BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902020000419073

Article

Development and validation of RP-HPLC method for simultaneous determination of lamivudine, stavudine, and zidovudine in perfusate samples: Application to the Single-Pass Intestinal Perfusion (SPIP) studies

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A reversed-phase high performance liquid chromatography (RP-HPLC) method with ultraviolet detection was developed and validated for the simultaneous quantification of antiretroviral drugs lamivudine (3TC), stavudine (d4T), and zidovudine (AZT) in perfusate samples obtained from the Single-Pass Intestinal Perfusion studies. The chromatographic analysis was performed using a Gemini C18 column and didanosine as internal standard (IS). The following parameters were considered for the validation procedure: system suitability, accuracy, precision, linearity and selectivity. The limits of detection were 0.32 μg/mL for 3TC, 0.11 μg/mL for d4T and 0.45 μg/mL for AZT and the limits of quantification were 1.06 μg/mL for 3TC, 0.38 μg/mL for d4T and 1.51 μg/mL for AZT. Repeatability and intermediate precision ranged from 1.05 to 1.31 and 1.50 to 1.87, respectively, and are expressed as percent of relative standard deviation (RSD). Based on these results, the developed and validated RP-HPLC method can be used for simultaneous determination of 3TC, d4T, and AZT in perfusate samples. Furthermore, this method is simple and adequate for measurements of the antiretroviral drugs in the same sample, since those compounds are mostly co-administered. Besides, this work can be used as an initial base for the development of similar methods in the same conditions presented in our study.

Keywords: Antiretroviral drugs. Chromatographic method. RP-HPLC. Permeability. SPIP.

INTRODUCTION

Antiretroviral drugs are widely used for the treatment of infection caused by human immunodeficiency virus (HIV). Today, six drug classes have been approved by the Food and Drug Administration (FDA): protease inhibitors (PI), non-nucleoside reverse transcriptase inhibitors (NNRTI), nucleoside reverse transcriptase inhibitors (NRTI), fusion inhibitors (FI), entry inhibitors (EI), and integrase strand transfer inhibitors (INSTI) (FDA, 2019a). NRTI are prodrugs and require intracellular phosphorylation to become an active triphosphate form. These triphosphate derivatives inhibit the activity of HIV-1 reverse transcriptase by competing with the endogenous nucleosides to cause DNA chain termination. Lamivudine (3TC), stavudine (d4T), and zidovudine (AZT) are examples of NRTI drugs and they are widely studied to assess their properties as solubility and permeability, despite some characteristics are still unclear (Figure 1) (FDA, 2019a; Balimane, Sinko, 1999; Strauch *et al*., 2011).

In August 2000, the FDA published a guidance based on Biopharmaceutics Classification System (BCS) proposed by Amidon and colleagues for biowaiver processes of class I drugs. Thereafter, the permeability studies have grown as well as methods for quantitative analysis of substances contained in permeability samples (Amidon *et al*., 1995; CDER/FDA, 2017).

Among different techniques for permeability assessment, PAMPA (Parallel Artificial Membrane Permeability Assay), Caco-2 (human colon carcinoma cell line), MDCK (Madin-Darby canine kidney cells)

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and *ex vivo* study using isolated tissue (everted gut sac or diffusion cells as Ussing chamber and Franz cells) are some of models used for drug prediction through intestinal membrane (Balimane, Chong, Morrison, 2000; CDER/FDA, 2017; Dezani *et al*., 2013; Reis *et al*., 2013).

Besides *in vitro* and *ex vivo* methods, the Single-Pass Intestinal Perfusion (SPIP, also known as *in situ* intestinal perfusion) is widely used and it is recommended by the FDA guidance for permeability studies to predict oral absorption of drugs (CDER/FDA, 2017; Dezani *et al*., 2016; Reis *et al*., 2013).

Generally, the SPIP is performed in small rodents and it has been used for absorption and transport mechanism studies of drugs throughout the gastrointestinal tract. In humans, the intestinal perfusion is also described for the establishment of local absorption rate (Dezani *et al.,* 2017; Lennernäs, 1998; Lennernäs, Nylander, Ungell, 1997).

Chromatographic methods with ultraviolet (UV) detection, tandem mass spectrometry (MS) and radioimmunoassay for determination of antiretroviral drugs are generally used for quantification in plasma, urine, blood serum or other biological fluids in pharmacokinetic and bioequivalence studies (Aymard *et al*., 2000; Fan, Stewart, 2002; Huang *et al*., 2004; Kaul *et al*., 1996; Kenney *et al*., 2000; Kumar *et al*., 2013; Moyer *et al*., 1999; Porta *et al*., 2008; Sarasa *et al*., 2000).

Although some methods have been reported in the literature for pharmacokinetic/bioequivalence studies of antiretroviral drugs, there are few analytical methods described for samples obtained from SPIP studies of antiretroviral drugs. In addition, a method is developed for each drug but not for simultaneous determination of compounds. Normally, the matrix used is plasma and some differences such as constitution of mobile phase and wavelengths may vary as well as sample preparation, pH of mobile phase, and oven temperature. Up to now, HPLC methods used for permeability evaluation are rarely reported for daily application in SPIP studies (Kano *et al*., 2005; Verweij-Van Wissen, Aarnoutse, Burger, 2005).

The aim of this study was to describe the development and the analytical validation of a simple and selective reversed-phase high-performance liquid chromatography (RP-HPLC) method with UV detection for simultaneous quantification of 3TC, d4T and AZT and its application for the SPIP assays.

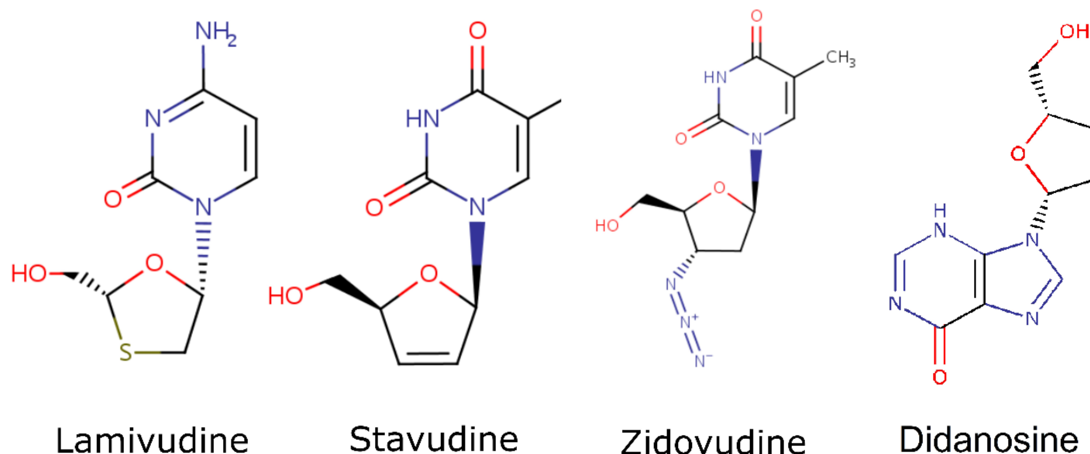

FIGURE 1 – Chemical structures of the antiretroviral drugs used in this study. Didanosine was used as internal standard (FDA, 2019a).

EXPERIMENTAL

Material and methods

Chemicals and reagents

Active pharmaceutical ingredient (API) working standards of 3TC (potency = 99.7%), d4T (potency = 101.0%), AZT (potency = 99.8%) and didanosine (ddI, potency = 99.6%) were kindly donated by Fundação para o Remédio Popular (FURP, São Paulo, Brazil) and Cristália Produtos Químicos Farmacêuticos (São Paulo, Brazil). Chromatographic grade methanol, acetonitrile, potassium phosphate monobasic and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). High purity deionized water was obtained from Milli-Q purification system (Millipore, MA, USA).

Sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), mannitol, polyethylene glycol 4000 (PEG-4000), and D-glucose were used for perfusion solution preparation (Dezani *et al*., 2016; Jain *et al*., 2007).

Instrumentation and chromatographic conditions

HPLC system (Merck-Hitachi LaChrom®, USA) equipped with pump (L-7100), autosampler (L-7200), column oven (L-7300), ultraviolet (UV) detector (L-7400) and vacuum degasser (L-7612) was used for all analysis. The connection between all system components and the specific software was made by an interface D-7000 for data collection and processing (chromatographic station software, HPLC system manager, version 4.1, 1994-2001, P/N 810866201).

A Gemini C18 Phenomenex® column (150 mm x 4.6 mm x 5 μm) was used as stationary phase protected with a guard column C18 (4 x 3.0 mm i.d., Phenomenex®). The isocratic mobile phase consisted of a mixture of phosphate buffer (20 mM), acetonitrile and methanol in the ratio of 90:7:3 (v/v/v, %) and the pH was adjusted to 4.5 using orthophosphoric acid. The mobile phase was filtered using a 0.45 μm PVDF membrane filter and degassed prior to use. UV detector signal was set at a wavelength of 270 nm and the chromatographic determination was performed under isocratic condition with a constant flow rate of 0.7 mL/min. The column oven temperature was kept at 35 ºC and the injection volume was 30 μL.

SPIP study and sample preparation

The intestinal permeability of the antiretroviral drugs was assessed using the SPIP method in male Wistar rats. The experimental protocol was approved by the Ethics Committee on Animal Use of the University of São Paulo under protocol number 235.

The perfusion solution was prepared with NaCl (48 mM), KCl (5.4 mM), Na_2HPO_4 (28 mM), NaH_2PO_4 (43 mM), mannitol (35 mM), PEG-4000 (1 g/L) and anhydrous D-glucose (10 mM). All the components were solubilized in purified water (Milli-Q) and the pH was adjusted to 6.5 (Dezani *et al.,* 2016, Dezani *et al.,* 2017; Jain *et al*., 2007; Reis *et al*., 2013).

Concentration of drugs in perfusion solution was determined by dividing the highest prescribed dose by 250 mL (CDER/FDA, 2017; Dahan, West, Amidon, 2009; Dezani *et al*., 2016, Dezani *et al.,* 2017; Kim *et al*., 2006). Thus, the final concentration in perfusion solution for each drug was: 1.20 mg/mL for 3TC (300 mg as highest prescribed dose), 0.16 mg/mL for d4T (40 mg as highest prescribed dose), 1.20 mg/mL for AZT (300 mg as highest prescribed dose) (FDA, 2019b).

All procedures performed in animals followed those described by Dezani and colleagues (2016). Briefly, rats were anesthetized with an intramuscular injection of ketamine-xylazine mixture (0.1 g/kg and 0.02 g/kg, respectively) and placed on a heated surface. A segment of 10 cm of the jejunum was cannulated at both ends. The drug-free perfusion solution was pumped at 0.5 mL/min in order to clean any residual debris. Then, the perfusion solution containing the compound (3TC, d4T or AZT) was perfused throughout the intestinal lumen at a constant flow rate of 0.2 mL/min for 120 min. Samples were collected at 15, 30, 45, 60, 75, 90, 105, and 120 min from the distal portion of the jejunum and stored at -20 ºC until HPLC analysis (Dezani *et al*., 2016, Dezani *et al.,* 2017).

Regarding sample preparation, the perfusate samples were prepared after centrifuging them at room temperature (3500 rpm for 10 minutes), as follows: 150 μL of sample, 150 μL of IS didanosine solution (125 μg/mL) and 1200 μL of mobile phase (phosphate buffer 20 mM, acetonitrile and methanol, 90:7:3 v/v/v, %). The mixtures were vortex-mixed and analyzed by HPLC under chromatographic conditions described above.

For effective permeability (P_{eff}) results, chromatographic data were applied in the following equation:

$$P_{eff} = -\frac{Q_{in}}{A}\ x \ln\frac{C_{out\ corrected}}{C_{in}}$$

where: P_{eff} is the effective permeability in cm/s; Q_{in} is the inlet perfusate influx in mL/min; A is the intestinal area available for absorption considering radius and length ($2\pi rl$); $C_{out\ corrected}$ is the corrected outlet drug concentration, and C_{in} is the inlet drug concentration.

Stock standard solutions

Stock standard solution was prepared by transferring 250 mg of d4T into a 100 mL volumetric flask. A mixture of purified water and methanol (80:20 v/v, %) was added for solubilization. The same procedure was made for 3TC (625 mg) and AZT (625 mg). Thus, the concentration of the stock solutions prepared were 2.5 mg/mL for d4T, 6.25 mg/mL for 3TC and AZT.

Working standard solutions

Series of working standard solutions were diluted in water and methanol mixture (80:20 v/v, %) from each stock standard solution, producing a concentration range of 0.5-50 µg/mL for d4T, 10-200 µg/mL for 3TC and 10-200 µg/mL for AZT. All working standard solutions were used for HPLC method development and the same procedure was made using the perfusion solution instead of water and methanol mixture in order to get the calibration curve concentration levels in the validation procedure. After preparation, all solutions were sonicated for 30 min prior to use.

Didanosine (ddI) solution was prepared using a mixture of water and methanol (80:20, v/v) and used as IS in the concentration of 125 µg/mL.

Method validation

The HPLC method validation was performed according to the ICH guideline (ICH, 2005). Blank perfusate solution was collected from the distal portion of the cannulated jejunum and used to assess the amount of chromatographic interference at the retention times of each drug.

Each drug diluted into the perfusate solution to obtain the concentration levels of the calibration curve was used for the chromatographic validation and the quantitative analysis were performed based on the relationship between peak ratios (drug/IS) and concentration in µg/mL.

Validation parameters considered for this work include: system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and selectivity. The purpose is to demonstrate that the chromatographic method is adequate for sample measurements.

System suitability test parameters were evaluated in order to ensure the performance of the HPLC system. To guarantee that the chromatographic system is working properly during the analysis, parameters as capacity factor (*k'*), selectivity factor (α), resolution (*R*), column efficiency (number of theoretical plates), and tailing factor (*T*) were evaluated during method development and validation (Mukherjee, Bera, 2012; Zakeri-Milani *et al.,* 2005).

Capacity factor evaluation (*k'*) is a measure of the retention time of a substance contained in a sample as a function of combination of mobile phase and column. Parameter *k'* can be calculated by the following equation (Zakeri-Milani *et al.,* 2005):

$$k'_{(A)} = (tA - t0)/t0$$

where: *tA* is the retention time of the substance and *t0* is the retention time for an unretained compound (*t0* = 2.8 min in the present work). A good separation is achieve when the *k'* is in the range of $0.5 < k' < 10$.

Selectivity factor (α) corresponds to the separation of two compounds contained in the same sample. For compounds A and B, α is defined as a ratio between the retentions of the two compounds and is represented by the following equation (Zakeri-Milani *et al.,* 2005):

$$\alpha = k'_A/k'_B$$

where: *k'* is the capacity factor of each compound.

Resolution (*R*) is the measure of the degree of separation between close peaks. For two compounds, A and B, in the same chromatographic run, *R* can be calculated by the following equation (Zakeri-Milani *et al.,* 2005):

$$R = 2(tA - tB)/(wA + wB)$$

where: *tA* and *tB* correspond to their retention times; *wA* and *wB* correspond to the width of the base of the component peaks. A complete separation between peaks results in an *R* value of, at least, 1.5.

Column efficiency is related to the number of theoretical plates and it represents the efficiency of the stationary phase and how well is the integrity of the column packing. Tailing and/or fronting may indicate that the column is not suitable for chromatographic analysis. The number of theoretical plates can be calculated by using the equation below (Zakeri-Milani *et al.,* 2005):

$$N = 16(tR/w)^2$$

Tailing factor (*T*) was the last parameter evaluated for system suitability and it refers to peak asymmetry, i.e., the peak should present a shape of normal Gaussian distribution. Parameter *T* can be calculated by the following equation (Zakeri-Milani *et al.,* 2005):

$$T = w_{0.05}/2f$$

where: $w_{0.05}$ is the distance from the leading edge to the tailing edge of the peak measured at a point 5% of the peak height from the baseline and *f* is the time from width start point at 5% of peak height to retention time. A *T* value of 1 means that the peak is symmetric. The acceptance criteria for *T* parameter is $0.5 \leq T \leq 2$.

The linearity was evaluated by comparison of peak ratios (drug/IS) *versus* concentration of the compound for linear regression by least-square method. LOD corresponds to the lowest concentration of drug that can be detected by the instrument and its value is equivalent of three times of the standard deviation of the injected perfusate sample. LOQ is the lowest concentration of the drug that can be quantified with precision and accuracy and can be calculated based on chromatograms that present signals greater than ten times of the signal of an injected perfusate sample (ICH, 2005; Russo, Barbato, Grumetto, 2016).

For accuracy assessment, three different concentration levels were established for each drug: high, intermediate and low concentration. Thus, 25 µg/mL (low), 125 µg/mL (intermediate) and 150 µg/mL (high) were considered for 3TC; 1 µg/mL (low), 20 µg/mL (intermediate) and 30 µg/mL (high) were considered for d4T; and 25 µg/mL (low), 125 µg/mL (intermediate) and 150 µg/mL (high) were considered for AZT. The values obtained from the analysis were compared to the nominal amount of the drug. The results were expressed as the mean of three determinations.

The accuracy was calculated as follows: *Accuracy (%) = As/Na x 100*, where *As* is the amount of the drug (d4T, 3TC or AZT) determined in the solution and *Na* is the nominal amount of the drug (d4T, 3TC or AZT).

Precision of the method was checked by determination of three concentration levels within the same day (repeatability) and in consecutive days (intermediate precision) and they were calculated as follow: *RSD (%) = SD/Cm x 100*, where *RSD* is the relative standard deviation, *SD* is the standard deviation, and *Cm* is mean concentration. Intermediate precision was performed by analyzing the samples with two different analysts in different days.

Respective peak ratios (drug/IS), dilution factors, and samples were taken into consideration for quantitative analysis of d4T, 3TC and AZT in the perfusate solutions from SPIP studies.

RESULTS AND DISCUSSION

Since the gut has mucus layer and it may contain food residues, a simple, precise and accurate quantification method is needed for analysis of drugs contained in the intestinal fluid in order to avoid interferences of any kind. In the SPIP method, the perfusate may contain food debris that can interfere in the chromatographic analysis. On the other hand, as the intestinal tissue remains viable during all period of SPIP experiment, there are no many debris in the samples since there is less handling of the jejunum (Dezani *et al.,* 2017).

Method development and optimization

Although chromatographic methods can be found in the literature, some parameters had to be discussed about the analytical method development described in this study. As the three antiretroviral drugs are structurally similar, the chromatographic parameters had to be optimized carefully to ensure the analytes were fully resolved. One of the major challenges of this study was to include the AZT in the same run with 3TC and d4T due to its elution difficulty in the same conditions of the other NRTI of this study.

Studies have been published using method developed in LC-MS/MS, but the UV detection is

more available. In some cases, the quantification of drugs contained in perfusate samples does not require a very sensitive method as LC-MS/MS due to high concentration of drugs in perfusate solution.

Few interferences were noticed during the development of the chromatographic method. Extraction procedures tend to be simple by adding acetonitrile in comparison to solid-phase or liquid-liquid extraction. In our study, centrifuging procedure was sufficient to prepare the chromatographic run, which means low costs involved in the SPIP experiments.

For method development, the influence of perfusate solution as matrix was evaluated in order to check interferences in the chromatograms. The comparison between blank perfusate solution and a mixture of water:methanol (80:20% v/v) was considered and no extra peaks were observed.

Didanosine was used as IS in this study due to similarities in its chemical structure in comparison with the NRTI antiretroviral drugs. Besides, ddI presented as stable compound during the analytical development.

Since the antiretroviral drugs are administered simultaneously, an efficient separation method is required. Parameters such as sample matrix, buffer pH and concentration were evaluated. An isocratic mobile phase delivered at a flow rate of 0.7 mL/min provided good separation of the peaks and the concentration of 20 mM of phosphate buffer solution in the mobile phase was considered to avoid crystallization in the chromatographic system.

The mobile phase containing only phosphate buffer and methanol did not present a good definition of the peaks. Thus, the acetonitrile was included in the proportion of 7% and a good resolution of the three drugs was achieved.

Chromatographic conditions were optimized in order to get a single run capable to detect the three antiretroviral drugs simultaneously. Proportions of mobile phase were tested and a proportion of 7% of acetonitrile and 3% of methanol in aqueous solution of phosphate buffer 20 mM was optimal for detection of d4T, 3TC, AZT and ddI in the same run using a Gemini C18 column. The flow of 0.7 mL/min and the mobile phase determined resulted in a good separation of the compounds in comparison with different ratios of the same components. Besides, the wavelength was set in order to get peaks with adequate resolution.

As d4T, 3TC, AZT and ddI exhibit significant absorbance at the wavelength of 270 nm, it was set as detection wavelength for the simultaneous quantification of all compounds in perfusate solutions.

Method validation

System suitability was evaluated in order to make sure the HPLC system is able to measure the compounds contained in perfusate samples. Five parameters were evaluated including: capacity factor (*k'*) selectivity factor (α), resolution (*R*), column efficiency (number of theoretical plates), and tailing factor (*T*). Table I shows the results obtained as well as the acceptance criteria for each parameter evaluated.

TABLE I – Results regarding the parameters evaluated for system suitability of the chromatographic method developed for simultaneous determination of 3TC, d4T, and AZT

System Suitability		Drug			
Parameter	**Acceptance Criteria**	**3TC**	**d4T**	**AZT**	**ddI**
Capacity factor (*k'*)	$0.5 < k' < 10$	0.71	1.79	9.21	1.36
Selectivity factor (α)	Does not apply	Separation between AZT and 3TC: α = 12.97 Separation between d4T and ddI: α = 1.32			
Resolution (*R*)	Greater than 1.5	Separation between AZT and 3TC: R = 30.9 Separation between d4T and ddI: R = 3			
Column efficiency (number of theoretical plates)	Does not apply	2304	6084	4356	10067
Tailing factor (*T*)	$0.5 \leq T \leq 2$	1.2	1.1	1.1	1.3

Drugs: 3TC: lamivudine; d4T: stavudine; AZT: zidovudine

System suitability results shown in Table I indicate that the chromatographic method is suitable to be developed for perfusate sample analysis.

The ICH guideline was used for chromatographic method development and validation with respect to selectivity, linearity, LOD and LOQ, accuracy and precision (ICH, 2005). The results obtained were shown as mean, standard deviation (SD) and relative standard deviation (RSD).

Selectivity of the method for 3TC, d4T and AZT was assessed by observing chromatograms of blank perfusion solution and the calibration curve with drug solubilized in the perfusate solution. The drug-free solution chromatogram shows that there was no interference in the retention times of the compounds, as shown in the Figure 2.

The developed method using chromatographic conditions showed to be selective for d4T, 3TC and AZT contained in samples from SPIP. The retention times for each compound were: 4.8 min for 3TC, 7.8 min for d4T, 28.6 min for AZT and 6.6 min for ddI, as shown in the Figure 3. No extra peaks were observed.

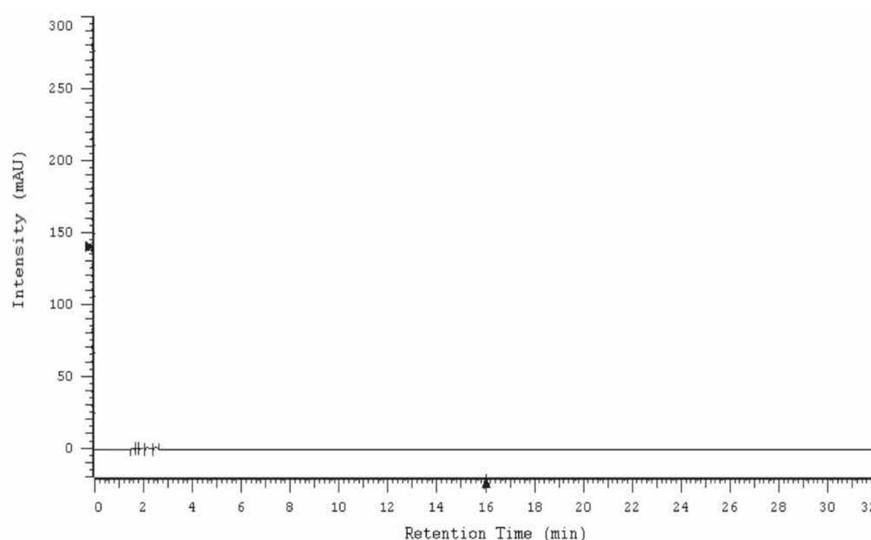

FIGURE 2 – Chromatogram of drug-free perfusion solution. Determination using UV detector set at a wavelength of 270 nm (see *Instrumentation and chromatographic conditions* section for details regarding chromatographic condition).

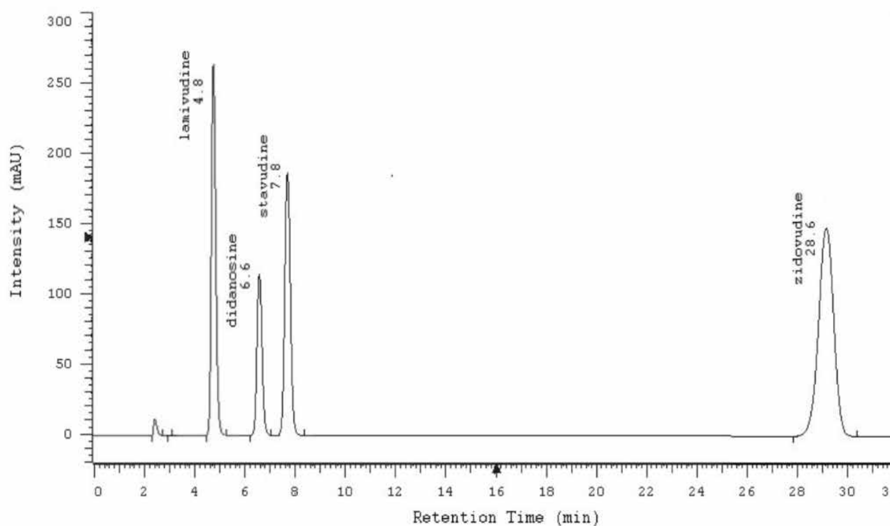

FIGURE 3 – Chromatogram of the peaks and retention times of antiretroviral drugs and ddI (IS). Determination using UV detector set at wavelength of 270 nm (see *Instrumentation and chromatographic conditions* section for details regarding chromatographic condition).

Although the total run time takes 32 min, the three antiretroviral drugs (3TC, d4T and AZT) were separated efficiently in the same chromatographic run, which is different from those studies reported in the literature for each drug (one drug at a time). Thus, the method developed and described in this study allowed to save time for determination of the drugs. The perfusate presented a minimal interference in each run performed.

The sample preparation with a volume required for analysis allowed to obtain a good result and resolution. It is important to consider that an extraction procedure is required for plasma samples before HPLC analysis, which make the procedure more expensive and laborious. As a consequence, a low drug recovery may be obtained (Aymard *et al*., 2000).

Standard curves were prepared by spiking each drug in the drug-free perfusion solution to obtain different concentration levels. Table II shows the results of the parameters evaluated for the HPLC method validation.

The parameters of the chromatographic method were evaluated in order to get the best response of the antiretroviral drugs in the chromatograms, as shown in Table II. The results presented a good repeatability and intermediate precision for all drugs evaluated.

The comparison between standard curve (free of matrix) and calibration curve (matrix) in the perfusion solution showed no difference in the chromatographic parameters between them. In addition, the analysis of perfusate samples from the SPIP experiments did not affect the P_{eff} results.

The LOD of the chromatographic method was evaluated based on the standard deviation of the response and the slope (ICH, 2005). LOD values were less than 0.45 µg/mL for all compounds (Table II).

The reproducibility and linearity were evaluated by injections in triplicate of the calibration curves of the drugs. The calibration curves were prepared in the perfusion solution and adjusted to pH 6.5, exactly the same solution used for *in situ* perfusion studies. Calibration curves assessment were evaluated by plotting the peak ratio (drug/IS) *versus* concentration.

The calibration curves for all substances tested were linear, as shown in Table II, varying from the smallest range of 0.5-50 µg/mL for d4T to the greatest range of 10-200 µg/mL for 3TC and AZT.

As shown in Table II, the correlation coefficient (r^2) for all antiretroviral drugs tested was greater than 0.999 and the RSD values of repeatability and intermediate precision for antiretroviral drugs were less than 1.9% for all concentrations tested.

TABLE II – Results of the RP-HPLC method validation for antiretroviral drugs 3TC, d4T, and AZT

	3TC	**d4T**	**AZT**
Concentration range (µg/mL)	10-200	0.5-50	10-200
Slope	0.0186	0.2193	0.0160
Intercept	0.0118	-0.0074	0.0174
r^2	0.9997	0.9999	0.9998
Repeatability RSD* (%)	1.31	1.05	1.07
Intermediate precision RSD* (%)	1.87	1.56	1.50
Accuracy (%)	100.97	102.38	101.41
LOD** (µg/mL)	0.32	0.11	0.45
LOQ** (µg/mL)	1.06	0.38	1.51

*The reported RSD (relative standard deviation) values are the mean values of three levels of concentration for each drug (25 µg/mL, 125 µg/mL and 150 µg/mL for 3TC; 1 µg/mL, 20 µg/mL and 30 µg/mL for d4T; and 25 µg/mL, 125 µg/mL and 150 µg/mL for AZT).
**Where 3TC: lamivudine; d4T: stavudine; AZT: zidovudine; LOD: limit of detection; LOQ: limit of quantification.

The Gemini C18 Phenomenex® column shown a good stability demonstrated by a very stable retention times and unchanged peak areas for all compounds.

Simultaneous determination of drugs using RP-HPLC in samples from SPIP is simple to be performed in comparison with the simultaneous determination of drugs in plasma or blood serum. Sample preparation techniques are simple, fast, clean, and present low costs in comparison with other biological samples. The perfusion solution preparation is extremely important for *in situ* perfusion studies, since any component may interfere in chromatograms during the experiments, which may lead to problem in validation parameters such as selectivity, accuracy, and precision.

The permeability samples require that low permeable compounds could be detected by an adequate analytical method, as HPLC. Thus, the LOQ would be able to detect low concentrations of drugs in permeability studies. Furthermore, samples are obtained from perfusate and the method must be able to detect slight variations of drug, which is achieved by LOD, LOQ and sensitivity of the method (Patil *et al*., 2012).

Application of chromatographic method to the samples from SPIP studies

The developed RP-HPLC method was applied for quantification of 3TC, d4T and AZT in perfusion samples. The method showed adequate for quantification of the drugs and the effective permeability (P_{eff}) results are presented in the Table III. All experiments were performed considering n=7.

TABLE III – P_{eff} results of 3TC, d4T, and AZT obtained from the SPIP experiments (Dezani *et al.,* 2016)

Drug	**P_{eff} x 10^{-5} (cm/s) ± SD***
3TC*	3.08 ± 0.26
d4T*	3.96 ± 0.47
AZT*	4.17 ± 0.43

** Where 3TC: lamivudine; d4T: stavudine; AZT: zidovudine; SD: standard deviation*

Permeability studies of antiretroviral drugs using the SPIP method in rats allowed to get some similar results to those reported in the literature, even though some conditions have been modified. In addition, the similarity in the permeability results between *in situ* perfusion and *in vitro* methods can indicate that the compound is primary transported by a passive mechanism. On the other hand, when results are different between both methods, some mechanisms can be involved such as uptake and efflux carriers, which requires a good chromatographic method for determination of drugs in these conditions in order to get reliable results (Dezani *et al*., 2016).

The developed method was based on that published by Kano and colleagues (2005). In that work, a chromatographic method was developed for 3TC determination in human plasma. However, as the treatment of HIV infection involves traditionally the use of more than one drug, a chromatographic method with co-elution is necessary for monitoring compounds in different biological fluids.

In comparison with other methods described in the literature for determination of antiretroviral drugs, the perfusate requires a simple procedure for sample cleanup. The centrifuging or filtration are the simple methods used for cleanup of samples from SPIP, which make the preparation less laborious and less expensive for permeability studies (Zakeri-Milani *et al.,* 2005).

No extra peaks were observed in the chromatographic run and parameters as repeatability and intermediate precision presented a good result, as shown in Table II.

Many methods in the literature report a complex sample preparation. For plasma, precipitation and solid-phase extraction are the most described for sample purification. That involves costs and it is time-consuming and laborious. Furthermore, recovery problems can be observed in some cases and, as the matrix is very dirty, the acquisition of high noise signal in the chromatographic method can be a problem as well. Thus, purchasing of aggressive solvents and cartridge for solid-phase extraction should be considered as an additional cost for laboratorial procedures. For urine, similar procedures for sample cleanup are used and some drawbacks are reported, although the experiment is not invasive: sample preparation is complex as plasma, it is time-consuming process, laborious, high-noise signal can be observed, expensive and these studies are not recommended for drugs highly metabolized without previous investigation of metabolites excreted in the urine.

On the other hand, centrifuging or filtration is sufficient for sample cleanup, presents low-noise signal and there are minimal recovery problems. The sample preparation is clean, timesaving and less laborious. Furthermore, is safe for the environment and for those people that are working on the sample preparation.

Chromatographic methods for antiretroviral drugs described in the literature are intended to be applied for complex matrices, especially plasma, serum and urine. Many interferences in these type of samples require a good cleanup procedure.

For many drugs, studies are performed in animals and humans in order to get information about their

bioavailability and pharmacokinetics, which involve invasive procedures and ethical issues. In some cases, these studies are not enough for understanding on absorption processes of compounds orally administered. Permeability studies have been developed since the establishment of classification systems and the SPIP constitutes the closest method of *in vivo* condition. Permeation studies have been developed to elucidate the transport mechanisms of drugs.

The method described in this work showed some advantages over the others published. This method uses ddI as IS, a commercially available compound and chemical structure similar to the molecules used in this study. Determination of three compounds in the same chromatographic run and the LOD and linearity showed to be adequate for the three drugs 3TC, d4T and AZT quantification in perfusate samples considering the highest dose dissolved in the perfusion solution based on the maximum concentration available in the luminal content.

Chromatographic methods in HPLC with UV detection are available for a great number of laboratories, which allows using the method described in this study for SPIP. For routine procedures, HPLC method can be used for antiretroviral drugs. In addition, the chromatographic method developed and validated in our study is able to provide a good response for quantification of drugs in samples from SPIP and it is simple and fast. Besides, the HPLC represents low costs and it is easy to handle (Dezani *et al*., 2016).

CONCLUSION

A RP-HPLC method was developed and validated for simultaneous determination of 3TC, d4T and AZT in samples from SPIP studies. Parameters as system suitability, accuracy, linearity, precision, selectivity and limits of detection and quantification were evaluated considering a HPLC method with UV detection. This method is used for the simultaneous determination of three antiretroviral drugs and presented a relatively short run time, which may be applicable for daily routine in permeability studies. The great advantage is the use of this method for quantification of three drugs in the same method, which is possible to save time in comparison with other methods described in the literature for quantification of each compound.

The chromatographic method presented in this study was validated following the ICH guidance (ICH, 2005) and it demonstrated effective for simultaneous drug quantification of antiretroviral drugs 3TC, d4T and AZT in perfusate samples. Thus, the present chromatographic method can be used for daily laboratorial procedures for SPIP studies as a standardized tool for analysis. Furthermore, this method can be used as an initial base for the development of similar methods for antiretroviral drugs quantification in daily routine.

ACKNOWLEDGEMENTS

The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil, Grant # 473329/2009-3) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil, fellowship) for financial support and Fundação para o Remédio Popular (FURP, Brazil) and Cristália Produtos Químicos Farmacêuticos for donating the chemical substances (lamivudine, stavudine, zidovudine, and didanosine).

CONFLICT OF INTEREST

The authors report no conflict of interest associated with this manuscript.

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Received for publication on 21st January 2019
Accepted for publication on 12nd July 2019