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The development and validation of an UHPLC–MS/MS method for the rapid quantification of the antiretroviral agent dapivirine in human plasma

Background: Dapivirine is a non-nucleoside reverse transcriptase inhibitor designed to prevent HIV-1 viral replication and subsequent propagation. A sensitive method is required to quantify plasma concentrations to assess drug efficacy. **Results:** Dapivirine-spiked plasma was combined with acetonitrile containing deuterated IS and was processed for analysis. The method has an analytical measuring range from 20 to 10,000 pg/ml. For the LLOQ, low, mid and high QCs, intra- and inter-assay precision (%CV) ranged from 5.58 to 13.89% and 5.23 to 13.36%, respectively, and intra- and inter-day accuracy (% deviation) ranged from -5.61 to 0.75% and -4.30 to 6.24%, respectively. **Conclusion:** A robust and sensitive LC–MS/MS assay for the high-throughput quantification of the antiretroviral drug dapivirine in human plasma was developed and validated following bioanalytical validation guidelines. The assay meets criteria for the analysis of samples from large research trials.

An estimated 34 million people are living with HIV/AIDS worldwide, with approximately 2.5 million new infections occurring annually [1]. While prevention methods have long focused on behavioral strategies, including abstinence, monogamy, and the use of barrier methods such as male and female condoms, these approaches are not always an option in specific populations, due to both gender and cultural pressures [2–4]. Since heterosexual transmission is the most common mode of HIV infection globally, drug-mediated approaches have been pursued for HIV prevention [2,4,5].

While **antiretroviral** drugs have shown efficacy in the treatment of HIV infection, antiretroviral drugs have also been successfully used for HIV prevention in a variety of settings, including pre-exposure prophylaxis in HIV-uninfected individuals (PrEP) and as ‘treatment as prevention’ in HIV-infected individuals, following either oral or vaginal administration [6–12]. Topical microbicides are vaginally or rectally administered products designed to prevent HIV transmission at the site of acquisition [2,13–15]. Microbicidal compounds may be applied in various formulations, including hydroxyethyl cellulose-based gels, silicone elastomer or thermoplastic rings, or hydroxypropyl methyl cellulose-containing films [4,13,14,16–19]. For example, in ring formulations, topical microbicides may

provide continuous, controlled and long-term delivery of pharmaceutical compounds, to both the local and/or systemic physiological environment [2].

Based on the success of tenofovir-containing PrEP products, additional agents are under development to increase the barrier to resistance when used as combination products. **Dapivirine** (also known as TMC120), is a hydrophobic, substituted diarylpyrimidine analog that acts as a **non-nucleoside reverse transcriptase inhibitor** (NNRTI) of HIV, and was originally developed by Tibotec Pharmaceuticals Ltd (Cork, Ireland) [16,19,20]. The NNRTI exhibits its anti-HIV activity through the noncompetitive binding and subsequent inhibition of the viral reverse transcriptase enzyme. While there is evidence that dapivirine undergoes cytochrome P450-mediated metabolism, structural metabolites have not been reported to date [21]. Dapivirine has demonstrated significant anti-infective efficacy against HIV-1 strains *in vitro*, as well as success *in vivo* in a hu-SCID mouse model [21–24]. Recent clinical trials indicate that topical administration of dapivirine is safe and well-tolerated when applied as a vaginal gel or ring [18–20,25]. As such, dapivirine is currently being pursued as a topical microbicide for HIV prevention in two large Phase III clinical trials [26,101].

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Key Terms

Antiretroviral: Medications that are used for the treatment of infection by retroviruses.

Dapivirine: A highly hydrophobic non-nucleoside reverse transcriptase inhibitor that is being pursued as a topical agent for HIV management and prevention.

Non-nucleoside reverse transcriptase inhibitor: Small molecule that allosterically binds to HIV reverse transcriptase at a site other than the active site of the enzyme.

Assay validation: Experiments conducted to assure the precision, accuracy and overall validity of an analytical assay.

To better understand the PK and PD of the NNRTI dapivirine, robust analytical methods are required to quantitate the drug *in vivo*. Although different dosing regimens and formulations have been used for the topical application of dapivirine, plasma drug concentrations are typically low, ranging from 39 pg/ml to 1.1 ng/ml [16,17,20]. Therefore, highly sensitive methods are required to quantitate plasma dapivirine concentrations. Previous approaches for the detection and quantification of dapivirine from pharmacological delivery systems and biological matrices include HPLC–UV and LC–MS/MS analyses [17,27,28]. In contrast to previously published approaches, the LC–MS/MS method described herein offers several key advantages; including rapid, low-cost sample preparation and robust UHPLC–MS/MS analysis, as well as increased sensitivity when compared with existing HPLC–UV methods. Additionally, this is the first report of a fully validated UHPLC–MS/MS method for the quantification of dapivirine in human plasma. Although analytical measuring ranges were determined for previously published LC–MS/MS methods, full chromatographic and validation metrics were not provided in terms of acceptance criteria. The described analytical method is currently being implemented for dapivirine quantification in several forthcoming clinical trials and has shown the robustness necessary for high-throughput sample analysis.

Experimental**■ Chemicals**

Stock solutions of dapivirine (TMC120; -[[4-[(2,4,6-trimethylphenyl)amino]-2-pyrimidinyl]amino]benzonitrile) and its isotopically labeled IS ($^2\text{H}_4$ -dapivirine) were acquired in powder form from the International Partnership for Microbicides (IPM) and reconstituted in acetonitrile at stock concentrations of 1 and 0.5 mg/ml, respectively. The structure for dapivirine, $\text{C}_{20}\text{H}_{19}\text{N}_5$, is depicted in **Figure 1**. Drug-free human K2 EDTA plasma was purchased from Bioreclamation (PA, USA). HPLC-grade

and LC–MS-grade acetonitrile and LC–MS-grade water were acquired from Fisher Scientific (PA, USA). Proteomics-grade formic acid was purchased from Proteochem (CO, USA).

■ Preparation of reagents and standards

Dapivirine stock solution was diluted in acetonitrile to working solutions of 1 $\mu\text{g/ml}$ (10^6 pg/ml), 0.1 $\mu\text{g/ml}$ (10^5 pg/ml) and 0.01 $\mu\text{g/ml}$ (10^4 pg/ml). Each master and working stock was stored at -20°C in 2 ml glass vials. Calibration standards containing final concentrations of 20, 50, 100, 250, 500, 1000, 2500, 5000 and 10,000 pg/ml were prepared by spiking human K2 EDTA plasma with appropriate volumes of working solutions. An IS mixture of deuterated dapivirine ($^2\text{H}_4$ -dapivirine) was prepared as a 5 ng/ml IS spiking solution in acetonitrile for a final, postextraction concentration of 2.5 ng/ml deuterated standard.

■ Sample preparation

Sample preparation involved combining 150 μl of spiked plasma with 50 μl of IS spiking solution to each well of an Eppendorf 96 deep-well plate (NY, USA). Samples were then vortexed for 5 s and transferred to a 96-well Captiva 0.45 μm protein precipitation filtration plate (Agilent Technologies, CA, USA). Following the transfer of sample to the filtration plate, 500 μl HPLC-grade acetonitrile was added to each well and the mixture was incubated for 5 min at room temperature. The analyte of interest and IS were eluted into glass inserts placed into a 96-well plate via the application of vacuum pressure. Eluents were evaporated under a dry nitrogen stream and precipitates were reconstituted in 100 μl of 1:1 0.1% formic acid in water:0.1% formic acid in acetonitrile (both solvents were LC–MS-grade); 10 μl of reconstituted samples were subjected to chromatographic separation and mass spectrometric analysis.

■ Instrument & acquisition parameters

Chromatographic separation was achieved using a Waters Acquity UHPLC system consisting of a binary solvent manager, a sample manager, a column heater/cooler (which was not used) and a 50 μl injection loop (MA, USA). The Acquity autosampler was maintained at 4°C and was programmed to draw 10 μl of sample for chromatographic separation.

Analytical separation was achieved using a Waters BEH C8, 50×2.1 mm UHPLC column, with a 1.7 μm particle size. The column was maintained at ambient temperature. Mobile

Figure 1. Non-nucleoside reverse transcriptase inhibitor dapivirine ($\text{C}_{20}\text{H}_{19}\text{N}_5$).
*Denotes positions of deuterium atoms for the deuterated $^2\text{H}_4$ -dapivirine IS.

phase A for both loading and eluting pumps consisted of water with 0.1% formic acid, while mobile phase B consisted of acetonitrile with 0.1% formic acid. Dapivirine and its deuterated IS were eluted under gradient conditions from 20% acetonitrile containing 0.1% formic acid to 50% additive-containing acetonitrile. Please see **TABLE 1** for the UHPLC conditions used for separation of dapivirine. The column eluent was directed to the mass spectrometer from 0.20 to 4.00 min and diverted to waste at all other times. The total analytical run time for this method is 5.00 min.

Detection of dapivirine was performed using an API 5000 tandem mass spectrometer (AB SCIEX, CA, USA) with an ESI source operated in positive ionization mode. Instrument parameters were optimized including collision-associated dissociation gas setting (6), curtain gas setting (35), Gas 1 (40), Gas 2 (40), ion spray voltage (5500 V), source temperature (600°C) and dwell time (50 ms). Analytes were monitored in SRM mode. The ion transitions for dapivirine were 330.4→158.1 (quantifier ion) and 330.4→119.1 (qualifier ion). The ion transition for the isotopically labeled IS was 334.3→119.0. Although a quantifier and qualifier ion were monitored for dapivirine, analyte quantification was determined based on the 330.4→158.1 transition. A qualifier ion is monitored to reduce misidentification of dapivirine from potential interfering substances, background ions and concomitant medications. The described SRM transitions were determined by direct infusion of a stock solution of dapivirine and the IS into the ionization source, and optimization of aforementioned mass spectrometric parameters to achieve appropriate analytical sensitivity.

■ Data evaluation

Analyst® 1.5 Software (Version 1.5.1 Build 5218; AB SCIEX) was used to acquire and analyze the chromatographic data. All calculations for data reporting were performed using the Analyst 1.5.1 Software. Microsoft Office Excel 2010 was used to determine intra- and inter-assay means, SD and %CV, as well as percent deviation from theoretical concentrations (% deviation [%DEV]). Outliers were defined as values greater than two average standard deviations away from the mean.

■ Method validation

The UHPLC–MS/MS method was validated based on the recommendations published by the US FDA and in compliance with the National Institute of Allergies and Infectious Diseases

Table 1. UHPLC schematic for detection of dapivirine in human EDTA plasma.

Step	Start time (min)	Flow (ml/min)	%A [†]	%B [‡]
1	0.00	1.0	80	20
2	1.50	1.0	50	50
3	2.00	1.0	50	50
4	2.20	1.0	5	95
5	3.70	1.0	80	20
6	3.80	1.0	80	20

[†]H₂O, 0.1% formic acid.

[‡]Acetonitrile, 0.1% formic acid.

(NIAID) Division of AIDS (DAIDS) Clinical Pharmacology Quality Assurance (CPQA) program [29,30]. **Assay validation** metrics assessed include intra- and inter-assay precision and accuracy, linearity, extraction efficiency, selectivity, and matrix effects and stability.

■ Precision, accuracy & recovery

Intra-assay precision was evaluated through the analysis of six injections of dapivirine QC concentrations of 20, 60, 800 and 8500 pg/ml. These concentrations represent the LLOQ, low QC, mid QC and high QC values, respectively. Mean, SD and %CV was assessed at each level. Inter-assay precision was determined through analysis of the aforementioned drug concentrations measured over three independent analytical runs. Observed values were based on the run-specific calibration curve. The mean of each set of concentrations, as well as the SD and %CV, were evaluated following the bioanalytical validation recommendations published by the FDA [29].

Intra-assay accuracy was performed using the previously described dapivirine QC levels. Accuracy is represented as % DEV, and is determined as the difference between mean observed QC concentrations and the theoretical concentration divided by the theoretical concentration; the result is then multiplied by 100.

■ Calibration curve generation & analysis

For calibration curve generation, calibration standards were analyzed at the beginning and end of each analytical method, with the first set of calibrators run in ascending order (20 to 10,000 pg/ml) and the latter set in descending order. Calibration curve analysis was calculated using the ratio of the peak area of analyte and IS with a 1/×2 weighted quadratic regression. Precision and accuracy were determined for each calibration standard over three independent analytical runs, while recovery was assessed as percentage of the target value.

■ Dilutional integrity

The dilutional integrity of plasma specimens containing 30,000 pg/ml (three-times the ULOQ) was tested by performing four-, eight- and 16-fold dilutions with drug-free EDTA plasma. Precision and accuracy were determined by setting theoretical values at the calculated diluted concentrations (7500, 3750 and 1875 pg/ml, respectively). To further establish the dilutional integrity of specimens, twofold and fourfold dilutions were performed on mid and high QC levels, and precision and accuracy were determined by setting theoretical values at the calculated diluted concentrations (400 and 200 pg/ml for the mid QC, and 4250 and 2150 pg/ml for the high QC). Samples were prepared and analyzed in replicates of four.

■ Stability

To assess stability, plasma samples at the previously described QC levels were measured under several conditions. First, samples were measured directly, and then post-72 h at 4°C in the UHPLC autosampler (injection matrix stability). Secondly, samples were incubated in plasma matrix for 24 h at room temperature, and then extracted and prepared as described. Samples were prepared in triplicate and compared with a freshly prepared standard curve (sample matrix stability). Plasma samples spiked with the low, mid and high QC levels underwent three freeze–thaw cycles. Frozen samples were allowed to thaw at room temperature and were subsequently frozen for 12–24 h. Freeze–thaw stability was assessed by comparing freshly made QC samples to the QC samples that had undergone the freeze–thaw process. Freeze–thaw studies were conducted in replicates of four. For all of the aforementioned precision studies, a percentage difference was determined, which is a measure of the difference between stability tested sample and freshly prepared or analyzed QC, divided by the freshly prepared QC and subsequent multiplication by 100.

■ Selectivity

To assess assay selectivity, five lots of drug-free plasma were extracted as previously described and subjected to LC–MS/MS analysis in SRM mode using the Analyst® 1.5 Software. Manual and automated review was performed at the expected retention times for dapivirine and its isotopically labeled IS. All drug-free plasma samples were overlaid and inspected for false

positive signals from dapivirine or its IS, at their respective retention times.

■ Matrix effect characterization/extraction efficiency

Quantitative assessment of matrix effects on analyte ionization was performed following the experiments described by Matuszewski *et al.* [31]. To assess matrix effects, extraction efficiency and overall processing efficiency, three sets of QC materials were prepared at low, mid and high QC concentrations. An unextracted sample set was prepared in a 1:1 ratio of water containing 0.1% formic acid:acetonitrile containing 0.1% formic acid. To assess matrix effects, and efficiency of the sample preparation method, a second set of QCs and IS was spiked into postextracted drug-free plasma. Finally, to assess overall efficiency, a pre-extracted set was prepared and processed following the previously described standard protocol. Raw peak areas for dapivirine and the IS were analyzed, and used to calculate overall matrix effects, recovery efficiency and processing efficiency. Matrix effects were calculated as the ratio of average peak area of postextracted QC samples to unextracted QC samples multiplied by 100. Extraction efficiency was calculated as the ratio of average peak area of pre-extracted QC samples to postextracted QC samples multiplied by 100. Finally, processing efficiency was calculated as average peak area of pre-extracted QC samples to unextracted QC samples multiplied by 100. Assessment of matrix effects was performed across five independent lots of drug-free plasma to assess any potential ion suppression or enhancement.

Results

■ LC

Chromatographic separation of the NNRTI dapivirine was achieved using a gradient elution and a Waters UHPLC BEH C8 column. Similar to other NNRTIs, dapivirine is hydrophobic, and was eluted using reversed-phase gradient conditions with water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B). Specifically, dapivirine and its isotopically labeled IS were subjected to a solvent gradient from 20 to 50% mobile phase B and eluted at 43% organic solvent (1.15 min) (FIGURE 2). After analyte elution, the gradient was quickly ramped to 95% mobile phase B and held for 1.5 min to wash the column. The system was re-equilibrated to initial

Figure 2. Chromatogram of (A) dapivirine and (B) $^2\text{H}_4$ -dapivirine, with retention time demarcated at 1.15 min.

conditions with a 0.3 min gradient step from 95% mobile phase B to 80% aqueous solvent, followed by holding at 80% aqueous solvent for 1.2 min to ensure column re-equilibration.

■ MS

In order to optimize ESI conditions for dapivirine and its IS, both compounds were infused into the mass spectrometer ionization source. Both compounds were ionized in positive and negative modes, and showed optimal ionization in positive mode. The ‘compound optimization’ option in the Analyst software was used to obtain values for SRM transition m/z ratios, collision energy, collision exit potential and declustering potential for dapivirine and its IS. Instrument source ionization conditions were optimized manually by infusing each compound at 10 $\mu\text{l}/\text{min}$ into a 1 ml/min flow of (50/50) mobile phase A/mobile phase B (MPA/MPB) from the UHPLC system (commonly known as teeing in). The instrument ionization source conditions that were optimized manually in this fashion included the curtain gas, nebulizing gas 1 and gas 2, ionization voltage and source temperature. Using optimized mass spectrometric parameters, the protonated $[\text{M}+\text{H}]^+$ forms for both dapivirine and $^2\text{H}_4$ -dapivirine were monitored and determined. MS analysis of dapivirine shows the presence of the dapivirine precursor or parent ion at 330.3 m/z , which is isolated in the first resolving quadrupole before being transferred to the collision cell, where ion collision-induced dissociation occurs to produce the dapivirine product ion spectrum shown in **FIGURE 3**. The most abundant product ion from dapivirine collision-induced dissociation is observed at 158.1 m/z , which was monitored as

the dapivirine quantifier ion; the 119.1 m/z product ion was monitored for qualitative purposes. **FIGURE 3** also shows the structure of the dapivirine parent ion (330.3 m/z) and the suggested structure of the primary fragment ion at 158.1 m/z . The primary product ion for $^2\text{H}_4$ -dapivirine m/z 334.3 precursor ion (data not shown) was found at m/z 119.0, which was used as the quantifier ion for dapivirine IS.

■ Precision & accuracy

Accuracy and precision data for intra- and inter-day plasma samples for dapivirine are presented in **TABLE 2**. As per the recommendations published by the FDA and in compliance with the NIAID/DAIDS CPQA program, intra- and inter-assay precision (%CV) must be $\leq 20\%$ at the LLOQ, and $\leq 15\%$ for low, mid and high QC levels. The same limits also apply to accuracy, which is indicated as %DEV. Across intra- and inter-assay analyses, precision ranged from 5.58 to 13.89%, and 5.23 to 13.36%, respectively. Accuracy ranged from -5.61 to 0.75% for intra-day studies, and -4.30 to 6.24% for inter-assay analysis. All observed values were within the accepted limits of variation and deviation. This method is currently being implemented in a high-throughput manner to support several large clinical trials. Longitudinal evaluation of QC levels indicate that this method is robust and reproducible, with cumulative %CVs ranging from 7.63 to 13.30% for QC samples analyzed over a 2 month period (data not shown).

■ Calibration curve analysis

The plasma calibration curve was constructed to span an analytical measuring range of 20 pg/ml to

Figure 3. Product ion spectrum of dapivirine m/z 330.3 shows the predominant dapivirine product ions at m/z 158.1 and 119.1. The former product ion at m/z 158.1 was monitored as the dapivirine-quantifier ion, while the latter product ion at m/z 119.1 was monitored for qualitative purposes only. DPV: Dapivirine.

10,000 pg/ml. Standard curves were constructed using weighted ($1/x^2$) quadratic regression of calibrators, as previously described in the methods. During the development of this assay, the use of a quadratic regression with $1/x^2$ weighting showed the most consistent and best fit for the 3-log analytical measuring range of this assay. The peak area ratio (peak area dapivirine/peak area 4H_2 -dapivirine), intercept, and quadratic and linear coefficients ($y = ax^2 + bx + c$; y = peak area ratio, x = concentration, a = quadratic coefficient, b = linear coefficient and c = intercept) were used to determine the concentrations of QC and unknown specimen concentrations. A representative calibration curve generated

using quadratic regression of calibrators with $1/x^2$ weighting is depicted in **Figure 4**. The average regression ($n = 3$) was found to be >0.993 . The LLOQ was defined as the lowest calibrator that resulted in a $\%CV \leq 20\%$. The $\%CV$ at the LLOQ ($n = 6$; 20 pg/ml) was 15.12%. Precision for the remaining calibrators ranged from 2.77 to 9.51% (**Table 3**). Furthermore, the recovery observed for the mean back-calculated concentrations for the three calibration curves (containing a forward and backwards set of standards) ranged from -2.80 to 5.33%.

When establishing the analytical measuring range of the assay, studies were also conducted to ensure dilutional integrity of clinical

Table 2. Intra- and inter-assay precision and accuracy results.

QC level	Intra-assay precision and accuracy [†]				Inter-assay precision and accuracy [‡]			
	Mean (pg/ml)	SD (pg/ml)	%CV	%DEV	Mean (pg/ml)	SD (pg/ml)	%CV	%DEV
LLOQ (20 pg/ml)	20.2	2.8	13.89	0.75	21.2	2.8	13.36	6.24
Low (60 pg/ml)	56.6	4.7	8.33	-5.61	57.4	4.1	7.13	-4.30
Mid (800 pg/ml)	786	44	5.58	-1.77	775	40	5.23	-3.16
High (8500 pg/ml)	8552	520	6.08	0.61	8734	462	5.29	2.76

[†] $n = 6$ for each level of QC; representative data from a single analytical run.

[‡] $n = 17$ for LLOQ materials; $n=18$ for low-, mid- and high-QC materials; complex precision and accuracy from three analytical runs.

%DEV: % deviation.

Figure 4. Calibration curve of dapivirine from human plasma. The analytical measuring range of the assay is 20–10,000 pg/ml. Insert shows the analytical measuring range from 20 to 500 pg/ml. The curve is fit to the calibrators with a quadratic regression using $1/x^2$ weighting. The equation of the line is: $y = 8.08 \times 10^{-5}x^2 + 0.00111x + 0.00483$, with an r^2 value of 0.9962.

research specimens that were both sample limited or above the ULOQ. Therefore, a plasma sample was spiked with three-times the ULOQ of dapivirine (30,000 pg/ml), and diluted four-, eight- and 16-fold. Four replicates were analyzed per dilution. Dilutional integrity analysis indicated %CVs ranging from 3.2 to 6.8% and accuracies ranging from 11.6 to 13.5%, which are within acceptable limits. To assess analysis of sample-limited specimens, twofold and fourfold dilutions were performed on mid- and high-QC levels and subsequently analyzed. The precision ranged from 2.0 to 7.4% and accuracy ranged from -8.13 to -4.06% (data not shown).

■ Stability

Several experiments were conducted to assess stability of dapivirine, both in a reconstitution buffer containing 1:1 water containing 0.1% formic acid:acetonitrile containing 0.1% formic acid, as well as in the plasma matrix. A summary of stability studies are presented in [TABLE 4](#). For reconstitution (or injection matrix) stability analysis, specimens prepared in the previously described reconstitution buffer were reanalyzed 72 h post-initial analysis. Samples were maintained at 4°C in the sample manager over that time. Low, mid and high QC levels ($n = 6$) were compared with the original standard curve

Table 3. Dapivirine recovery results.

Calculated concentration (pg/ml)	n	Observed mean (pg/ml)	% Recovery	SD	%CV
20	6	20.0	100.0	3.02	15.12
50	6	50.4	100.8	4.79	9.51
100	5	100	100.0	7.23	7.24
250	5	243	97.2	8.40	3.46
500	6	494	98.8	22	4.38
1000	6	992	99.2	37	3.78
2500	6	2633	105.3	146	5.55
5000	6	4917	98.3	308	6.27
10000	6	9922	99.2	275	2.77

Table 4. Dapivirine stability studies.

QC level	Freeze–thaw stability (n = 4)			Sample matrix stability (n = 4)			Injection matrix stability (n = 6)		
	Control mean	Treated mean	% Difference	Control mean	Treated mean	% Difference	Control mean	Treated mean	% Difference
Low (60 pg/ml)	55.1	58.5	6.22	58.7	63.7	8.46	60.8	57.0	-6.31
Mid (800 pg/ml)	767.8	781.75	1.82	761.3	774.7	1.75	831.2	800.5	-3.69
High (8500 pg/ml)	8923	8790	-1.49	9340	9247	-1.00	7560	7475	-1.12

analyzed 3 days prior. To assess overall acceptability, the percentage difference was calculated, comparing initially analyzed QC levels with QC concentrations determined 72 h later. The percentage difference ranged from -6.31 to -1.12% and was deemed acceptable. For sample matrix analysis, QC levels prepared in plasma were incubated at ambient temperature for 24 h in replicates of four. Comparison of room temperature-incubated samples to freshly prepared QC plasma specimens yielded differences ranging from -1.0 to 8.5%. Freeze–thaw studies were also conducted in replicates of four and compared with freshly prepared calibrators and QC levels. Differences between samples post-three rounds of freeze–thaw and freshly made QC samples ranged from -1.49 to 6.22%. For all stability studies, percent differences fell well within the FDA recommended acceptance criteria of less than $\pm 15\%$ difference from original value.

Studies were also conducted for long-term stability and stock solution stability. QC levels prepared and stored at -20°C were shown to be stable for a minimum of 2 weeks in sample matrix, yielding percentage differences from freshly prepared samples ranging from -1.68 to 5.29% (data not shown). Furthermore, stock solution stability of dapivirine was determined on aliquots prepared for 24 h at room temperature and 6 months at -20°C in acetonitrile. The overall percentage difference when compared with a freshly prepared stock solution was 0.36% when maintained at room temperature for 24 h, and -3.44% when stored for 6 months at -20°C (data not shown).

■ Selectivity & matrix effects

FIGURE 5 shows chromatograms for blank human plasma free of dapivirine and its deuterated standard. No interfering peaks from endogenous compounds were observed at the retention times (1.14 min) of dapivirine and $^4\text{H}_2$ -dapivirine. The specificity of the LC–MS/MS method was evaluated by analyzing human plasma samples from

five different lots to investigate potential interferences. No significant response was observed in the LC region for any of the blank samples analyzed. A representative overlay of chromatographic spectra depicting a lot of blank human plasma and the same lot spiked with 20 pg/ml dapivirine is shown in FIGURE 4. The response ratio (peak area) of the LLOQ was at least six-times higher than blank plasma.

Analysis of matrix effects assesses potential ion suppression or enhancement by endogenous compounds on the analytes of interest. In addition to assessing ion suppression or enhancement, extraction and overall processing efficiency were also determined. Peak responses for both dapivirine and $^4\text{H}_2$ -dapivirine from extracted specimens were compared with both neat standard solutions spiked into blank plasma (postextracted) as well as neat standards prepared in mobile phase (unextracted). The peak areas for both analyte and IS are summarized in TABLE 5. Matrix effects of solutions prepared at low, mid and high QC concentrations are within the acceptance range of $\pm 15\%$ difference from theoretical nominal concentration. It should be noted that, comparing post-extracted to unextracted samples, we observe recoveries ranging from 89.5 to 95.1% for dapivirine and 87.1 to 94.7% for $^4\text{H}_2$ -dapivirine. These data suggest that minimal ion suppression is observed for dapivirine. Matrix effects, recovery efficiency and processing efficiency of the isotopically labeled IS imply that any observed ion suppression is consistent for both analyte and IS, thereby reducing any relative matrix effects and impact on accurate analyte quantitation.

Discussion

The method described above allows for the rapid and robust quantification of the anti-retroviral agent dapivirine in human plasma. This is the first report describing the full validation and overview of analytical metrics of a UHPLC–S/MS method for the topical PrEP

NNRTI. The method performs well analytically, and plasma concentrations may be quantified over a dynamic analytical measuring range from 20 to 10,000 g/ml, with extended linearity to 30,000 g/ml. The LC–MS/MS method meets all of the acceptance criteria recommended by the FDA Guidance for Industry: Bioanalytical Method Validation [29]. Furthermore, the described method also follows the guidance of the NIAID DAIDS CPQA program [30]. The CPQA is a quality assurance initiative to provide support to pharmacology specialty

laboratories conducting HIV/AIDS translational research. For the development and validation of LC–MS/MS methods, the program also endorses the FDA Bioanalytical Method Validation Guidelines.

The described LC–MS/MS method offers several key advantages over previously described methods. Using this LC–MS/MS method, dapivirine is efficiently extracted via protein precipitation using acetonitrile and requires very little incubation time to isolate the NNRTI from the plasma matrix. Other LC–MS/MS methods use

Figure 5. Selectivity of dapivirine and 2H_4 -dapivirine transitions. (A) Chromatograms of drug-free human plasma illustrating the extracted ion current of the dapivirine transition (upper panel) and the 2H_4 -dapivirine transition (lower panel). (B) Overlay of dapivirine at the LLOQ (20 pg/ml) spiked into blank K2 EDTA plasma and blank plasma. The relative intensity is six-times the background level of the blank plasma (upper panel). Extracted ion current for the 2H_4 -dapivirine transition is depicted in the lower panel.

Table 5. Matrix effects, recovery efficiency and processing efficiency of dapivirine and deuterated dapivirine IS ($^4\text{H}_2$ -dapivirine).

QC level	DPV peak area		⁴ H ₂ -DPV peak area		Matrix effects		Recovery efficiency		Processing efficiency		
	Unextracted	Post-extracted	Pre-extracted	Post-extracted	DPV	⁴ H ₂ -DPV	DPV	⁴ H ₂ -DPV	DPV	⁴ H ₂ -DPV	
Low (60 pg/ml)	16,320	15,525	14,800	73,480	69,960	95.1	87.1	95.1	87.1	90.7	82.9
Mid (800 pg/ml)	44,180	39,540	37,040	74,140	67,440	89.5	89.0	93.7	91.0	83.8	81.0
High (8500 pg/ml)	334,200	316,600	321,600	76,320	73,500	94.7	94.5	101.6	96.3	96.2	91.0

DPV: Dapivirine.

DPV: Dapivirine.

time-consuming and manual isolation methods, including liquid–liquid extraction and several evaporation steps [17,19,32]. While these extraction processes may result in decreased background signals due to more thorough removal of endogenous compounds, there may also be an overall loss of analyte. Another advantage of this method is that the implementation of a gradient chromatographic scheme and optimized mass spectrometric metrics facilitate a robust and specific dapivirine signal at the LLOQ, making further sample cleanup unnecessary. The method described herein involves a straightforward sample preparation that is performed in a high-throughput manner on a 96-well protein precipitation plate, with a total preparation time of less than 90 min.

Several observations were noted during the development of the quantitative LC–MS/MS dapivirine method. Prior to choosing a BEH C8 column, several reversed-phase columns were tested for separation of dapivirine, including a BEH C18 column. During the C18 column wash and re-equilibration step transition from 5 to 80% aqueous, remnant drug was eluted from the column (data not shown). The lipophilic nature of dapivirine prevented the NNRTI from being readily washed from the C18 column [32,33]. To eliminate column carry-over and to optimize chromatographic separation and elution of the NNRTI, a column with a less sorbent C8 stationary phase (as compared with the octadecyl carbon C18 column) was implemented, which allowed for weaker interactions between the analyte and the hydrophobic stationary phase. Although the transition to a less sorbent column decreased column carry-over of dapivirine, it was necessary to increase the flow rate to 1.0 ml/min and extend the gradient to 95% mobile phase B, as well as hold at 95% B for 1.5 min to scrub residual dapivirine from the column, further reducing carryover.

While it may be perceived that the LLOQ is a limitation of this method, particularly in the assessment of plasma dapivirine PK, literature has indicated that our LLOQ is sufficient for the detection of the antiretroviral following topical administration. For example, in a Phase I safety trial, administration of dapivirine at 50 μM in a gel formulation had quantifiable levels (>50 pg/ml, the lower limit of the assay in that study) in 75% of study participants [18]. In two additional Phase I safety trials (IPM001 and IPM008), 25 or 200 mg dapivirine from intravaginal rings were evaluated over 7 days

and found to be well tolerated [20]. The 200 mg intravaginal ring resulted in mean plasma dapivirine levels of 39 pg/ml throughout the observation period. Subsequent Phase I randomized trials also assessed dapivirine in a gel formulation applied twice-daily for 42 days; in gels containing 0.02% of the NNRTI, mean plasma levels were 474 pg/ml after 28 days on study [19]. A later study assessing dapivirine PK in a 0.02% gel formulation yielded plasma concentrations from 23–80 pg/ml (day 1 post-insertion) and 31–471 pg/ml (day 10 post-insertion) [16]. Lastly, plasma dapivirine concentrations were determined 24 h after application of a 0.05% gel formulation and found to be approximately 250 pg/ml, and levels did not fall below 70 pg/ml [17]. These findings support the utility of the described LC–MS/MS method for the quantification of dapivirine for PK studies.

Based on the aforementioned tolerability and efficacy data, two Phase III clinical trials evaluating dapivirine vaginal rings are currently underway [26,101]. Although circulating plasma levels of topically administered dapivirine are low, the described LC–MS/MS method is being used in several clinical studies and is

sensitive enough to detect plasma dapivirine following topical microbicide dosing. Many of the previously published methods are unable to reach the LLOQ described herein [18,20,27,32]. Therefore, this method may be applied for the quantification of plasma dapivirine levels in clinical trials from a variety of topical formulations.

Future perspective

Although antiretroviral agents have long been used for the management of HIV/AIDS, increased attention has been placed on their role in the prevention of HIV transmission following both oral and topical administration. The NNRTI dapivirine has shown promise in early Phase I clinical trials and is being pursued as a topical microbicide in Phase III clinical trials for the prevention of HIV transmission. Consequently, the development of rapid and robust tests for the quantitation of dapivirine concentrations in plasma will be utilized to better understand the PK of the antiretroviral agent, and may also be used to monitor adherence to treatment regimens. The utility of LC–MS/MS platforms combine high selectivity and sensitivity for the

Executive summary

Background

- Antiretroviral agents have shown efficacy in the treatment of HIV infection, and have been used successfully for HIV prevention in a variety of settings, including pre-exposure prophylaxis.
- The non-nucleoside reverse transcriptase inhibitor dapivirine is a hydrophobic molecule currently being pursued as a microbicidal agent for the prevention of HIV transmission.
- There are no published papers that comprehensively describe the development, optimization and validation metrics of a LC–MS/MS assay for dapivirine quantitation.

Experimental

- A novel LC–MS/MS that detects the non-nucleoside reverse transcriptase inhibitor dapivirine in plasma is described.
- The LC–MS/MS method was validated following the US FDA Bioanalytical Method Validation Guidelines (2001).

Results

- LC and MS parameters were optimized for the analytical separation and ionization of dapivirine, respectively.
- For dapivirine, both a quantifier and qualifier ion was monitored.
- The developed and validated method covers a three-log analytical measuring range, and has a LLOQ of 20 pg/ml and a ULOQ of 10,000 pg/ml.

Discussion

- This method offers rapid and robust detection of dapivirine via LC–MS/MS analysis.
- This method can be utilized for high-throughput use, as sample preparation is minimal and can be conducted using a 96-well plate-based format.
- Due to the hydrophobicity of the molecule, chromatographic conditions were optimized to minimize carryover.

Conclusion

- This method has been validated as per the recommendations of the FDA Bioanalytical Method Validation Guidelines (2001), and has also been reviewed by oversight organizations.
- This highly selective and sensitive method may be used for the rapid quantitation of dapivirine in plasma.

identification and accurate quantitation of small molecules in a rapid manner. Thus, continued meaningful progress will be made in the development and application of LC–MS/MS methods to support both clinical and research studies.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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