.1.1. Chemicals

Abacavir sulfate (99.4%), atazanavir sulfate (99.6%), nelfinavir mesylate (98.3%), ritonavir (99.4%), saquinavir mesylate (99.4%), tenofovir disoproxil fumarate (99.1%), and zidovudine (99.0%) were purchased from the United States Pharmacopeial Convention (Rockville, USA), darunavir ethanolate (100.0%), didanosine (>99%), efavirenz (>99%), emtricitabine (>99%), etravirine (97.1%), lopinavir (>99%), nevirapine (>99%) and raltegravir potassium salt (>99%) from Alsachim (Strasbourg, France), indinavir (97.2%), lamivudine (99.7) and stavudine (99.6%) from the European Directorate for the Quality of Medicines "EDQM" (Strasbourg, France). The four preservatives used in this study were obtained from the following suppliers: buthylated hydroxyanisol "BHA" (96%) and methyl paraben "nipagin" (99%) from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany), buthylated hydroxytoluene "BHT" (≥99%) from Merck Schuchardt OHG (Hohenbrun, Germany), and propyl paraben "nipasol" (101.9%) from Fagron N.V. (Waregem, Belgium). Ammonium formate (99%) was purchased from Alfa Aesar (Karlsruhe, Germany), methanol (HPLC gradient grade) and hydrochloric acid (37%) from Merck (Darmstadt, Germany). The ultrapure water was produced with a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA).

Table 1
Grouping of analytes per pKa(s) and molecular weights in different sub-group solutions for DoE.

Sub-group	Analytes	pKa (s)	Molecular weight (g/mol)
Sub-group 1	ABC	5.01	335.37
	BHA	8.9	180.25
	ddI	9.12	236.23
	d4T	9.95	224.21
	EFV	10.2	315.67
Sub-group 2	NVP	2.8	266.30
	IDV	3.8; 6.2	711.87
	RAL	6.3	444.42
	AZT	9.68	267.24
	BHT	12.23	220.36
Sub-group 3	FTC	2.65	247.25
	TDF	3.75	635.51
	3TC	4.3	229.26
	NFV	6.00; 11.06	663.90
	NpG	8.4	152.15
Sub-group 4	SQV	1.1; 7.1	670.84
	LPV	2.8	628.81
	RTV	3.48	720.94
	NpS	7.91	180.20

2.2. Sample preparation

2.2.1. Sample solutions for methods development

Three types of solution were concerned:

2.2.1.1. Individual sample solutions for preliminary data information. Fifteen individual sample solutions at 100 μg mL^{-1} of ABC, ddI, EFV, FTC, IDV, 3TC, LPV, NFV, NVP, RAL, RTV, SQV, TDF, d4T, and AZT were prepared in methanol by dissolving approximately 10.0 mg of each individual analyte in 100.0 mL volumetric flasks, and completing to volume with the same solvent. For non-easily soluble compounds in methanol, 10 min of ultrasonic bath were necessary to allow total dissolution.

These solutions served in collecting preliminary data information on respective UV spectra and retention times at apex (t_R). The individual sample solutions for BHA, BHT, NpG and NpS were not prepared as the needed preliminary information was already known and available. Collecting the retention time of the single compounds prior to optimization was useful in order to divide the compounds into subgroups and avoid peak overlapping that would be observed when testing all the compounds together. Indeed, in order to properly compute a DS, all peaks should be fully monitored for the whole range of experimental conditions.

2.2.1.2. Sub-group solutions for design of experiments (DoE). The 15 ARV compounds and the 4 excipients were split into four subgroups differing from one to another by respective molecular weights and dissociation constants "pKa(s)" as shown in Table 1. We tried as much as possible to have sub-groups containing molecules with different analytical data to easy the experimental design.

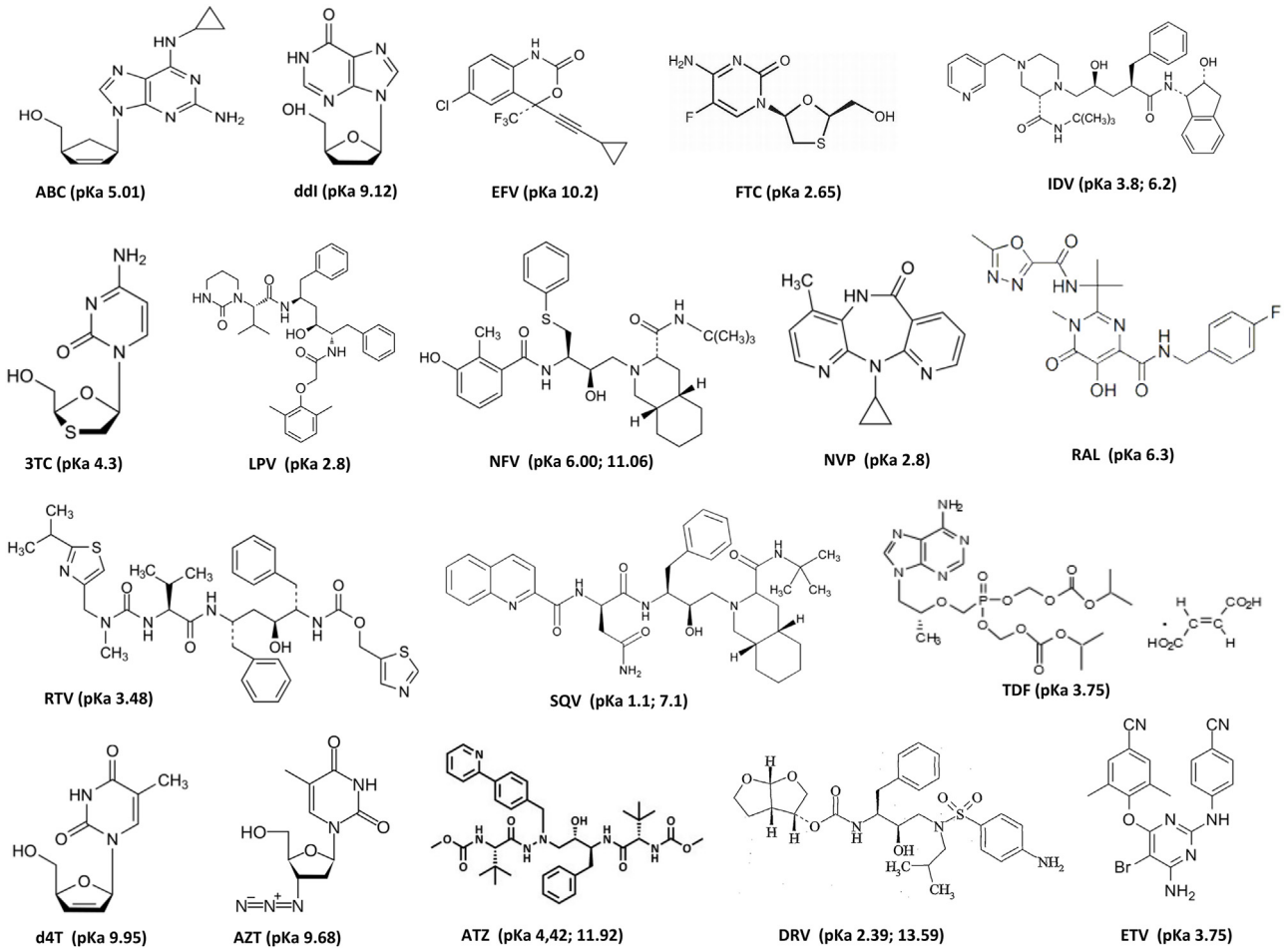

Fig. 1. Molecular structures and pKa(s) of the 18 antiretroviral medicines.
Legend:
ABC: Abacavir, ATZ: atazanavir, ddI: didanosine, DRV: darunavir, d4T: stavudine, EFV: efavirenz, ETV: etravirine, FTC: emtricitabine, IDV: indinavir, LPV: lopinavir, NFV: nelfinavir, NVP: nevirapine, RAL: raltegravir, RTV: ritonavir, SQV: saquinavir, TDF: tenofovir, ZDV or AZT: zidovudine, 3TC: lamivudine.

Stock solutions per sub-group were prepared at 1 mg mL^{-1} of each analyte in methanol, and 10 min of ultrasonic bath were necessary to allow total dissolution of non-easily soluble compounds. Then, aliquots from sub-group stock solutions were diluted with the same solvent to have final working solutions at 200 μg mL^{-1} for design of experiments.

2.2.1.3. Overall solution for the screening method. A mixture of aliquots was prepared by mixing together 2.0 mL from each sub-group stock solution in a 10.0 mL volumetric flask in order to have a final solution of 200 μg mL^{-1} of each analyte. This overall working solution was used for testing the optimal analytical conditions of the screening method and also for assessing other predicted methods to check their capability in separating the analytes in the overall mixture solution.

2.2.1.4. Additional sub-group of other ARV compounds after design of experiments. Another sub-group i.e. Sub-group 5 composed by three additional ARV agents namely ATZ (pKa 4.42; 11.92), DAR (pKa 2.39; 13.59) and ETV (pKa 3.75) was prepared in the same conditions as done for the four previous sub-groups in order to have final working solutions at 200 μg mL^{-1} (see Section 2.2.1.2). In fact, the present study including the planed DoE was initially based on 15 ARV medicines used in Rwanda by 2013 and inventoried from the national list of essential medicines published at that period [16]; After having constructed the DoE, we were informed that in 2015 the Government of Rwanda that served us as a model of developing country using ARV medicines at a large scale has updated its national list of essential medicines by introducing the three ARV above mentioned in the ART for adult and pediatric patients [17,18]. This new sub-group was used to check whether the analytical method for the analysis of solid dosage forms will be suitable to detect the three compounds which are among the most used in pharmaceutical solid dosage forms.

2.2.2. Sample solutions for calibration and validation

Two sets of solutions, one for calibration standards (CS) and another for validation standards (VS) were prepared, by dissolving in methanol approximately 7.0 mg of FTC, 10.0 mg of TDF, and 20.0 mg of EFV in each volumetric flask of 20.0 mL and completed to volume with the same solvent to obtain stock solutions at the concentration of 350 μg mL^{-1}, 500 μg mL^{-1} and 1000 μg mL^{-1} of FTC, TDF and EFV, respectively.

Then, appropriate and subsequent dilutions were performed using methanol in order to obtain three final calibration standards (CS) at three different concentration levels, and three validation standards (VS) at five concentration levels daily.

A) Calibration standards

The corresponding concentration levels for CS were as following:

Level 1 (50%): 42 μg mL^{-1} (FTC), 60 μg mL^{-1} (TDF) and 120 μg mL^{-1} (EFV);

Level 3 (100%): 84 μg mL^{-1} (FTC), 120 μg mL^{-1} (TDF) and 240 μg mL^{-1} (EFV);

Level 5 (150%): 126 μg mL^{-1} (FTC), 180 μg mL^{-1} (TDF) and 360 μg mL^{-1} (EFV).

B) Validation standards

The validation standards (VS) were prepared considering the tablets matrix in order to simulate as much as possible the drug formulation and evaluate the matrix effect on the analytes. A matrix stock solution composed by major excipients of the studied medicine was prepared by dissolving titanium dioxide, talc, croscarmellose sodium, magnesium stearate, microcrystalline cellulose, sodium laurylsulfate and hyprolose in methanol in order to obtain 0,1 mg mL^{-1}, 0,1 mg mL^{-1}, 5 mg mL^{-1}, 0,5 mg mL^{-1}, 25 mg mL^{-1}, 1 mg mL^{-1} and 0,5 mg mL^{-1} respectively. This solution contains an equivalent amount of the major excipients as compared to the real galenic formulation for which the analytical method is validated for.

For VS, one stock solution containing FTC at 350 μg mL^{-1}, TDF at 500 μg mL^{-1} and EFV at 1000 μg mL^{-1} was prepared in methanol as done for CS. To this stock solution was added 545 μL of the matrix stock solution in a volumetric 20.0 mL flask and completed to volume with methanol. This solution was filtered through 0.45 μm PTFE syringe filtration disks before diluting the aliquots for experiments.

Appropriate dilutions were performed with methanol in order to obtain final validation standard solutions at five different concentration levels, and three independent solutions per concentration level:

Level 1 (50%): 42 μg mL^{-1} (FTC) – 60 μg mL^{-1} (TDF) and 120 μg mL^{-1} (EFV);

Level 2 (75%): 63 μg mL^{-1} (FTC) – 90 μg mL^{-1} (TDF) and 180 μg mL^{-1} (EFV);

Level 3 (100%): 84 μg mL^{-1} (FTC) – 120 μg mL^{-1} (TDF) and 240 μg mL^{-1} (EFV);

Level 4 (125%): 105 μg mL^{-1} (FTC) – 150 μg mL^{-1} (TDF) and 300 μg mL^{-1} (EFV);

Level 5 (150%): 126 μg mL^{-1} (FTC) – 180 μg mL^{-1} (TDF) and 360 μg mL^{-1} (EFV).

In final, three independent solutions ($n = 3$) were prepared for each concentration level ($m = 3$ for CS, $m = 5$ for VS), and all these preparations were repeated for three days corresponding to three series ($p = 3$).

For the analyses of medicine samples, the concentrations of reference standard solutions were identical to Level 3 (100%) of the VS: 84 μg mL^{-1} of FTC, 120 μg mL^{-1} of TDF and 240 μg mL^{-1} for EFV. For the sample to be analyzed, powdered portions of the tablets were taken and treated in the same way as reference solutions to give theoretically final expected concentrations of 84 μg mL^{-1} (FTC), 120 μg mL^{-1} (TDF) and 240 μg mL^{-1} (EFV). The solutions were freshly prepared, and they were filtered through 0.45 μm PTFE syringe filtration disks prior to their analysis onto the liquid chromatographic system.

2.2.3. Sample preparation for FTC/TDF/EFV tablets

20 tablets from each sample batch were weighed and finely powdered. Then, three portions containing approximately 80.0 mg of FTC, 120.0 mg of TDF, and 240.0 mg of EFV for the sample containing FTC/TDF/EFV were accurately weighed and dissolved in 100.0 mL volumetric flasks with methanol. One minute of mechanical shaking with vortex was enough to dissolve the studied analytes; and further appropriate dilutions were done with the same solvent to have final working solutions containing 80/120/240 μg mL^{-1} of the three active ingredients respectively in sample A, and 80/120 μg mL^{-1} in sample B containing only FTC and TDF. But before injecting the sample solutions in the chromatographic system, they were filtered through 0.45 μm PTFE syringe filtration disks.

In parallel, two independent standard solutions were prepared at the same concentration levels (i.e. 80/120/240 μg mL^{-1}) of FTC, TDF, and EFV reference substances prepared in methanol too.

2.3. Instrumentation and chromatographic conditions

The optimization of the methods was performed on a HPLC system comprising a Waters 2695 separation module coupled to a Waters 2996 photodiode array detector from Waters Corporation (Eschborn, Germany). The analytical column was XBridge Shield (100 mm × 4.6 mm ID), packed with C18 stationary phase (3.5 μm, dp) from Waters Corporation.

The analytes were monitored photometrically at a wavelength of 210 nm while chromatographic data of experimental conditions were recorded from 200 to 400 nm. The collected chromatographic data were basically the UV spectra of the analytes, retention times (at the beginning, apex and end), peaks' areas and peaks' heights. The injection volume was 10 μL and the mobile phase flow rate was 1 mL min^{-1}, the temperature of the column compartment was fixed at values between 25 °C and 35 °C depending on the DoE value to be tested or optimized method. The temperature of sample compartment was fixed at 15 °C.

Measurements of pH were performed with a SevenEasy S20 pH meter (Mettler Toledo, Columbus, OH, USA). The buffer solutions of the mobile phase consisted of ammonium formate with pH adjusted between 2.7 and 4.8, of ammonium acetate with pH adjusted between 4.8 and 6.0, and of ammonium hydrogen carbonate with pH adjusted between pH 6.0 and 10.0. The adjustment depended on the values to test for DoE and for the optimal analytical conditions. The pH was adjusted with 6N hydrochloric acid or with ammonium hydroxide 27% while the concentration of the buffers was 10 mM.

2.4. Software

Empower 2.0 software (Waters Corpoartion, MA, USA) for Windows was used to control the HPLC system, to record the signals from the detector and to interpret the generated chromatograms. The gradient steps were calculated using a freeware HPLC Calculator v3.0 developed by Guillarme et al. [19]. JMP 12.2.0 (SAS Institute Inc., NC, USA) and R 3.2.2 (GNU project/Free Software Foundation, MA, USA) softwares were used for generating the DoE trials and experimental data treatment. The accuracy profiles as well as the statistical calculations including the validation results and uncertainty estimates were obtained thanks to e-noval® V3.0 software (Arlenda, Belgium).

3. Results and discussions

3.1. Design of experiments

The strategy of design of experiments (DoE) was applied to collect data through laboratory tests and to define design spaces (DS) related to different optimal analytical conditions for the screening method or specific methods for the analysis of ARV compounds and the studied excipients in different dosage forms. In this study, a D-Optimal design was used to allow simultaneous optimization of the methods in few experimental runs. Each experiment was performed in duplicate considering the mean of the results.

The pH of the aqueous part of mobile phase, the gradient time (TG) and the column temperature (T°) were selected as the factors to investigate the appropriate analytical conditions within the selected levels of those factors as shown in Table 2. All the factor

Table 2
Factors and corresponding levels selected for the D-Optimal Design.

Factors	Levels					
pH	2.70	4.53	5.85	6.35	8.18	10.00
Gradient time (TG, min.)	7.25		15.25		23.25	
Temperature (T°, °C)	25.0		30.0		35.0	

Table 3
Experimental matrix of D-Optimal design for the investigation of analytical factors.

Trial	Experimental set up		
	T° (°C)	TG (min)	pH
1	35.0	15.25	4.53
2	30.0	23.25	4.53
3	35.0	7.25	10.00
4	25.0	7.25	10.00
5	35.0	15.25	8.18
6	30.0	23.25	10.00
7	30.0	15.25	2.70
8	25.0	7.25	2.70
9	35.0	23.25	10.00
10	25.0	15.25	10.00
11	35.0	7.25	2.70
12	25.0	23.25	2.70
13	30.0	15.25	6.35
14	25.0	7.25	5.85
15	30.0	15.25	6.35
16	30.0	7.25	8.18
17	30.0	15.25	6.35
18	25.0	23.25	8.18
19	35.0	23.25	2.70

levels were symmetrical. For pH, the symmetry was between values 2 and 8, with 5 the central level and additional points between that central and the extreme points. Since the majority of the compounds to test are basic another point (pH 10) was added to further investigate the separation of those compounds. Because the chromatographic column was able to support that pH level, it was assessed and treated as an augmented level of the initial DoE.. . .. . . Volatile salts were preferred for potential further transfer to LC–MS in case of investigation of potential "abnormal peak", i.e. in counterfeit medicines. A total of 19 experimental conditions were randomly generated with R 3.2.2 software (see Table 3). For each trial run, three retention times (t_R), at the beginning, the apex and the end of each peak analyte were recorded as analytical responses from the chromatogram; Retention time at apex was used for peak identification while retention times at the beginning and at end were used to model the responses and express the separation (S). The sample solutions of each sub-group were analyzed throughout these 19 experimental trials.

Prior to run the experimental trials, preliminary tests were carried out at pH 2.7 and pH 10.0, TG = 7.25 min and T° = 35 °C on individual sample solution of each compound. This allowed recording the information on UV–vis spectra and t_R of each peak analyte useful for easy identification and information on the chromatographic behavior in terms of elution speed.

To prevent any co-elution of the peak analytes during the experiments, we took the precaution of dispatching the analytes according to their differences on pKa(s) of at least 1 unit pKa and molecular weight of 10 g mol^{-1}. This allowed good chromatographic separation of the compounds and therefore allowed recording precise data on respective three retention times (beginning, apex and end) of each peak analyte since with co-eluting peaks this would not be possible.

3.2. Influence of the analytical factors on the chromatographic behavior of the analytes

The responses (retention times at beginning, apex and end) from the experimental data were modeled by the following multivariate multiple linear model with quadratic for factors other than pH and interactions between factors as shown by the following equation:

$$Y = \beta_o + \beta_1.\mathrm{pH} + \beta_2.\mathrm{pH}^2 + \beta_3.\mathrm{pH}^3 + \beta_4.\mathrm{T_G} + \beta_5.{\mathrm{T_G}}^2 + \beta_6.\mathrm{T}^\circ + \beta_7.{\mathrm{T}^\circ}^2 + \beta_8.\mathrm{pH}.\mathrm{T_G} + \beta_9.\mathrm{pH}.\mathrm{T}^\circ + \beta_{10}.\mathrm{T}^\circ.\mathrm{T_G} + \beta_{11}.\mathrm{pH}.\mathrm{T}^\circ.\mathrm{T_G} + E$$

$$Y = XB + E$$

With βo, . . . β11, the model parameters and E the estimated error.

As can be noticed in Fig. 2, pH and TG have an important influence on the three retention times of the compounds: Obviously the decrease of the TG significantly decreases the three retention times of the compounds, but often at the expense of peak separation due to peak co-elution. It was also noticed that pH variation of the mobile phase led to significant changes in retention times of some compounds such as 3TC, ABC, NpG, NpS, TDF, RAL, d4T, IDV, SQV, and AZT, but also the variation of pH of the mobile phase can cause the inversion of peaks and therefore change the elution order of peaks (see Fig. 2a). For example the elution order at pH 2.7 will start with 3TC while at pH 10.0 it will start with ddI. Other compounds such as ABC, NpG, RAL, NpS, IDV, etc. will have a change in the elution order upon variation of the pH. Moreover, some compounds are subject of co-elution at some pH levels; for example NVP/NpS at around pH 10.0, AZT/NpG at around pH 9.7, 3TC/ddI and RAL/ABC at around pH 8.5, ddI/3TC/FTC/d4T, NpG/ABC, RAL/NVP, and NpS/TDF at around pH 7.0, ddI/FTC/d4T, NpG/ABC, NpS/TDF, and SQV/NFV/LPV at around pH 5.0, AZT/ABC and NpG/NVP at around pH 2.7. Therefore there is need of carefulness while optimizing the methods involving those compounds in a drug formulation.

Concerning T°, one can noticed that when increasing between 25 °C and 35 °C, a slight retention times decrease of all peak analytes was observed without any co-elution or change of peaks elution order.

Based on these observations, the ideal would be to run the chromatographic analysis at higher TG for better peaks separation thus their easier identification; however, this becomes a compromise with the analysis time that should be reduced in order to obtain the results as rapid as possible for allowing taking decision on analyzed batch products especially in case of failure (out-of-specifications).

3.3. Methods optimization

The optimization of the methods was done thanks to R 3.2.2 freeware [20] available for most computer systems. It allowed to develop Bayesian models on the prior recorded results of the DoE and to predict different optimal analytical conditions for the expected analytical methods using Monte Carlo simulations. To each expected method, the following were computed: design spaces, graphs of the t_R variation versus T°, pH and TG as well as the corresponding predicted chromatogram. Then, the predicted optimal conditions were tested in practice while maintaining constant the other analytical conditions: column, flow rate, injection volume and detection wavelength as mentioned in the section of materials and methods. As mobile phase, classic and affordable solvents were privileged i.e. methanol LC grade and buffers mentioned earlier (see Section 2.3).

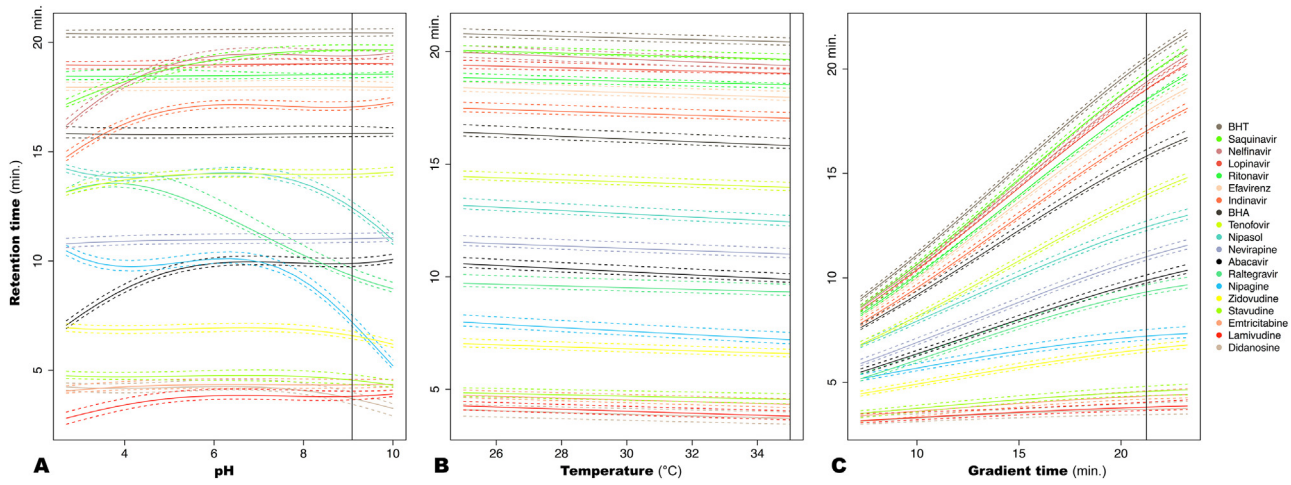

Fig. 2. Modelled retention times (t_R) of different compounds with respect to factors of experimental design: (a) pH, (b) temperature (T°, °C), and (c) Gradient time (TG, min).

Legend: BHT, Saquinavir, Nelfinavir, Lopinavir, Ritonavir, Efavirenz, Indinavir, BHA, Tenofovir, Nipasol, Nevirapine, Abacavir, Raltegravir, Nipagine, Zidovudine, Stavudine, Emtricitabine, Lamivudine, Didanosine

Table 4
Optimal analytical conditions and operating range within DS for the separation of the molecules.

	OptimalP(S > 0)	pH	TG (min)	T° (°C)
Screenig method for 18 compouds	0.2%	9.09(8.70–9.30)	21.25(21.0–21.6)	35.0(34.5–35.0)
Method for Liquid dosage forms	95%	3.61(3.60–6.00)	9.25 (9.0–23.3)	25.0(25.0–35.0)
Method for Solid dosage forms	95%	2.70(2.70–3.70)	7.25 (7.0–23.3)	25.0(25.0–35.0)

Three kinds of optimization were needed in order to obtain the optimal conditions on T°, pH, TG and probability of peaks separation P(S > 0) for the studied analytical methods presented in Table 4.

3.3.1. *First optimization: screening method*

To sustain the optimization of the screening method through the evaluation of quality chromatogram [7,8], we selected the separation between peaks of the critical pair as a critical quality attribute (CQA). Then, as proposed by Lebrun et al . [9], we used in this study the separation criterion (*S*) defined as the difference between the t_R at the beginning of a "n" th eluting peak (t_RB) and the t_R of the end of the "n-1" th eluting peak (t_RE) of the critical peak pair. The critical pair is defined by min(S), for a given operating condition. In the present study, it was not constituted by the same peak pairs; it has changed every time within the domain according to the experimental conditions. A screening method able to detecting 15 ARV and 4 preservatives compounds was optimized. The optimized experimental conditions were selected inside the design of experiments by means of the DS and considering the CQA defined as S > 0. These were expressed in terms of probability with the optimal conditions indicated in Table 4 including the probability surfaces to have S > 0 (see Fig. 3). As can be observed over the experimental domain that we investigated, it is noticed that the probability of peak separation P(S > 0) is very low (around 0.2%) the range of the optimal conditions is narrow to allow separation of all peaks, reversely when the probability is very high (95%), the range of the optimal conditions is large (see section 3.3.3.). Such a low probability may signify that a full separation (S > 0) among all the compounds is hardly found. The main reason over this low probability relies in the "too complex" situation depicted hereby Indeed, numerous compounds are encompassed in the design space computed. Besides that, another explanation is the chromatographic behavior of compounds such as 3TC, ddI, d4T and FTC which are similar and that co-elute around 5 min (see Fig. 4.a). Moreover, this probability is lessened by the calculated uncertainty of prediction represented by the gray zones around all analytes, and therefore allowing to predict a relatively higher risk of having other co-eluting peaks for compounds that have similar chromatographic behavior especially in case of changes of pH and TG as discussed in the previous section about the influence of the studied factors (pH, T°, TG). One can notice potential risk of peak co-elution of AZT and NpG at around 7 min; RAL and ABC at around 10 min; and RTV, LPV, NFV and SQV at around 19 min.

Despite this very low probability of peak separation, the 19 compounds were sufficiently separated to allow their identification from an injected solution containing 15 ARV and the 4 studied preservatives (cf. Fig. 4.b). As predicted, the compounds with similar chromatographic behavior, 3TC, FTC and d4T presented a co-elution at around 3 min whereas NFV and SQV had an inversion of peaks order while slightly co-elution at around 18 min which correspond to the aforementioned gray zone of uncertainty of prediction.

From the observed results, it can be noticed that the predicted and experimental chromatograms are quite comparable in terms of peaks elution order and retention times (beginning, apex and end) supported by the calculated coefficient of determination ($R^2 = 0.9946$) between the predicted t_R and observed t_R that was very good demonstrating the accuracy of the prediction of the compounds in the screening method.

The small changes observed may be explained by the uncertainty of prediction due to the variation of the chromatographic conditions especially on pH factor that was found to have a significant and different influence on the chromatographic behavior of some compounds by relatively changing their t_R. Some relationship

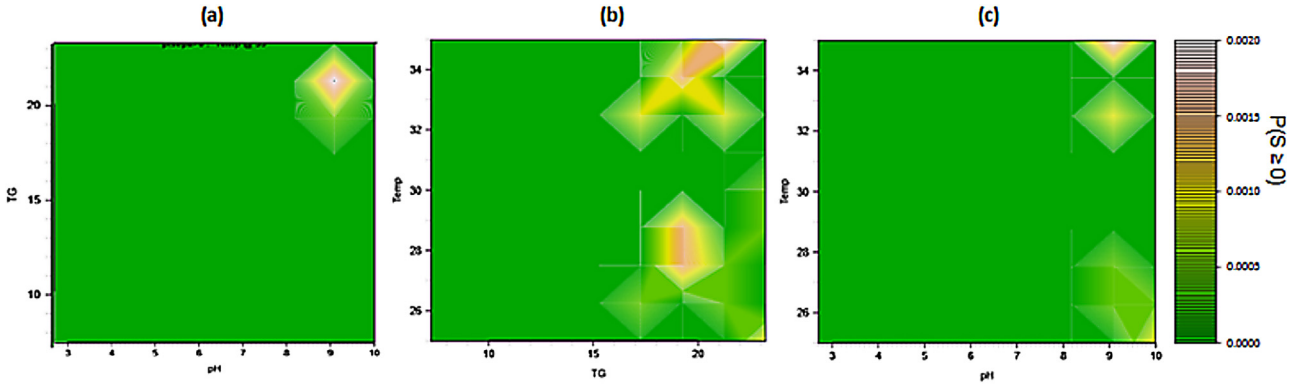

Fig. 3. Probability surfaces to reach S ≥ 0: (a) pH versus TG (min.), (b) TG (min.) versus T° (°C), (c) pH versus T° (°C).

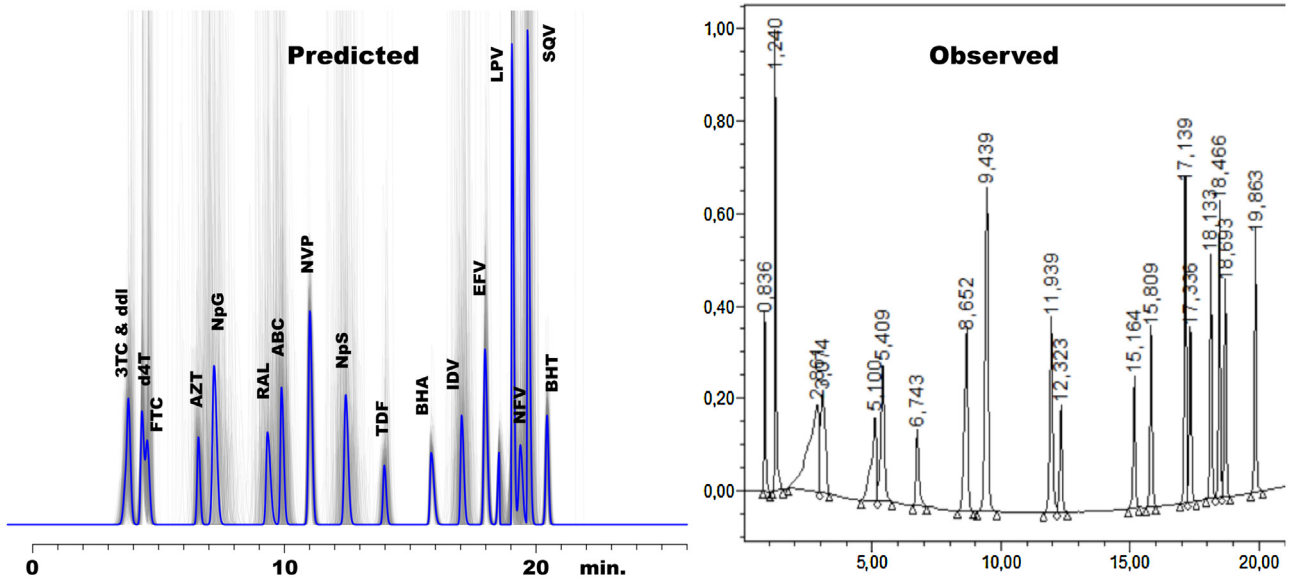

Fig. 4. (a) Predicted chromatogram for the screening method. (b) Experimental chromatogram for the screening method on the overall solution.
Legend per elution order:
(0) Fumaric acid from tenofovir disoproxil fumarate; (1) ddI; (2) 3TC/FTC; (3) d4T; (4) AZT; (5) NpG; (6) RAL; (7) ABC; (8) NVP; (9) NpS; (10) TDF; (11) BHA; (12) IDV; (13) EFV; (14) RTV; (15) LPV; (16) SQV; (17) NFV; (18) BHT
Experimental conditions: Column: XBridge Shield (C18), 100 × 4.6 mm; Flow: 1 mL min^{-1}, T°: 35 °C, λ = 210 nm, Gradient elution:

For 18 compounds	Methanol (%)	Buffer (%)	Elution phase type
0.00	5	95	Gradient
21.25	95	5	
25.25	95	5	Isocratic plate
25.65	5	95	Rebalancing initial
37.25	5	95	mobile phase

observed between pH and t_R were increasing quadratic regression and sigmoid, whereas others were decreasing regression or even no regression at all. The compounds concerned were ddI, 3TC, d4T, AZT, NpG, RAL, ABC, NpS, IDV and NFV at pH > 9 that is the optimal level of pH for the screening method. As a warning to have reproducible results, the analysts should be careful while adjusting the pH of the buffer solution in order to minimize the risk of variation in retention times (beginning, apex and end) and/or peaks elution order. Other precaution is to have a sufficient re-equilibration time of the column after gradient before a next run as indicated in Fig. 5.

We tried also to investigate the probable cause of the fronting peaks observed at 2.86 min and at 5.10 min. For that, we run separately the solutions of the four sub-groups using the same analytical conditions of the screening method. As can be seen in overlayed chromatograms in Fig. 6 no peak co-elution was observed inside the sub-group or between sub-groups and no fronting phenomenon occurred. It was noticed that d4T co-elutes with both 3TC and FTC when the three compounds are mixed, but 3TC and FTC are well separated when they are mixed in one solution. On the other hand, the peak of AZT in the chromatogram of sub-group solution 2 was found very good without any fronting peak. We understood that the fronting phenomenon on this analyte in the overall mixture solution is caused by the presence of NpG that co-elutes with AZT in this analytical method. Note that the chromatograms of Figs. 4 .b and 6 have similar peaks elution order and very closer retention times (at apex).

3.3.2. Second optimization: generic method dedicated to liquid dosage forms

In a following step, we were interested to the development of methods which are applicable to specific dosage forms containing the studied ARVs. A generic method for the analysis of ARV

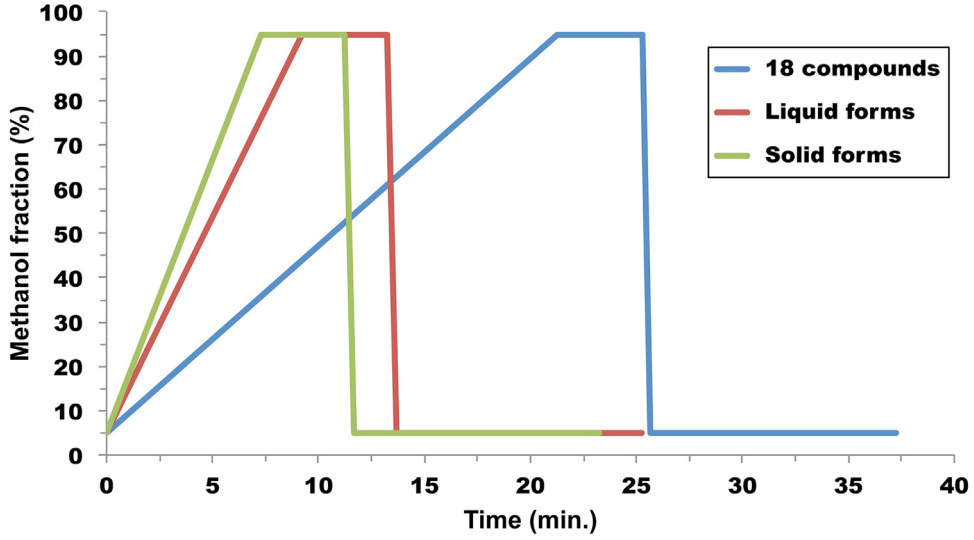

Fig. 5. Gradient profiles for the screening method of 18 compounds, liquid dosage forms, and solid dosage forms methods.

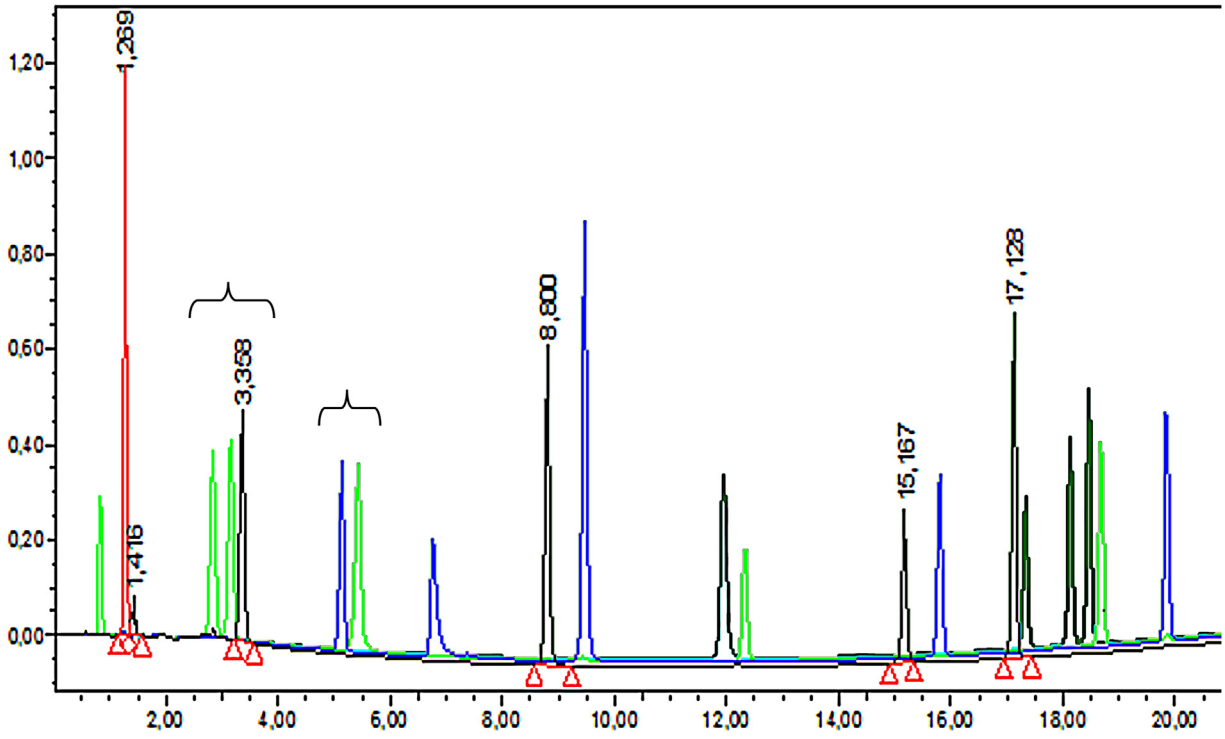

Fig. 6. Overlaying chromatograms of the 4 sub-groups containing the studied 15 ARV agents and 4 excipients, analysed with the screening method.
Legend per elution order:
Blue peaks belong to the ARV sub-group 2 solution (AZT, RAL, NVP, IDV, BHT);
Green peaks to sub-group 3 (fumaric acid, 3TC, FTC, NpG, TDF, NFV);
Cyan peaks to sub-group 4 (NpS, RTV, LPV, SQV),
Red and Black peaks to sub-group 1 (ddI, d4T, ABC, BHA, EFV)
Experimental conditions: the same as for Fig. 4

medicines in liquid dosage forms was optimized at the predicted optimal conditions given in Table 4. It was developed on the basis of earlier DoE/DS database on the 3 ARV (3TC, FTC and NFV) and the 4 studied preservatives (BHA, BHT, NpG, NpS) generally used in liquid dosage formulations. These ARV were selected on the basis of their medical prescription in pediatric formulations that are susceptible of containing NpG and NpS as antimicrobial preservatives and/or BHA, BHT as antioxidants, the latter being generally used in the manufacture of medicines that contain fatty compounds or oils to prevent their oxidation.

For this method we focused on the analysis of different liquid dosage forms either in single or combined active ingredients, and on monitoring the content of the four studied excipients in pediatric formulations such as EPIVIR® (3TC), ZERIT® (d4T), Mezivir® (AZT), VIRAMUNE® (NVP), Ziagen® (ABC), Emtriva® (FTC), and various other ARV medicines containing among others NpG and NpS [21–26]. On the other hand, a recent reflection paper by the Euro-

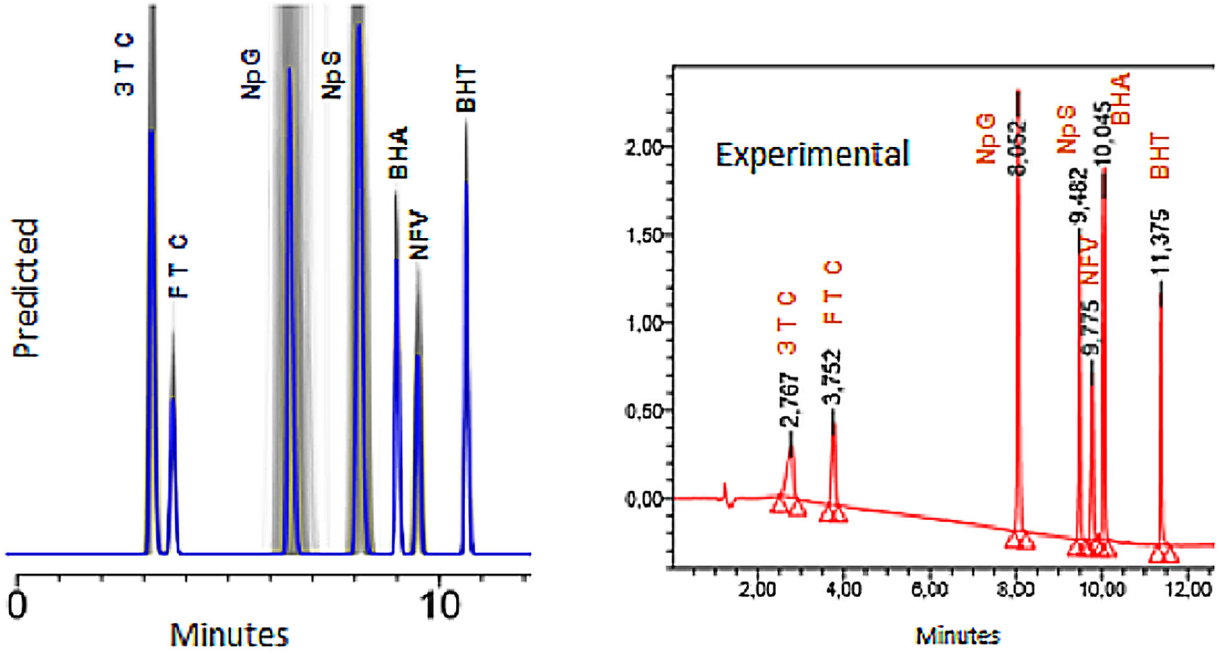

Fig. 7. Predicted and experimental chromatograms of the method for the analysis of liquid dosage forms.
Experimental conditions: Column: XBridge Shield (C18), 100 × 4.6 mm; Flow: 1 mL min^{-1}, T°: 25 °C, λ = 210 nm, Gradient elution:

For liquid dosage forms	Methanol (%)	Buffer (%)	Elution phase type
0.0	5	95	Gradient
9.25	95	5	
13.25	95	5	Isocratic plate
13.65	5	95	Rebalancing initial
25.25	5	95	mobile phase

Table 5
Predicted and observed retention times (t_R at apex) on the method for the analysis of liquid dosage forms.

Compound	Predicted t_R (min.)	Observed t_R (min.)	Difference(min.)
3TC	3.17	2.77	−0.40
FTC	3.68	3.75	0.07
NpG	6.44	8.05	1.61
NpS	8.09	9.48	1.39
BHA	8.97	10.05	1.08
NFV	9.48	9.78	−0.30
BHT	10.62	11.38	0.76

pean Medicines Agency (EMA) on the use of NpG and NpS as excipients in human medicinal products for oral use stipulated that in oral pharmaceutical formulations, association of those excipients are used with concentrations generally ranging from the authorized content from 0.015% to 0.2% for NpG and from 0.02% to 0.06% for NpS. Based on the current posology of medicines containing these conservatives, concentrations of 0.2% and 0.06% would correspond to maximal intakes of approximately 140 mg/day and 50 mg/day, respectively [27]. These can serve as acceptable limits of the two antimicrobial preservatives in various formulations. Thus far, to allow monitoring the BHA and BHT excipients in medicines, we considered as specification the acceptable daily intake (ADI) limits fixed by the Joint FAO/WHO Expert Committee on Food Additives. The ADI for BHA was: 0–0.5 mg/kg body weight/day, and for BHT: 0–0.3 mg/kg body weight/day [28–30] which would be compared to the posology of medicines and the medical prescriptions in order to avoid consuming more than the recommended ADIs. In this way our interest was also to fulfill the above requirements at least by detecting these conservatives. As can be seen in Table 5 and in Fig. 7, the peaks of the ARV are resolved as well of the 4 preservatives in 12 min. The predicted and experimental results were found comparable in terms of peaks elution order except BHA and NFV for which an inversion of peak elution was observed. We noticed a change of more than 1 min in retention time (at apex) of NpG, NpS and BHA mainly due to the prediction uncertainty and sensitivity of the compounds on slight changes of pH of the aqueous mobile phase.

All ARV components among the most found in liquid dosage forms can be quantified without any interference with the 4 studied excipients. The method for liquid dosage forms is able to detect also the presence of the latter.

3.3.3. Third optimization: generic method dedicated to solid dosage forms

Solid dosage forms are very often used in combination of several ARVs at fixed-dose combinations "FDC" among which are listed 3TC/d4T/NVP, 3TC/AZT/NVP, FTC/TDF/EFV and 3TC/TDF/EFV. In this context, we oriented our third optimization for a generic method starting from the same database previously constructed for all the 19 compounds. The predicted optimal conditions (see Table 4) allowed to obtaining the predicted chromatograms for the 4 FDCs (see Fig. 8). We tested in practice the conditions of one FDC (i.e. FTC/TDF/EFV) and we obtained the experimental results quite similar in terms of peaks elution order and retention times (at apex) as illustrated in Fig. 9.

We noticed that the variation in t_R was less than 0.6 min, and all peaks were separated, very sharp and symmetric allowing to identify even the fumaric acid at 2.74 min. This analyte is released from tenofovir disoproxil fumarate (TDF).

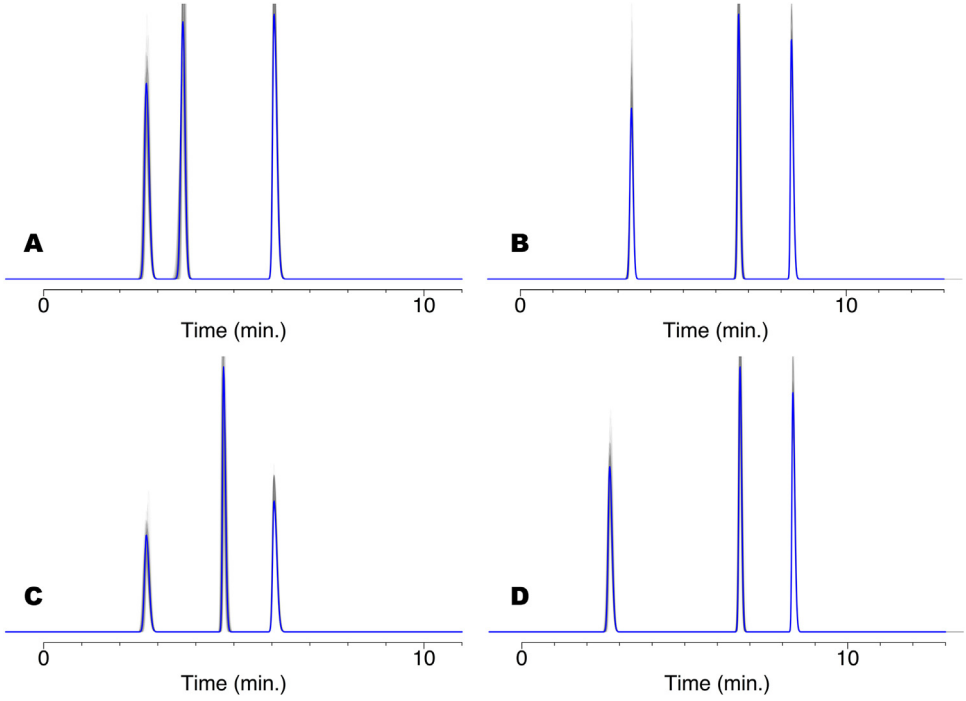

Fig. 8. Predicted chromatograms of the 4 fixed-dose combinations of 3TC, AZT, d4T, EFV, FTC, NVP, and TDF in solid dosage forms.
Experimental conditions: Column: XBridge Shield (C18), 100 × 4.6 mm; Flow: 1 mL min^{-1}, T°: 25 °C, λ = 210 nm, Gradient elution:

For solid dosage forms	Methanol (%)	Buffer (%)	Elution phase type
0.0	5	95	Gradient
7.25	95	5	
11.25	95	5	Isocratic plate
11.65	5	95	Rebalancing initial
23.25	5	95	mobile phase

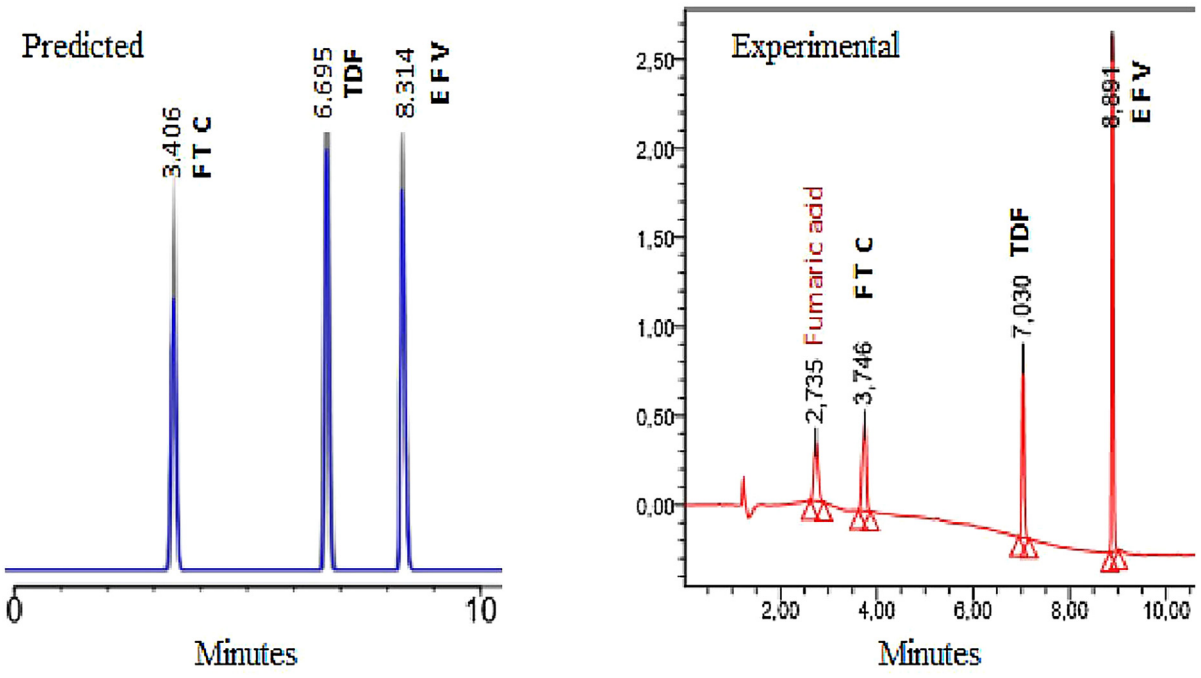

Fig. 9. Predicted and experimental chromatograms of FTC/TDF/EFV combination. Resolutions are 6 (between Fumarate and FT), 27 (between FTC and TDF) and 24 (between TDF and EFV).
Experimental conditions: the same as for Fig. 8

Table 6
Summary of the validation results.

Validation criteria	Conc. ($\mu g\,mL^{-1}$)	EFV	Conc. ($\mu g\,mL^{-1}$)	FTC	Conc. ($\mu g\,mL^{-1}$)	TDF
Trueness: Absolute bias ($\mu g\,mL^{-1}$) & (Relative bias) (%)	*120*	5.23 (4.36)	*42*	1.70 (4.02)	*60*	−1.83 (−3.07)
	180	12.77 (7.09)	*63*	−0.88 (−1.38)	*89*	4.06 (4.53)
	240	−3.95 (−1.64)	*85*	0.99 (1.17)	*119*	0.19 (0.16)
	300	−1.51 (−0.50)	*106*	2.57 (2.43)	*149*	−6.31 (−4.21)
	360	−1.58 (−0.44)	*127*	3.79 (2.99)	*179*	−0.84 (0.47)
Precision: Repeatability (RSD%) / Intermediate precision (RSD%)	*120*	3.82/4.03	*42*	3.15/3.15	*60*	3.17/3.17
	180	4.08/4.08	*63*	2.72/2.72	*89*	1.63/1.94
	240	1.52/1.72	*85*	2.28/2.28	*119*	1.74/1.74
	300	0.97/1.93	*106*	1.75/1.75	*149*	0.92/0.92
	360	1.82/2.04	*127*	1.82/1.82	*179*	1.50/2.04
Accuracy: 95% βE ($\mu g\,mL^{-1}$) & (Relative 95%βE) (%)	*120*	114.5–136.1 (−4.62 to 13.33)	*42*	41.1–46.9 (−2.82 to 10.85)	*60*	53.7–61.9 (−9.95 to 3.81)
	180	176.9–208.8 (−1.77 to 15.95)	*63*	58.8–66.3 (−7.29 to 4.53)	*89*	89.4–97.7 (−0.12 to 9.18)
	240	226.6–245.8 (−5.64 to 2.36)	*85*	81.4–89.8 (−3.78 to 6.13)	*119*	115.0–124.0 (−3.62 to 3.94)
	300	278.6–318.7 (−7.19 to 6.18)	*106*	104.3–112.3 (−1.38 to 6.24)	*149*	139.8–145.8 (−6.23 to 2.24)
	360	341.6–375.6 (−5.16 to 4.28)	*127*	125.7–135.7 (−0.96 to 6.93)	*179*	168.4–187.8 (−5.87 to 4.94)
Linearity:	Slope	0.9535	Slope	1.036	Slope	0.972
	Intercept	13.35	Intercept	1.42	Intercept	2.41
	R^2	0.992	R^2	0.995	R^2	0.992
Dosing range:						
Lower LOQ ($\mu g\,mL^{-1}$)		206.4	LLOQ	45.14	LLOQ	59.64
Upper LOQ ($\mu g\,mL^{-1}$)		360.2	ULOQ	126.9	ULOQ	178.9

βE = β-Expectation Tolerance Interval.
Between-run variance equaling or almost equaling to zero means that the intermediate precision equals to repeatability.

3.4. Method validation

After the optimization process, it was necessary to demonstrate that the developed analytical methods provide accurate assay results. In this study, we choose the method dedicated to the assay of one FDC composed by Emtricitabine (FTC), Tenofovir (TDF) and Efavirenz (EFV) in tablets formulations. These three molecules were selected for validation since they are used alone or in combination to avoid HIV resistance due to different action mechanisms. In addition, their chemical structures are different and representative of the most popular pharmaco-chemical groups available among the ARV. Emitricitabine belongs to analog of cytidine, tenofovir to analog of nucleotide of adenine and efavirenz is a non nucleoside of 4-benzyl and 4-benzoyl-3 dimethylamine pyridinones. Indeed, the action of these three compounds is strongly related to their specific structures. Thus it was very interesting to evaluate the ability of method to quantify these compounds through validation. The validation criteria commonly found in the document Q2(R1) of the International Conference on Harmonization (ICH) [15] were considered namely: selectivity, trueness, precision (repeatability and intermediate precision), accuracy, linearity, limit of detection (LOD)/limit of quantitation (LOQ), and dosing range.

At first, we ensured that the selectivity of the method was suitable Since no interference was observed at the tR of the peaks of interest here FTC, TDF and EFV, and the resolution between peaks was largely well above 1.5, one can conclude that this validation criteria was fulfilled.

At second, we applied the concept of total error strategy represented by accuracy profiles as decision tool on the fit-for-purpose of the method for its intended use [11–14].

By using the data of CS, the linear regression model was constructed and allowed obtaining the calculated result from VS. The residual sum of square (RSS) values were 4844, 408.5 and 1288 for EFV, FTC and TDF, respectively. Then, accuracy profiles for the three ARV compounds were drawn as can be seen in Fig. 10 with the results of validation criteria summarized in Table 6. The acceptance limits were set at ±10% according to the International Pharmacopoeia which is among the most used pharmacopoeias in Rwanda in the analysis of medicines [31].

From the back-calculated results of VS that are the experimental ones, trueness of the method 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 3 ARV and a mean experimental one. We were able to get information on systematic error that was found acceptable since the relative biases

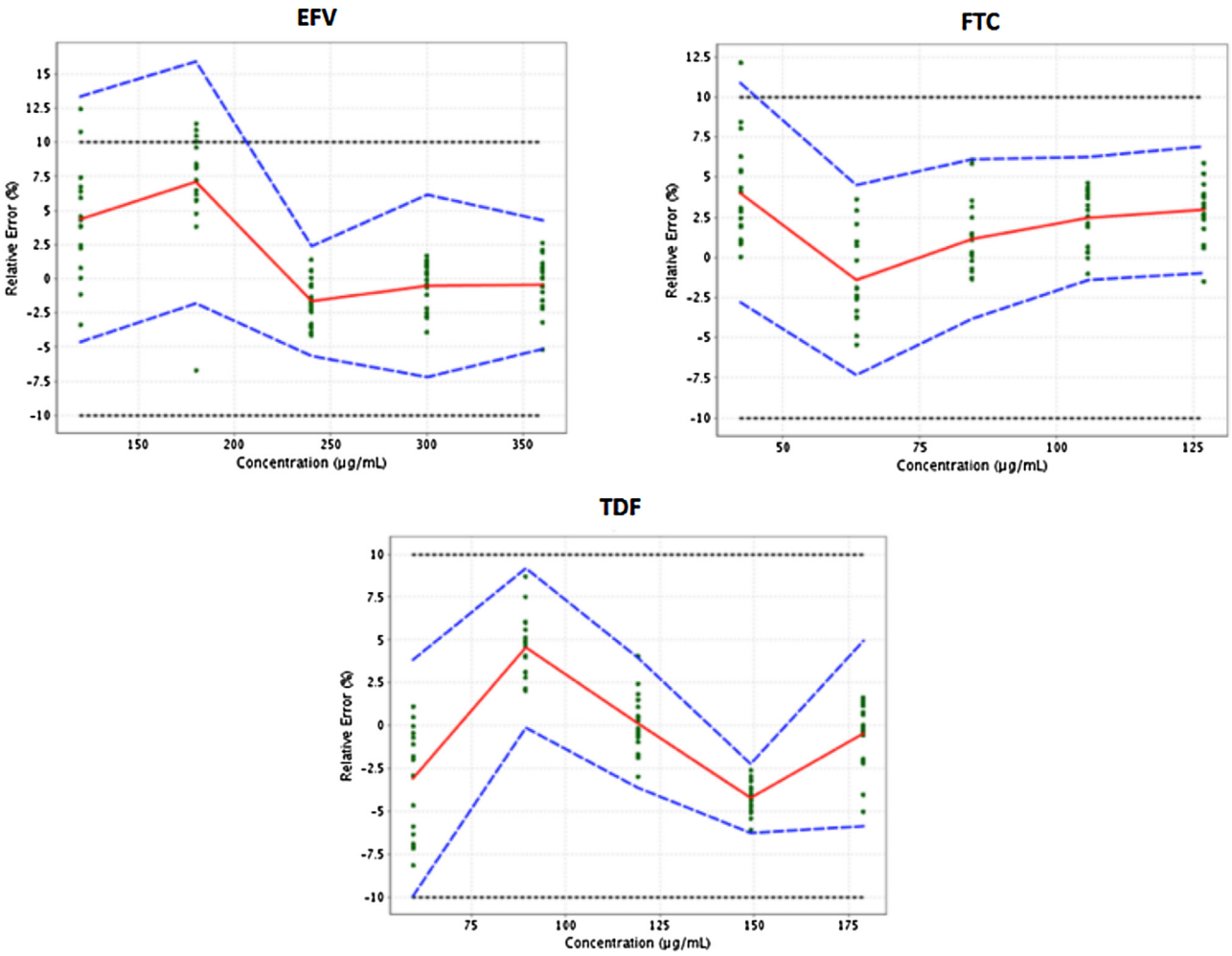

Fig. 10. Accuracy profiles for quantitative methods validation of EFV, FTC and TDF in tablet formulations. The plain red line represents the relative bias, the blue dashed lines the 95% β-expectation tolerance limits and the black dotted lines the 10% acceptance limits. The green dots express the relative error of the back-calculated concentrations plotted with respect to their targeted concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For 18 compounds	Methanol (%)	Buffer (%)	Elution phase type
0.00	5	95	Gradient
21.25	95	5	
25.25	95	5	Isocratic plate
25.65	5	95	Rebalancing initial
37.25	5	95	mobile phase
For liquid dosage forms	**Methanol (%)**	**Buffer (%)**	**Elution phase type**
0.0	5	95	Gradient
9.25	95	5	
13.25	95	5	Isocratic plate
13.65	5	95	Rebalancing initial
25.25	5	95	mobile phase
For solid dosage forms	**Methanol (%)**	**Buffer (%)**	**Elution phase type**
0.0	5	95	Gradient
7.25	95	5	
11.25	95	5	Isocratic plate
11.65	5	95	Rebalancing initial
23.25	5	95	mobile phase

were between – 1.64% and 7.09% for EFV; – 1.38% and 4.02% for FTC; – 4.21% and 4.53% for TDF, all in respect with the acceptable limit of ± 10%.

Method precision was also found acceptable since there was a closeness of agreement among measurements here the back-calculated results of VS obtained from multiple sampling of homogeneous samples of the three ARV. The relative standard deviation values for repeatability and for intermediate precision at the target 100% concentration level were acceptable with a maximum of 1.72% for EFV, of 2.28% for FTC and of 1.74% for TDF.

To demonstrate the method linearity, we assessed the relationship between the back-calculated results of VS (experimental ones) against the introduced concentrations of the 3 ARV in the samples. The linear regression model was fitted on the two types of concentrations, with a good linearity of the results illustrated (cf. Table 6) by the slopes close to 1.

Table 7
Assay results of two ARV medicines coded A and B from Benin and Rwanda. Results consist in the mean percentage of claimed nominal content and the relative standard deviation calculated on 3 independent samples. Specifications are set to 90.0%–110.0% of the claimed nominal content (mg).

Drug		EFV	FTC	TDF
A	Claimed	600 mg	200 mg	300 mg
	Assay in%	100.3 ± 1.04%	80.2 ± 0.78%	84.8 ± 1.17%
B	Claimed	0 mg	200 mg	300 mg
	Assay in%	–	93.1 ± 1.74%	91.5 ± 1.40%

Method accuracy taking into account the total error, *i.e.* systematic and random errors, was assessed from the accuracy profile shown in Fig. 10. In addition, as shown in Table 6, the relative β-expectation tolerance intervals are in general within a range of [−9.95, 9.18%] except level 1 of FTC, and level 1 and 2 of EFV. As the lower and upper tolerance bounds are included within the acceptance limits for all the targeted concentration levels (except the one mentioned i.e. Level 1 for FTC; level 1 and 2 for EFV), one can ensure that each future result will fall within the acceptable limits with a probability of at least 95% [32].

We estimated also the limit of detection (LOD) that is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. The reported values were: 6.30; 10.63 and 19.74 $\mu g\,mL^{-1}$ for FTC, TDF, and EFV respectively.

The lower limit of quantification (LLOQ) is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy meaning in the range of the specified acceptable limits of ±10%. The definition can also be applicable to the upper limit of quantitation (ULOQ) which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy in the range of the considered acceptable limits. The limits of quantitation were obtained by calculating the smallest and highest concentrations beyond which the accuracy limits or β-expectation limits go outside the acceptance limits. The intervals between the lower and the upper limits where the procedure achieves adequate accuracy allowed us to set the dosing ranges that were 45.14–126.9 $\mu g\,mL^{-1}$ for FTC; 59.64–178.9 $\mu g\,mL^{-1}$ for TDF and 206.4–360.2 $\mu g\,mL^{-1}$ for EFV.

The uncertainty is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand. In this study the relative expanded uncertainly (%) have been found less than 8%.

3.5. Application of the method

As the last step of the method lifecycle, the developed and validated method dedicated to solid dosage form was applied in routine for quality control of tablets samples. Two different brand pharmaceutical drugs were tested. They were sampled in Benin and in Rwanda, and claimed to contain FTC, TDF and EFV (sample A) and only FTC and TDF (sample B). The analytical results presented in Table 7 consisted in the mean percentages of the claimed nominal contents in each medicine and the relative standard deviations calculated on 3 independent samples preparations. Only Brand B satisfied to the acceptable content limits 90.0%–110.0%. For Brand A, only EFV content was found within these limits whereas the other active ingredients were present but out of the specifications. We can also notice the good precision of the method since the RSD are below 1.74%.

4. Conclusion

The main objective of the present study was to develop generic LC methods for qualitative and/or quantitative analysis of 18 ARV namely ABC, ddI, EFV, FTC, IDV, 3TC, LPV, NFV, NVP, RTV, SQV, d4T, TDF, AZT, RAL, ATZ, DRV, ETV and 4 major excipients (NpG, NpS, BHA and BHT) by applying DoE/DS methodology.

Firstly, 15 ARV and the excipients were selected for optimization using D-optimal design based on Gradient time, column temperature and pH of buffer solution selected as analytical factors, the experiments showed that the Gradient time and pH had significant effects on peak separations within the explored experimental domain.

By exploiting the generated DoE database, a screening method was predicted thanks to Monte Carlo simulations for simultaneous analysis of 15 + 4 compounds using low cost mobile phase composed by methanol and 10 mM ammonium hydrogen carbonate buffer. This method was found useful in the detection of suspected counterfeit/falsified or a mix up of the ARV studied.

Secondly, other methods for the analysis of solid and liquid dosage forms were optimized. For oral solutions or suspensions, a generic method using methanol and 10 mM ammonium formate buffer pH 3.61 was optimized for the analysis of 3DT, AZT, ABC, EFV, FTC, LPV/RTV, etc. together with at least one of the four studied excipients.

Thirdly, for the analysis of solid dosage forms another method was optimized to analyze four tablet fixed-dose combinations. The one for FTC/TDF/EFV was successfully applied in 3 cases: validation, application on real samples. The build DoE/DS database can serve in the prediction of other optimal analytical conditions for the analysis of any other fixed-dose combination or mono-component active ingredient with one or more of the four studied major excipients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.02.040.

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