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Simultaneous Measurement of Etravirine, Maraviroc and Raltegravir in Pigtail Macaque Plasma, Vaginal Secretions and Vaginal Tissue using a LC-MS/MS Assay

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Abstract

Etravirine (ETR), maraviroc (MVC) and raltegravir (RAL) are promising antiretroviral drugs being used in HIV treatment and may be interesting for prevention applications such as oral or topical pre-exposure prophylaxis. Here we describe a sensitive and accurate method for the simultaneous detection of ETR, MVC and RAL from pigtail macaque plasma, vaginal secretions, and vaginal tissue. This method is characterized by a straightforward precipitation extraction method, a limit of quantification $<0.5 \text{ ng mL}^{-1}$ for all three antiretrovirals bolstered by a corresponding internal standard for each drug analyte, and short run time. Quantification is performed using positive ion electrospray triple quadrupole mass spectrometry. This method was validated over clinically relevant ranges for the three ARV drugs in all three matrices: $0.1\text{--}100 \text{ ng mL}^{-1}$ for ETR, $0.05\text{--}100 \text{ ng mL}^{-1}$ for MVC and $1\text{--}100 \text{ ng mL}^{-1}$ for RAL. Our method is accurate and precise, with measured mean inter-assay precision (%CV) and accuracy (% bias) of 5.08% and 1.96%, respectively, while the mean intra-assay precision and accuracy were 3.44% and 1.08 %. The overall post-extraction recovery for ETR, MVC and RAL was $>94\%$ in all cases. We also show that extracted biological samples are stable after storage at room temperature or 4°C and after three freeze/thaw cycles. This is the first analytical method capable of quantifying ETR, MVC and RAL in biological matrices relevant for pre-clinical testing of oral or topical HIV prevention methods in pigtailed macaques.

Keywords

etravirine; maraviroc; raltegravir; plasma; vaginal tissue; vaginal secretions

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1. Introduction

Disparate rates of HIV acquisition between men and women have motivated development of oral and topical prevention methods intended as discreet, woman-initiated dosage forms. Poor performance of the 1% tenofovir gel in recent clinical trials has spurred the inclusion of new, more potent antiretrovirals into microbicides[1]. Furthermore, much like formulations for treatment of HIV, cocktails of multiple antiretrovirals with varied mechanisms of action may prove to have synergistic capacity and lower required drug dosage, with a lower risk for development of resistant HIV strains[2]. The majority of HIV prevention dosage forms in development, including pills[1], gels[3], rings[4], films[5] and fibers[6], aim to prevent acquisition of HIV in the genital tract[7]. HIV prevention formulations are typically tested first in non-human primate models for safety and efficacy, motivating development of quick, highly sensitive bioanalytical methods for the detection of antiretroviral cocktails in affected tissues, including vaginal tissue and secretions, as well as plasma.

Etravirine (TMC125, Intelence®) is non-nucleoside reverse transcriptase inhibitor (NNRTI) that is highly active against both wild-type and NNRTI-resistant HIV strains[8], has a higher genetic barrier to the development of resistance than currently available NNRTIs[9], and has been shown to be safe, tolerable and effective in the treatment-experienced patients[10]. Maraviroc (UK-427,857, Celsentri®, Selzentry®) is a selective CCR-5 antagonist with potent anti-HIV activity[11], that has been investigated for microbicide use alone in gel form[12] and in combination with dapivirine as a vaginal ring[13]. Raltegravir (MK-0518, Isentress®) is a novel HIV-1 integrase inhibitor that prevent proviral DNA-strand transfer[14], which has been shown to protect macaques from SHIV challenge when applied post-exposure in a topical gel[15].

To date, several methods have been published for the quantification of ETR, MVC and/or RAL in a variety of tissue matrices. Fayet et al. validated a LC-MS/MS assay for ETR, MVC and RAL, in addition to darunavir, in human plasma[16]. Other LC-MS/MS assays have been published for quantification of each drug alone, including ETR in human plasma and peripheral blood mononuclear cells and rat plasma[17-18], MVC in human plasma, urine and cerebrospinal fluid[19], and RAL in human plasma and peripheral blood mononuclear cells[20-21]. The combination of ETR, MVC and RAL has also been quantified using LC-MS/MS in human plasma in addition to a combination of eight other relevant antiretroviral drugs[22]. Currently, no LC-MS/MS assay has been validated to quantify ETR, MVC and RAL combinations in vaginal tissue and secretions, or plasma.

Here we present a validated analytical method for the simultaneous quantification of etravirine, maraviroc and raltegravir in pigtail macaque vaginal tissue, vaginal secretions and plasma using liquid chromatography coupled with tandem triple quadrupole mass spectrometry detection. A single step extraction is performed using acetonitrile precipitation of plasma, vaginal secretions and homogenized vaginal tissue, after the addition of ETR-¹³C₆, MVC-d₆ and RAL-d₆ as internal standards. The mixture is vortexed vigorously, centrifuged for 10 minutes at 10,000 RPM and filtered before a 2 µL injection into an EMD Chromolith® Performance RP-18e 100-3mm analytical column. Analytes are separated using a gradient method of water with 10 mM formic acid and 1:1 acetonitrile:methanol

with 10mM formic acid. Our method enables the simultaneous quantification of these three antiretroviral drugs for pharmacokinetic and pharmacodynamic preclinical studies in pigtail macaque studies.

2. Materials & Methods

2.1 Reagents and tissues

Etravirine (ETR) and maraviroc (MVC) drug standards were obtained from the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD, USA). Raltegravir (RAL) drug standard and maraviroc-d₆ (MVC-d₆), raltegravir-d₆ (RAL-d₆), and etravirine-¹³C₆ (ETR-¹³C₆) internal standards were purchased from Alsachim, Inc. (Illkirch-Graffenstaden, France). Acetonitrile (ACN), methanol (MeOH) and formic acid, all Optima-LC/MS grade, were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure water was obtained using a Milli-Q UF-Plus apparatus (Millipore, Burlington, MA, USA). Naïve pigtail macaque (*Macaca nemestrina*) plasma, vaginal secretions and vaginal tissue were purchased from the Washington National Primate Research Center (WaNPRC) Tissue Distribution Program (Seattle, WA, USA).

2.2 Instrumentation and equipment

The liquid chromatography system was composed of an I-Class Acquity UPLC (Waters Corporation, Milford, MA, USA) with a direct infusion syringe pump and temperature-controlled 96 vial autosampler maintained at 4 °C. Samples were separated on a Chromolith Performance RP-18e 100-3mm analytical column (Merck, Darmstadt, Germany) at ambient conditions. The chromatographic system was coupled to a Waters Xevo TQ-S tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) with a Micromass Zspray™ Atmospheric Pressure Ionization (API) Source. The LC-MS/MS system and data analysis was carried out using MassLynx® software (version 4.1) (Waters Corporation, Milford, MA, USA).

Processing of plasma, secretions and tissue was performed using a Precellys® 24 tissue homogenizer (Bertin Corporation, Rockville, MD, USA) (tissue only) and a Sorvall Legend 14 centrifuge (Thermo Scientific, Waltham, MA, USA).

2.3 Calibration standard, internal standard and quality control (QCs) solutions

Stock solutions of ETR, MVC and RAL were prepared at a concentration of 1 mg mL⁻¹ in DMSO, and then were combined and diluted with ACN to make working solutions at concentrations of 10,000 ng mL⁻¹, 200 ng mL⁻¹ and 2 ng mL⁻¹ (Table 1). Calibration standards were then diluted with appropriate amounts of ACN (neat standards), or plasma, vaginal secretions, or tissue to achieve a working calibration range from 0.01–100 ng mL⁻¹. Internal standards (ETR-¹³C₆, MVC-d₆, RAL-d₆) were likewise diluted as stock solutions in DMSO at 1 mg mL⁻¹. A working solution of all three internal standards was prepared at a concentration of 10 ng mL⁻¹ in ACN. Internal standards were added to all samples at a ratio of 1:10 (vol:vol) resulting in a final internal standard concentration of 1 ng mL⁻¹. Three separate quality control stock and working solutions were weighed and prepared separately from the calibration standards. QCs were prepared at concentrations of 0.5 ng mL⁻¹ (low), 5

ng mL⁻¹ (medium) and 50 ng mL⁻¹ (high). All solutions were prepared according to recommendations on bioanalytical method validation stating that the total added volume must be 10% of the biological sample[23]. Samples were prepared fresh for intraday experiments, and then aliquoted and stored at -80°C until analysis.

2.4 LC-MS/MS conditions

Mobile phase A was composed of 10 mM formic acid in ultrapure water and mobile phase B was composed of 10mM formic acid in 50:50 ACN:MeOH. Solvents were prepared before each set of analysis. The mobile phase gradient was delivered over 8.5 minutes and consisted of 98% A: 2% B for 1 minute, ramping to 100% B by 6 minutes, holding for one minute, and then transitioning back to 98% A: 2% B over 1.5 minutes. The flow rate was 0.5 mL min⁻¹ with an injection volume of 2 µL.

The mass spectrometer was operated in positive ion mode. The selected *m/z* transitions and typical retention time for each analyte are reported in Table 2. The ionization parameters were as follows: capillary voltage of 3 kV, source temperature of 150°C, cone gas flow of 150 L hr⁻¹, desolvation gas flow of 1000 L hr⁻¹ and collision gas flow of 0.15 mL min⁻¹. The spectrometer was engaged during minutes 2–7 of each run. All species were analyzed at a cone voltage of 50 kV and a collision energy of 28 V (ETR, MVC, ETR-¹³C₆, MVC-d₆) or 40 V (RAL, RAL-d₆).

2.5 Sample preparation

2.5.1 Plasma—Plasma samples were precipitated with cold ACN (with or without analytes depending on the sample), vortexed thoroughly, centrifuged for 10 minutes at 10,000 RPM, filtered using a 0.22 µm PVDF syringe filter (Millipore, Burlington, MA, USA) and added to a 200 µL HPLC vial.

2.5.2 Vaginal secretions—Secretion samples were collected with a Merocel ophthalmic sponge (Medtronic, Jacksonville, FL, USA), diluted with 300 µL of phosphate buffered saline (PBS) to thin the mucus, and then incubated for 1 hour at 4°C. Sponge and extraction buffer were then transferred to Spin-X centrifuge tubes with 0.22 µm cellulose acetate filters (Corning Inc., Corning, NY, USA). Secretion eluate was then precipitated with cold ACN (with or without analytes depending on the sample), vortexed thoroughly, centrifuged for 10 minutes at 10,000 RPM, filtered using a 0.22 µm PVDF syringe filter (Millipore, Burlington, MA, USA) and added to a 200 µL HPLC vial.

2.5.3 Vaginal tissue—Tissue biopsies were collected with a 5 mm biopsy punch and added to an eppendorf tube with 640 mg of 1.4 mm and six 2.8 mm zirconium oxide ceramic beads (Precellys, Bertin Corporation, Rockville, MD, USA). The samples were homogenized on the Precellys at 6,500 RPM for three cycles of 20 seconds. Homogenized samples were then centrifuged for 10 minutes at 10,000 RPM, and filtered using a 0.22 µm PVDF syringe filter (Millipore, Burlington, MA, USA) and added to a 200 µL HPLC vial.

2.6 Quantification

Quantitative analysis of the three antiretroviral drugs was performed using the internal standard method, relative to deuterated internal standard (e.g. ETR and ETR- $^{13}\text{C}_6$). Each level of the calibration curve was analyzed in triplicate at the beginning of the run, and one high and low standard was analyzed at the end of each run. An eight-point calibration curve was calculated and fit using linear regression of peak area ratios (drug peak area/internal standard peak area) versus concentration. The calibration was established over the range of 0.1–100 ng mL $^{-1}$ for ETR, 0.05–100 ng mL $^{-1}$ for MVC and 0.5 ng mL $^{-1}$ for RAL in all tissue matrices so as to cover the clinically relevant expected concentrations for microbicide application. Separate calibration curves were made for the bottom four and top four points of each curve. Chromatographic data acquisition, peak integration and quantification were performed in MassLynx® software (version 4.1) (Waters Corporation, Milford, MA, USA).

2.7 Accuracy and precision

The method validation procedure was based on recommendations published from the Conference on Analytical Methods Validation [23]. Replicate analysis (n=3) of QC samples at the low, medium and high concentrations were used for the intra-assay precision and accuracy determination in pigtail macaque plasma, vaginal secretions and vaginal tissue. Inter-assay accuracy and precision were determined by repeated analysis performed on three different occasions (Table 3). The concentration in each sample was determined using calibration standards prepared on the same day. The precision was calculated as the coefficient of variation (%CV) within a single run (intra-assay) and between three different assays (inter-assay), and the accuracy as the percentage of deviation between nominal and measured concentrations. The analytical series were considered valid and accepted only if the precision %CV between nominal and back-calculated concentrations of each calibration level and QC were less than $\pm 15\%$, and less than $\pm 20\%$ at the lowest QC level.

2.8 Limit of quantification and limit of detection

The limit of quantification (LOQ) for each drug analyzed was defined as the lowest calibration standard for which the standard curve returned accurate and precise results within the given limits. The limit of detection (LOD) was defined as the lowest calibration level for which a drug peak above the noise level was visible on the chromatogram, with a signal to noise of 3:1. LOQ and LOD for each drug can be found in Table 4.

2.9 Stability of antiretroviral drugs in plasma, vaginal secretions and vaginal tissue

Stability studies of ETR, MVC and RAL were performed to determine sample storage procedures should samples be prepared but the instrument unusable due to unforeseen circumstances. The stability studies were carried out as follows:

- a. Stability of samples at room temperature and 4°C: separate QC samples at low, medium and high concentrations of antiretrovirals in plasma, secretions and tissue were kept at room temperature or 4°C for 48 hours. Samples were analyzed immediately after preparation and after 48 hours at respective conditions, and antiretroviral drug concentrations were expressed as % deviation from the initial concentration.

- b. Stability of samples after three freeze/thaw cycles: QC samples at low, medium and high concentrations of antiretrovirals in plasma, secretions and tissue frozen at -80°C and then allowed to thaw at room temperature three times. Samples were analyzed immediately after preparation and after three freeze/thaw cycles, and antiretroviral drug concentrations were expressed as % deviation from the initial concentration.

2.10 Matrix effect and recovery

In the initial method validation steps, the matrix effect on chromatography for plasma, vaginal secretions and vaginal tissue was examined by observing whether blank matrix samples had any visible peaks at the expected retention times for each analyte, and whether matrix eluates spiked with analytes caused any shift or perturbation of the retention time for each analyte. Subsequently, the matrix effect for each analyte in each matrix was assessed. Three series of QC samples at low, medium and high concentrations in triplicate were prepared as follows:

- a. Neat standards of antiretroviral drugs subjected to the same extraction process, sans matrix;
- b. Plasma, vaginal secretion and vaginal tissue samples spiked with antiretroviral drugs and internal standards **after** extraction;
- c. Plasma, vaginal secretion and vaginal tissue samples spiked with antiretroviral drugs and internal standards **before** precipitation.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs and internal standards in the presence of plasma, vaginal secretion and tissue matrices (i.e. the matrix effect) was assessed by comparing the absolute peak areas of analytes either solubilized in extraction buffer (A), or added after (B) or before (C) the extraction process, based on the recommendations by Matuszewski et al[24, 25]. Mean peak area ratios (B_2 , C_2) were calculated as the ratio of drug to appropriate internal standard (e.g. ETR to ETR- $^{13}\text{C}_6$) within the same sample. The matrix effect (ME) is expressed as the ratio of peak areas of the analytes added to blank matrix after extraction to the neat standard (B/A), expressed as a percentage. The extraction yield (EY) is expressed as the ratio of peak areas of blank matrix samples spiked before and after extraction (C/B), expressed as a percentage. The overall recovery (OR) was expressed as the ratio of mean peak area ratios for each antiretroviral for samples spiked before extraction to those spiked after extraction (C_2/B_2). The process efficiency (PE) is expressed as the ratio of the peak area of analytes spiked before extraction to the peak area of analytes spiked into neat extraction buffer (C/A).

3. Results and Discussion

3.1 Chromatograms

We present a single method for simultaneous detection of ETR, MVC, RAL by liquid chromatography coupled with tandem mass spectrometry. Typical chromatographs of ETR, MVC and RAL at the lowest calibration level (Figure 2, a–c) and highest calibration level (Figure 2, d–f) of the neat standards reveal that peak retention time and shape are consistent

across the calibration range. Typical retention times for each analyte can be found in Table 2. The internal standards were spiked at a single concentration for all samples (1 ng mL^{-1}) and resulting chromatograms can be found in Figure 2(g–i). The chromatograms for the internal standards (ETR- $^{13}\text{C}_6$, MVC- d_6 and RAL- d_6) (Figure 2, g–i) reveal identical retention to the corresponding analyte (ETR, MVC and RAL). The separation occurs between 4–7 minutes of the 8.5-minute run, and is satisfactory for obtaining sufficient peak resolution for all analytes.

Figure 3 shows chromatograms for ETR, MVC and RAL at the medium level QC concentration (5 ng mL^{-1}) in each of the tested matrices (plasma, vaginal secretions and vaginal tissue). None of the matrices caused a shift in retention time or peak shape for any of the drug analytes, or internal standards (not shown). Changes in peak intensity due to the presence of matrix (matrix effects) are discussed in section 3.5.

3.2 Calibration curve and internal standard

Calibration curves for each antiretroviral drug (Table 1) were described by a split linear regression. Samples at a concentration of 1 ng mL^{-1} or above were analyzed using a calibration curve that included all eight calibration levels (regardless of LOQ), while samples at lower concentrations were analyzed using a calibration curve that included the four calibration levels from 0.01 – 1 ng mL^{-1} . This method allowed for the widest, reproducible range of quantification for each drug. Over the considered calibration range, the regression coefficient (r^2) was >0.99 for each curve. The back-calculated calibration standards were within $\pm 15\%$ (or $\pm 20\%$ at LOQ) of the nominal concentration.

Stable, isotopically labeled analogues were used as internal standards for each antiretroviral drug. Studies in analyte detection using LC-MS/MS have revealed that labeling compounds with three to eight ^2H or ^{13}C is the most common strategy to achieve co-elution of drug and internal standard, while minimizing cross-talk on the mass analyzer and compensating for small errors that may arise during processing and samples analysis [26, 27]. Based on availability and suitability, ETR- $^{13}\text{C}_6$, MVC- d_6 and RAL- d_6 were chosen and produced a corresponding internal standard for each antiretroviral drug that was shifted by +6 mass units. The individual internal standard for each ARV drug allows for precise observation of matrix-induced change retention time for each drug/internal standard combination and are appropriate for use across species.

3.3 Precision, accuracy, LOQ and LOD

Precision and accuracy as determined by the low, medium and high QC samples in plasma, vaginal secretions and vaginal tissue (Table 3). The levels of QC samples were chosen to reflect low, medium and high concentrations of the linear calibration range as determined with preliminary experiments, as well as anticipated clinically relevant drug concentrations. The mean inter-assay precision (%CV) and accuracy (% bias) were 5.08% and 1.96%, respectively. Intra-assay precision and accuracy were 3.44% and 1.08 %, respectively. The inter-assay and intra-assay deviations (bias) from nominal concentrations ranged from -14.87% to 16.68% and -19.08% to 16.72% , respectively. Overall, the quantification of ETR and MVC were more accurate and precise than RAL in all matrices. The lowest QC for

RAL (0.5 ng mL^{-1}) had a large deviation in %CV in plasma (17.54%), vaginal secretions (21.61%), and vaginal tissue (16.18%), which is likely due to effects from the matrix on the ionization efficiency of RAL. Heine et al. also observed an increased recovered concentration compared to nominal concentration for RAL in peripheral blood mononuclear cells (PBMCs) [17]. Increasing the organic precipitation volume to matrix ratio may decrease matrix effects, thereby increasing the range of quantification for RAL. For more conservative analyses, it may be appropriate to consider only concentrations of 1 ng mL^{-1} of RAL in matrix, while neat standards are viable down to 0.5 ng mL^{-1} .

Based on standard pharmacokinetics and pharmacodynamics for topical delivery we expect that secretion concentrations > tissue concentrations $\gg$ plasma concentrations, in comparison to oral delivery, wherein the order is reversed[28]. Thus, topical delivery will likely dictate the maximum concentration for vaginal secretions and tissue but minimum concentration for plasma, whereas oral delivery will define the maximum plasma concentration but minimum vaginal secretion and tissue concentrations. Topical gel and ring microbicide formulations of MVC have yielded concentrations of 10^0 – 10^1 ng mL^{-1} in plasma, 10^4 – 10^7 ng mL^{-1} in vaginal fluid and 10^2 – 10^4 ng mL^{-1} in vaginal tissue[12, 29–30]. Though ETR has not yet been tested as a microbicide, dapivirine, a chemically similar antiretroviral has been extensively tested as a microbicide, yielding concentrations of 10 – 10^3 ng mL^{-1} in vaginal tissue, 10^0 – 10^2 ng mL^{-1} in plasma and ng mL^{-1} in vaginal fluid[4, 31–33]. RAL concentration after administration as a topical, vaginal microbicide has not yet been reported in the literature. Orally delivery yields plasma concentrations of 10^2 – 10^3 ng mL^{-1} ETR [17], 10^2 – 10^3 ng mL^{-1} MVC [34], and 10^2 – 10^3 ng mL^{-1} RAL [35], but have not yet been quantified in the vaginal compartment. Given these reported values, we anticipate that this quantitative method for simultaneous detection of ETR, MVC and RAL will adequately span the clinically relevant range for all three drugs.

3.4 Stability of extracted antiretrovirals

The stability of ETR, MVC and RAL in plasma, vaginal secretions and vaginal tissue samples were evaluated under room temperature and 4°C storage conditions for 48 hours (Table 5.) The deviation after storage at room temperature ranged from -11.76% to 15.66% of the original concentration measured for ETR, MVC and RAL. For the samples stored at 4°C , deviations ranged from -9.66% to 12.74% . Given the analytical variation, these results indicate that ETR, MVC and RAL are stable in all matrices for at least 48 hours at either room temperature or 4°C . The stability of the three antiretrovirals was also evaluated after three freeze/thaw cycles, wherein samples were allowed to completely freeze at -80°C , before completely thawing at room temperature. Compared to previously measured concentrations for these samples, the deviations ranged from -9.58% to 15.48% for all three drugs. Thus, we expect that standards and samples may be frozen and thawed up to three times before analysis without risking skewed results due to drug degradation.

3.5 Matrix effect and recovery

The matrix effect, extraction yield, overall recovery, and process efficiency were analyzed for ETR, MVC and RAL in plasma, vaginal secretions and vaginal tissue (Table 6). The assessment of matrix effect was quantified as the ratio of mean peak area of analytes spiked

before extraction to that of the neat standard (ratio B/A, Table 6). A value above or below 100% indicates enhancement or suppression of ionization, respectively, by the given matrix[36]. Our results indicate that ETR and MVC are minimally affected by plasma (100–119% recovery) and vaginal secretions (98–120% recovery), while RAL undergoes significant ionization enhancement (185%–377% recovery) in these matrices. ETR is minimally affected (99%–106% recovery) in the vaginal secretions whereas MVC undergoes ionization suppression (45–52%), and RAL undergoes ionization enhancement (189%–384%). These differences may be due to inherent variations in pH and protein content in these matrices that alter the solubility and ionization efficiency for these physicochemically diverse drugs [37–39].

Extraction yield (EY) was evaluated by normalizing the samples spiked after extraction to those spiked before extraction (ratio C/B) for each matrix (Table 6). As expected, there was a loss of each drug and internal standard through the extraction process, although this was remarkably consistent across concentrations. Extraction in plasma and vaginal tissue yielded the greatest recovery of MVC, followed by ETR and RAL whereas vaginal secretions yielded highest recovery of ETR, followed by MVC and then RAL. Process efficiency (PE) as evaluated by normalizing peak areas of samples spiked before extraction to the corresponding neat standards, and echo the trends observed with the extraction yield for plasma samples. However, for vaginal secretions, MVC has a very low (~28%) but consistent PE, while RAL is high (94–190%). In vaginal tissue, the PE of ETR is similar to plasma and secretions, while the PE for MVC and RAL are both higher than expected, with increasing PE with decreasing concentration. These differences reflect how the different matrices and varied extraction protocols affect the drug ionization, and are attenuated by carefully timing the spiking of internal standard.

Most importantly, the overall recovery (OY) was assessed using peak adjusted ratios for each drug and corresponding internal standard (ratio C_2/B_2) in order to ensure that unknown concentrations from future study samples are quantified accurately. All drugs were within $\pm 15\%$ of the neat standard, indicating that samples in all matrices may be normalized to a neat standard with this assay, and that internal standards must be spiked before extraction to overcome the matrix effects and extraction inefficiencies associated with this assay.

4. Conclusions

We report a validated LC-MS/MS method for the simultaneous detection of ETR, MVC and RAL in pigtail macaque plasma, vaginal secretions and vaginal tissue. This analytical method should enable the pharmacokinetic study of these antiretrovirals applied through a topical, vaginal microbicide or oral treatment/prophylaxis. The high sensitivity and specificity attained allows for the quantification of as little as 0.2 pg for ETR, 0.1 pg for MVC, and 1 pg for RAL from a 2 μ L injection volume. These outcomes will allow for accurate quantification of these antiretroviral drugs in non-human primate, as well as human, pharmacokinetics studies. The combination of a corresponding internal standard for each drug analyte and quantification using a neat standard curve highlight the usability and robust nature of this analytical method. To our knowledge, this is the first LC-MS/MS method to

quantify these three antiretroviral drugs simultaneously in matrices appropriate for detection in reproductive tract tissues (vaginal secretions, vaginal tissue and plasma).

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Abbreviations

ACN	acetonitrile
ARV	antiretroviral
ETR	etravirine
HIV	human immunodeficiency virus
LC-MS/MS	liquid chromatography tandem mass spectrometry
MeOH	methanol
NNRTI	non-nucleoside reverse transcriptase inhibitor
MVC	maraviroc
RAL	raltegravir

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Highlights

- Simultaneous LC-MS/MS assay for etravirine, maraviroc, and raltegravir
- Drugs can be quantified in pigtail macaque plasma, vaginal tissue and secretions
- A simple precipitation method allows for >94% overall recovery of each drug
- Low limit of quantification enables detection after vaginal or oral delivery

Figure 1.
Chemical structures of antiretroviral drugs.

Figure 2.

Chromatograms for drugs (MVC, RAL, ETR) and internal standards (MVC-d₆, RAL-d₆, ETR-¹³C₆) at high and low concentrations. Neat chromatograms for 0.1 ng mL⁻¹ standards (ac), 100 ng mL⁻¹ standards (d–f) and 1 ng mL⁻¹ internal standards (g–i).

Figure 3.

Chromatograms for (ETR, MVC, RAL) in each matrix. Each drug was at a concentration of 5 ng mL⁻¹ in plasma (a-c), vaginal secretions (d-f) and vaginal tissue (g-i).

Table 1

Concentrations of stock solutions, working solutions and QC controls.

Solution	Stock Solution Solvent	Stock Solution Concentration	Working Solution Concentration *	Calibration Range	QC Controls
Drug (ETR, MVC, RAL)	DMSO	1 ng mL ⁻¹	10,000; 200; 2 ng mL ⁻¹	0.01–100 ng mL ⁻¹	0.5; 5; 50 ng mL ⁻¹
Internal Standard (ETR- ¹³ C ₆ , MVC-d ₆ , RAL-d ₆)	DMSO	1 mg mL ⁻¹	10,000 ng mL ⁻¹ ; 10 ng mL ⁻¹	N/A	N/A

* Obtained by dilution of stock solution with ACN

Table 2

Instrument method for the LC-MS/MS analysis for ETR, MVC and RAL with ETR-¹³C₆, MVC-d₆, and RAL-d₆ as internal controls.

Drug/Internal Standard	Precursor Ion (m/z)	Fragment Ion (m/z)	Dwell Time (s)	Cone Voltage (kV)	Collision Energy (V)	Retention Time (minutes)
ETR	435.22	162.98	0.079	50	28.0	6.74
ETR- ¹³ C ₆	441.20	162.98	0.079	50	28.0	6.74
MVC	445.35	109.10	0.079	50	28.0	4.33
MVC-d ₆	451.35	115.10	0.079	50	28.0	4.33
RAL	514.48	280.22	0.079	50	30.0	5.56
RAL-d ₆	520.48	280.22	0.079	50	30.0	5.56

Table 3

Precision and accuracy of the assay for the three antiretroviral drugs in pigtail macaque plasma, vaginal secretions and vaginal tissue at low, medium and high concentrations.

Drug/Matrix	Nominal Concentration (ng mL ⁻¹)	Inter-assay (n=3)			Intra-assay (n=3)					
		Concentration Found (ng mL ⁻¹)	±SD	Precision %CV	Accuracy Bias %	Concentration Found (ng mL ⁻¹)	±SD	Precision %CV	Accuracy Bias %	
Plasma	ETR	0.5	0.52	0.03	5.80	3.93	0.55	0.05	9.88	10.89
		5	4.93	0.12	2.36	-1.42	4.91	0.18	3.63	-1.80
		50	49.94	2.02	4.11	-0.12	47.58	1.26	2.66	-4.84
	MVC	0.5	0.51	0.01	1.66	1.07	0.50	0.02	3.17	-0.68
		5	5.29	0.15	2.81	5.73	5.18	0.14	2.73	3.52
		50	52.95	1.24	2.34	5.90	51.79	0.58	1.12	3.58
	RAL	0.5	0.50	0.09	17.54	0.47	0.44	0.02	4.42	-12.90
		5	5.39	0.07	1.36	7.77	5.46	0.20	3.65	9.28
		50	55.29	0.95	1.73	10.59	56.15	0.81	1.45	12.29
Vaginal Secretions	ETR	0.5	0.52	0.04	6.79	4.30	0.56	0.05	9.67	11.22
		5	5.11	0.05	1.03	2.10	5.07	0.33	6.50	1.38
		50	51.39	1.96	3.82	2.78	49.70	0.26	0.52	-0.60
	MVC	0.5	0.43	0.02	5.49	-13.47	0.43	0.00	0.68	-13.37
		5	4.60	0.41	8.99	-8.09	5.00	0.07	1.35	0.08
		50	42.57	2.52	5.92	-14.87	40.46	0.62	1.53	-19.08
	RAL	0.5	0.53	0.12	21.61	6.90	0.47	0.01	2.66	-6.62
		5	5.74	0.24	4.15	14.72	5.78	0.20	3.46	15.53
		50	58.34	3.49	5.99	16.68	58.36	2.46	4.21	16.72
Vaginal Tissue	ETR	0.5	0.51	0.00	0.69	2.60	0.51	0.02	3.80	1.82
		5	4.92	0.08	1.61	-1.56	4.89	0.19	3.98	-2.13
		50	48.90	1.94	3.98	-2.21	47.44	0.69	1.46	-5.11
MVC	0.5	0.56	0.02	3.23	11.81	0.56	0.05	9.03	12.52	

Drug/Matrix	Nominal Concentration (ng mL ⁻¹)	Inter-assay (n=3)				Intra-assay (n=3)			
		Concentration Found (ng mL ⁻¹)	±SD	Precision %CV	Accuracy Bias %	Concentration Found (ng mL ⁻¹)	±SD	Precision %CV	Accuracy Bias %
RAL	5	5.08	0.17	3.42	1.65	4.90	0.08	1.64	-1.98
	50	50.33	0.87	1.73	0.66	49.56	0.50	1.01	-0.88
	0.5	0.50	0.08	16.18	0.60	0.52	0.03	4.83	3.65
	5	4.70	0.04	0.76	-5.92	4.72	0.06	1.31	-5.54
	50	50.21	1.06	2.12	0.42	51.13	1.33	2.59	2.27

Table 4

Limit of detection and limit of quantification of antiretroviral drugs.

	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹) (pg)		Accuracy at LOQ (bias %)	Precision at LOQ (%CV)
		0.1	0.2		
Etravirine (ETR)	0.01	0.1	0.2	-1.22	18.28
Maraviroc (MVC)	0.01	0.05	0.1	-7.99	10.36
Raltegravir (RAL)	0.05	0.5	1	-4.62	5.33

Table 5

Stability of ETR, MVC, RAL in plasma, vaginal secretions and vaginal tissue after storage at room temperature, 4°C and three freeze(−80°C)/thaw cycles.

Matrix	Drug (ng mL ⁻¹)	Room Temperature (48 hours), %	4°C (48 hours), %	3X Freeze-thaw, %
Plasma	ETR	0.5	-4.43	1.82
		5	-6.07	-4.94
		50	-1.43	-1.46
	MVC	0.5	7.60	-3.25
		5	-3.76	-1.70
		50	-5.46	-0.78
	RAL	0.5	-12.78	-0.51
		5	0.14	3.51
		50	0.41	0.84
Vaginal Secretions	ETR	0.5	14.36	1.40
		5	2.18	-7.37
		50	1.80	-4.83
	MVC	0.5	15.66	-9.66
		5	10.69	7.36
		50	9.70	5.59
	RAL	0.5	-4.49	-0.38
		5	-2.87	7.03
		50	-1.04	5.67
Vaginal Tissue	ETR	0.5	3.78	3.57
		5	0.79	-8.27
		50	-7.71	-4.31
	MVC	0.5	-5.03	-1.93
		5	0.84	-4.65
		50	-2.45	0.74
		0.5	-5.03	-6.10
		5	0.84	-5.87
		50	-2.45	0.62

Matrix	Drug (ng mL ⁻¹)	Room Temperature (48 hours), %	4°C (48 hours), %	3X Freeze-thaw, %
RAL	0.5	-11.76	12.74	1.80
	5	1.57	1.97	1.02
	50	-1.24	3.11	2.13

Table 6

Matrix effect, extraction yield, process efficiency, and overall recovery of antiretroviral drugs.

Matrix	Compound	Nominal Conc. (ng mL ⁻¹)	Mean Peak Areas			Mean Peak Area Ratios			ME (%)	EY (%)	PE (%)	OR (%)
			A (n=3)	B (n=3)	C (n=3)	B ₂	C ₂	B/A				
Plasma	ETR	50	309365	308815	216646	52.68	53.98	100%	70%	70%	70%	102%
		5	30904	32003	21663	5.23	5.09	104%	68%	68%	68%	97%
		0.5	3168	3426	2222	0.56	0.52	108%	65%	65%	65%	94%
MVC		50	149541									
			8	1665409	1369574	53.78	53.45	111%	82%	82%	82%	99%
		5	148516	169360	140618	5.42	5.34	114%	83%	83%	83%	99%
RAL		0.5	13762	16419	15054	0.52	0.56	119%	92%	92%	92%	108%
		50	343857	635792	334203	40.48	41.84	185%	53%	53%	53%	103%
		5	23781	63743	33353	4.06	4.06	268%	52%	52%	52%	100%
ETR- ¹³ C ₆		0.5	1759	6623	3403	0.42	0.42	377%	51%	51%	51%	100%
		50	5801	5862	4013	-	-	101%	68%	-	-	-
		5	5868	6120	4256	-	-	104%	70%	-	-	-
MVC-d ₆		0.5	5813	6173	4254	-	-	106%	69%	-	-	-
		50	27979	30969	25625	-	-	111%	83%	-	-	-
		5	28021	31255	26325	-	-	112%	84%	-	-	-
RAL-d ₆		0.5	23674	31438	26730	-	-	133%	85%	-	-	-
		50	8492	15708	7988	-	-	185%	51%	-	-	-
		5	5473	15702	8208	-	-	287%	52%	-	-	-
Vaginal Secretions	ETR	0.5	3207	15595	8014	-	-	486%	51%	-	-	-
		50	309365	307674	210309	55.00	53.97	99%	68%	68%	68%	98%
		5	30904	31249	21237	5.34	5.36	101%	68%	69%	69%	100%
MVC		0.5	3168	3348	2207	0.54	0.54	106%	66%	70%	70%	101%
		50	149541									

Matrix	Compound	Nominal Conc. (ng mL ⁻¹)	Mean Peak Areas			Mean Peak Area Ratios			ME (%)	EY (%)	PE (%)	OR (%)
			A (n=3)	B (n=3)	C (n=3)	B ₂	C ₂	B/A				
RAL		5	8	678174	399876	41.51	45.94	45%		59%	27%	111%
			148516	65043	39438	4.15	4.71	44%				
			13762	7210	3935	0.47	0.46	52%				
ETR- ¹³ C ₆		50	343857	651437	321720	40.90	46.61	189%		49%	94%	114%
			23781	65916	32024	4.18	4.49	277%				
			1759	6747	3346	0.42	0.47	384%				
MVC-d ₆		50	5801	5594	3897	-	-	96%		70%	-	-
			5868	5853	3959	-	-	100%				
			5813	6242	4067	-	-	107%				
RAL-d ₆		50	27979	16338	8705	-	-	58%		53%	-	-
			28021	15658	8380	-	-	56%				
			23674	15487	8476	-	-	65%				
Vaginal Tissue	ETR	50	8492	15926	6902	-	-	188%		43%	-	-
			5473	15763	7130	-	-	288%				
			3207	15902	7061	-	-	496%				
MVC		50	309365	304446	208348	52.50	51.00	98%		68%	67%	97%
			30904	31048	21526	5.18	5.13	100%				
			3168	3177	2209	0.57	0.54	100%				
RAL		50	149541									
			8	1575348	1255628	50.82	50.78	105%				
			148516	160173	133328	5.21	5.21	108%				
			13762	16464	16186	0.55	0.63	120%				
ETR- ¹³ C ₆		50	343857	561681	299121	36.60	38.05	163%		53%	87%	104%
			23781	56126	29092	3.57	3.57	236%				
			1759	5906	3149	0.39	0.40	336%				
ETR- ¹³ C ₆		50	5801	5799	4085	-	-	100%		70%	-	-

Matrix	Compound	Nominal Conc. (ng mL ⁻¹)	Mean Peak Areas			Mean Peak Area Ratios			ME (%)	EY (%)	PE (%)	OR (%)
			A (n=3)	B (n=3)	C (n=3)	B ₂	C ₂					
	MVC-d ₆	5	5868	5990	4195	-	-	-	102%	70%	-	-
		0.5	5813	5613	4125	-	-	-	97%	73%	-	-
		50	27979	30996	24727	-	-	-	111%	80%	-	-
		5	28021	30722	25593	-	-	-	110%	83%	-	-
		0.5	23674	30018	25514	-	-	-	127%	85%	-	-
		50	8492	15346	7862	-	-	-	181%	51%	-	-
	RAL-d ₆	5	5473	15701	8157	-	-	-	287%	52%	-	-
		0.5	3207	15303	7784	-	-	-	477%	51%	-	-