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Development and validation of a HPLC method for the assay of dapivirine in cell-based and tissue permeability experiments

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ABSTRACT

Dapivirine, a non-nucleoside reverse transcriptase inhibitor, is being currently used for the development of potential anti-HIV microbicide formulations and delivery systems. A new high-performance liquid chromatography (HPLC) method with UV detection was developed for the assay of this drug in different biological matrices, namely cell lysates, receptor media from permeability experiments and homogenates of mucosal tissues. The method used a reversed-phase C18 column with a mobile phase composed of trifluoroacetic acid solution (0.1%, v/v) and acetonitrile in a gradient mode. Injection volume was 50 μL and the flow rate 1 mL/min. The total run time was 12 min and UV detection was performed at 290 nm for dapivirine and the internal standard (IS) diphenylamine. A Box-Behnken experimental design was used to study different experimental variables of the method, namely the ratio of the mobile phase components and the gradient time, and their influence in responses such as the retention factor, tailing factor, and theoretical plates for dapivirine and the IS, as well as the peak resolution between both compounds. The optimized method was further validated and its usefulness assessed for *in vitro* and *ex vivo* experiments using dapivirine or dapivirine-loaded nanoparticles. The method showed to be selective, linear, accurate and precise in the range of 0.02–1.5 μg/mL. Other chromatographic parameters, namely carry-over, lower limit of quantification (0.02 μg/mL), limit of detection (0.006 μg/mL), recovery (equal or higher than 90.7%), and sample stability at different storage conditions, were also determined and found adequate for the intended purposes. The method was successfully used for cell uptake assays and permeability studies across cell monolayers and pig genital mucosal tissues. Overall, the proposed method provides a simple, versatile and reliable way for studying the behavior of dapivirine in different biological matrices and assessing its potential as an anti-HIV microbicide drug.

1. Introduction

HIV/AIDS is one of the most important health, social and economic global burdens of our days. Prevention, particular to sexual transmission, is a keystone in the fight against infection and disease, but highly effective strategies are limited to condom use and sexual abstinence. Microbicides are products intended for vaginal or rectal administration around the time of sexual intercourse in order to avoid the transmission of HIV and potentially other pathogens [1]. Despite several initial disappointing results, recent success of a vaginal gel product containing 1% of the antiretroviral drug tenofovir boosted interest in the field [2]. Alongside tenofovir and other promising compounds, dapivirine (TMC120; 4-[[4-[2,4,6-trimethylphenyl)amino]-2-pyrimidinyl]-amino]-benzonitrile) (Fig. 1) is one of the leading antiretroviral agents currently being tested for the development of anti-HIV vaginal microbicides [1]. Preclinical testing of this potent non-nucleoside reverse transcriptase inhibitor provided encouraging results in terms of activity and toxicity for both vaginal and rectal applications [3–5]. Moreover, recent clinical studies indicate that dapivirine is safe and provides favorable pharmacokinetics when delivered either as a vaginal gel or ring [6–8]. Two phase III clinical trials testing dapivirine vaginal rings are currently underway [9,10].

Different dosage forms/delivery systems have been proposed for the vaginal administration of dapivirine. Alongside vaginal gels and rings, others such as films [11] and tablets [12] have also been suggested. Moreover, nanotechnology-based solutions have been

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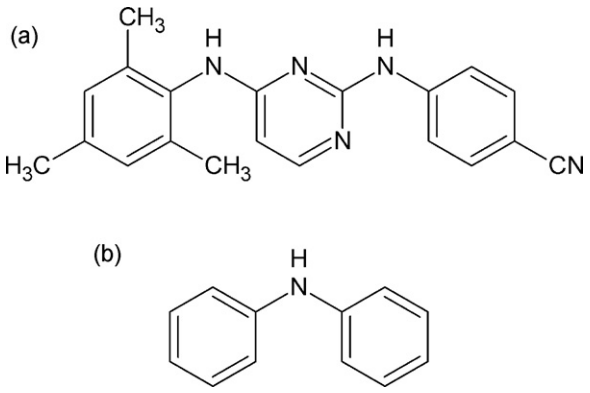

Fig. 1. Chemical structure of (a) dapivirine and (b) diphenylamine (internal standard).

advocated for the development of microbicides, namely because nanosystems may provide controlled release, increased intracellular levels, and overall enhanced mucosal delivery of microbicide drugs [13]. Our group has recently reported on the development and *in vitro* characterization of dapivirine-loaded polymeric nanoparticles [14], which required drug assay in different biological matrices. A few high-performance liquid chromatography (HPLC) methods with UV quantification have been briefly described in the literature for dapivirine mainly for evaluating drug content or drug release from different pharmaceutical dosage forms/delivery systems [15–17]. Two HPLC-UV methods validated according to ICH guidelines have been reported by our group for polymeric nanoparticles [18] and by Gupta et al. for combination vaginal tablets [19]. Also, liquid chromatography tandem mass spectrometry (LC–MS/MS) methods have been used for clinical study samples (genital tissue, cervicovaginal lavage and plasma) [6,7,20] but no details were provided on the method procedure. In one pre-clinical study by Nuttall et al. [21] an LC–MS/MS method was described for dosing dapivirine in the plasma and cervicovaginal tissue of macaques and rabbits but validation parameters were not included.

To our best knowledge, no previous report in the literature detailed on the concurrently development and validation of a quantitative bioanalytical HPLC method for assaying dapivirine, namely in cell-based or tissue permeability experiments. In this article, we describe the successful development, validation and application of a bioanalytical reversed-phase HPLC-UV method for dosing dapivirine in cell lysates and permeability experiments across cell monolayers or mucosal tissues, using diphenylamine (DPA; Fig. 1) as an internal standard (IS).

2. Materials and methods

2.1. Materials

Dapivirine (99.8%, w/w) was a kind offer from the International Partnership for Microbicides (Silver Spring, MD, USA). DPA and HPLC-grade acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). HPLC-grade trifluoroacetic acid (TFA) was acquired from Sigma–Aldrich (St. Louis, MO, USA), Hank's Balanced Salt Solution (HBSS) from Invitrogen (Carlsbad, CA, USA), and poloxamer 407 (Pluracare® F127 Prill) from BASF (Ludwigshafen, Germany). Water was prepared in-house by using a Simplicity® UV Ultrapure Water System (Millipore Corporation, Billerica, USA). All other chemicals and solvents were of analytical grade or equivalent. Prior to use, individual mobile phase solvents were degassed in an ultrasonic bath for 15 min.

2.2. Cells and mucosal tissues

Caco-2 colorectal epithelial cells, VK2/E6E7 human vaginal epithelial cells, HeLa human cervical cells, J774A.1 mice monocyte/macrophages, and CaSki human cervical carcinoma cells were obtained from ATCC (Manassas, VA, USA). DC-100 dendritic cells were purchased from MatTek Corp. (Ashland, MA, USA), and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from buffy coats of healthy adult donors. Cells were maintaining and used during experiments with appropriate culture media, as previously described [5,14]. Pig vaginal and rectal mucosal tissues were obtained from a local slaughter house immediately after sacrifice. Tissues were maintained and transported in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), and used within 2 h.

2.3. Liquid chromatography instrumentation

Chromatographic runs were performed using a Dionex Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with LPG-3400AB quaternary pump, WPS-3000TBPL analytical autosampler, TCC-3000SD thermostated column compartment, and DAD-3000 diode array detector. Results from runs were acquired and processed with the Chromeleon® chromatography management system (Version 6.80; Dionex Corporation). Separation was performed at 20 °C using a Waters Xterra® RP18 separation column (Waters Corporation, Milford, MA, USA) with 5 μm particle size, 4.6 mm internal diameter and 150 mm in length, and a Merck LiChrospher® 100 RP-18 guard column (Merck) with 5 μm particle size.

2.4. Analytical matrices

Matrices processed for analysis included cell lysates and the receptor media collected from either basolateral chambers of Transwell® Permeable Supports (Corning, Inc., Tewksbury, MA, USA) or from receptor chambers of Franz cells during permeability experiments across confluent cell monolayers or mucosal tissues, respectively. Also, mucosal tissues used during permeability experiments were collected and processed for drug assay.

Lysates from different cell types were obtained by treating monocultures grown in 6-well plates with a lysis buffer (10 mM Tris–HCl, pH 7.4, 2 mM EDTA, pH 8.0, 150 mM NaCl, 0.876% (w/v) Brij® 97, 0.125% (w/v) Tween® 20, and one tablet/50 mL of protease inhibitor cocktail (cOmplete, Mini, EDTA-free; Roche Diagnostics, Indianapolis, IN, USA)) for 30 min at 4 °C. In the case of cell monolayers grown in Transwell® Permeable Supports, the support membranes were carefully detached from Transwell® plates, placed in 6-well plates and treated with the lysis buffer. In all cases, the lysis buffer was spiked with appropriate amounts of IS. Receptor media, comprising HBSS added with 0.2% (w/v) poloxamer 407, was obtained at different time-points from the basolateral chamber of Transwell® Permeable Supports bearing either Caco-2 or CaSki cell monolayers, or from the receptor chamber of Franz cells supporting mucosal tissue. Mucosal tissues from permeability studies were washed thoroughly with HBSS and collected for further processing. Pools of cell lysates, basolateral media or mucosal tissues were established and used for validation purposes except if stated otherwise.

2.5. Stock solution and calibration standards

Stock solutions of dapivirine and IS were prepared in ACN at a concentration of 1 mg/mL. Working solutions of different concentrations were prepared immediately before sample preparation by dilution of stock solutions with ACN.

Calibration standards were prepared differently for cell lysates, receptor media and mucosal tissues. Drug-free cell lysates (200 μL and containing the IS) were centrifuged (13,000 rpm, 10 min, 4 °C) and obtained supernatants mixed for 1 min with ACN (400 μL) containing different amounts of dapivirine by vortexing. Samples were again centrifuged (13,000 rpm, 10 min, 4 °C), newly obtained supernatants collected and filtered by 0.20 μm PTFE syringe filters (National Scientific, Rockwood, TN, USA), and assayed. In the case of receptor media, drug-free samples (300 μL) obtained from simulated permeability experiments were mixed by vortexing for 1 min with the same volume of ACN containing the drug and IS at the desired concentrations. Samples were then centrifuged (13,000 rpm, 10 min, 4 °C), supernatants collected and filtered by 0.20 μm PTFE syringe filters, and assayed. As for mucosal tissues, drug-free samples (around 500 mg) were processed by adding 500 μL of ACN containing the drug and IS and homogenizing for 10 min in a Ultra-Turrax T25 homogenizer (Janke and Kunkel Type, IKA, Staufen, Germany). Tissue homogenates were then centrifuged (13,000 rpm, 10 min, and 4 °C), supernatants collected and filtered by 0.20 μm PTFE syringe filters, and assayed. Calibration standards for all matrices were prepared at a final concentration of 0.1 μg/mL in IS.

2.6. Development and optimization of chromatographic conditions

Chromatographic analysis was performed in gradient mode. The mobile phase, comprising TFA 0.1% (v/v) and ACN, was pumped at 1 mL/min. After sample injection (50 μL), the initial TFA 0.1%:ACN ratio was progressively changed over a period of time (further referred to as ramp time) until reaching a final TFA 0.1%:ACN ratio. After the ramp time, the final TFA 0.1%:ACN ratio was kept constant until minute nine, changing back again to the initial TFA 0.1%:ACN ratio over one minute and kept constant until the end of the run in order to stabilized the column for subsequent runs. Total run time was 12 min. UV detection of both dapivirine and IS was at 290 nm.

A 3-factor, 3-level Box-Behnken experimental design was used to optimize the experimental HPLC factors (or variables), namely the initial and final TFA 0.1%:ACN ratios, and the ramp time. Evaluated responses included the retention factor (k'), tailing factor (T), peak resolution (R), and theoretical plates (N) for both dapivirine and IS. Optimization was performed using a cell lysate pool matrix. Minitab® software (v. 15.1.20.0; Minitab Inc., State College, PA, USA) was used for the generation and analysis of the experimental design.

2.7. Method validation

The method was validated in order to concur with the FDA [22] and the recently released EMA [23] guidelines on the validation of bioanalytical methods. Evaluated parameters included selectivity and carry-over effect, lower limit of quantification (LLOQ) and limit of detection (LOD), linearity of calibration curve, accuracy, precision, recovery and stability. Selectivity was studied by running at least 10 different blank individual samples (i.e. not pooled) of tested matrices and by comparing results with chromatograms obtained for drug- and IS-spiked samples. Also, the interference of blank nanoparticles and individual components of nanoparticles [14] was studied by spiking blank samples. Carry-over effect was evaluated by injection of a spiked sample at the maximum considered concentration of dapivirine (1.5 μg/mL) followed by a blank sample and evaluation of the responses at the retention time of dapivirine and IS. The LLOQ and LOD were determined based on the signal-to-noise (S/N) ratio. The calibration curve was obtained in triplicate from the response (ratio between the area of dapivirine and IS peaks) against the nominal concentration of dapivirine of eight standard solutions (0.02, 0.03, 0.06, 0.1, 0.3, 0.6, 1, 1.5 μg/mL) by the least squares linear regression. Linearity was evaluated by visual analysis of the plots, calculation of the correlation coefficient (R^2), back calculation of concentrations of individual calibration standards, and analysis of the response factors (i.e. ratio between the response and nominal concentration of each standard sample). Within-run (or intra-day) and between-run (or inter-day) accuracy and precision were determined using five different spiked samples for each of four different concentration levels (LLOQ, low level, middle level and high level). Accuracy was expressed as the measured concentration and recovery (percentage of the nominal concentration), while precision was defined by the relative standard deviation (RSD). Recovery was determined at four concentration levels (LLOQ, low level, middle level and high level) by comparing the results for extracted standard samples with those of non-extracted standard samples, i.e. blank samples to which the drug and IS were added only after sample processing. The stability of dapivirine in different matrices was assessed at two levels (low and high), namely after 72 h at room temperature, three 24 h freeze–thawing cycles (−20 °C/20 °C) over 7 days, and 14 days at −20 °C. Also, stability was determined for prepared samples stored in the autosampler during 48 h, and for the stock solutions of dapivirine and IS (1 mg/mL) stored at room temperature and away from light during one month.

2.8. Method applicability

Developed method was used to study cell-associated drug levels and cell monolayer/mucosal tissue permeability of dapivirine either as free drug or associated to poloxamer-modified polycaprolactone (PEO-PCL) nanoparticles. Dapivirine-loaded PEO-PCL nanoparticles were produced by nanoprecipitation as previously described [14]. In the case of cell-based studies, dapivirine or dapivirine-loaded PEO-PCL nanoparticles were dispersed in cell culture media at a concentration of 1 μM (expressed in dapivirine) and incubated with cells ($0.5–3.75 \times 10^5$/well) in 6-well plates for 1 h at 37 °C/5% CO_2. Cells were then washed trice with PBS, pH 7.4, and cell lysates obtained and processed as described for calibration standards. Protein content of cell lysates was also assessed using a colorimetric-based commercial kit (Pierce® BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA).

The permeability of free or nanoparticle-associated dapivirine was studied across cell monolayers (CaSki or Caco-2) grown in Transwell® Permeable Supports and pig vaginal or rectal mucosal tissues mounted in Franz cells. In the case of monolayers, the free drug or nanoparticles were dispersed in 1.5 mL of HBSS at a final dapivirine concentration of 5 μM and placed in the apical chamber; the basolateral chamber was filled with 2.5 mL of HBSS added with 0.2% (w/v) poloxamer 407. In the case of mucosal tissues, the free drug or nanoparticles were dispersed in a simulated vaginal fluid (SVF) [24] or PBS pH 7.4 at a concentration of 0.02% (w/v) in dapivirine and placed in the donor chamber, while the receptor chamber was filled with 8 mL of PBS pH 7.4 added with 0.2% (w/v) poloxamer 407. Samples from the basolateral chamber or receptor chamber were collected after 2 h, processed as described for calibration standards and assayed for dapivirine. Apparent permeability coefficients (P_{app} in cm s^{-1}) were calculated according to the following equation:

$$P_{app} = \frac{Q}{A \times C \times t} \quad (1)$$

where Q is the total amount of permeated drug (μg), A the diffusion area, C the concentration of dapivirine (μg cm^3) in the apical or donor chamber at time zero, and t the time of experiment (s). Further, monolayers mounted on Transwell® supports or mucosal tissues from Franz cells were thoroughly washed with HBSS, processed as stated for calibration standards and assayed

Table 1
Quadratic model fit and regression analysis for studied responses.

Response	Quadratic model equation[a]	R^2
k' (dapivirine)	$Y = 2.02167 + 0.44125A + 0.195B + 0.32125C - 0.03646A^2 + 0.03854B^2 - 0.07396C^2 + 0.09875AB + 0.20375AC$	0.9960
k' (IS)	$Y = 4.455 + 0.24437A + 0.86625B + 1.03062C - 0.10688A^2 + 0.26187B^2 - 0.07938C^2 + 0.05AB + 0.17625AC + 0.04BC$	0.9976
R (dapivirine/IS)	$Y = 18.9467 - 0.5662A + 1.4281B + 2.8306C - 0.6883A^2 - 0.9796C^2 - 0.2988AB - 0.47CB$	0.9711
N (dapivirine)	$Y = 8370.4 + 3475.9A - 824.6B - 1313C + 594.3A^2 - 250.4BC$	0.9887
N (IS)	$Y = 30{,}173 + 4109A - 4173B + 4562C - 2557A^2 - 3340B^2 - 4315C^2 - 1665AB + 3620AC + 1288BC$	0.9717

[a] Only statistically significant terms are presented ($p < 0.05$); factors are coded as presented in Table S2.

for dapivirine content. Cell lysates obtained from monolayers were also assayed for soluble protein content.

3. Results and discussion

3.1. Development and optimization of chromatographic conditions

An initial method (Table S1; Supplementary Data) was established after performing different screening runs based on previously reported HPLC-UV methods for assaying dapivirine [15–19]. The column used in this work was selected specifically due to its low silanol activity [25] since dapivirine is known to interact strongly with silanol groups, resulting in peak tailing [18]. DPA, which has been previously used successfully as an IS in different chromatographic methods for dosing diverse aromatic amino compounds [26,27], was tested as the IS. Its solubility in both organic solvents and water was deemed suitable in order to allow processing analytical matrices diversely. Also, DPA presents several common chemical structural features with dapivirine (Fig. 1) and demonstrated satisfactory chromatographic profiles (stable signal, good peak shape, and good resolution with dapivirine) under the chromatographic conditions selected for dapivirine.

However, dapivirine and IS responded differently to different ratios of TFA 0.1% and ACN. Dapivirine required higher content of TFA 0.1% in order to achieve adequate retention, while higher ACN content was needed in order to allow the elution of the IS. Thus, a gradient method was preferred. Also, preliminary experiments identified the initial and final TFA 0.1%:ACN ratios and the ramp time as being the most critical factors on the outcome of the method. A response surface methodology was further used in order to study the influence of these factors, as well as their interactions, in the outcome of the HPLC method. Changes to HPLC parameters selected as critical (tested levels are presented in Table S2; Supplementary Data) were best fitted by the quadratic model in predicting the outcome of k' and N of dapivirine and IS, and R (dapivirine/IS) by using a 3-factor, 3-level Box-Behnken factorial design (results for the different test runs are presented in Table S3; Supplementary Data). This response surface design has been previously used to optimize different HPLC methods [28–30] and was chosen specifically due to its ability to avoid extreme combinations of studied factors while allowing using a reduced number of experiments when only 3 variables are considered [31]. Fitted quadratic model equations are presented in Table 1. Values for R^2 of calculated quadratic models seem to support their good ability to predict the behavior of chromatographic parameters such as k' and N of both dapivirine and IS, and R between dapivirine and IS. However, the correlation with T of both dapivirine and IS was not significant. Quadratic relationships between same factors (A^2, B^2 and C^2) and interactions between different factors (AB, AC and CB) were observed in all cases of k' and N and R responses but in different proportions, reinforcing the value of using a multifactorial design in the optimization steps of the described method. For instances, k' for IS was influenced by all factors, and possible quadratic relationships and interactions between same and different factors, while N for dapivirine was only affected by individual factors, quadratic initial TFA 0.1%:ACN ratio and the interaction between final TFA 0.1%:ACN ratio and ramp time. Alongside the establishment of quadratic model equations, the 3D surface plots representing the interactions among studied factors were generated and analyzed for each response (Figs. S1–S3; Supplementary Data).

The method variables were then adjusted in order to optimize k' and N according to pre-established value ranges (Table S4; Supplementary Data) by using the Response Optimizer function of the Minitab® software. The software allows obtaining different combinations of values for studied factors in order to achieve a set of optimized responses. Three different optimal variations to the tested factors were defined and verified experimentally in order to validate the fitted models; the same experimental validation was also performed for nine different variations of the tested factors in order to confirm the predictability of generated models (Table S5; Supplementary Data). All models were able to adequately predict experimental results with an average absolute error of 2.0%. In all cases, the absolute error was equal or less than 6.7% in all cases (Table S5; Supplementary Data). The optimized setting comprising initial and final TFA 0.1%:ACN ratios of 65:35 and 27:73, respectively, and a ramp time of 6.5 min (optimized setting 1 in Table S5; Supplementary Data) was further considered for validation purposes and for all defined matrices, i.e. cell lysates, receptor media and mucosal tissues. Chromatographic performance parameters for the optimized method did not differ among matrices, being presented in Table 2.

3.2. Method validation

The optimized chromatographic method was validated for the proposed matrices according to the FDA [22] and EMA [23] guidelines on validation of bioanalytical methods. Selectivity was assessed by comparing representative chromatograms of blank samples with those of samples spiked with dapivirine and IS (Fig. 2). Similar chromatographic profiles were observed for all matrices. The absence of interfering components co-eluting with dapivirine and IS indicated that the method was selective. Signals in the region of the peak of dapivirine were well below the maximum 20% level of the LLOQ, as defined by the EMA guideline [23]. In the case of

Table 2
Chromatographic performance parameters for the optimized method (mean ± SD; $n = 3$).

Parameter	Result	Acceptance criteria
Dapivirine retention time (min)	5.02 ± 0.01	N.D.
IS retention time (min)	8.23 ± 0.01	N.D.
Solvent retention time (min)	1.44 ± 0.01	N.D.
k' (dapivirine)	2.38 ± 0.04	$2 < k' < 10$
k' (IS)	4.54 ± 0.05	$2 < k' < 10$
T (dapivirine)	1.03 ± 0.01	$0.8 < T < 1.5$
T (IS)	1.02 ± 0.01	$0.8 < T < 1.5$
R (dapivirine/IS)	18.1 ± 0.1	$R > 2$
N (dapivirine)	12,332 ± 292	$N > 2000$
N (IS)	35,195 ± 577	$N > 2000$

N.D. – not defined.

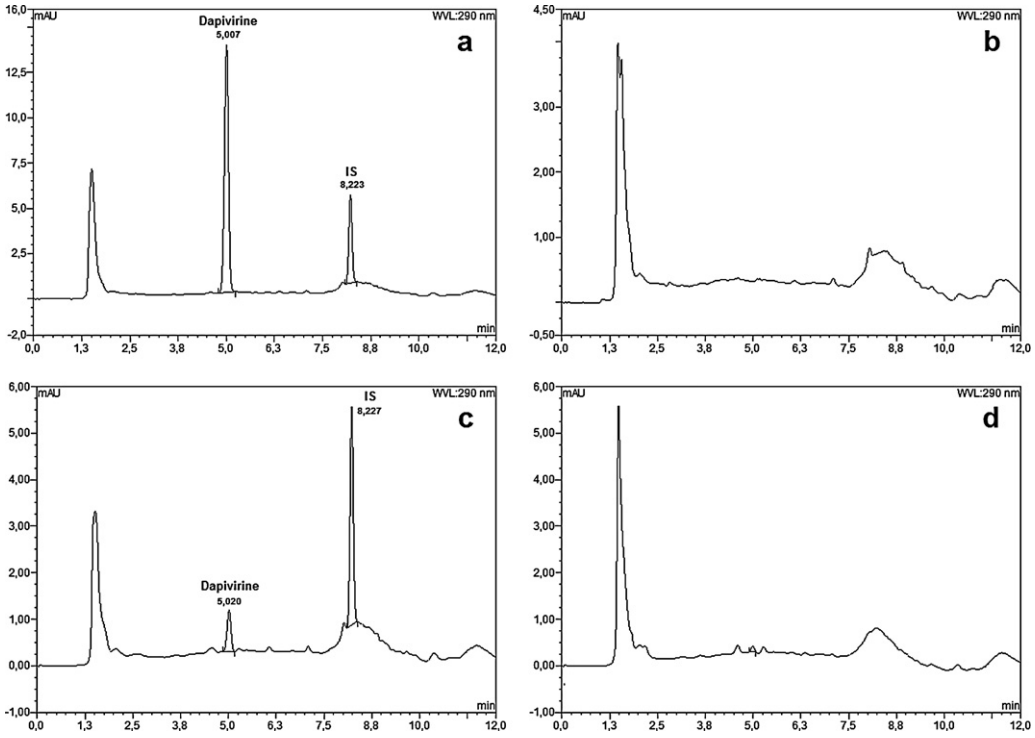

Fig. 2. Chromatograms of (a) spiked sample with 0.3 μg/mL dapivirine and 0.1 μg/mL IS, (b) blank sample, (c) spiked sample at LLOQ of dapivirine and 0.1 μg/mL IS, and (d) carry-over. Presented samples are from cell lysates.

IS, interference values were lower than the maximum accepted by this last guideline (5%). Moreover, selectivity was also maintained when formulation components of nanoparticles and nanoparticles were spiked in matrices. The absence of further peaks in spiked samples seems to indicate that spiked compounds do not absorb at around 290 nm or are not retained in the column, being eluted with the solvent.

Peak areas at the retention time of dapivirine were around or less than 7% of the one for the LLOQ of dapivirine when blank samples were analyzed after running a sample containing 1.5 μg/mL dapivirine and IS (Fig. 2d). Also, no signal for the IS was observed under the same conditions. This indicated that the carry-over effect was negligible and inferior to the 20% and 5% limits for dapivirine and IS, respectively, as defined by the EMA guideline [23].

The LLOQ and LOD were determined by sequential dilution of samples ($n = 5$) with blank matrices and analysis of the S/N ratio. Ratios of 10:1 and 3:1 are routinely considered for calculating the LLOQ and LOD, respectively [32]. Thus, LLOQ was determined as 0.02 μg/mL and LOD estimated at 0 0.006 μg/mL. These values are considerably lower than the ones determined for a HPLC-UV method previously developed by our group (LLOQ = 0.24 μg/mL and LOD = 0.08 μg/mL as determined from the standard deviation of the response and the slope of the calibration curve) [18].

Calibration curves were established in the range of 0.02–1.5 μg/mL in order to include the LLOQ and fulfill the array of concentrations expected during the application of the method. Analysis of linearity was performed by the visual inspection of the calibration curves, as well as the calculation of the regression equation and R^2 (Table 3). Individual R^2 values for each replicate of the calibration curve were equal or higher than 0.9991 for all considered matrices, thus suggesting good linearity in the considered range. Also, the back calculated concentrations of individual calibration standards presented deviations equal or less than 15% (equal or less than 20% for the LLOQ) from the nominal values (Table S6; Supplementary Data), as defined by both FDA and EMA guidelines [22,23]. However, and even if commonly used for assessing the quality of linear regressions, the use of R^2 as a true pointer of linearity has been questioned [33]. Therefore, the assessment of linearity was reinforced by the analysis of the response factors plot. The near zero slope of the curve and RSD values below 2% across all concentrations tested reinforced the linearity of the calibration curve (Table 3) [34].

The within-run and between-run accuracy and precision results are presented in Table 4. In the case of accuracy, the percentage deviation of the mean measured concentration from the nominal concentration for all levels ranged from −1.4% to +9.5% in within- and between-runs and for both considered matrices. These values are below the maximum 15% deviation allowed (20% in the case of LLOQ) by both FDA and EMA guidelines [22,23]. In the case of precision, the within- and between-run RSD values varied from 1.7% to 10.2% for all levels and both matrices, again within the 15% (20% for the LLOQ) deviation considered acceptable as per FDA and EMA guidelines. Thus the method was accepted as accurate and precise.

Table 5 presents the recovery values for dapivirine of the developed method upon sample processing. Values ranged from 90.7% to 102.0% when free dapivirine was used and were comparable between different matrices. In order to discard possible differences in drug extraction during sample processing, drug recovery was also tested when using dapivirine-loaded nanoparticles. Results showed no differences when compared to the free drug, with recovery values in the range of 93.2–98.4% (Table 5). Overall, these results indicated that the extraction of the drug was nearly complete for all matrices during sample processing. Lastly, the stability of dapivirine in different matrices and under different conditions is presented in Table 6. Deviations from nominal concentrations were less or equal than 4.2%, below the maximum of 15% defined in by EMA [23]. Also, dapivirine and IS were stable in stock solutions of 1 mg/mL, presenting recovery percentages of 101.7% (RSD = 1.3%) and 100.8% (RSD = 1.7%), respectively, upon 3 months at room temperature and protected from light.

Table 3
Results for linear regression and response factors analysis.

	Cell lysates	Receptor media	Mucosa tissues
Regression equation[a]	$R = 12.069(\pm0.059) \times C - 0.0281(\pm0.0400)$	$R = 10.816(\pm0.025) \times C + 0.0008(\pm0.0168)$	$R = 12.414(\pm0.048) \times C - 0.0299(\pm0.0325)$
R^2	0.99985	0.99998	0.99991
Response factors slope	0.1304	−0.1603	0.2762
Response factors RSD	1.6%	1.9%	1.8%

[a] Mean results ($n = 3$; standard error is indicated in brackets); R – response (area ratio between dapivirine and IS); C – concentration (μg/mL).

Table 4
Accuracy and precision results ($n = 5$).

Matrix	Nominal concentration (μg/mL)	Within-run			Between-run[a]		
		Measured concentration (μg/mL)	Recovery (%)	RSD (%)	Measured concentration (μg/mL)	Recovery (%)	RSD (%)
Cell lysates	0.02	0.022	108.8	8.4	0.021	107.0	10.2
	0.05	0.052	104.9	5.7	0.053	106.5	5.6
	0.75	0.770	102.7	4.6	0.740	98.7	5.8
	1.2	1.18	98.6	3.3	1.19	99.3	4.7
Receptor media	0.02	0.022	109.5	5.0	0.021	106.5	7.6
	0.05	0.052	103.9	5.3	0.051	101.7	4.3
	0.75	0.768	102.3	1.7	0.768	102.4	2.6
	1.2	1.23	102.5	2.2	1.22	101.8	3.1
Mucosal tissues	0.02	0.021	106.4	10.3	0.021	107.1	7.4
	0.05	0.052	103.8	8.7	0.052	104.2	6.3
	0.75	0.731	97.4	6.1	0.742	99.0	7.1
	1.2	1.25	104.0	5.3	1.20	100.2	5.4

[a] Three runs determined in different days.

3.3. Method applicability

The ability of dapivirine-loaded PEO-PCL nanoparticles (200 nm, zeta potential −30 mV and 13% (w/w) drug loading) [14] to promote enhanced intracellular/cell associated drug levels as compared to free dapivirine are presented in Fig. 3. Overall, significantly higher drug levels were achieved with nanoparticles, except for CaSki and VK2/E6E7 cells. In particular, the noticeably higher drug levels obtained in HIV target cells (PBMCs, macrophages and dendritic cells) seem particular relevant and suggest that nanoparticles were able to provide targeted delivery of dapivirine to these cells in a passive fashion.

In the case of cell monolayer permeability experiments, small but significant differences ($p < 0.05$; 2-tailed Student's t-test for independent samples) were observed for dapivirine when associated to nanoparticles or in the free form. Dapivirine presented P_{app} values (mean ± SD) of $3.70 \times 10^{-6} \pm 0.36 \times 10^{-6}$ and $4.44 \times 10^{-6} \pm 0.26 \times 10^{-6}$ cm s^{-1} for CaSki and Caco-2 cell monolayers, respectively, when dapivirine-loaded nanoparticles were used; in the case of the free drug, these results increased to $4.84 \times 10^{-6} \pm 0.28 \times 10^{-6}$ and $5.26 \times 10^{-6} \pm 0.38 \times 10^{-6}$ cm s^{-1} for CaSki and Caco-2 cell monolayers, respectively. Even if higher, the values found in this study are in order with those previously reported for free dapivirine in Caco-2 cell monolayers ($2.14 \times 10^{-6} \pm 0.81 \times 10^{-6}$ cm s^{-1}) [35].

The retention of dapivirine at cell monolayers/mucosal tissues was also determined. The amount of dapivirine assayed in CaSki cell monolayers was 0.56 ± 0.06 and 0.16 ± 0.02 μg/mg of protein for nanoparticles and free drug, respectively. In the case of Caco-2, a similar trend was observed: 0.70 ± 0.06 and 0.38 ± 0.08 μg/mg of protein for nanoparticles and free drug, respectively. These data indicate that nanoparticles were able to improve drug retention at the monolayers. In the case of both vaginal and rectal mucosal tissues, the amount of associated drug was successfully determined but characterized by a wide variation between different samples with values ranging from 0.8 to 20.6 μg/g of tissue. Previous work by Akil et al. [36] using human cervical tissue mounted in Franz cells found retention levels of dapivirine of 33–96 μg/g of tissue, which are higher than the ones found in this study. These differences may be related to the diverse set-up (e.g. the amount of dapivirine in the donor compartment was 100 μg) and tissue origin. Also, we did not observe differences when using dapivirine-loaded nanoparticles or

Table 5
Recovery results ($n = 5$).

Matrix	Nominal concentration (μg/mL)	Free-dapivirine		Dapivirine-loaded nanoparticles	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Cell lysates	0.02	92.7	9.2	97.3	3.8
	0.05	102.0	5.4	93.6	5.9
	0.75	90.7	6.7	98.4	4.7
	1.2	90.7	6.7	93.4	3.3
Receptor media	0.02	97.1	4.9	95.6	7.7
	0.05	94.9	5.3	96.6	2.7
	0.75	95.1	1.7	93.2	3.2
	1.2	93.0	2.2	94.3	3.6
Mucosal tissues	0.02	100.2	5.6	97.4	6.8
	0.05	97.6	5.8	96.7	2.9
	0.75	93.9	5.7	98.4	6.5
	1.2	94.1	5.2	96.0	2.7

Table 6
Stability of dapivirine samples in different matrices and conditions ($n = 3$).

Condition	Matrix	Nominal concentration (μg/mL)	Recovery (%)	RSD (%)
72 h/room temperature	Cell lysates	0.05	97.3	4.9
		1.2	101.2	1.7
	Receptor media	0.05	100.6	4.0
		1.2	99.7	2.0
	Mucosal tissues	0.05	99.5	4.0
		1.2	98.9	1.7
14 days/−20 °C	Cell lysates	0.05	100.5	14.0
		1.2	98.1	3.0
	Receptor media	0.05	102.2	1.9
		1.2	98.0	5.6
	Mucosal tissues	0.05	99.0	13.5
		1.2	102.4	7.2
Freeze–thawing[a]	Cell lysates	0.05	97.0	4.6
		1.2	97.8	1.2
	Receptor media	0.05	104.2	8.0
		1.2	100.8	4.0
	Mucosal tissues	0.05	108.5	4.1
		1.2	100.8	5.3
48 h/in autosampler	Cell lysates	0.05	103.3	4.4
		1.2	101.3	4.4
	Receptor media	0.05	100.9	2.0
		1.2	101.6	2.1
	Mucosal tissues	0.05	105.3	5.0
		1.2	101.7	5.6

[a] Three freeze–thawing cycles, where samples were frozen at −20 °C for at least 24 h and thawed at 20 °C (performed over 7 days).

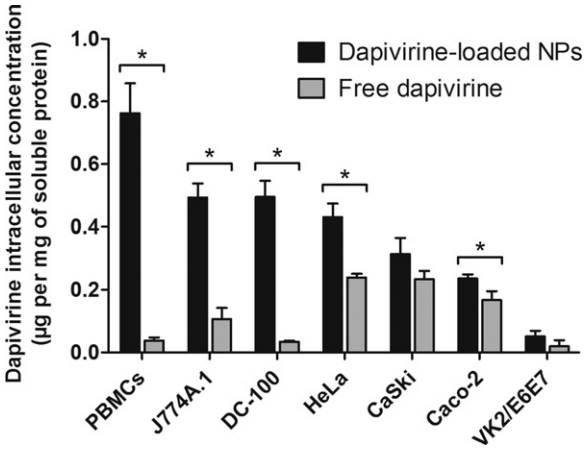

Fig. 3. Intracellular/cell associated levels of dapivirine in different cell types for drug-loaded PEO-PCL nanoparticles (NPs) and the free drug after 1 h incubation (dapivirine initial concentration was 1 μM). Columns represent mean values and vertical bars the standard deviation ($n = 3$). (*) denotes a significant difference ($p < 0.05$) of mean results as assessed by 2-tailed Student's *t*-test for independent samples.

free drug, or among different types of tissue (vaginal or rectal). The broad variation found is most probably related to inconsistencies in tissue preparation, particularly sample thickness, and deserves further work in refining the Franz cell permeability set-up.

4. Conclusions

A new bioanalytical HPLC-UV method was developed and validated for the assay of the antiretroviral drug dapivirine. The Box-Behnken experimental design was shown useful in optimizing the proposed method, allowing for multivariate analysis with a minimal number of experiences. An optimized method was validated according to both FDA and EMA guidelines on bioanalytical assays and was shown to be selective, linear, accurate and precise in the range of 0.02–1.5 μg/mL. Also, the amount of carry-over effect, LLOQ, LOD, recovery and sample stability were determined and found adequate for the purposes of the method and/or in accordance with guidelines. The method was successfully applied to the assay of dapivirine in cell-based assays and permeability experiments through both cell monolayers and mucosal tissues. Thus, the hereby proposed HPLC-UV method provides a simple, versatile and reliable approach to the assay dapivirine in different biological matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.10.034.

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