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Original Article

Development and validation of ultraviolet spectrophotometric methods for lamivudine and zidovudine quantification in dissolution test

[Desarrollo y validación de métodos espectrofotométricos ultravioleta para la cuantificación de lamivudina y zidovudina en pruebas de disolución]

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Abstract

Context: In the development of a good analytical method, the selection of appropriate conditions for quantifying drugs is essential. The method validation should be determined before conducting the dissolution test.

Aims: To develop and validate two analytical methods by UV spectrophotometry to quantify lamivudine and zidovudine in dissolution test.

Methods: The dissolution conditions were 900 mL of dissolution medium (pH 1.2, pH 4.5, and pH 6.8), using paddles, at 75 rpm, and sampling time points of 5, 10, 15, 20, and 30 minutes. The analytical methods were developed by scanning analytical solutions in the UV region. The proposed methods were validated in accordance with the ICH guideline and the United States Pharmacopoeia.

Results: Lamivudine showed wavelengths at 280 nm, 273 nm, and 270 nm in buffers at pH 1.2, pH 4.5, and pH 6.8, respectively; while zidovudine showed a wavelength at 266 nm in all three buffers. Regression analysis confirmed linearity (r2>0.998). Placebos and diluents showed no analytical interference. LOD and LOQ were lower than the linearity range. Recovery percentages were within 95% to 105%. RSD values were below 2% and 7% for repeatability and intermediate precision, respectively. No changes introduced modified the method response. The analytical solutions were stable until 24 hours. The results showed that Whatman No 41, No 42, and PVDF 0.45 µm filters can be used.

Conclusions: The proposed analytical methods demonstrated linearity, specificity, accuracy, and precision. In addition, LOD and LOQ, robustness, stability of the analytical solution, and filter test showed satisfactory results.

Keywords: analytical methods; drug dissolution; reverse transcriptase inhibitors; spectrophotometry.

Resumen

Contexto: En el desarrollo de un buen método analítico, la selección de las condiciones adecuadas para cuantificar los fármacos es esencial. La validación del método debe determinarse antes de realizar las pruebas de disolución.

Objetivos: Desarrollar y validar dos métodos analíticos por espectrofotometría UV para cuantificar lamivudina y zidovudina en pruebas de disolución.

Métodos: Las condiciones de disolución fueron de 900 mL de medio de disolución (pH 1,2; pH 4,5 y pH 6,8), utilizando paletas, a 75 rpm, y puntos de tiempo de muestreo de 5, 10, 15, 20 y 30 minutos. Los métodos analíticos se desarrollaron mediante el escaneo de las soluciones analíticas en la región UV. Los métodos propuestos fueron validados de acuerdo con la directriz ICH y la Farmacopea de los Estados Unidos.

Resultados: Lamivudina mostró longitudes de onda a 280 nm, 273 nm y 270 nm en tampones a pH 1,2; pH 4,5 y pH 6,8, respectivamente; mientras que zidovudina mostró una longitud de onda de 266 nm en los tres tampones. El análisis de regresión confirmó la linealidad (r2>0,998). Los placebos y diluyentes no mostraron interferencia analítica. El LOD y LOQ fueron más bajos que el rango de linealidad. Los porcentajes de recuperación estuvieron dentro del 95% al 105%. Los valores de RSD fueron inferiores al 2% y 7% para la repetibilidad y la precisión intermedia, respectivamente. Ningún cambio introducido modificó la respuesta del método. Las soluciones analíticas fueron estables hasta las 24 horas. Los resultados mostraron que se pueden utilizar filtros Whatman No 41, No 42 y PVDF 0,45 µm.

Conclusiones: Los métodos analíticos propuestos demostraron linealidad, especificidad, exactitud y precisión. Además, LD y LC, la robustez, la estabilidad de la solución analítica y la prueba de filtro mostraron resultados satisfactorios.

Palabras Clave: métodos analíticos; disolución de fármacos; inhibidores de la transcriptasa inversa; espectrofotometría.

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INTRODUCTION

Lamivudine and zidovudine are nucleoside reverse transcriptase inhibitors that have activity against the human immunodeficiency virus (HIV). They have been widely used to prevent or prolong the onset of acquired immunodeficiency syndrome (AIDS), which can cause potentially fatal complications (Wang et al., 2019).

Both drugs are well absorbed in the gastrointestinal tract after oral administration, and according to the Biopharmaceutics Classification System (BCS), lamivudine is a class III drug (high solubility and low permeability) (Strauch et al., 2011), while zidovudine is a class I drug (high solubility and high permeability) (Soares et al., 2013).

Dissolution testing is a physicochemical test that has been extensively used for batch quality control and during the development of solid oral dosage forms (tablets and capsules) (Chen et al., 2017). The results of dissolution testing can often be correlated with the biopharmaceutical behavior of a drug (Reppas et al., 2014). This is important in comparative studies of dissolution test of solid oral dosage forms (generics and reference products) that are candidates for the waive of bioequivalence studies (biowaiver) (Friedel et al., 2018).

In the development of a good analytical method for dissolution testing, the selection of appropriate conditions for the quantification of the dissolved drug is essential (Gao and Sanvordeker, 2014). Ultraviolet spectrophotometry and, in some cases, high-performance liquid chromatography (HPLC) are typically used (Friedel et al., 2018). Due to the large number of samples generated in a dissolution test, the analytical system must be relatively fast to achieve high sample throughput (Gray and Rosanske, 2014).

In the United States Pharmacopeia, the method described for dissolution tests of lamivudine and zidovudine tablets uses ultraviolet spectrophotometry (at 280 nm) and HPLC technique, respectively (USP, 2019a; 2019b). In the British Pharmacopoeia, the methods provided use ultraviolet spectrophotometry at 280 nm and 266 nm, for lamivudine and zidovudine, respectively (BP, 2018a; 2018b). Some published works have reported methods of quantification of lamivudine and zidovudine tablets in dissolution studies through UV spectrophotometric and HPLC (Fernandes et al., 2003; Hwisa et al., 2013; Mandloi et al., 2009; Ozturk et al., 2015; Stuart et al., 2014).

The aim of this work was to develop and validate analytical methods by UV spectrophotometry for the quantitative analysis of lamivudine and zidovudine in dissolution test; as alternative, simple, and relatively less expensive methods than official methods.

MATERIAL AND METHODS

Materials and reagent

Secondary reference standards: lamivudine (lot LRAC5118 and purity of 99.6%) and zidovudine (lot LRAC2710 and purity of 99.7%) were obtained from Sigma-Aldrich (United States).

Pharmaceutical products: lamivudine 150 mg tablets (lot 2050760; expiration 05/2022) and zidovudine 300 mg tablets (lot 2111440; expiration 11/2023) were obtained from AC Farma Laboratories SA (Peru).

Placebos were prepared with all the excipients in the appropriate proportions for lamivudine 150 mg tablets (microcrystalline cellulose, sodium starch glycolate, magnesium stearate, and opadry YS-1-7706-G) (Strauch et al., 2011), and zidovudine 300 mg tablets (microcrystalline cellulose, sodium starch glycolate, magnesium stearate, povidone K30, opadry OY-7300) (Soares et al., 2013).

Reagents: potassium chloride, sodium acetate trihydrate, glacial acetic acid 100%, and monobasic potassium phosphate were obtained from Supelco (Germany); while hydrochloric acid 37% was from J.T. Baker (United States), and sodium hydroxide 97% was from Macron (Sweden). All reagents used were American Chemical Society (ACS) grade.

Dissolution profile

The dissolution profiles were performed in a dissolution apparatus (Varian 705 DS, United States), using paddles (USP apparatus II) rotating at 75 rpm and 900 mL of dissolution medium (buffers pH 1.2, pH 4.5, and pH 6.8) which were prepared in accordance with United States Pharmacopoeia (USP 42). The buffers were degassed and kept to the temperature of 37 ± 0.5°C. Manual sampling aliquots of 5.0 mL were removed at 5, 10, 15, 20, and 30 minutes; using Whatman No. 41 quantitative filter paper discs coupled to polypropylene filter holders of 25 mm (Pall Life Sciences, United States).

Method development

Different concentrations of lamivudine and zidovudine were prepared from appropriate dilution of the standard stock solutions with each of the buffers to achieve an optimal absorption measurement range of 0.1-1.0 AU (AU as absorbance units).

The solutions were scanned in the wavelengths range of 200 nm to 400 nm using a UV-VIS spectro-

photometer (Lambda 25 Model, Perkin Elmer, United States). A rectangular quartz cell of 10 mm path length was used for these studies.

For quantification of lamivudine by UV spectrophotometry, the wavelengths were 280 nm, 273 nm, and 270 nm for buffers pH 1.2, pH 4.5, and pH 6.8, respectively; while the wavelength found for zidovudine was 266 nm in all three buffers.

Method validation

The validation of the analytical methods was performed according to the International Harmonization Conference (ICH) Q2 (R1) guidelines and the recommendations of the USP 42 for the validation of analytical methods, considering the parameters: linearity, range, specificity, limits of detection and quantification, accuracy, precision, robustness, stability of analytical solution, and filter test, for each analyte (ICH, 2005; USP, 2019c; 2019d).

Linearity and range

Five points in triplicate ranging between 2.5-15 µg/mL (pH 1.2) and 2.5–20 µg/mL (pH 4.5 and pH 6.8) were prepared from a stock solution containing 0.4 mg/mL of lamivudine reference standard dissolved in distilled water.

In the case of zidovudine, the range of 5-20 µg/mL was established. Five points in triplicate were prepared in all three buffers, from a stock solution containing 0.42 mg/mL of zidovudine reference standard, previously dissolved in methanol (equivalent to 5% of the final volume) and diluted with distilled water.

Specificity

The specificity was intended to evaluate if any compound present in the formulation or the dissolution medium showed any signal or interference that could affect the quantification of the analyte. The discriminatory capacity was confirmed by comparing of the scans in a range of 200 nm to 400 nm obtained from references standards, tablets samples, placebos (mixture of excipients), and diluents (buffers pH 1.2, pH 4.5, and pH 6.8).

The interference should not exceed 2%, if it exceeds the value, it is necessary to modify the method.

Limit of detection and quantification

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can be detected, while the quantification limit (LOQ) is the lowest concentration of the analyte that can be measured with acceptable accuracy and precision.

The LOD and LOQ were determined by the method based on extrapolation of the calibration line to zero concentration (AEFI, 2001), using [1]:

$$C_L = \frac{|a| + k\ x\ |S_{bl}|}{|b| x \sqrt{n}} \qquad [1]$$

Where C_L: analyte concentration in LOD or LOQ; a: term independent of the calibration curve obtained by representing the response of the method (y-axis) versus the analyte concentration (x-axis); k: constant value, equal to 3 for LOD or 10 for LOQ; S_{bl}: term independent of the calibration curve obtained by representing the standard deviations of the response of the method (y-axis) versus the concentrations studied (x-axis); b: slope of the calibration curve obtained by representing the response of the method (y-axis) versus the analyte concentration (y-axis); n: number of data.

Accuracy

The accuracy was determined by the recovering of known amounts of reference standard added to the placebo at the beginning of the dissolution.

Three concentrations were prepared in triplicate, covering the entire linearity range (low, medium, and high).

The recovery percentage was calculated using [2]:

$$\%R = \frac{X_H}{X_A} x\ 100 \qquad [2]$$

Where %R: percentage of recovery; X_H: amount of drug found; X_A: amount of drug added.

Precision

The precision of the developed method was evaluated through repeatability and intermediate precision.

The repeatability of the method was prepared from nine samples divided into three concentration levels (low, medium, and high) and analyzed on the same day.

For the determination of the intermediate precision, six samples were prepared, which were analyzed in two different days with different equipments: 1) UV-Vis spectrophotometers Lambda 25 Model, Perkin Elmer, United States and 2) UV-Vis spectrophotometers Thermo scientific, Orion Aquamate 8000, United States. The final concentrations of the samples were 12.7-16.7 µg/mL for lamivudine and 16.7 µg/mL for zidovudine.

The repeatability and intermediate precision of the method were evaluated using the relative standard deviation (RSD) values.

Robustness

The robustness of the method was determined by analyzing three samples under a variety of conditions. For this purpose, small changes were included such as a) wavelength, b) dilution of the samples and c) medium without degassing.

The impact of the variations was measured using the absolute difference.

Stability of analytical solution

For this purpose, two groups of samples were analyzed: a) freshly prepared samples and b) samples kept at 25°C for 24 h. The effect of the stability of the samples in the different buffers was evaluated by the absolute difference.

Filter test

The filter test was performed by comparing of centrifuged samples (6000 rpm, 10 min, 15°C) and filtered samples with different types of filters: a) Whatman No. 41 filter paper quantitative, b) Whatman No. 42 filter paper quantitative, and c) polyvinylidene fluoride (PVDF) membrane filters with a pore size of 0.45 µm. It was evaluated by the absolute difference.

Statistical analysis

The results obtained such as arithmetic mean, standard deviation and relative standard deviation were evaluated according to the acceptance criteria established by ICH guidelines and USP 42. Linearity of the proposed method was confirmed by one-way analysis of variance (ANOVA), considering a confidence interval of 95%.

RESULTS AND DISCUSSION

The development of alternative, simple, and rapid UV spectrophotometric methods for the quantification of dissolved drug in studies of dissolution test will reduce the analysis time, labor, cost of reagents and materials, provided that the method selected is suitable for the drug under study (Gray and Rosanske, 2014).

Lamivudine and zidovudine are UV absorbing molecules, due to specific chromophores in their structures that absorb at a certain wavelength. This characteristic has allowed its application in quantitative determinations by the UV spectrophotometric method (Wang et al., 2006). The wavelength maximum absorbance (λmax) of the drugs for analysis was determined by scanning standard solutions of drugs in the UV region from 200 nm to 400 nm.

The UV absorption scan of lamivudine showed a peak at 280 nm, 273 nm, and 270 nm in buffers pH 1.2, pH 4.5, and pH 6.8, respectively; while zidovudine showed a peak at 266 nm in all three buffers, as shown in Fig. 1.

After selecting the optimal wavelength and the appropriate concentrations of both drugs in the different buffers, the analytical methods were validated according to the procedures and limits specified in the ICH Q2 (R1) guidelines and USP 42 (ICH, 2005; USP, 2019c; 2019d).

The concentration range evaluated for the dissolution profile covers the lowest and highest expected concentrations. The analytical methods for both drugs showed determination coefficients greater than 0.998 in all three buffers (pH 1.2, pH 4.5, and pH 6.8). All results of regression analysis are presented in Table 1. These results confirm that the relationship between concentration and response is linear at the established ranges (Fig. 2).

The UV absorption scans of standard and sample solutions of lamivudine showed peaks at 280 nm (pH 1.2), 273 nm (pH 4.5), and 270 nm (pH 6.8), which were not obtained with placebos and diluents (buffers), and therefore, there was no interference due to the excipients used in the formulation, thus demonstrating the specificity of the analytical methods. The results of zidovudine also showed the absence of analytical interference in placebos and diluents (Fig. 3).

The determination of LOD and LOQ for lamivudine were found to be between 0.064-0.158 µg/mL and 0.147-0.274 µg/mL, respectively. For zidovudine, the LOD and LOQ varied between 0.058-0.095 µg/mL and 0.134-0.273 µg/mL, respectively. The results of LOD and LOQ are shown in Table 1.

For the accuracy of the method, recovery experiments were performed at three concentration levels (low, medium, and high), and the final recovery was calculated. All the results obtained complied with the recovery limit of 95% to 105% established by USP 42. The recovery percentages of lamivudine for each level studied were between 98.4% and 100.6% in all three buffers; while zidovudine showed recovery percentages between 97.4% and 100.3%, which confirm the accuracy of the methods.

The repeatability of the method was confirmed by RSD values, which were less than 2% in all three buffers. This result confirms that the repeatability of the method is good.

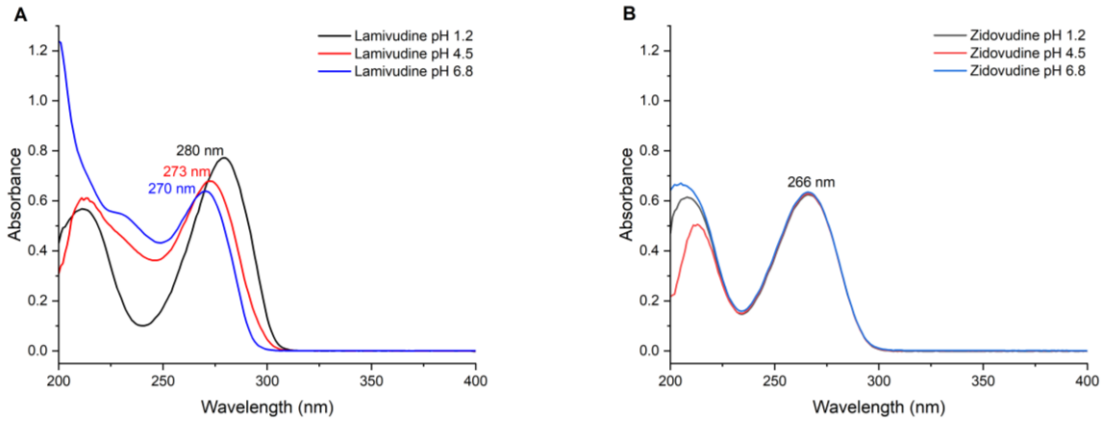

Figure 1. Ultraviolet spectra of (A) lamivudine (15 µg/mL), and (B) zidovudine (16.7 µg/mL) in buffers at pH 1.2, pH 4.5, and pH 6.8.

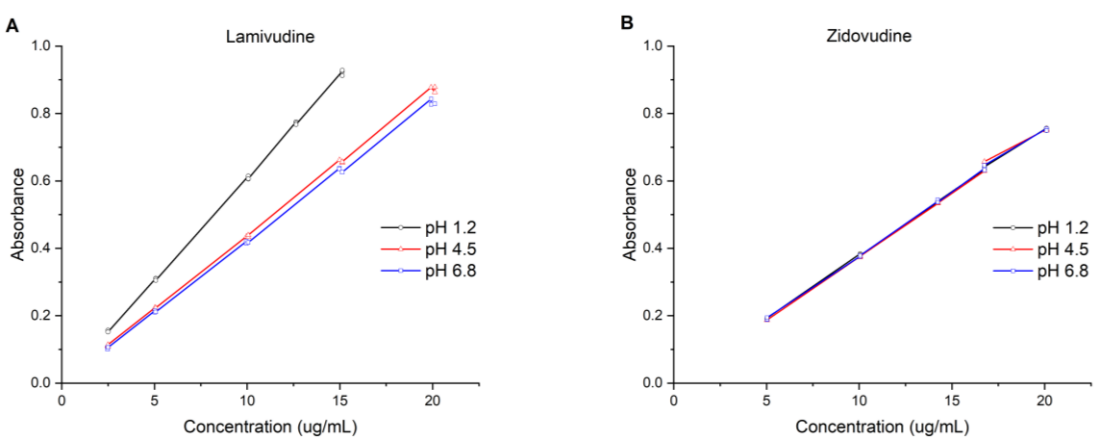

Figure 2. Linearity curves of (A) lamivudine, and (B) zidovudine in buffers at pH 1.2, pH 4.5, and pH 6.8.

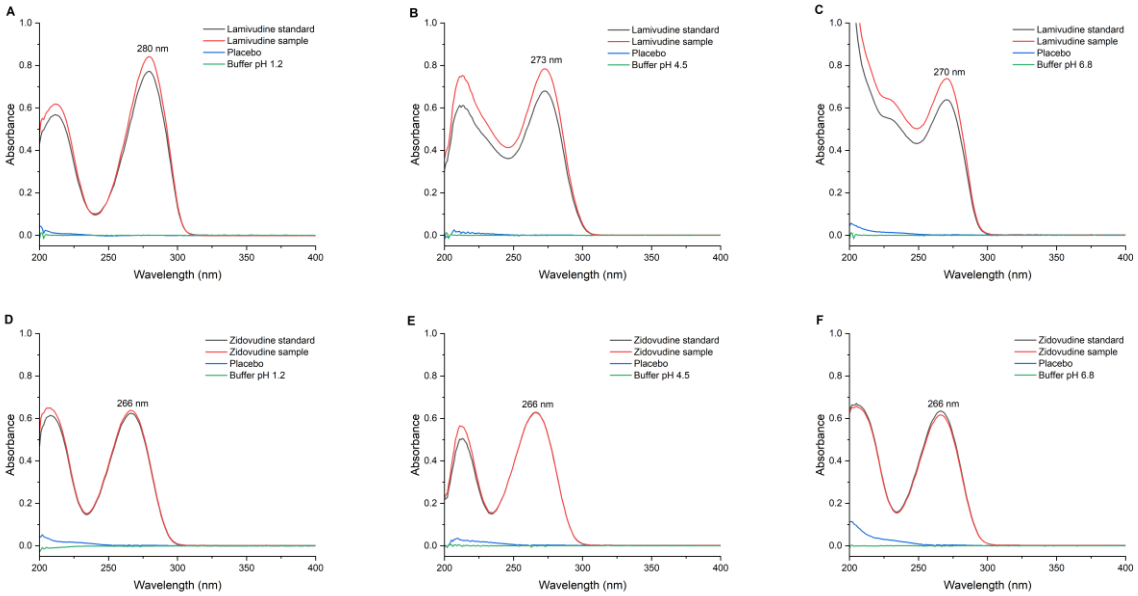

Figure 3. Absorption spectra ranging from 200 nm to 400 nm for lamivudine (A, B, C) and zidovudine (D, E, F) in buffers at pH 1.2, pH 4.5, and pH 6.8.

Table 1. Linearity, range, and limits of detection and quantification using UV spectrophotometric methods.

Lamivudine			
Acceptance criteria	**pH 1.2**	**pH 4.5**	**pH 6.8**
Range	2.5 to 15 µg/mL	2.5 to 20 µg/mL	2.5 to 20 µg/mL
Regression equation[a]	y=0.0608x+0.0006	y=0.0434x+0.0030	y=0.0417x+0.0017
Slope does not include zero	0.0603 to 0.0614	0.0428 to 0.0439	0.0411 to 0.0423
Intercept includes zero	-0.0054 to 0.0066	-0.0035 to 0.0096	-0.0057 to 0.0091
Determination coefficient (r^2) not less than 0.98	0.9997	0.9996	0.9994
p-value lower than 0.05	$1.0x10^{-24}$	$2.1x10^{-23}$	$1.9x10^{-22}$
Response factor, RSD no more than 5%	1.1%	1.7%	1.5%
Limit of detection (LOD)	0.100 µg/mL	0.064 µg/mL	0.158 µg/mL
Limit of quantification (LOQ)	0.274 µg/mL	0.147 µg/mL	0.168 µg/mL
Zidovudine			
Acceptance criteria	**pH 1.2**	**pH 4.5**	**pH 6.8**
Range	5-20 µg/mL	5-20 µg/mL	5-20 µg/mL
Regression equation[a]	y=0.0375x+0.0043	y=0.0378x-0.0022	y=0.0376x+0.0026
Slope does not include zero	0.0370 to 0.0379	0.0369 to 0.0386	0.0369 to 0.0382
Intercept includes zero	-0.0018 to 0.0105	-0.0145 to 0.0102	-0.0065 to 0.0116
Determination coefficient (r^2) not less than 0.98	0.9996	0.9985	0.9992
p-value lower than 0.05	$1.1x10^{-23}$	$8.1x10^{-20}$	$1.6x10^{-21}$
Response factor, RSD no more than 5%	0.8%	1.3%	1.1%
Limit of detection (LOD)	0.058 µg/mL	0.058 µg/mL	0.095 µg/mL
Limit of quantification (LOQ)	0.134 µg/mL	0.152 µg/mL	0.273 µg/mL

[a] Based on three calibration curves

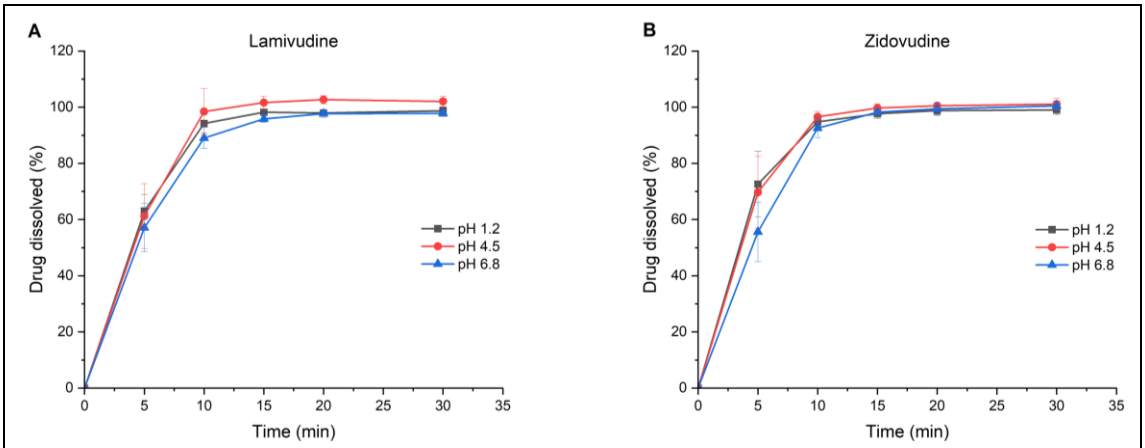

Figure 4. Dissolution profiles of (A) lamivudine and (B) zidovudine in buffers at pH 1.2, pH 4.5, and pH 6.8.
Data are expressed as mean ± SD (n=6).

For intermediate precision analysis, dissolution profiles of drug immediate-release tablets were performed on different days and different spectrophotometers, with sampling times of 5, 10, 15, 20, and 30 minutes. The RSD values at the sampling times of 5 and 10 minutes were less than 20%, while the other points were less than 10% (WHO, 2017). As shown in Fig. 4, both drugs complied with the requirement for products exhibiting very rapid dissolution, namely, more than 85% of the drugs were dissolved within 15 minutes in all three buffers (WHO, 2017). Similar results were reported by (Ozturk et al., 2015; Reddy et al., 2014; Strauch et al., 2011).

Table 2. Accuracy, repeatability, and intermediate accuracy using UV spectrophotometric methods.

Lamivudine					
Parameter	**Acceptance criteria**		**pH 1.2**	**pH 4.5**	**pH 6.8**
Accuracy	Recovery from 95% to 105%	2.2 ug/mL	99.5 ± 1.4[a]	98.6 ± 0.4[a]	98.4 ± 1.0[a]
		14.2 ug/mL	98.5 ± 0.9[a]	100.6 ± 0.7[a]	100.2 ± 1.2[a]
		20.2 ug/mL	99.9 ± 0.6[a]	100.1 ± 0.6[a]	99.4 ± 0.4[a]
	RSD no more than 2%		0.6%	1.1%	0.3%
Repeatability	RSD no more than 5%		1.0%	1.3%	0.5%
Intermediate precision	RSD no more than 10%	5 min	0.8%	3.6%	2.9%
		10 min	2.7%	0.7%	6.5%
		15 min	1.2%	0.6%	5.1%
		20 min	1.4%	0.9%	4.0%
		30 min	0.8%	0.4%	3.9%
Zidovudine					
Parameter	**Acceptance criteria**		**pH 1.2**	**pH 4.5**	**pH 6.8**
Accuracy	Recovery from 95% to 105%	5.3 ug/mL	100.3 ± 1.9[a]	97.7 ± 1.5[a]	100.2 ± 0.7[a]
		14.9 ug/mL	99.4 ± 1.1[a]	98.4 ± 2.2[a]	99.0 ± 0.4[a]
		21.1 ug/mL	99.2 ± 0.4[a]	97.4 ± 0.2[a]	99.0 ± 0.4[a]
	RSD no more than 2%		0.6%	0.5%	0.7%
Repeatability	RSD no more than 5%		1.2%	0.9%	0.8%
Intermediate precision	RSD no more than 10%	5 min	5.5%	1.3%	0.1%
		10 min	0.01%	1.1%	1.6%
		15 min	1.7%	0.4%	0.5%
		20 min	2.2%	0.2%	0.5%
		30 min	2.6%	0.2%	0.03%

[a]Data are expressed as mean ± SD (n=3).

The intermediate precision analysis at all sampling times, showed RSD values below 7% for both drugs, demonstrating the intermediate precision of the method.

The results of the accuracy, repeatability and intermediate precision method are shown in Table 2.

For robustness of the method, no change introduced modified the method response beyond the allowed limit. These results indicate that the method can admit small and deliberate changes, demonstrating the robustness of the method used.

The study of the stability of the analytical solutions in the different buffers showed that lamivudine and zidovudine solutions are stable until 24 hours at room temperature after their preparation. This is particularly beneficial due to the large number of samples, which are collected in dissolution studies.

The sample filtration test was evaluated by comparing centrifuged samples (unfiltered) with three different filters. The results showed that any of these filters can be used for studies of dissolution test.

The results of the robustness, stability of the analytical solution, and filter test are shown in Table 3.

This study allows us to understand the key role of well-characterized and validated analytical methods, following the principles and procedures of validation of analytical methods described in the ICH guideline to generate reliable estimates of analyte concentrations (ICH, 2005).

The development and validation of analytical methods following the procedure proposed here will increase the reliability of the results in comparative studies of lamivudine or zidovudine dissolution test from immediate-release tablets.

Table 3. Robustness, stability of analytical solution and filter test using UV spectrophotometric methods.

Lamivudine					
Parameter	**Acceptance criteria**		**pH 1.2**	**pH 4.5**	**pH 6.8**
Robustness	Absolute difference no more than 2%	C_1-C_0	0.01%	0.2%	0.1%
		C_2-C_0	0.3%	0.2%	0.04%
		C_3-C_0	0.3%	0.3%	0.9%
Stability of analytical solution	Absolute difference no more than 2%	24h-0h	0.4%	0.5%	0.9%
Filter test	Absolute difference no more than 2%	SF-F_1	0.2%	0.4%	0.04%
		SF-F_2	0.3%	0.2%	0.03%
		SF-F_3	0.1%	0.02%	0.2%
Zidovudine					
Parameter	**Acceptance criteria**		**pH 1.2**	**pH 4.5**	**pH 6.8**
Robustness	Absolute difference no more than 2%	C_1-C_0	0.02%	0.3%	0.02%
		C_2-C_0	0.3%	0.2%	0.02%
		C_3-C_0	0.6%	0.4%	0.4%
Stability of analytical solution	Absolute difference no more than 2%	24h-0h	1.2%	0.4%	0.01%
Filter test	Absolute difference no more than 2%	SF-F_1	0.3%	0.8%	1.0%
		SF-F_2	0.3%	0.2%	0.8%
		SF-F_3	1.0%	0.7%	0.6%

C_0: initial condition; C_1: different wavelength (±2 nm); C_2: different dilution of samples; C_3: buffer without degassing; SF: centrifuged samples; F_1: Whatman No. 41 filter paper; F_2: Whatman No. 42 filter paper; F_3: PVDF 0.45 µm filter.

CONCLUSION

Two analytical methods were developed by ultraviolet spectrophotometry for the quantification of lamivudine and zidovudine in dissolution test of solid oral dosage forms. The results obtained during the validation of both analytical methods demonstrated linearity, specificity, accuracy, and precision. In addition, the limits of detection and quantification, robustness, stability of the analytical solution, and filter test showed satisfactory results. Therefore, these methods could be considered in comparative studies of the dissolution test of lamivudine and zidovudine in immediate-release tablets.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Perez-Chauca E	Alva-Plasencia PM	Ferraz HG
Concepts or ideas	x	x	x
Design	x	x	x
Definition of intellectual content	x	x	x
Literature search	x		
Experimental studies	x		
Data acquisition	x		
Data analysis	x		
Statistical analysis	x		
Manuscript preparation	x		
Manuscript editing	x	x	x
Manuscript review	x	x	x

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