Published in final edited form as:

J Pharm Biomed Anal. 2014 September ; 98: 407–416. doi:10.1016/j.jpba.2014.06.018.

Dual Quantification of Dapivirine and Maraviroc in Cervicovaginal Secretions from Ophthalmic Tear Strips and Polyester-Based Swabs via Liquid Chromatographic-Tandem Mass Spectrometric (LC-MS/MS) Analysis

Teresa L. Parsons, PhD,

Department of Medicine, Johns Hopkins University, 600 N. Wolfe St., Osler 505, Baltimore, MD, 21287, USA

Joshua F. Emory, PhD,

Department of Medicine, Johns Hopkins University, 600 N. Wolfe St., Osler 500, Baltimore, MD, 21287, USA

Lauren A. Seserko, BS,

Department of Medicine, Johns Hopkins University, 600 N. Wolfe St., Osler 500, Baltimore, MD, 21287, USA

Wutyi S. Aung, MPH, and

Department of Medicine, Johns Hopkins University, 600 N. Wolfe St., Blalock 563, Baltimore, MD, 21287, USA

Mark A. Marzinke, PhD*

Departments of Medicine¹ and Pathology², Johns Hopkins University, 1800 Orleans St., Sheikh Zayed Tower, B1020-G, Baltimore, MD, 21287, USA

Abstract

Background—Topical microbicidal agents are being actively pursued as a modality to prevent HIV viral transmission during sexual intercourse. Quantification of antiretroviral agents in specimen sources where antiviral activity is elicited is critical, and drug measurements in cervicovaginal fluid can provide key information on local drug concentrations. Two antiretroviral drugs, dapivirine and maraviroc, have gained interest as vaginal microbicidal agents, and rugged methods are required for their quantification in cervicovaginal secretions.

Methods—Cervicovaginal fluid spiked with dapivirine and maraviroc were applied to ophthalmic tear strips or polyester-based swabs to mimic collection procedures used in clinical

© 2014 Elsevier B.V. All rights reserved

*Corresponding Author: Mark A. Marzinke, PhD, DABCC, Departments of Medicine and Pathology, Johns Hopkins University, 1800 Orleans St., Sheikh Zayed Tower, B1020-G, Baltimore, MD 21287, USA, mmarzin1@jhmi.edu, Phone: 443-287-7516, Fax: 410-955-0767.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

studies. Following sample extraction and the addition of isotopically-labeled internal standards, samples were subjected to liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analysis using a Waters BEH C8, 50 × 2.1 mm, 1.7 µm particle size column, on an API 4000 mass analyzer operated in selective reaction monitoring mode. The method was validated according to FDA Bioanalytical Method Validation guidelines.

Results—Due to the disparate saturation capacity of the tested collection devices, the analytical measuring ranges for dapivirine and maraviroc in cervicovaginal fluid on the ophthalmic tear strip were 0.05 to 25 ng/tear strip, and 0.025 to 25 ng/tear strip, respectively. As for the polyester-based swab, the analytical measuring ranges were 0.25 to 125 ng/swab for dapivirine and 0.125 to 125 ng/swab for maraviroc. Dilutional studies were performed for both analytes to extended ranges of 25,000 ng/tear strip and 11,250 ng/swab. Standard curves were generated via weighted ($1/x^2$) linear or quadratic regression of calibrators. Precision, accuracy, stability and matrix effects studies were all performed and deemed acceptable according to the recommendations of the FDA Bioanalytical Method Validation guidelines.

Conclusions—A rugged LC-MS/MS method for the dual quantification of dapivirine and maraviroc in cervicovaginal fluid using two unique collection devices has been developed and validated. The described method meets the criteria to support large research trials.

Keywords

Dapivirine; Maraviroc; Cervicovaginal Fluid; Validation; Antiretroviral

1. Introduction

Despite the multiple modalities available for the treatment and management of HIV/AIDS, more than 34 million individuals are currently infected with the virus, with more than 2.5 million new infections occurring annually [1]. There are numerous antiretroviral (ARV) drugs approved by the Food and Drug Administration (FDA) for disease treatment, and maximal viral suppression is typically observed in response to combinatorial ARV therapies [2]. In addition to disease management, ARV drugs have also been effective in HIV prevention in a number of settings, including pre-exposure prophylaxis (PrEP) in HIV uninfected individuals, as well as treatment as prevention in HIV-infected individuals [3, 4]. However, there are numerous impediments to treatment success, including high-pill burden or oral therapeutic regimens, lack of regimen adherence, variable drug efficacy and potential ARV drug resistance, and decreased drug concentrations at the site of transmission [5–7]. Based on these challenges, topical administration of microbicides has been pursued as a viral prevention strategy.

Since heterosexual transmission accounts for the highest proportion of HIV-1 infection globally, topical application of vaginal microbicides has been pursued to facilitate increased localized drug concentrations and subsequently, decreased transmission events [8–15]. One of the first studies that assessed the effectiveness of a topical microbicide was the Center for the AIDS Program of Research in South Africa (CAPRISA) 004 trial, a two-arm double-blinded placebo-controlled study to assess the safety and efficacy of the nucleotide reverse transcriptase inhibitor (NRTI) tenofovir [12]. The study concluded that topical

administration of tenofovir as a gel formulation reduced HIV acquisition by 39% overall, and 54% in women with high adherence to microbicide application.

With the success of the CAPRISA 004 trial, as well as additional tenofovir-containing PrEP studies, other drugs have also been evaluated as vaginal microbicide agents, including the non-nucleoside reverse transcriptase inhibitor (NNRTI) dapivirine and the CCR-5 antagonist maraviroc. These agents elicit their antiviral activity through the noncompetitive binding and subsequent inhibition of the viral reverse transcriptase enzyme and the abrogation of the viral protein interaction with the host cell CD4 receptor via allosteric binding to the host cell CCR-5 co-receptor molecule, respectively [16–18]. While maraviroc has been well tolerated and elicits viral suppression following oral administration, dapivirine has primarily been pursued as a microbicide agent [11, 19–21]. Topical administration of dapivirine was well tolerated in both reservoir and matrix-based silicone elastomeric ring formulations, and localized concentrations were several orders of magnitude higher than required for *in vitro* viral inhibition [10, 22]. Following the insertion of a matrix-based ring containing 25 mg of dapivirine, average concentrations at the $C_{\max}$ ranged from 850.7 – 1,913 $\mu\text{g/g}$ within cervicovaginal fluid, depending on where the fluid was collected in relation to the ring; the reservoir ring released less drug and the $C_{\max}$ ranged from 7.6–14.4 $\mu\text{g/g}$ following the application of the reservoir-formulated ring [10]. There have been other formulations used in phase I studies, including gel and film-based delivery devices, to assess tolerability and the compartmentalized pharmacokinetics of the NNRTI [9, 11, 23]. Conversely, several studies in nonhuman primates have pursued the topical application of maraviroc, where it has shown protection from infection at the site of transmission [15, 24]. Currently, ongoing studies are looking at the safety, efficacy and compartmentalized pharmacokinetics of both dapivirine and maraviroc as vaginal microbicides independently, or in combination [25, 26].

Methods with a wide analytical measuring range are required for the quantification of drugs in luminal fluids for compartmentalized pharmacokinetic analysis. Cervicovaginal secretions are heterogeneous fluids collected within the vagina, and are comprised of cervical mucus containing mucins and glycoproteins, as well as sloughed vaginal epithelial cells, lymphocytes, eosinophils and a number of other cell types [27]. Further, cervicovaginal fluid is typically acidic due to the presence of *Lactobacillus*-containing microflora. One important pre-analytical consideration is the collection device used for the acquisition of cervicovaginal fluid. Previously implemented collection devices include Tear Flo_{TM} Test Strips and Alpha Med Tear Test Ophthalmic Strips, as well as a variety of swabs and sponges [11, 23, 28].

Due to the unique composition of the matrix, as well as the analytical measuring range that may be encountered following topical administration of these drugs, optimized methods are required for the expedient and accurate quantification of these analytes in cervicovaginal secretions. While literature has referenced the quantification of dapivirine in cervicovaginal matrices with lower limits of quantification (LLOQ) ranging from 20 ng/g cervicovaginal fluid to 40 pg/collection device, and maraviroc quantification with a LLOQ of 50 ng/ml, the full validation metrics have never been described [10, 11, 14, 22]. Further, a method for the dual quantification of both the NNRTI dapivirine and the CCR-5 antagonist maraviroc has not been previously reported. Our group has previously developed and validated liquid

chromatographic-tandem mass spectrometric (LC-MS/MS) assays for the quantification of dapivirine and maraviroc in plasma, with lower limits of quantification of 20 pg/ml and 500 pg/ml, respectively [29, 30]. However, a method for quantification in cervicovaginal fluid must be amended for a wide analytical measuring range, with extensive linearity to accommodate previously reported localized drug concentrations. The described analytical method is currently being implemented for dapivirine and maraviroc quantification in cervicovaginal secretions in a number of completed and ongoing clinical trials, and meets the needed criteria for accurate and precise drug measurements.

2. Experimental

2.1. Chemicals

Dapivirine, also known as TMC120 ($\text{-}[[4\text{-}[(2,4,6\text{-Trimethylphenyl})\text{amino}]\text{-}2\text{-pyrimidinyl}]\text{amino}]\text{benzonitrile}$), as well as the deuterated internal standard $^2\text{H}_4\text{-dapivirine}$, were obtained from the International Partnership for Microbicides (IPM) in powdered form. Maraviroc (4,4-Difluoro-N-[(1S)-3-[(3-exo)-3-[3-methyl-5-(1-methylethyl-d6)-4H-1,2,4-triazol-4-yl]-8-azabicyclo[3.2.1]oct-8-yl]-1-phenylpropyl]cyclohexanecarboxamide), and its isotopically labeled internal standard, $^2\text{H}_6\text{-maraviroc}$, were purchased from Toronto Research Chemicals (TRC, North York, ON). The chemical structures for both dapivirine ($\text{C}_{20}\text{H}_{19}\text{N}_5$) and maraviroc ($\text{C}_{29}\text{H}_{41}\text{F}_2\text{N}_5\text{O}$) are depicted in Figure 1. Blank cervicovaginal fluid was obtained from healthy human subjects consented to an institutional review board (IRB)-approved protocol for biological sample collection. HPLC-grade and LC-MS/MS grade acetonitrile and LC-MS grade water were purchased from Fisher Scientific (Pittsburgh, PA). Proteomics-grade formic acid was purchased from Proteochem (Denver, CO).

2.2. Preparation of reagents and standards

Stock solutions of dapivirine and maraviroc were prepared in acetonitrile at final concentrations of 1 mg/ml each. Stock solutions of labeled internal standards were prepared in acetonitrile at final concentrations of 0.5 mg/ml ($^2\text{H}_4\text{-dapivirine}$) and 1 mg/ml ($^2\text{H}_6\text{-maraviroc}$). The dapivirine and maraviroc stock solutions were diluted with acetonitrile to generate working solutions of 100, 10, 1 and 0.1 $\mu\text{g/ml}$ for both compounds. Cervicovaginal fluid was collected using an Insteade Softcup (Evoform Inc. San Diego CA) from healthy volunteers as per the aforementioned IRB- approved protocol. Specifically, secretions were self-collected by placing the Softcup in the vaginal cavity for 30 seconds; post-removal, the apparatus was transferred to a conical tube and centrifuged at $300 \times g$ for 10min at room temperature (25°C). Following centrifugation, collected fluid was diluted 20-fold with water. Calibrators and quality control samples were prepared by spiking working solutions into diluted cervicovaginal fluid. The calibrator solutions were prepared at concentrations of 2, 5, 10, 25, 100, 250, 750 and 1000 ng/ml for dapivirine, and 1, 2, 5, 10, 50, 125, 300 and 1000 ng/ml for maraviroc. Calibrator solutions were maintained in 2.0 ml glass vials and stored at -80°C . Calibrator solutions were then placed onto two collection devices used in clinical research studies: Tear Flo_{TM} sterile tear flow test strips (Fisher Scientific) and polyester-tipped Dacron swabs, Puritan[®] (Fisher Scientific). Twenty five microliters were placed on the Tear Flo_{TM} tear strip and strips were allowed to dry for 15 minutes in a $12 \times 75\text{ mm}$

glass tube prior to sample processing. The final calibrator concentrations on each tear strip for dapivirine were 0.05, 0.125, 0.250, 0.625, 2.5, 6.25, 18.75, and 25 ng/tear strip. Final maraviroc concentrations were 0.025, 0.05, 0.125, 0.25, 1.25, 3.13, 7.5 and 25 ng/tear strip.

The same calibrator stock solutions in diluted cervicovaginal fluid were spiked onto polyester-based Dacron swabs. To generate swabs containing cervicovaginal fluid, 125 µl of calibrator solutions were added to generate final concentrations of 0.25, 0.625, 1.25, 3.13, 12.5, 31.3, 93.8 and 125ng/swab for dapivirine and 0.125, 0.25, 0.625, 1.25, 6.25, 15.6, 37.5 and 125ng/swab for maraviroc. Polyester swabs were stored in glass vials at -20°C.

Quality controls (QCs) were also prepared in diluted cervicovaginal fluid at the lower limit of quantification (LLOQ), as well as low, mid and high levels. Concentrations at the LLOQ, low, mid and high QC levels were 2, 6, 75 and 850 ng/ml for dapivirine, and 1, 3, 40 and 850 ng/ml for maraviroc, respectively. On the tear strip, final concentrations at the LLOQ, low, mid and high QC levels were 0.05, 0.15, 1.88, and 21.25 ng/tear strip for dapivirine, and 0.025, 0.075, 1, and 21.25 ng/tear strip for maraviroc, respectively. On the polyester Dacron swab, final concentrations at the LLOQ, low, mid and high QC levels were 0.25, 0.75, 9.38, and 106ng/swab for dapivirine, and 0.125, 0.375, 5, and 106ng/swab for maraviroc, respectively.

2.3. Sample Preparation

Once thawed, cervicovaginal fluid containing analytes of interest applied to the tear strip was extracted via the addition of 2 ml acetonitrile. Samples were vigorously vortexed and incubated at room temperature for 20 min. One milliliter of the solution was transferred to a 12 × 75 mm glass test tube. Following the extraction of the analyte from the collection device, the internal standard solution (10 ng/ml ²H₄-dapivirine and ²H₆-maraviroc) was added and samples were vigorously vortexed. Solutions were evaporated to dryness under a nitrogen stream and reconstituted in 0.1 ml acetonitrile:water (60:40). Ten microliters of solution was used for LC-MS/MS analysis.

Cervicovaginal fluid collected on polyester-based swabs was extracted in 1.5 ml methyl-*tert*-butyl ether:methanol (50:50) solution containing 25% ammonium hydroxide. Following vigorous vortexing, samples were incubated in the pH-adjusted organic solvent for 1 h. Swabs were then removed, and the solution was then evaporated to dryness under a nitrogen stream. The resulting residue was reconstituted in 125 µl of water:acetonitrile (70:30). Following this, 50 µl of the extracted solution was combined with 50 µl internal standard solution (25 ng/ml ²H₄-dapivirine and 5 ng/ml ²H₆-maraviroc) and 100 µl of a 70:30 water-acetonitrile mixture. Ten microliters of solution was used for LC-MS/MS analysis.

2.4. Separation and Instrument Acquisition Parameters

Chromatographic separation of dapivirine and maraviroc was performed using a Waters BEH C8, 50 × 2.1 mm UPLC column with a 1.7 µm particle size, on a Waters Acquity UPLC system as previously described [29, 30]. A mobile phase system consisting of water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B) was used for separation. Briefly, the chromatographic method included a

gradient elution from 20% to 50% mobile phase B over 1.0 minute, and was maintained at 50% organic for 0.5 minutes. Both dapivirine and maraviroc were eluted during this time. Following, there was a gradient increase to 95% mobile phase B over 0.5 minutes, where the column was maintained with a high percentage of organic solvent flowing through it for 1.5 minutes. The column was then re-equilibrated to an aqueous environment via a gradient transition to 80% mobile phase A over 1.2 minutes. The flow rate throughout the chromatographic run was 1,000 μ l per minute and the run time was 5.0 minutes.

Dual detection of dapivirine and maraviroc was performed using an API 4000 tandem mass spectrometer (AB SCIEX, CA, USA) with an ESI source operated in positive ionization mode. Analytes were monitored in selective reaction monitoring (SRM) mode. The monitored ion transitions for dapivirine and its internal standard were m/z 330.4 $\rightarrow$ 158.1 and m/z 334.3 $\rightarrow$ 119.0, respectively; the transitions for MVC and its internal standard were m/z 515.5 $\rightarrow$ 390.2 and m/z 520.6 $\rightarrow$ 389.1, respectively.

2.5. Data Evaluation

All data were acquired and analyzed using Analyst[®] 1.5 Software (Version 1.5.1 Build 528) (AB SCIEX). Calculations performed to determine validation metrics, including precision, accuracy, stability, and matrix effects, were performed using Microsoft Office Excel 2010. Outliers were defined by Grubb's Outlier Test.

2.6. Method Validation

The bioanalytical method for dual dapivirine and maraviroc quantification was validated based on the recommendations of the Food and Drug Administration (FDA) Guidance for Industry, Bioanalytical Method Validation [31]. The validation of this assay involved assessment of intra- (within) and inter- (between) assay precision and accuracy, linearity, selectivity, stability, carryover, and matrix effects.

2.6.1. Precision and Accuracy Studies—Precision and accuracy studies were evaluated via the testing of collection device-specific dapivirine and maraviroc containing quality control materials at the aforementioned concentrations. Within-run precision was determined through the analysis of six injections of materials extracted from cervicovaginal fluid materials spiked onto tear strips or polyester swabs. The observed means, standard deviations (SDs) and the percent coefficients of variations (%CVs) were calculated at all QC levels ($n = 6$). Between-run precision was evaluated through the testing of QC samples over three independent runs; further, all observed values were determined from run-specific calibration curves. The %CV was used to illustrate assay precision. Accuracy was determined as the percent deviation (% DEV), which is a measure of the difference between mean observed and theoretical QC concentrations divided by the level-specific theoretical QC concentration and finally multiplied by 100. Both intra- and inter-assay accuracy were determined from six injections within an analytical run and between three independent runs, in the same manner as precision studies were performed.

2.6.2. Calibration Curve Analysis—For calibration curve generation, calibration standards were analyzed at the beginning and end of each analytical run. The first set of

calibrators were analyzed in ascending order and the second set in descending order. Calibration curves were calculated using the ratio of the peak area of the analyte to the internal standard using either a weighted $1/x^2$ linear or quadratic regression. Precision and accuracy were determined for each calibration standard over three independent analytical runs.

2.6.3. Dilutional Integrity—Extended linearity beyond the established analytical measuring range of the assay was also evaluated. Dilutional integrity on cervicovaginal fluid applied to a tear strip was performed by preparing solution 1,000-times more concentrated than the highest calibrator. Similar to calibrators and QC samples, 25 μ l was applied to a tear strip for a final concentrations of 25,000 ng/tear strip for both dapivirine and maraviroc. Following extraction from the collection device, samples were diluted with blank cervicovaginal fluid.

For samples added to polyester swabs, cervicovaginal fluid containing dapivirine and maraviroc concentrations three- and 90-times the highest calibrator were prepared. Final concentrations for both drugs on the collection device were 375ng/swab and 11,250ng/swab at 3-times and 90-times the upper limit of quantification, respectively. The samples were diluted with water following extraction from the Dacron swab.

2.6.4. Stability—Stability was assessed under a number of conditions to ensure the fidelity of sample analysis. For injection matrix stability studies, samples were extracted from tear strip or polyester swab collection devices and directly measured, and re-analyzed 3 (swab) or 5 (tear strip) days after being maintained at 4°C. Sample matrix stability was performed by incubating thawed cervicovaginal fluid-spiked tear strip and polyester-based swab QC samples at room temperature for 72 h. Samples were then extracted and prepared as described above. Freeze-thaw stability studies were performed on QC-spiked collection devices subjected to three freeze thaw cycles at –20°C. Stability under these conditions was assessed by comparing observed values to freshly prepared QC materials applied to either tear strips or polyester swabs, followed by immediate extraction and analysis. For all stability studies, specimens were prepared and analyzed in replicates of four, with the exception of the injection matrix stability study which was performed using six replicates, and the low QC for sample matrix stability on the polyester-based swab, which was performed in triplicate. Stability was analyzed through the calculation of a percent difference (% difference), which is a measure of the difference between a stability challenged sample and a freshly prepared or analyzed QC specimen divided by the freshly prepared QC sample; this result is then multiplied by 100. For all stability challenges, acceptability was determined as a % difference <15% between observed and reference QC value.

2.6.5. Selectivity—Assay selectivity was determined by the analysis of 6 independent lots of drug-free cervicovaginal fluid from healthy volunteers. Six lots of cervicovaginal fluid were extracted from the specific collection device and subjected to LC-MS/MS analysis. Review of chromatograms was performed to verify that no interfering substances were observed.

2.6.6. Matrix Effect Characterization/Extraction Efficiency—Matrix effects, extraction efficiency and processing efficiency were determined following the experiments described by Matuszewski and colleagues[32]. Briefly, three sets of samples were prepared in six independent lots of drug-free cervicovaginal fluid. An un-extracted set was prepared at low, mid and high QC concentrations in the absence of matrix. A post-extracted set was prepared following the extraction of cervicovaginal secretions from the tear strip or polyester-based swab and then spiked with antiretroviral agents. A pre-extracted set contained drug-containing cervicovaginal fluid applied to the tear strip or polyester-based swab and extracted as previously described. Raw peak areas for the analytes and internal standards were analyzed and used to calculate overall matrix effects (a comparison of post-extracted samples to un-extracted samples), extraction efficiency (a comparison of pre-extracted samples to post-extracted samples) and processing efficiency (a comparison of pre-extracted samples to un-extracted samples) observed for both analytes.

3. Results

3.1. Method Development

For dapivirine and maraviroc quantification in cervicovaginal fluid using ophthalmic tear strips or polyester-based swabs, the same chromatographic and mass spectrometric conditions were implemented as previously described for drug quantification in plasma [29, 30]. However, significant development was required for the preparation of a specimen for LC-MS/MS analysis, as the matrix may be acquired using a variety of collection devices. Several previous and ongoing studies have looked at the acquisition of cervicovaginal secretions using tear strips or polyester-based swabs. A volume of liquid was added to the collection device to achieve saturation of the collection device; optimal spiking volumes to saturate the tear strip and polyester-based Dacron swab were 25 μ l and 125 μ l, respectively. Extraction of dapivirine and maraviroc from the tear strip was optimally achieved using 100% acetonitrile and incubating the tear strip in organic solvent for 20 minutes prior to evaporating the extracted material to dryness and reconstituting in a 60:40 mixture of water:acetonitrile. A variety of extraction solutions and reconstitution mixtures were tested, including varying ratios of water and methanol, DMSO, and isopropanol. The aforementioned conditions were optimal for the extraction of both ARVs from the tear strip, and were determined via the comparison of peak areas of samples extracted from the tear strip to neat solutions prepared in solvent (**data not shown**).

Extraction of the antiretrovirals from the polyester-based Dacron swabs was more challenging, particularly for dapivirine. Dapivirine is a highly hydrophobic NNRTI, and is highly adsorptive to plastic collection devices, including the polyester-based Dacron swab [29]. As a result, stringent extraction conditions were employed to remove dapivirine from the polyester Dacron swab. Method development indicated that the addition of ammonium hydroxide was required to maximize analyte extraction from the polyester-based collection device (Supplemental Table 1). Example chromatograms of dapivirine and maraviroc, and their isotopically-labeled internal standards, extracted from cervicovaginal fluid, are shown in Figure 2.

3.2. Method Validation

3.2.1. Precision and Accuracy—Intra- and inter-assay precision and accuracy were observed at quality control (QC) levels in cervicovaginal secretions and applied to tear strips and polyester-based Dacron swabs. Although a single set of quality control stock solutions were generated for the validation of dapivirine and maraviroc quantification in cervicovaginal fluid acquired using either collection devices, the differing volumes applied to each collection device resulted in QC solutions with different final concentrations. The intra- and inter-assay precision of dapivirine in cervicovaginal fluid and the fluid applied to the tear strip was assessed at LLOQ (0.05 ng/strip), low (0.15 ng/strip), mid (1.88 ng/strip) and high (21.3 ng/strip) QC levels and ranged from 4.3% to 8.5% and 5.8% to 9.1%, respectively. Intra- and inter-assay accuracy for dapivirine in this matrix and collection device ranged from 0.9% to 7.4% and 1.5% to 8.4%, respectively. Intra- and inter-assay precision and accuracy for maraviroc collected on the tear strips at LLOQ (0.025 ng/strip), low (0.075 ng/strip), mid (1.0 ng/strip) and high (21.3 ng/strip) QC levels ranged from 7.0% to 14.2% and 9.4% to 15.9% (precision), and –11.6% to 7.7% and –10.4% to 1.1% (accuracy), respectively.

The intra- and inter-assay precision of dapivirine in cervicovaginal fluid and the fluid applied to the polyester-based Dacron swab was assessed at LLOQ (0.250ng/swab), low (0.750ng/swab), mid 9.38ng/swab) and high (106ng/swab) QC levels and ranged from 1.7% to 12.9% and 8.2% to 11.2%, respectively; intra- and inter-assay accuracy ranged from –8.4% to 7.6% and –8.9% to 6.3%, respectively. Intra- and inter-assay precision and accuracy for maraviroc collected on the polyester-based swabs at LLOQ (0.125ng/swab), low (0.375ng/swab), mid (5.00ng/swab) and high (106ng/swab) QC levels ranged from 1.7% to 8.5% and 3.3% to 6.9% (precision), and –2.4% to 2.3% and –4.9% to 1.8% (accuracy), respectively. The observed values for both analytes in cervicovaginal fluid applied to each collection device are acceptable according to the guidelines recommended by the FDA Bioanalytical Guidelines, Guidance for Industry [31]. A full summary of precision and accuracy is illustrated in Table 1.

3.2.2. Calibration Curve Analysis—For cervicovaginal fluid applied to the tear strip, the analytical measuring ranges for dapivirine and maraviroc are 0.05 to 25 ng/strip and 0.025 to 25 ng/strip, respectively. The dapivirine standard curve was generated using weighted ($1/x^2$) quadratic regression of calibrators, while the maraviroc standard curve was constructed using weighted ($1/x^2$) linear regression of calibrators. The average regressions for dapivirine and maraviroc in cervicovaginal fluid and the fluid collected on the tear strip, from three independent runs, were 0.997 and 0.994, respectively (**data not shown**). Calibrator precision at the lowest calibrator (the LLOQ concentrations) for dapivirine and maraviroc were 4.3% and 8.8%, respectively; this is well within the recommended precision of 20% at the lowest calibrator; remaining calibrators ranged from 2.2% to 6.9% for dapivirine and 7.2% to 10.2% for maraviroc. The accuracy of calibration standards, which was determined as the mean back-calculated drug concentrations from the three standard curves, ranged from –8.1% to 6.1% for dapivirine and –5.1% to 4.0% for maraviroc.

Similar findings were observed for ARV quantification in cervicovaginal secretions collected on the polyester-based Dacron swab. One notable exception was that both dapivirine and maraviroc standard curves were generated using weighted ($1/x^2$) linear regression analysis. At the lowest calibrator, the observed precision was 5.5% and 4.3% for dapivirine and maraviroc, respectively; the remaining calibrators ranged in precision from 3.7% to 10.3% for dapivirine and 1.8% to 5.5% for maraviroc. Accuracy of standards ranged from -7.8% to 6.2% for dapivirine and -3.7% to 5.1% for maraviroc. Example calibration curves for dapivirine and maraviroc in cervicovaginal secretions on both the tear strip and polyester-based swab are illustrated in Figure 3.

3.2.3. Dilutional Integrity—While maraviroc may be administered orally or topically, dapivirine has been pursued as a topical microbicide in a number of completed and ongoing clinical trials. As a result, a quantitative assay to determine dapivirine and maraviroc concentrations in cervicovaginal secretions must exhibit extended linearity to quantify high drug concentrations. The dilutional integrity of both antiretrovirals in cervicovaginal fluid on the tear strip or polyester-based Dacron swab was tested by assessing drug concentrations at 1,000 and 90 times the upper limit of quantification, respectively. On the tear strip, dapivirine and maraviroc were applied for drug concentrations of 25,000 ng/strip for each drug; following 1,000, 4,000 and 8,000 dilutions, precision and accuracy of dapivirine ranged from 2.7% to 5.6% and -2.6% to 7.4%, respectively; precision and accuracy of maraviroc ranged from 5.9% to 7.1% and -5.9% to 4.4%, respectively. On polyester-based Dacron swabs, antiretroviral drugs were applied for final concentrations of 4,500 ng/swab; following 5,000- and 10,000-fold dilutions, precision and accuracy ranged from 1.3% to 1.5% and 6.7% to 8.8% for dapivirine and 5.1% to 11.3% and -9.1% to -7.7% for maraviroc. These data are within the limits recommended by the FDA, and illustrate that samples above the highest calibrator may be diluted for analysis.

3.2.4. Stability Analyses—The validation of the described methods test drug quantification under a variety of stability challenges, as study samples may undergo freeze-thaw events, maintained at room temperature prior to storage, or re-analyzed post-extraction. Stability was assessed by the percent difference between stability challenged QC samples and freshly extracted or tested samples. Both dapivirine and maraviroc were stable when challenged with three freeze-thaw cycles, when drug-containing cervicovaginal fluid was maintained on the tear strip and polyester-based swab for 72 hours at room temperature, and following extraction from each collection device and maintained in the appropriate reconstitution solution for 72 (swab) or 120 hours (tear strip). Across both collection devices, freeze-thaw stability, sample matrix stability and injection matrix stability ranged from -10.2% to 7.5%, -8.6% to 11.8% and -1.3% to 2.8% for dapivirine, respectively, and -6.2% to 6.6%, -0.1% to 14.3% and -7.6% to 13.8% for maraviroc, respectively (Table 2). Additional long-term stability studies demonstrated dapivirine and maraviroc stability on the tear strip or polyester-based swab for 10 days and 1 month, respectively (**data not shown**).

3.2.5. Matrix Effects and Selectivity Studies—Similar to previously reported observations describing the selectivity of dapivirine and maraviroc in plasma, no interfering

peaks were observed in drug-free cervicovaginal fluid at the retention times at which dapivirine, maraviroc and the respective internal standards, elute (Figure 4) [29, 30]. Further, to assess the potential influence of endogenous compounds on analyte suppression or enhancement, quantitative matrix effects studies were performed following the guidelines of Matuszewski and colleagues [32]. Peak responses of dapivirine and maraviroc, and their respective internal standards from the described procedure (pre-extracted set) were compared to a neat formulation (un-extracted set) as well as cervicovaginal fluid spiked post-extraction from either the ophthalmic tear strips or polyester-based swabs (post-extracted set).

A summary of matrix effects, which compared the average peak responses of post-extracted samples to un-extracted specimens, as well as comparisons of the pre-extracted set to the post-extracted set (recovery efficiency) and pre-extracted set to the un-extracted set (processing efficiency) is described in Table 3. Dapivirine showed minimal ion suppression across cervicovaginal fluid lots when collected on the tear strip, and relative matrix effects were negligible, as illustrated by $\sim 5\%$ difference between peak responses for dapivirine and $^2\text{H}_4$ -dapivirine. The extraction of dapivirine from this collection device and its overall processing efficiency were also acceptable. While a greater degree of ion suppression was observed in samples collected on the polyester-based swabs, the relative matrix effects were still minimal, with an average percent difference of $\sim 15\%$ between analyte and internal standard, which is acceptable. The differences in overall recovery and processing efficiencies between dapivirine and the $^2\text{H}_4$ -dapivirine internal standard is due to the addition of the isotopically-labeled standard post-cervicovaginal fluid extraction from the collection device, resulting in increased recovery and processing of the internal standard as compared to the analyte. However, since drug quantification is based on the peak area ratio of drug to internal standard, and calibrators are prepared in the same manner as QC and unknown specimens, the determination of drug concentrations are not impacted by the differential recovery and processing efficiencies. Further, these efficiencies do not differ greatly across QC levels. Similar results were observed with maraviroc, with the only distinction that maraviroc showed greater ion suppression in cervicovaginal fluid collected on the tear strips compared to dapivirine (Table 4).

4. Discussion

The method described herein illustrates the development and bioanalytical validation of an LC4-MS/MS method for the dual quantification of dapivirine and maraviroc in cervicovaginal secretions acquired using either an ophthalmic tear strip or a polyester-based Dacron swabs. While the assays are specifically optimized for drug extraction and sample preparation, and have collection device-specific analytical measuring ranges, all assays have been validated according to the recommendations of the FDA Guidance for Industry, Bioanalytical Method Validation guidelines [31]. Further, the described method utilizes previously detailed LC-MS/MS conditions for dapivirine and maraviroc quantification in plasma [29, 30].

For topically applied drugs, including those administered as vaginal or rectal microbicidal agents, while plasma concentrations reflect systemic drug exposure, luminal fluids, such as rectal fluid and cervicovaginal fluid, can provide significant insight regarding localized drug

concentrations at the potential site of viral transmission [33]. Since 2004, dapivirine has been exclusively assessed as a microbicidal agent, and studies have indicated that the NNRTI is safe and well-tolerated in a variety of topical formulations [8–10]. Consequently, the NNRTI is being pursued as a vaginal microbicidal agent in two ongoing Phase III clinical trials [25, 26]. While maraviroc has been explored in oral formulations for HIV management, the aforementioned Microbicide Trials Network Protocol 013 (MTN-013) is assessing the safety and tolerability of the dual topical administration of maraviroc and dapivirine [19, 25]. Therefore, the measurements of dapivirine and maraviroc in cervicovaginal fluid is critical in order to characterize their distributions at the potential site of viral transmission.

Pre-analytical variables were considered in the development and validation of this assay. Cervicovaginal fluid has been collected using a variety of collection devices, including ophthalmic tear strips, polyester-based swabs, sponges, or via direct aspiration [10, 11, 15, 22, 24, 34–35]. It should be noted that the development and validation of an assay is not only drug specific, but also specific to the collection device. The methods described herein demonstrate the need for collection device-optimized protocols for drug isolation and analysis. LC-MS/MS methods for dapivirine and maraviroc were developed in cervicovaginal secretions applied to tear strips and polyester-based swabs to accommodate several phase III clinical trials assessing drug concentrations disparately, or in combination [25, 26, 36]. However, due to the low volume of cervicovaginal secretions that are acquired using these collection devices, calibrators and quality control samples were prepared using cervicovaginal secretions collected via the Instead Softcup and diluted 20-fold with water. The dilution of cervicovaginal fluid was performed to provide sufficient material for development, validation, and implementation studies. The assays do not discriminate between vaginal lumen locations, which may be perceived as a limitation of the assay. However, since the validation is produced in a luminal fluid matrix collected from the cavity where secretions are released, the authors feel the described methods can reflect drug concentrations in fluids collected from any vaginal lumen location, including the introitus, mid vagina or cervix.

When cervicovaginal fluid is collected on the tear strip, dapivirine and maraviroc may be quantified across an analytical measuring range of 0.05 to 25 ng/strip and 0.025 to 25 ng/strip, respectively. Dynamic ranges are collection device-specific because of the initial volumes applied to calibrators and quality controls. It should be noted that interpreting drug concentrations for pharmacokinetic analysis may be difficult if data are reported as drug concentration per collection device. Consequently, for clinical studies, it is recommended that the weight of the collection device pre- and post-cervicovaginal fluid collection is recorded, to determine the net weight of the fluid on the device; this information may be used to determine the quantity of drug per weight of fluid. Data reporting in this format allows for the normalization of fluid collected on the ophthalmic tear strip or polyester-based swab. [9–11, 23].

Since drug concentrations in fluids may be significantly higher than systemic plasma concentrations, extended dilutional integrity studies are required during assay validation. Dapivirine concentrations in cervicovaginal fluid, when administered as 0.05% gel

formulations, were $125.9 \pm 81.5 \mu\text{g/g}$ and $273.1 \pm 140.9 \mu\text{g/g}$ at the C_{max} [23]. The application of 25 mg dapivirine in intravaginal ring formulations yielded cervicovaginal fluid concentrations ranging from 1,118 to 2,673 $\mu\text{g/g}$ 8–24 h post-insertion [10]. Further, studies assessing maraviroc concentrations 0.5–2 h post- hydroxylethyl cellulose gel administration, ranged from 10^4 – 10^7 ng/ml in cervicovaginal fluid [15]. Dilutional analysis is therefore required to assess the ability to accurately quantify drug concentrations beyond the highest calibrator. However, the dynamic range for the assays was set to achieve maximum sensitivity so that these methods could also be utilized in the quantification of drug concentrations following oral administration, which is more relevant for maraviroc, as it is currently used as part of oral antiretroviral therapies.

Although the described assays demonstrate the bioanalytical and validation of two antiretroviral agents in cervicovaginal secretions, due to the unavailability of samples, and the lack of use of dapivirine in clinical settings, it was not possible to acquire non-study cervicovaginal samples to assess the ruggedness of the method. However, this method is being actively applied to previously described trials to assess the compartmentalized pharmacokinetics of these analytes. Secondly, the described assay utilized diluted cervicovaginal fluid for the generation of calibrators and QC solutions. We felt this was an appropriate surrogate for direct cervicovaginal fluid due to volume constraints and that the material would be applied to a collection device prior to extraction. As indicated by our matrix studies, overall matrix effects were less a cause of concern than the optimization of drug extraction from a collection device. Another limitation of the assay is the addition of the internal standard during sample preparation. While the authors acknowledge it would be optimal to apply the internal standard at the start of sample extraction, it was not possible to add the internal standard solution mixture to a saturated ophthalmic tear strip or polyester-based swab. Therefore, internal standards were added following extraction of analytes from the aforementioned collection devices.

5. Conclusions

LC-MS/MS methods have been developed and validated for the dual quantification of dapivirine and maraviroc in cervicovaginal fluid collected on ophthalmic tear strips or polyester-based swabs. It should be noted that the analytical measuring ranges for drug quantification are device-specific. The method involves straightforward and minimal sample processing for high-throughput sample analyses. Validations of these assays were conducted according to FDA recommendations, with additional dilutional studies, indicating the method is rugged, accurate, and reproducible. Furthermore, the methods described herein illustrate a roadmap for the development and optimization of quantitative LC-MS/MS assays in alternative specimen sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Microbicide Trials Network (MTN) sponsored by the NIH/DAIDS grant UM1 AI068633. This research was also supported by the HIV Prevention Trials Network, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID), the National Institute of Mental Health (NIMH), and the National Institute of Drug Abuse (NIDA), Office of AIDS Research, of the National Institutes of Health (NIH), Department of Health and Human Services (DHHS), grant UM1-AI068613. Finally, this work was supported by the NIH/NIAID grant U19AI0882639-03. We are also grateful for essential analytical equipment provided by a grant from the James B. Pendleton Charitable Trust.

References

1. Global report: UNAIDS report on the global AIDS epidemic 2012. 2012. <http://www.unaids.org/en/resources/publications/2012>
2. Volberding PA, Deeks SG. Antiretroviral therapy and management of HIV infections. *Lancet*. 2010; 376:49–62. [PubMed: 20609987]
3. Weber J, Tatoud R, Fidler S. Postexposure prophylaxis, preexposure prophylaxis or universal test and treat: the strategic use of antiretroviral drugs to prevent HIV acquisition and transmission. *AIDS*. 2010; 24:S27–S39. [PubMed: 21042050]
4. Cohen MS, Muessig KE, Smith MK, Powers KA, Kashuba ADM. Antiviral agents and HIV prevention: controversies, conflicts and consensus. *AIDS*. 2012; 26:1585–1598. [PubMed: 22507927]
5. Tang MW, Shafer RW. HIV-1 antiretroviral resistance: scientific principles and clinical applications. *Drugs*. 2012; 72:e1–e25. [PubMed: 22686620]
6. Buscher A, Hartman C, Kallen MA, Giordano TP. Impact of antiretroviral dosing frequency and pill burden on adherence among newly diagnosed, antiretroviral-naïve HIV patients. *Int J STD AIDS*. 2010; 23:351–355. [PubMed: 22648890]
7. Williams AB, Amico KR, Bova C, Womack JA. A proposal for quality standards for measuring medication adherence in research. *AIDS Behav*. 2013; 17:284–297. [PubMed: 22407465]
8. Jespers VA, Van Roey JM, Beets GI, Buvé AM. Dose-ranging phase 1 study of TMC120, a promising vaginal microbicide, in HIV-negative and HIV-positive female volunteers. *J Acquir Immune Defic Syndr*. 2007; 44:154–158.
9. Nel AM, Coplan P, van de Wijgert JH, Kapiga SH, von Mollendorf C, Guebels E, Vyankandondera J, Rees HV, Masenga G, Kiwelu I, Moyes J, Smythe SC. Safety, tolerability, and systemic absorption of dapivirine vaginal microbicide gel in healthy, HIV-negative women. *AIDS*. 2009; 23:1531–1538. [PubMed: 19550287]
10. Nel AM, Smythe SC, Young K, Malcolm K, McCoy C, Rosenberg Z, Romano J. Safety and pharmacokinetics of dapivirine delivery from matrix and reservoir intravaginal rings to HIV-negative women. *J Acquir Immune Defic Syndr*. 2009; 51:416–423.
11. Nel AM, Coplan P, Smythe SC, McCord K, Mitchnick M, Kaptur PE, Romano J. Pharmacokinetic assessment of dapivirine vaginal microbicide gel in healthy, HIV-negative women. *AIDS Res Hum Retroviruses*. 2010; 26:1181–1190. [PubMed: 20854207]
12. Karim QA, Karim SSA, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany ABM, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mltosha M, Morris L, Taylor D. on behalf of the CAPRISA 004 Trial Group. Effectiveness and safety of a tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science*. 2010; 329:1168–1174. [PubMed: 20643915]
13. Fetherston SM, Boyd P, McCoy CF, McBride MC, Edwards KL, Ampofo S, Malcolm RK. A silicone elastomer vaginal ring for HIV prevention containing two microbicides with different mechanisms of action. *Eur J Pharm Sci*. 2012; 48:406–415. [PubMed: 23266465]
14. Malcolm RK, Fetherston SM, McCoy CF, Boyd P, Major I. Vaginal rings for delivery of HIV microbicides. *Int J Womens Health*. 2012; 4:595–605. [PubMed: 23204872]
15. Malcolm RK, Forbes CJ, Geer L, Veazey RS, Goldman L, Klasse PJ, Moore JP. Pharmacokinetics and efficacy of a vaginally administered maraviroc gel in rhesus macaques. *J Antimicrob Chemother*. 2013; 68:678–683. [PubMed: 23111849]

16. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*. 1996; 86(3):367–377. [PubMed: 8756719]
17. Moore JP, Kitchen SG, Pugach P, Zack JA. The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. *AIDS Res Hum Retroviruses*. 2004; 20(1):111–126. [PubMed: 15000703]
18. Fletcher P, Harman S, Azijn H, Armanasco N, Manlow P, Perumal D, deBethune MP, Nuttall J, Romano J, Shattock R. Inhibition of human immunodeficiency virus type 1 infection by the candidate microbicide dapivirine, a non nucleoside reverse transcriptase inhibitor. *Antimicrob Agents Chemother*. 2009; 53:487–495. [PubMed: 19029331]
19. Fätkenheuer G, Nelson M, Lazzarin A, Konourina I, Hoepelman AI, Lampiris H, Hirschel B, Tebas P, Raffi F, Trottier B, Bellos N, Saag M, Cooper DA, Westby M, Tawadrous M, Sullivan JF, Ridgway C, Dunne MW, Felstead S, Mayer H, van der Ryst E. MOTIVATE 1 and MOTIVATE 2 Study Teams. Subgroup analyses of maraviroc in previously treated R5 HIV-1 infection. *N Engl J Med*. 2008; 359(14):1442–1455. [PubMed: 18832245]
20. Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, Horban A, Nadler J, Clotet B, Karlsson A, Wohlfeiler M, Montana JB, McHale M, Sullivan J, Ridgway C, Felstead S, Dunne MW, van der Ryst E, Mayer H. MOTIVATE Study Teams. Maraviroc for previously treated patients with R5 HIV- infection. *N Engl J Med*. 2008; 359(14):1429–1441. [PubMed: 18832244]
21. Neff CP, Ndolo T, Tandon A, Habu Y, Akkina R. Oral pre-exposure prophylaxis by anti-retroviralsaltegravi and maraviroc protects against HIV-1 vaginal transmission in a humanized mouse model. *PLoS One*. 2010; 21:e15257. [PubMed: 21203568]
22. Romano J, Variano B, Coplan P, van Roey J, Douville K, Rosenberg Z, Temmerman M, Verstraeten H, Van Bortel L, Weyers S, Mitchnick M. *AIDS Res Hum Reteroviruses*. 2009; 25:483–488.
23. Nel AM, Smythe SC, Habibi S, Kaptur PE, Romano JW. Pharmacokinetics of 2 dapivirine vaginal microbicide gels and their safety vs. hydroxyethyl cellulose-based universal placebo gel. *J Acquir Immune DeficSyndr*. 2010; 55:161–169.
24. Malcolm RK, Lowry D, Boyd P, Geer L, Veazey RS, Goldman L, Klasse PJ, Shattock RJ, Moore JP. Pharmacokinetics of a CCR5 inhibitor in rhesus macaques following vaginal, rectal and oral application. *J Antimicrob Chemother*. 2014; 69:1325–1329. [PubMed: 24381072]
25. International Partnership for Microbicides. [accessed 15.04.14] First efficacy trial of a microbicide ring to prevent HIV is underway. available from: <http://www.ipmglobal.org/publications/first-efficacy-trial-microbicide-ring589 prevent-hiv-underway>
26. Microbicides Trials Network. ASPIRE – a study to prevent infection with a ring for extended use. available from: <http://www.mtnstopshiv.org/news/studies/mtn020/background>.
27. Ferguson LM, Rohan LC. The importance of the vaginal delivery route for antiretrovirals in HIV prevention. *TherDeliv*. 2011; 2:1535–1550.
28. Verma NA, Lee AC, Herold BC, Heller MJ. Topical Prophylaxis for HIV Prevention in Women: Becoming a Reality. *Curr HIV/AIDS Rep*. 2011; 8:104–113. [PubMed: 21424725]
29. Seserko LA, Emory JF, Hendrix CW, Marzinke MA. The development and validation of an UHPLC-MS/MS method for the rapid quantification of the antiretroviral agent dapivirine in human plasma. *Bioanalysis*. 2013; 5:2771–2783. [PubMed: 24256358]
30. Emory JF, Seserko LA, Marzinke MA. Development and bioanalytical validation of a liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for the quantification of the CCR5 antagonist maraviroc in human plasma. *Clin Chim Acta*. 2014; 431:198–205. [PubMed: 24561264]
31. US Department of Health and Human Services UF. Center for Veterinary Medicine: Guidance for Industry: Bioanalytical Method Validation. Rockville, MD, USA: FDA; 2001. Center for Drug Evaluation and Research. updated February 2012.
32. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at pictogram per milliliter concentrations. *Anal Chem*. 1998; 70:882–889. [PubMed: 9511465]

33. Else LJ, Taylor S, Back DJ, Khoo SH. Pharmacokinetics of antiretroviral drugs in anatomical sanctuary sites: the male and female genital tract. *AntivirTher.* 2011; 16:1149–1167.
34. Dumond JB, Yeh RF, Patterson KB, Corbett AH, Jung BH, Rezk NL, Bridges AS, Stewart PW, Cohen MS, Kashuba AD. Antiretroviral drug exposure in the female genital tract: implications for oral pre- and post-exposure prophylaxis. *AIDS.* 2007; 21:1899–1907. [PubMed: 17721097]
35. Bunge K, Macio I, Meyn L, Noguchi L, Parniak MA, Schwartz JL, Moncla B, Hillier S. The safety, persistence, and acceptability of an antiretroviral microbicide candidate UC781. *J Acquir Immune DeficSyndr.* 2012; 60:337–343.
36. HIV Prevention Trials Network. [accessed 15.04.14] HPTN-069, Phase II randomized, double-blind, study of safety and tolerability of maraviroc, maraviroc + emtricitabine, maraviroc + tenofovir, or tenofovir + emtricitabine for preexposure prophylaxis to prevent HIV transmission in at-risk men who have sex with men and in at-risk women: http://www.hptn.org/research_studies/hptn069.asp.

Highlights

- Development of an LC-MS/MS method for dapivirine and maraviroc quantification in cervicovaginal fluid
- Drug quantification in cervicovaginal fluid collected on multiple devices
Validation of LC-MS/MS assays following FDA guidelines

Figure 1. Structures of (A) the non-nucleoside reverse transcriptase inhibitor dapivirine ($C_{20}H_{19}N_5$) and (B) the CCR-5 antagonist maraviroc ($C_{29}H_{41}F_2N_5O$). * denotes position of deuterium atoms for the isotopically-labeled internal standards used for analyte quantification.

Figure 2. Representative chromatograms of (A) dapivirine and (C) $^2\text{H}_4$ -dapivirine, with retention time demarcated at 1.14 min; and (B) maraviroc and (D) $^2\text{H}_6$ -maraviroc, with retention time noted at 0.60 min, from cervicovaginal fluid extracted from polyester-based swabs.

Figure 3.

Calibration curves of dapivirine (DPV) from cervicovaginal fluid applied to (A) an ophthalmic tear strip or (C) a polyester-based swab. The analytical measuring range for (A) is 0.05 to 25 ng/tear strip and (C) is 0.25 to 125 ng/swab. Panels (B) and (D) illustrate calibration curves of maraviroc (MVC) from cervicovaginal fluid applied to (A) an ophthalmic tear strip or (D) a polyester-based swab. The analytical measuring range for (B) is 0.025 to 25 ng/tear strip and (D) 0.125 to 125 ng/swab. The curves are fit to the calibrators with either a (A) quadratic or (B-D) linear regression using $1/x^2$ weighting. Representative slopes of the line are illustrated within the figure

Figure 4.

Selectivity of dapivirine and maraviroc transitions. (A–B) Chromatograms illustrating the extracted ion current of the dapivirine transition from drug-free cervicovaginal fluid applied to (A) an ophthalmic tear strip or (B) a polyester-based swab. Chromatograms are overlaid with a sample spiked with the lower limit of quantification, (A) 0.05 ng/tear strip and (B) 0.25 ng/swab; the LLOQ is depicted green. (C–D) Chromatograms illustrating the extracted ion current of the maraviroc transition from drug-free cervicovaginal fluid applied to (C) an ophthalmic tear strip or (D) a polyester-based swab. Chromatograms are overlaid with a sample spiked with the lower limit of quantification, (C) 0.025 ng/tear strip and (D) 0.125 ng/swab; the LLOQ is depicted in purple.

Table 1

Intra-assay and inter-assay precision and accuracy results.

Dapivirine								
QC Level	Intra-Assay Precision and Accuracy				Inter-Assay Precision and Accuracy			
	Mean (ng/device)	SD (ng/device)	% CV	% DEV	Mean (ng/device)	SD (ng/device)	% CV	% DEV
Tear Strip								
LLOQ (0.05 ng/strip)	0.051	0.003	6.6	2.1	0.051	0.004	6.9	1.5
Low (0.15 ng/strip)	0.161	0.007	4.3	7.3	0.163	0.010	5.8	8.4
Mid (1.88 ng/strip)	1.90	0.12	6.1	0.9	1.93	0.11	5.9	2.7
High (21.3 ng/strip)	22.9	1.94	8.5	7.4	22.0	2.00	9.1	3.4
Polyester-swab								
LLOQ (0.250 ng/swab)	0.270	0.035	12.9	7.6	0.265	0.030	11.2	6.3
Low (0.750 ng/swab)	0.688	0.013	1.7	-8.4	0.683	0.075	10.8	-8.9
Mid (9.38 ng/swab)	9.47	0.575	6.1	1.0	9.73	0.800	8.2	3.6
High (106 ng/swab)	100	7.65	7.6	-5.7	108	11.7	10.8	1.7
Maraviroc								
Tear Strip								
LLOQ (0.025 ng/strip)	0.027	0.002	8.2	7.7	0.025	0.004	15.9	-1.2
Low (0.075 ng/strip)	0.066	0.005	7.0	-11.6	0.067	0.006	9.4	-10.4
Mid (1.0 ng/strip)	0.929	0.083	8.9	-7.1	0.928	0.090	9.7	-7.2
High (21.3 ng/strip)	20.8	2.94	14.2	-2.6	21.5	2.49	11.6	1.1
Polyester-swab								
LLOQ (0.125 ng/swab)	0.125	0.010	8.5	-0.7	0.125	0.008	6.9	0.5
Low (0.375 ng/swab)	0.385	0.010	2.3	2.3	0.370	0.018	4.9	-1.5
Mid (5.00 ng/swab)	5.05	0.125	2.7	0.9	5.10	0.175	3.3	1.8
High (106 ng/swab)	104	1.73	1.7	-2.4	101	5.40	5.3	-4.9

Table 2

Stability challenges for dapivirine and maraviroc in cervicovaginal fluid.

Dapivirine									
QC Level	Freeze-Thaw Stability n=4			Sample Matrix Stability ^b n=4			Injection Matrix Stability ^a n=4		
	Control Mean	Treated Mean	% Difference	Control Mean	Treated Mean	% Difference	Control Mean	Treated Mean	% Difference
Tear Strip									
Low (0.15 ng/strip)	0.153	0.164	7.5	0.153	0.157	2.8	0.161	0.161	0.1
Mid (1.88 ng/strip)	1.92	1.89	-1.3	1.92	1.92	0.1	1.90	1.93	1.5
High (21.3 ng/strip)	20.8	18.7	-10.2	20.8	20.9	0.4	22.5	23.2	2.8
Polyester-swab									
Low (0.750 ng/swab)	0.705	0.68	-3.6	0.663	0.740	11.8	0.688	0.683	-0.8
Mid (9.38 ng/swab)	8.60	7.93	-7.8	9.18	9.05	-1.5	9.48	9.35	-1.3
High (106 ng/swab)	91.8	90.8	-1.2	106	97.3	-8.6	100	99.0	-1.3
Maraviroc									
Tear Strip									
Low (0.075 ng/strip)	0.071	0.074	4.0	0.071	0.076	7.2	0.068	0.078	13.8
Mid (1.0 ng/strip)	0.940	0.950	0.5	0.940	1.03	8.9	0.900	0.902	0.8
High (21.3 ng/strip)	21.7	20.4	-6.2	21.7	23.7	8.9	20.5	21.0	2.3
Polyester-swab									
Low (0.375 ng/swab)	0.375	0.400	6.6	0.350	0.400	14.3	0.385	0.368	-4.6
Mid (5.00 ng/swab)	4.83	4.68	-3.1	5.05	5.05	-0.1	5.05	4.80	-4.7
High (106 ng/swab)	104	97.5	-6.1	95.3	99.8	4.6	104	95.8	-7.6

^a Injection matrix studies were performed 5 days post- initial testing of dapivirine and maraviroc applied to ophthalmic tear strips and 3 days post-initial testing of drugs applied to polyester- based swabs.

^b For sample matrix challenges, one of the challenged QCs at the low level was excluded as it deviated more than 15% from other challenged samples.

Table 3
Matrix Effects, recovery efficiency and processing efficiency of dapivirine and maraviroc in cervicovaginal fluid.

Dapivirine						
QC Level ^a	% Matrix Effects		% Recovery Efficiency		% Processing Efficiency	
	Analyte	Internal Standard	Analyte	Internal Standard	Analyte	Internal Standard
Tear Strip						
Low (0.15 ng/strip)	91.3	93.0	99.1	101	90.3	94.1
Mid (1.88 ng/strip)	91.8	93.6	100	103	91.9	96.2
High (21.3 ng/strip)	95.6	97.9	98.8	97.9	94.4	95.9
Polyester-swab						
Low (0.750 ng/swab)	47.2	59.4	71.4	121	33.7	72.1
Mid (9.38 ng/swab)	52.1	64.7	63.3	108	33.0	69.8
High (106 ng/swab)	61.1	79.4	69.4	116	42.4	91.9
Maraviroc						
Tear Strip						
Low (0.075 ng/strip)	68.5	71.5	110	146	75.3	104
Mid (1.0 ng/strip)	82.8	84.6	103	132	85.4	111
High (21.3 ng/strip)	84.7	88.5	82.1	110	69.5	97.7
Polyester-swab						
Low (0.375 ng/swab)	65.2	78.2	98.0	120	63.8	93.5
Mid (5.00 ng/swab)	71.3	83.1	95.5	118	68.1	97.7
High (106 ng/swab)	72.6	86.9	102	126	74.1	109

^aMatrix Effects for ophthalmic tear strip-applied drugs were performed upon 6 independent cervicovaginal fluid lots; polyester-based Dacron swab studies were performed across 5 independent lots of cervicovaginal fluid.