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## Green HPLC quantification method of lamivudine, zidovudine and nevirapine with identification of related substances in tablets

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### ABSTRACT

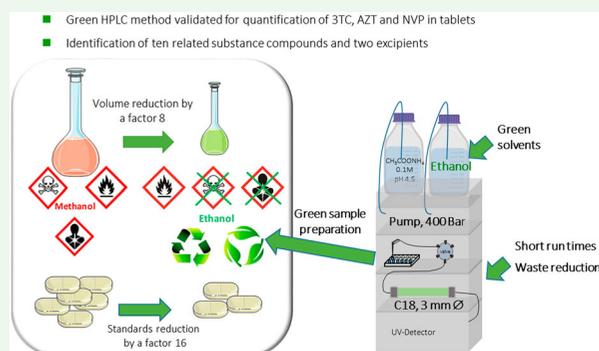
A green HPLC method for lamivudine (3TC), zidovudine (AZT) and nevirapine (NVP) determination in fixed-dose combination tablets was developed using ethanol as both one of the mobile phases and the solvent for sample preparation. This method was validated according to ICH Q2(R1) guideline. Additionally, the method was adapted to complete the analysis of five related substances described in the *International Pharmacopoeia* (Ph. Int.), five other known related substances and two excipients. The separation was obtained with a C18 column (ARV4 5  $\mu$ m 250  $\times$  3.0 mm, Interchim) using a gradient mode with 0.1M ammonium acetate buffer (pH 4.5) and ethanol as mobile phase at 35°C, a flow rate of 0.4 mL/min, at 270 nm and an injection volume of 10  $\mu$ L. The combination of ethanol (biodegradable and low-cost alternative solvent) with the use of a column diameter of 3 mm instead of 4.6 mm as used in pharmacopoeias, makes the wastes of the analysis more environmentally friendly and allows the use of a conventional HPLC pump (<400 bar) for easy implementation in quality control in countries with limited resources. Assessment of the method greenness was evaluated using three analytical tools: Analytical Eco-scale, AGREE metrics and Analytical Method Greenness Score (AMGS).

### ARTICLE HISTORY

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### KEYWORDS

Zidovudine; lamivudine; nevirapine; green HPLC; method validation



**Abbreviations:** ACN: acetonitrile; API: active principal ingredient; AZT: zidovudine; AZT imp EP A: zidovudine impurity European Pharmacopoeia A (stavudine); AZT imp EP B: zidovudine impurity European Pharmacopoeia B; AZT imp EP C: zidovudine impurity European Pharmacopoeia C (thymine); AZT imp EP E: zidovudine impurity European Pharmacopoeia E (thymidine); AZT imp EP G: zidovudine impurity European Pharmacopoeia G; MeOH: methanol; NVP: nevirapine; NVP imp EP A: nevirapine impurity European Pharmacopoeia A; NVP imp EP B: nevirapine impurity European Pharmacopoeia B; NVP imp EP C: nevirapine impurity European Pharmacopoeia C; Ph. Int.: International Pharmacopoeia; 3TC: lamivudine; 3TC imp EP C: lamivudine impurity European Pharmacopoeia C (salicylic acid); 3TC imp EP E: lamivudine impurity European Pharmacopoeia E (cytosine)

### Introduction

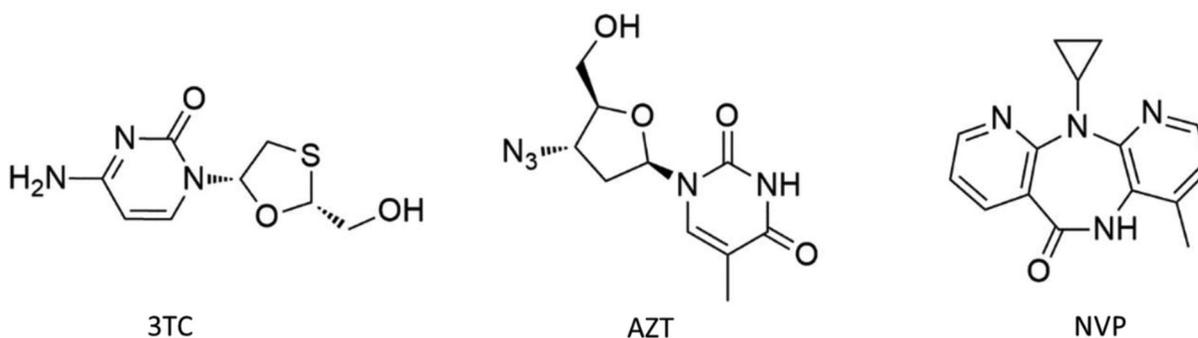
Antiretroviral drugs, like the Nucleoside Reverse Transcriptase Inhibitors (NRTIs) lamivudine (3TC) and

zidovudine (AZT) and the non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) nevirapine (NVP) (Figure 1) are widely prescribed to treat HIV infections in

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**Figure 1.** Chemical structures of lamivudine (3TC), zidovudine (AZT) and nevirapine (NVP).

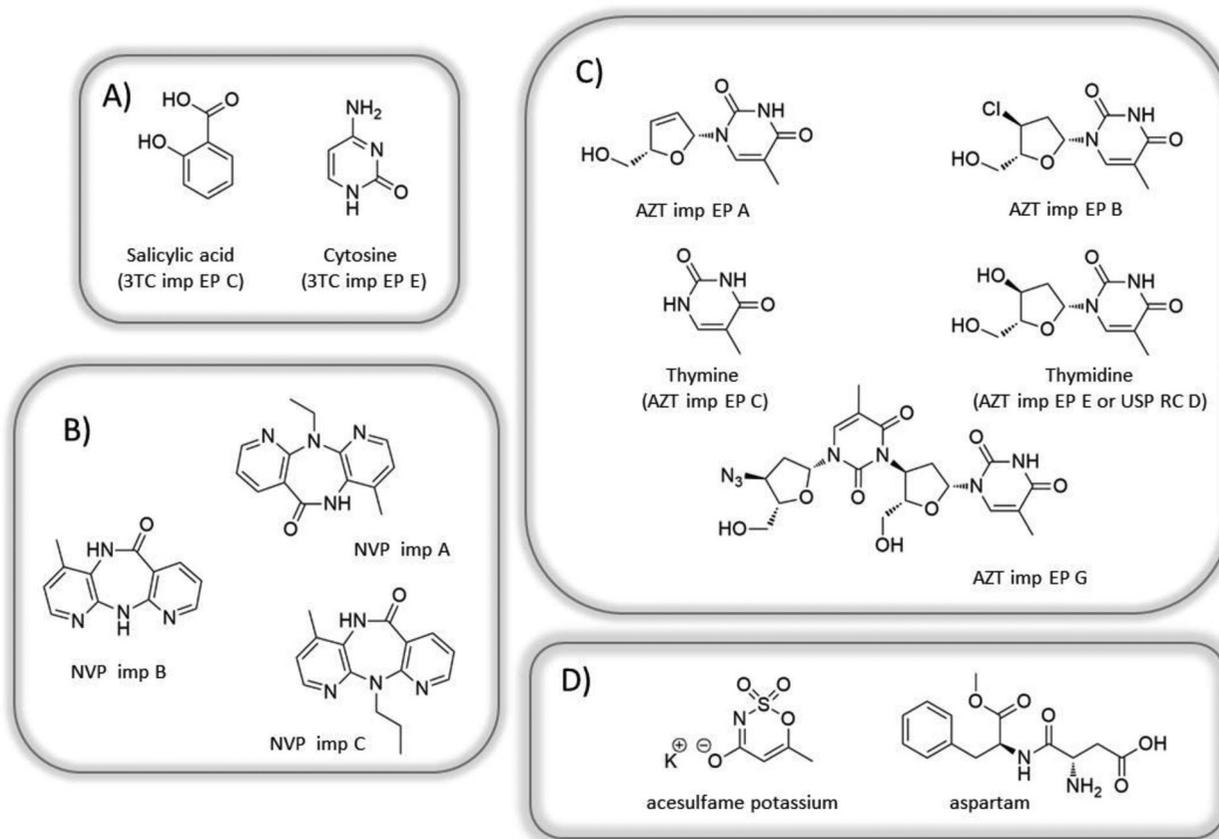
resource-limited countries. A fixed-dose combination of these three drugs appeared in 2005 (1) and now it is included in the WHO's list of essential medicines (2) and is available in adult and pediatric doses. A number of HPLC quantification methods of 3TC, AZT and NVP in pharmaceutical forms for performing pharmaceutical control analysis and combating the emergence of counterfeit medicines can be found in the literature (3–6) as well as in the official HPLC methods in pharmacopoeias. The European, United States (USP) and international (Ph. Int.) pharmacopoeias contain single monographs for each active principal ingredient (API). The USP and Ph. Int. also integrate the monographs for pharmaceutical products and the simultaneous dosage of 3TC, AZT and NVP in tablets is only available in Ph. Int. (7). These methods used acetonitrile (ACN) or methanol (MeOH), the most widely used solvents in RP-HPLC and acetate or phosphate buffer as mobile phases. However, methanol and ACN solvents are included on the EPA hazardous waste (8) and on ICH Q3C guideline (R8) (9). They should be properly disposed or recycled because discharging untreated wastewater containing ACN into natural water systems can cause serious ecological environmental damage. Minimizing ACN or MeOH consumption in analytical scientific process is therefore crucial in increasing the sustainability of pharmaceutical analysis (10, 11), and even more so in resource-limited countries where waste management and treatment constitutes a serious problem (12). At present, Eco-Friendly methods are developed using several chromatographic methods and types of pharmaceutical drugs (11, 13–16). To best of our knowledge, to date no green HPLC method has been proposed for determining 3TC, AZT or NVP in pharmaceutical forms, whether separately or simultaneously. The objective of the present work was to develop a green HPLC method for the simultaneous analysis of 3TC, AZT and NVP and its application in tablets for both paediatric and adult patients with the ethanol integration as a mobile phase. Additionally, the method was adapted to complete the analysis of

five related substances described in the Ph. Int., five other known related substances and two excipients (Figure 2). For that, ethanol (a biodegradable solvent, less toxic than ACN and MeOH) was successfully incorporated as the organic mobile phase using a typical HPLC pump (< 400 bar pressure). At the same time, the column diameter reduction (3 mm instead of 4.6 mm as used in Pharmacopoeias) was used to minimize waste generation and, therefore, waste management. Sample processing was also optimized using ethanol as a solvent, with the minimization of standard quantities and volume solutions. The proposed method should be easily implemented in quality control laboratories and in particular in the laboratories of resource-limited countries.

## Materials and methods

### Chemicals and reagents

Working reference standards of 3TC, AZT and NVP were obtained from Sigma-Aldrich, Saint-Quentin-Fallavier, France. The percentages of purity for AZT, 3TC and NVP are 99.7%, 99.6% and 99.9%, respectively. Cytosine (3TC imp EP E), thymine (AZT imp EP C), thymidine (AZT imp EP E or USP RC D) and NVP imp EP B were obtained from LGC Standards SARL, Molsheim, France. Salicylic acid (3TC imp EP C) was purchased from Sigma-Aldrich, Saint-Quentin-Fallavier, France. Stavudine (AZT imp EP A), AZT imp EP B CRS (Z1904000), NVP for peak identification CRS (Y0000521) and AZT for system suitability CRS1 (Y0001641) were obtained from the European Directorate for the Quality of Medicines (EDQM, Strasbourg, France). Analytic grade was used for ethanol (Carlo Erba, Val-de-Reuil, France), ammonium acetate and glacial acetic acid (Sigma/Merck, Saint-Quentin-Fallavier, France). Pharmaceutical forms (tablets) were provided by the Centre Humanitaire des Métiers de la Pharmacie (CHMP, Clermont-Ferrand, France) at two different dosages: 30 mg 3TC, 60 mg



**Figure 2.** Chemical structures of related substances of (A) lamivudine (3TC), (B) nevirapine (NVP), (C) zidovudine (AZT); and (D) chemical structures of excipients analysed.

AZT and 50 mg NVP for paediatric formulations; and 150 mg 3TC, 300 mg AZT and 200 mg NVP for adult. For the development phase, different formulations from different manufacturers were analysed.

### Instrumentation and separation conditions

The HPLC system consisted of an UHPLC 1260 Infinity II Prime LC (Agilent Technologies, Santa Clara, USA) equipped with a quaternary pump (800 bars), a degasser, a thermostatted auto sampler and column compartment and a photodiode array (PDA) detector, with a dwell volume of 875  $\mu$ L and a C18 stationary phase of 250  $\times$  3.0 mm, 5  $\mu$ m (Uptisphere C18-ARV4, Advion Interchim Scientific, Montluçon, France). Data were processed using OpenLAB CDS Agilent software.

Separation was achieved in a gradient mode with ammonium acetate buffer (0.1M; pH 4.5) as mobile phase A and ethanol as mobile phase B. The gradient profile (% v/v) consisted of an isocratic part at 4% B during 3 min followed by a linear increase to 45% of phase B at 31 min (slope of 1.4), a return to the initial conditions in 2 min that was kept during 7 min for

column equilibration. Analysis was completed in 40 min. The flow rate was set at 0.4 mL/min. The column and the auto sampler temperatures were maintained at 30°C and 25°C, respectively. Detection was monitored at 270 nm and the injection volume was 10  $\mu$ L.

### Sample preparation

#### Standard solutions

In a 100 mL volumetric flask, approximately 37.50 mg of 3TC WRS, 75 mg of AZT WRS and 50 mg of NVP WRS were accurately weighed. Afterward, 15 mL of ethanol were added with sonication of the solution for 15 min, which improves the dissolution rate of the drugs. Then, the mobile phase A was used to adjust the volume. After filtration through a 0.20  $\mu$ m H-PTFE syringe filter, 1 mL of the previous solution was introduced in a 10 mL volumetric flask and the mix of ethanol and mobile phase A at a ratio of 15:85 (diluent solvent) was used to adjust the volume. Standard concentrations were 37.50  $\mu$ g/mL of 3TC, 75  $\mu$ g/mL of AZT and 50  $\mu$ g/mL of NVP.

### Assay solutions

Twenty tablets were weighed and powdered. In a 25 mL volumetric flask, a quantity of the powdered tablets containing 9.38 mg of 3TC, 18.75 mg of AZT and 12.50 mg of NVP was accurately weighed. A volume of 3.8 mL of ethanol was added followed by sonication for 15 min. Then, the mobile phase A was used to make up to volume. After filtration through a 0.20 µm H-PTFE syringe filter, a dilution was made to get a final concentration of 37.50 µg/mL of 3TC, 75 µg/mL of AZT and 50 µg/mL of NVP in a 10 mL volumetric flask using the diluent solvent.

### System suitability solution

In a 10 mL volumetric flask, approximately 1 mg of the related substances thymidine RS (AZT imp EP E), AZT imp EP B RS and NVP imp EP B RS were accurately weighed. Following this, 5 mL of diluent solvent were added in the flask followed by a sonication for 15 min. Then, the diluent solvent was used to make up to volume. The solution was filtered through 0.20 µm H-PTFE filter. Further, a dilution was made to get a final concentration of 6.0 µg/mL of each related substances in a 10 mL volumetric flask using the standard solution.

### Method validation

The validation of the method was performed according to ICH Q2(R1) guideline (17). The following parameters were studied: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness. Linearity, precision and accuracy were performed on two different column batches, on three days and by two different analysts.

## Results and discussion

### Method development

Different conditions were tested to obtain a HPLC method as green as possible using a typical pump (maximum 400 bar) to quantify the three APIs. The related substances: thymidine, AZT imp EP B and NVP imp EP B used for system suitability in Ph. Int (7) were included in method development. Ethanol was chosen as the green mobile phase B. Ethanol has similar chromatographic characteristics to ACN or methanol, such as a complete miscibility with water, and it has a higher eluotropic strength than methanol, allowing the use of less organic solvent (18, 19). However, its use with conventional HPLC systems (400 bar) is challenging because of its high viscosity. To resolve this issue, the use of a

C18 column of 5 µm particle size, a 3 mm internal diameter and 250 cm in length allowed us to propose a method that remains accessible to laboratories with limited resources. The main parameter that was optimized was the gradient programme. First, the pressure of the system was checked using a gradient with water as mobile phase A and ethanol as mobile phase B. A linear increase from 5% to 90% B with a flow rate of 0.4 mL/min at 30°C during 60 min was witnessed. This experience results in a maximum pressure of 305 bar for 50% B. The flow rate was then fixed to 0.4 mL/min in order not to exceed the pressure limit value of a conventional HPLC. The chromatographic methods for the analysis of 3TC, AZT and NVP described in the literature use acetate or phosphate buffer as mobile phases. Here, we use acetate buffer for its greener properties. We compare different ionic strength (0.05, 0.075 and 0.1 M) of the ammonium acetate buffer (pH 4.5). The 0.1 M ammonium acetate buffer was retained as the mobile phase A for the next optimization because finer peaks and better resolution were obtained. Different gradients were tested (from 4% to 80% EtOH) with a column temperature of 30 °C and a mobile phase flow rate of 0.4 mL/min. The resolutions were the criteria used to choose the best gradient slope. Table 1 shows that steeper slopes correlated with smaller resolutions. The resolution between thymidine and 3TC was similar between the slope of 1.4 and the slope 1.2 ( $R = 4.4$  and  $4.5$ , respectively). However, the slope of 1.4 was selected based on the retention time diminution of the last peak (NVP) which changed from 28.8 min to 25.8 min. An isocratic step of 3 min was added before the gradient to facilitate the method of transfer between different laboratories because the dwell volume may differ, as no two instruments are alike. Next, the influence of temperature was studied at 25°C, 30°C and 35°C. A good separation with a reduction of analysis times was obtained at 35 °C (Table 1).

**Table 1.** Development results: Retention times (RT) of three APIs and resolutions (R) between thymidine [1] and 3TC [2], AZT [3] and AZT imp EP B [4], NVP imp EP B [5] and NVP [6].

Chromatographic conditions	RT (min)			R		
	3TC	AZT	NVP	[1–2]	[3–4]	[5–6]
Slope 1.2, 30 °C	9.9	17.1	28.8	4.5	5.6	5.2
Slope 1.4, 30 °C	9.5	15.9	25.8	4.4	5.3	4.7
Slope 2.0, 30 °C	9.0	14.0	21.1	4.1	4.8	3.8
Slope 2.5, 30 °C	8.6	12.8	18.5	3.8	4.4	3.2
Iso <sup>a</sup> 3 min, Slope 1.4, 25 °C	12.1	19.2	29.2	4.5	5.4	4.0
Iso <sup>a</sup> 3 min, Slope 1.4, 30 °C	11.8	18.9	28.9	4.5	5.2	4.3
Iso <sup>a</sup> 3 min, Slope 1.4, 35 °C	11.2	18.2	28.3	4.8	5.4	5.6

<sup>a</sup>Iso: isocratic part.

## Method validation

### Specificity

The specificity was studied with respect to related substances and the excipients present in the tablet forms. Five standards of related substances, described in Ph. Int., were analysed: 3TC imp EP E (cytosine), AZT imp EP C (thymine), AZT imp EP E (thymidine), AZT imp EP B and NVP imp EP B. To complete the list of related substances, additional related substances were injected: stavudine (AZT imp EP A), salicylic acid (3TC imp EP C) and EDQM solutions allowed us to identify retention times for NVP imp EP A, NVP imp EP C and AZT imp EP G (Figure 3). The chromatograms show an excellent separation between APIs and all impurities injected (resolutions > 1.5, Table 2).

Tablets for adult and child use from several manufacturers (Mylan, Strides, Cipla and Hetero) were analysed in order to verify the specificity form for different excipient compositions of 3TC-AZT-NVP. The chromatographic profiles bore a number of similarities from one manufacturer to another. However, in the case of Mylan 30 mg 3TC tablets, two peaks (E1 and E2) potentially corresponding to excipients, were observable (Figure 4).

In order to verify this hypothesis, LC-MS was performed. Full scan in negative mode allows us to highlight that the (E1) and (E2) peaks correspond to

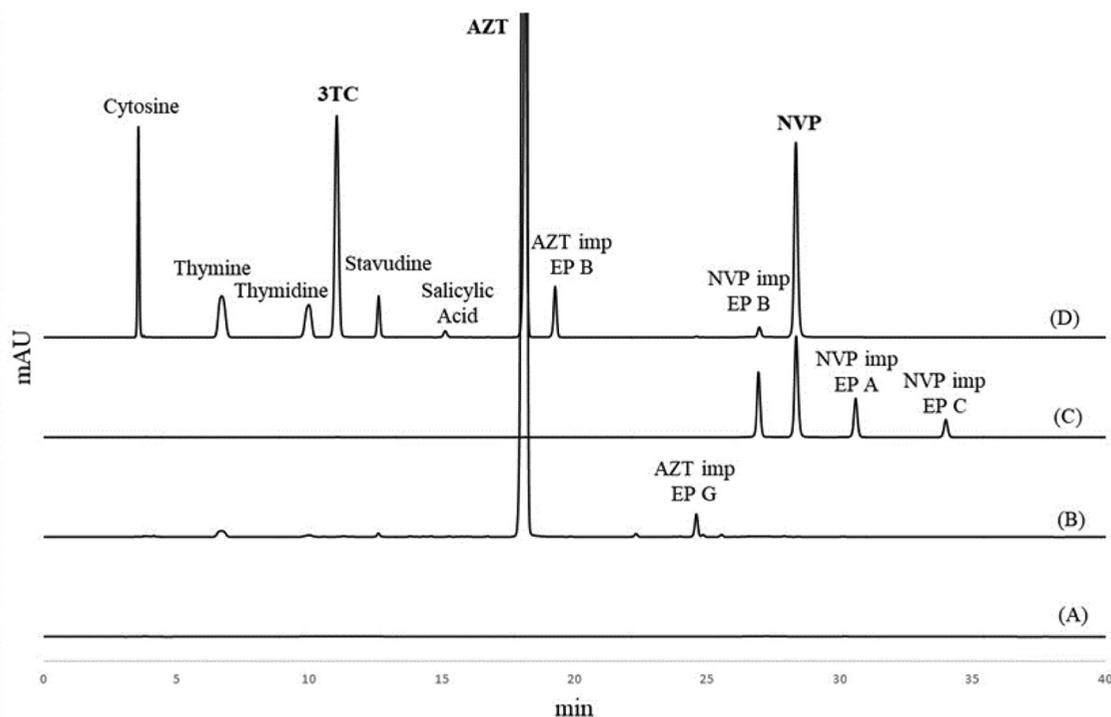
acesulfame potassium and aspartame – excipients indicated in the patient notice of this manufacturer – respectively (Figure 5). A posteriori, these two excipients have been injected and the retention times fit with LC-MS results. Resolutions between APIs and each peak were up to 1.5 (Table 2).

The green analytical method is specific for ten related substances and two excipients in pharmaceutical forms.

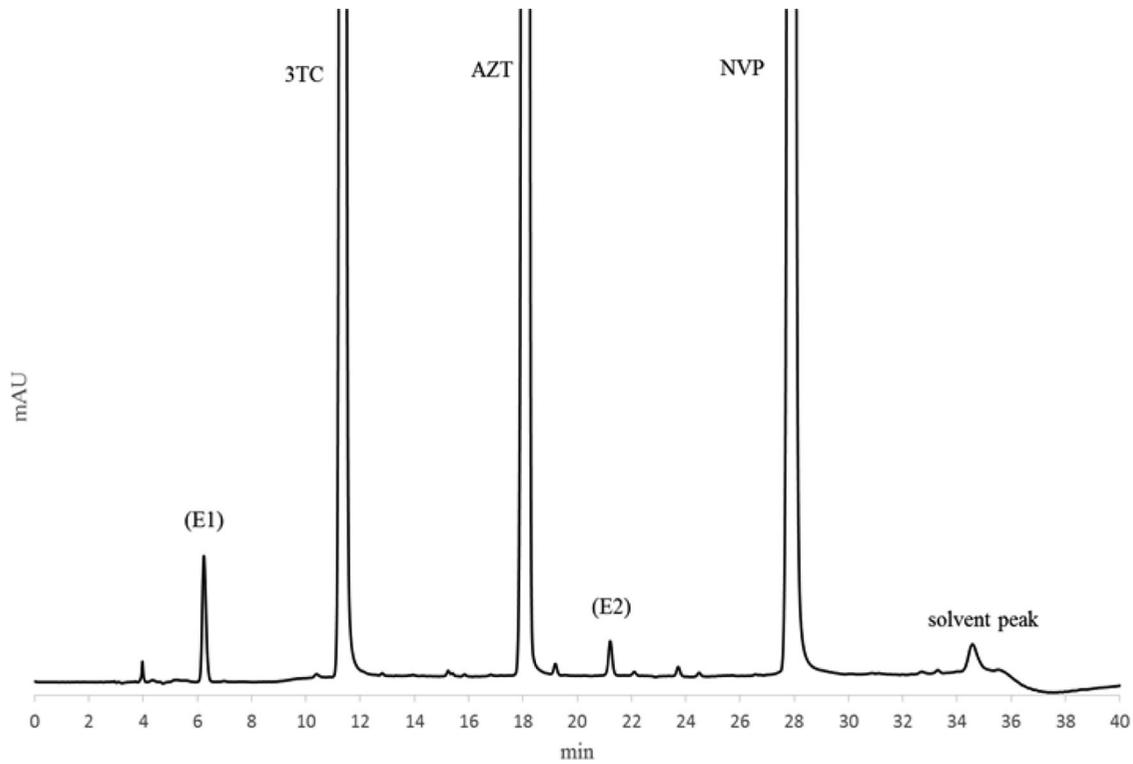
**Table 2.** Retention times for APIs, excipients and some related substances: TRR of related substances calculated with respect to associated API (3TC<sup>a</sup>, AZT<sup>b</sup>, NVP<sup>c</sup>).

Compounds	TR (min)	TRR (min)	Resolution
Cytosine (3TC imp EP E)	3.6	0.32 <sup>a</sup>	N/A
Acesulfame K (excipient)	6.1	N/A	13.6
Thymine (AZT imp EP C)	6.8	0.37 <sup>b</sup>	1.7
Thymidine (AZT imp EP E or USP RC D)	10.1	0.55 <sup>b</sup>	5.8
3TC	11.1	1.00 <sup>a</sup>	2.4
Stavudine (AZT imp EP A)	12.7	0.70 <sup>b</sup>	6.1
Salicylic acid (3TC imp EP C)	15.2	1.37 <sup>a</sup>	11.2
AZT	18.2	1.00 <sup>b</sup>	13.1
AZT imp EP B	19.4	1.07 <sup>b</sup>	5.5
Aspartame (excipient)	21.4	N/A	9.0
AZT imp EP G	24.7	1.36 <sup>b</sup>	14.2
NVP imp EP B	27.1	0.95 <sup>c</sup>	10.3
NVP	28.4	1.00 <sup>c</sup>	5.8
NVP imp EP A	30.7	1.08 <sup>c</sup>	9.0
NVP imp EP C	34.1	1.20 <sup>c</sup>	13.1

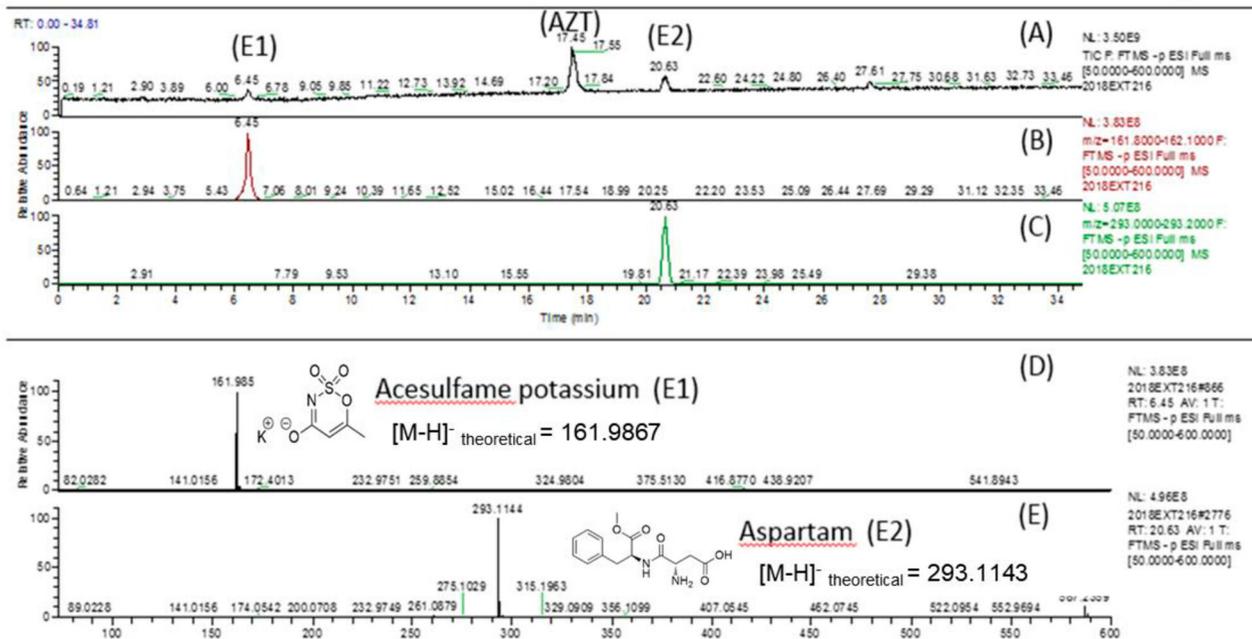
N/A: not applicable.



**Figure 3.** Chromatograms of APIs and related substances. (A) Blank, (B) EDQM, AZT for system suitability CRS1 solution, (C) EDQM, NVP for peak identification solution, (D) System suitability solution (The intensities vary from 0 to 1000 mAU except for AZT in (B) chromatogram).



**Figure 4.** Chromatogram for tablets of Mylan 30 mg 3TC. (The intensities vary from 0 to 950 mAU).



**Figure 5.** LC-MS analysis of Mylan 30 mg 3TC tablets: (A) Full scan chromatogram, (B, D) Extracted ion chromatogram m/z 162 and spectrum, (C, E) Extracted ion chromatogram m/z 293 and spectrum. UHPLC system Ultimate 3000 RSLC equipped with MS system Orbitrap Q-Exactive (ThermoScientific) with parameters (-) ESI full scan mode, sheath gas  $N_2$  flow rate 50 AU, Sweep gas  $N_2$  flow rate 3 AU, Aux. gas  $N_2$  flow rate 13 AU, discharge voltage  $-3.0$  kV, capillary temperature  $320^\circ\text{C}$ , Aux. gas temperature  $425^\circ\text{C}$ , resolution 35000, AGC target  $1e6$ ; scan range 65–750 m/z).

### Linearity

Linearity was evaluated by preparing five standard solutions in the range from 80% to 120% of nominal concentration (37.5 µg/mL for 3TC, 75.0 µg/mL for AZT and 50.0 µg/mL for NVP) (17). Each solution was injected in duplicate and three sets were performed ( $n = 6$  for each level). Response functions:  $\text{area} = f(\text{theoretical concentrations})$  were determinate for each API. Data were analysed with least squares linear regression (Table 3). All correlation coefficients ( $r$ ) were greater than 0.999 for each API. To validate linearity relations for each API, the measured concentration was expressed as a function of the theoretical concentration. Slopes were all close to 1, therefore, the method is linear on the range 80%–120% for each API.

The ratio between intercept and area of 100% for each API were evaluated. The ratios were less than 0.5%, allowing the use of one-point calibration at 100% for quantification as in pharmacopoeia methods. This type of calibration fits with green analytical chemistry by preparing less solution and therefore consuming less solvent and less standard.

### Limit of quantitation (LOQ) and limit of detection (LOD)

The limit of detection (LOD) and the limit of quantitation (LOQ) (Table 4) were calculated by the equations:  $\text{LOQ} = 10 \text{ Sa}/b$  and  $\text{LOD} = 3.3 \text{ Sa}/b$ , where  $\text{Sa}$  is the standard deviation of the intercept of the calibration curve and  $b$  is the slope of the calibration curve.

### Precision

The inter-assay precision study was performed on three days, by two analysts and on three different column batches per day. Three levels of standard concentrations were prepared in duplicate per day. This represents six replicates per level. To assess the variability found in a quality control laboratory, the R.S.D (%) was determined. The inter-assay R.S.D should not exceed 2.0%

The intra-assay precision study was performed on the same day by injecting two independent solutions of each level two times. This represents four replicates per level. R.S.D (%) calculated on these results represents the overall variability of the method (from sample preparation to acquisition). The intra-assay R.S.D should not exceed 2.0%.

Table 5 shows intra and inter-assay precision results obtained on standard solutions for each API. Intra-day precision and inter-day R.S.D (%) are all not greater than 0.5% and 0.8% respectively. These results, not greater than 2%, confirm the precision of the developed method.

### Accuracy

The accuracy of the method was determined using the standard solutions. Quantities of standard solution, corresponding to 80%, 100% and 120% of the target concentrations, were prepared for each API. Three solutions for each level were prepared and analysed twice. Recoveries were calculated for each level and each API ( $(\text{found concentration}/\text{introduced concentration}) \times 100$ ). Mean recoveries should be between 98% and 102% (20, 21). For each level, mean recoveries ranged from 99.95% and 100.27% (Table 6). These results indicate that the method ensures high accuracy for the analysis of these APIs in tablets.

### Robustness

Robustness of the method was evaluated by controlled variation in chromatographic conditions including pH of buffer from 4.3 to 4.7, column oven temperature from 33 to 37°C and percentage of water in ethanol (95% EtOH to absolute EtOH). For this study, a system suitability solution was injected. Coefficients of variation on API areas were not more than 1.7% and deviations of retention time were not more than 2.8% for each condition. Whatever the variations tested, resolutions for each API were no less than 3.4 between Thymidine and 3TC, 5.3 between AZT and AZT imp EP B and 5.1 between NVP and NVP imp EP B. This method remains effective over the range of conditions tested.

### Applications to pharmaceutical forms

The developed and validated green analytical method was applied to quantify 3TC, AZT and NVP in tablets for both adult and child use from 4 manufacturers. Table 7 shows the recovery results of each API based on the theoretical quantity (mg) per tablet. All results obtained were included in the specification range 90%–110% of Ph. Int. (7). This green method, being applicable to different tablets, could be used in QC laboratories.

### Assessment of greenness of analytical HPLC method

Three green metrics tools were used to calculate the greenness of the analytical method compared against the official method described in Ph. Int. (7). The first calculation was performed with the method published by Galuszka et al. (22). The authors proposed a semi-quantitative tool for evaluating the greenness of analytical methods called the Eco-Scale, which is based on the attribution of penalty points according to amount and hazard of chemical compound, energy used and waste generated. The total of penalty points is subtracted

**Table 3.** Validation of linearity.

API	3TC	AZT	NVP
Range (µg/mL)	30.0-45.0	60.0-90.0	40.0-60.0
Linear response function: peak area = f (theor. conc.)			
Regression equation	Y = 124259x	Y = 103645x	Y = 76240x
Y = ax+b	+4.951	-16.998	+6.372
R <sup>2</sup>	0.9994	0.9995	0.9994
R	0.9997	0.9997	0.9997
(a/peak area at 100%)×100	0.1%	0.2%	0.2%

**Table 4.** Limit of detection (LOD) and the limit of quantitation (LOQ).

API	3TC	AZT	NVP
LOQ(µg/mL)	1.83	3.58	6.18
LOD (µg/mL)	0.60	1.18	2.04

from the value of 100, which corresponds to the ideal green analysis. Any score above 75 is said to be an excellent green standard. Scores between 50 and 75 are said to be acceptable. This study is based on criteria such as solvents, instrumentation and waste volume.

Table 8 shows the score obtained by both the Ph. Int. method and the green analytical method. The scores obtained were 63 and 75, respectively. The eco-scale tool highlights that the new method is greener than that described in Ph. Int.

The Analytical GREENness calculator tools developed by Pena-Pereira et al. (23) take into consideration the 12 principles of green analytical chemistry and propose and scale from 0–1 points. The results obtained for the proposed method and the Ph. Int. method are shown in Figure 6.

An excellent score was obtained for criterion 7, which evaluates the volume of waste generated. The reduction of the column's diameter allows for a decreased flow rate thereby reducing solvent waste through consuming less mobile phase (24). Moreover, the proposed green HPLC method has an analysis time gain of 8 min by injections compared to the Ph. Int. method (7). The sample treatment was optimised and the concentration of the stock solutions was reduced, but the final solution concentration was maintained. The solvent volume was

**Table 5.** Precision of standard solutions.

API	Level (%)	Target conc. (µg/mL)	Intra-assay precision (n = 4/level)			Inter-assay precision (n = 6/level)		
			Mean conc. (µg/mL)	Standard deviation	R.S.D (%)	Mean conc. (µg/mL)	Standard deviation	R.S.D (%)
3TC	80	30.00	29.96	0.07	0.24	29.92	0.19	0.65
	100	37.50	37.60	0.12	0.32	37.39	0.13	0.36
	120	45.00	45.11	0.12	0.26	45.05	0.22	0.49
AZT	80	60.00	59.99	0.18	0.29	59.97	0.35	0.59
	100	75.00	75.06	0.09	0.13	74.75	0.45	0.60
	120	90.00	90.30	0.26	0.29	90.09	0.57	0.64
NVP	80	40.00	39.83	0.17	0.43	40.19	0.07	0.17
	100	50.00	50.13	0.05	0.09	49.79	0.37	0.74
	120	60.00	60.43	0.17	0.28	60.04	0.45	0.75

**Table 6.** Accuracy of standard solutions (n = 6/level).

API	Level (%)	Mean recovery (%)	R.S.D (%)
3TC	80	99.95	0.26
	100	100.07	0.27
	120	100.18	0.32
AZT	80	100.14	0.24
	100	99.97	0.14
	120	100.25	0.36
NVP	80	100.24	0.28
	100	100.00	0.24
	120	100.27	0.35

**Table 7.** Results obtained by green analytical method. Recoveries (%) of 3TC, AZT and NVP for tablets from different manufacturers (n = 2).

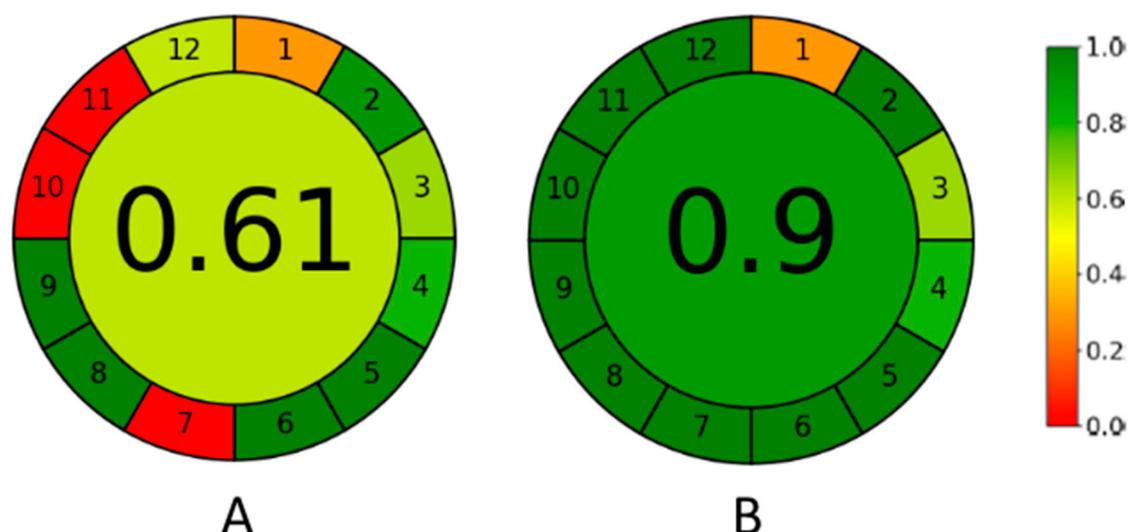
Sample	3TC	(%)	AZT	(%)	NVP	(%)
	(%)	R.S.D	(%)	R.S.D	(%)	R.S.D
Mylan 30 mg 3TC	98.29	1.0	97.97	0.4	95.34	0.8
Cipla 30 mg 3TC	99.96	0.5	99.47	0.1	98.33	0.1
Strides 30 mg 3TC	97.83	0.2	98.11	0.2	96.27	0.2
Mylan 150 mg 3TC	98.84	0.2	99.16	3.9	94.25	1.7
Cipla 150 mg 3TC	97.78	0.6	98.65	0.3	97.81	0.3
Strides 150 mg 3TC	99.47	1.3	100.18	0.8	98.40	1.3
Hetero 150 mg 3TC	98.54	0.6	97.83	1.3	95.25	0.6

**Table 8.** Eco-scale comparison between proposed method and Ph. Int method (7), based on calculation outlined by Galuszka et al. (22).

HPLC Method	Solvent sample preparation	Mobile phase	Instrument	Penalty	Eco-scale total score
Ph. Int.	12	16	9	38	63
Proposed method	8	8	9	26	75

then reduced by a factor of 8, contributing to the generation of less waste. The quantity of standards was divided by a factor of 16, reducing both the cost and the environmental impact of analyses.

The improved scores for criteria 10 (reagents obtained from renewable), 11 (toxic reagents) and 12



**Figure 6.** AGREE metric scores of (A) Ph. Int. method, and (B) proposed method.

(operator's safety), are associated to the use of ethanol. Ethanol was used effectively as an organic mobile phase without compromising separation performance, and employed a typical HPLC pump (< 400 bar pressure). This solvent is included in the class 3 of ICH Q3C guideline (R8) (9) characterized by a low toxic potential and a lower risk to human health in comparison to methanol, which is a solvent that has to be limited in pharmaceutical products because of its inherent toxicity (class 2).

The greenness score obtained with the Analytical Method Greenness Score (AMGS) tool from Hicks et al. (25) was 152.47 and 235.80 for the proposed method and the Ph. Int method, respectively. Smaller scores are associated with safer and more environmentally friendly methods. The main difference was for the solvent energy and environmental health safety (EHS) scores (5.98 vs 19.41 and 32 vs 79.01). Once again, the greenness gain was represented through the use of ethanol, the reduction in the time required for analysis and a far smaller consumption of solvents.

## Conclusion

A new green analytical method was optimized and validated to be easily applied in QC laboratories in less economically developed countries. This method therefore emphasized in particular the choice of a classic column that can be used on a traditional HPLC system. The analytical method was developed by decreasing the column diameter (3 mm instead of 4.6 mm) and reducing both the analysis time and the solvent volume of the prepared solutions. A particular strength of our findings was the successful use of

ethanol, a biodegradable and non-toxic solvent. This method also enables the quantification of 3TC, AZT and NVP. In addition to the three APIs, this one method of analysis identified ten related substance compounds (Cytosine, Thymine, Thymidine, Stavudine, Salicylic acid, AZT imp EP B, AZT imp EP G, NVP imp EP B, NVP imp EP A, NVP imp EP) and two excipients (acesulfame K and aspartam).

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No potential conflict of interest was reported by the author(s).

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