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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

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Abstract

Background—Maraviroc is a CCR5 antagonist that has been utilized as a viral entry inhibitor in the management of HIV-1. Current clinical trials are pursuing maraviroc drug efficacy in both oral and topical formulations. Therefore, in order to fully understand drug pharmacokinetics, a sensitive method is required to quantify plasma drug concentrations.

Methods—Maraviroc-spiked plasma was combined with acetonitrile containing an isotopically-labeled internal standard, and following protein precipitation, samples were evaporated to dryness and reconstituted for liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analysis. Chromatographic separation was achieved on a Waters BEH C8, 50 × 2.1 mm UPLC column, with a 1.7 μm particle size and the eluent was analyzed using an API 4000 mass analyzer in selected reaction monitoring mode. The method was validated as per FDA Bioanalytical Method Validation guidelines.

Results—The analytical measuring range of the LC-MS/MS method is 0.5-1000 ng/ml. Calibration curves were generated using weighted 1/x² quadratic regression. Inter- and intra-assay precision was 5.38% and 5.98%, respectively; inter- and intra-assay accuracy (%DEV) was 10.2% and 8.44%, respectively. Additional studies illustrated similar matrix effects between maraviroc and its internal standard, and that maraviroc is stable under a variety of conditions. Method comparison studies with a reference LC-MS/MS method show a slope of 0.948 with a Spearman coefficient of 0.98.

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Conclusions—Based on the validation metrics, we have generated a sensitive and automated LC-MS/MS method for maraviroc quantification in human plasma.

Keywords

Maraviroc; HIV; CCR5 antagonist; assay validation; LC-MS/MS

1. Introduction

It is currently reported that an estimated 34 million people are infected with human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) globally, with more than 2 million new infections occurring annually [1]. A primary treatment modality in the management and treatment of infected individuals is the use of antiretroviral therapies (ARTs). While many classes of antiretroviral drugs target HIV infectivity post-viral entry, agents that target HIV prior to viral entry have also been pursued in disease management [2,3]. There are a number of molecular events required for the entry of HIV into CD4-expressing cells, including monocytes and T-cell lymphocytes. Following binding of the HIV envelope protein gp120 to the CD4 receptor, the viral envelope undergoes a conformational change to facilitate binding of the virus-host cell complex to either the chemokine receptor 5 (CCR5) or chemokine receptor 4 (CXCR4) co-molecules [4]. Currently, maraviroc, marketed in the United States by Pfizer as Selzentry®, is the only CCR5 antagonist approved by the FDA for use in combination with other antiretroviral drugs in the management of treatment-experienced and naïve patients who are infected with the CCR5-tropic HIV-1 virus.

Mechanistically, maraviroc binds to the CCR5 co-receptor allosterically, thereby abrogating further interaction of the gp120-CD4 receptor complex and preventing membrane fusion [3,5,6]. In the MVC versus optimized therapy in viremic antiretroviral treatment-experienced patients studies (MOTIVATE 1 and 2), which were two parallel phase III placebo-controlled trials, efficacy of once or twice-daily administered drug was assessed over a 48-week period [7, 8]. Both trials concluded that administration of maraviroc resulted in significantly greater virologic suppression (42%-47% HIV RNA suppressed to <50 copies/ml), increased CD4+ T cell counts (113-128 cells/μl v. 54-69 cells/μl in placebo-arms), and tolerance of the drug, as indicated by the absence of any adverse events [7-9]. While maraviroc has largely been administered as an oral formulation, studies have also pursued its potential as a topical microbicide in mouse and nonhuman primate modes, demonstrating protection from infection at the site of transmission [10-12]

Pharmacokinetically, in healthy volunteers, the CCR5 antagonist was rapidly absorbed following oral administration with an absolute bioavailability of 23% for a 100 mg dose, and an estimated bioavailability of 33% from a 300 mg dose [13]. Plasma C_{max} was reached within 0.5-4 h post-dose, followed by reaching steady state, at which point maraviroc is transported throughout the circulation primarily (76%) bound to plasma proteins, chiefly albumin and α 1-acid glycoprotein [14, 15]. In healthy male volunteers, the mean C_{max} , T_{max} and AUC_{0-12} after 300 mg maraviroc administered orally twice daily at day 12 were 854 ng/ml (32% CV), 2.6 ± 1.5 h and 3609 ng h/ml (32% CV) and exhibited a half-life of $16.4 \pm$

2.3 h [14]. The major modalities of maraviroc metabolism are N-dealkylation and oxidation [14].

To better understand the pharmacokinetics and pharmacodynamics of maraviroc in large clinical trial settings, high-throughput analytical methods are required to quantitate the drug in vivo. A robust method is required for maraviroc quantification as the drug may be applied orally or topically. As observed in non-human primate models, vaginal administration of maraviroc in a gel formulation elicits high localized concentrations in cervicovaginal fluid, but systemic plasma concentrations are significantly lower (< 100 ng/ml) [12]. Further, according to the Department of Health and Human Services, Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, the suggested minimum target trough concentration for maraviroc in ART-experienced patients with drug resistance is >50 ng/ml [16]. Previous approaches for the detection and quantification of maraviroc from biological matrices include high-performance liquid chromatography with ultra violet detection (HPLC-UV) and liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analyses [17-22]. Many of these assays have lower limits of quantitation (LLOQ) ranging from 1 to 11 ng/ml, which may not be sufficient for monitoring systemic plasma drug concentrations following topical administration. 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 maraviroc quantification in several forthcoming clinical trials and has shown the robustness necessary for high-throughput sample analysis.

2. Materials and Methods

2.1 Chemicals

Stock solutions of 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 ($^2\text{H}_6$ -maraviroc) were acquired in powder form from Toronto Research Chemicals (TRC, North York, ON) and independently reconstituted in acetonitrile to stock concentrations of 1 mg/ml. The structure for maraviroc, $\text{C}_{29}\text{H}_{41}\text{F}_2\text{N}_5\text{O}$, is depicted in Fig. 1. Drug-free human K2 EDTA plasma was purchased from Bioreclamation (Colmar, PA). HPLC-grade and LC/MS- grade acetonitrile and LC/MS-grade water were acquired from Fisher Scientific (Pittsburgh, PA). Proteomics-grade formic acid was purchased from Proteochem (Denver, CO).

2.2 Preparation of reagents and standards

Maraviroc stock solution was diluted in acetonitrile to working solutions of 0.1, 1, 10, and 100 $\mu\text{g/ml}$. Calibration standards at 0.1, 1, 5, 10, 50, 100, 250, 500 and 1000 ng/ml were prepared by spiking human K2 EDTA plasma with appropriate volumes of working solutions. An internal standard mixture of deuterated maraviroc ($^2\text{H}_6$ -maraviroc) was prepared as a 10 ng/ml internal standard spiking solution in acetonitrile. Each master and working stock was stored at -20°C in 2 ml glass vials.

2.3 Sample Preparation

Sample preparation involved combining 50 μ l of spiked plasma with 50 μ l of internal standard spiking solution to each well of an Eppendorf 96 deep-well plate. Samples were then vortexed for 5 seconds and transferred to a 96-well Captiva 0.45 μ m protein precipitation filtration plate (Agilent Technologies). Following the transfer of the sample to the filtration plate, 500 μ l HPLC-grade acetonitrile was added to each well and the mixture was incubated for five minutes at room temperature. The analyte of interest and internal standard 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 μ l of 1:1 0.1% formic acid in water: 0.1% formic acid in acetonitrile (both solvents were LC/MS-grade); 10 μ l of reconstituted samples were subjected to chromatographic separation and mass spectrometric analysis.

2.4 Instrument and Acquisition Parameters

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

Chromatographic separation was achieved using a Waters BEH C8, 50 $\times$ 2.1 mm UPLC column, with a 1.7 μ m particle size. The column was maintained at ambient temperature. Mobile 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. Maraviroc and its deuterated internal standard were eluted under gradient conditions from 20% acetonitrile containing 0.1% formic acid to 50% additive-containing acetonitrile (Table 1). The column eluent was directed to the mass spectrometer from 0.20–4.00 min and diverted to waste at all other times. The total analytical run time for this method is 5.0 min.

Detection of maraviroc was performed using an API 4000 tandem mass spectrometer (AB SCIEX, Foster City, CA) with an electrospray ionization source 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 selected reaction monitoring (SRM) mode. Due to the high signal observed for the maraviroc parent ion (m/z 514.5), detection was performed targeting the ^{13}C isotope of the maraviroc parent ion (m/z 515.5). The ion transitions were m/z 515.5 $\rightarrow$ 390.2 for maraviroc and m/z 520.6 $\rightarrow$ 389.1 for the isotopically-labeled internal standard. Analyte-specific ionization parameters included declustering potentials of 86 and 80 V for maraviroc and $^2\text{H}_6$ - maraviroc, respectively, as well as collision energy of 29 V and collision cell exit potential of 10 V for both analytes. The described SRM transitions were determined by direct infusion of a stock solution of maraviroc and the internal standard into the ionization source and optimization of aforementioned mass spectrometric parameters to achieve appropriate analytical sensitivity.

2.5 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 (% DEV). Outliers were defined as values >2 SD deviations away from the mean. All outliers that were identified using this criterion were also identified as an outlier using the Grubbs' test for outliers.

3. Method Validation

The LC-MS/MS method was validated based on the recommendations published by the Food and Drug Administration (FDA) Guidance for Industry, Bioanalytical Method Validation [23]. The validation metrics assessed include intra- and inter-assay precision and accuracy, linearity, extraction efficiency, selectivity and matrix effects and stability. Further, method comparison and carryover analyses were performed.

3.1 Precision and Accuracy

Intra-assay (within-run) precision was evaluated through the analysis of six injections of maraviroc quality control (QC) concentrations of 0.5, 1.5, 50 and 850 ng/ml. These concentrations represent the lower limit of quantitation (LLOQ), as well, as low, mid and high QC values, respectively. Observed means, SDs and % CVs were assessed at each level. Inter-assay (between-run) precision was determined through analysis of the aforementioned drug concentrations measured over three independent analytical runs. Observed values were based on run-specific calibration curves. Within-run accuracy was performed using the previously described maraviroc QC levels. Accuracy is represented as % deviation (% 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. This approach has previously been implemented by our group in the analysis of the NNRTI dapivirine [24].

3.2 Calibration Curve 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 (0.5 ng/ml to 1000 ng/ml) and the latter set in descending order. Calibration curve analysis was calculated using the ratio of the peak area of analyte and internal standard with a $1/x^2$ weighted quadratic regression. Precision and accuracy were determined for each calibration standard over three independent analytical runs. The lower limit of quantitation for this assay was defined as the lowest concentration that could be detected with acceptable precision (% CV $\leq 20\%$) and accuracy (% DEV $\pm 20\%$). The functional limit of quantitation of this assay was assigned with the lowest calibrator that met these standards.

3.3 Dilutional Integrity

Studies were performed on plasma samples containing 3,000 ng/ml (3 times the upper limit of quantitation) of maraviroc that were diluted 4-fold, 8-fold and 16-fold dilutions with drug

free EDTA plasma. Precision and accuracy was determined by setting theoretical values at the calculated diluted concentrations (750, 375 and 188 ng/ml, respectively). Further, 2-fold and 4-fold dilutions were performed on mid and high QC levels, and precision and accuracy were determined by setting theoretical values at the calculated diluted concentrations (25 and 12.5 ng/ml for the mid QC and 425 and 212.5 ng/ml for the high QC). Samples were prepared and analyzed in replicates of 4.

3.4 Carryover

Carryover was determined via analysis of a high and low standard run in multiple sequences. The high standard (H) contained 750 ng/ml maraviroc and the low (L) standard was at a concentration of 5 ng/ml. Standards were run in the following order: L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H9, H10, L11. Standard means and SDs were determined and carryover acceptability criteria were assessed as per the guidelines described in the CLSI protocol EP10-A2 [25]. Carryover was also assessed qualitatively by running a post-injection blank following a high calibrator and assessing if the observed signal in the blank specimen was <20% that of a signal observed at the LLOQ.

3.5 Stability

Stability was assessed following the recommendations described in the FDA Guidance for Industry, Bioanalytical Method Validation guidelines. Briefly, plasma samples at the previously described QC levels were measured under several conditions. First, samples were measured directly, and reanalyzed post-72 h at 4°C in the UPLC autosampler (injection matrix stability). Secondly, samples were incubated in the plasma matrix for 10 d at room temperature and then extracted and prepared as described. Samples were prepared in replicates of four and compared to a freshly prepared standard curve (sample matrix stability). QC samples prepared in plasma were also subjected to three freeze thaw cycles at -20°C. Freeze thaw stability was assessed by comparing freshly made QC samples to the QC samples that had undergone the previously described process in replicates of four. For all of the aforementioned precision studies, a % difference was determined, which is a measure of the difference between the stability tested sample and freshly prepared or analyzed QC divided by the freshly prepared QC, with subsequent multiplication by 100. Acceptance criteria for all stability studies were <15% difference between from the originally measured QC value. The only exception was stock solution stability, which assessed the stability of standard solutions; the acceptance criterion was <10% difference of measured stock solution from freshly prepared solution (nominal).

3.6 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 maraviroc and ²H₆- maraviroc.

3.7 Matrix Effect Characterization/Extraction Efficiency

Quantitative assessment of matrix effects on analyte ionization was performed following the experiments described by Matuszewski and colleagues [26]. 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 un-extracted sample set was prepared in a 1:1 ratio of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid. To assess matrix effects and the efficiency of the sample preparation method, a second set of QCs and internal standard was spiked into post-extracted 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 maraviroc and the internal standard 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 post-extracted QC samples to un-extracted QC samples multiplied by 100. Extraction efficiency was calculated as the ratio of average peak area of pre-extracted QC samples to post-extracted QC samples multiplied by 100. Finally, processing efficiency was calculated as average peak area of pre-extracted QC samples to un-extracted QC samples multiplied by 100. Assessment of matrix effects was performed across 5 independent lots of drug free plasma to assess any potential ion suppression or enhancement.

3.8 Method Comparison

Twelve drug-free plasma specimens were spiked with various concentrations of maraviroc, spanning the previously described analytical measuring range. Specimens were split aliquots of equal volume and analyzed as unknowns. One set was analyzed using the described method. Comparison analysis was performed at the University of Alabama-Birmingham, via LC-MS/MS. The comparative method had an analytical measuring range of 5-5,000 ng/ml. Obtained results were analyzed using Deming regression analysis and Spearman correlation. In addition to testing spiked specimens, the appropriateness of the described LC-MS/MS method was also assessed using remnant patient specimens from the University of Alabama-Birmingham. Ten de-identified remnant specimens were extracted in duplicate and analyzed. Specimens originated from individuals receiving orally administered maraviroc as part of an ART regimen.

Further, the CPQA offers a proficiency testing program that contains both target values and peer means for small molecule quantification. The CCR5 antagonist maraviroc is included in the CPQA proficiency testing program. Using the validated LC-MS/MS method for maraviroc quantification, the laboratory participated in a proficiency testing challenge. The percent deviation from target was calculated as the observed concentration subtracted by the theoretical concentration, divided by the theoretical concentration, and finally multiplying that value by 100.

4. Results

4.1 Liquid Chromatographic-Tandem Mass Spectrometric (LC-MS/MS) Parameters

Maraviroc was chromatographically separated using a gradient elution and a Waters UPLC BEH C8 column. The CCR5 antagonist and its isotopically labeled internal standard were

separated using reversed-phase liquid chromatography and eluted under a solvent gradient from 20% to 50% mobile phase B at 31.2% organic solvent (0.53 minutes) (Fig. 2). Due to observed carryover during development, an extensive wash step was performed by ramping to 95% organic solvent and holding for 1.5 minutes. The total analytical run time for this assay is 5 minutes.

Following the determination of optimized mass spectrometric conditions via the direct infusion of maraviroc into the mass analyzer, the protonated $[M+H]^+$ forms for both maraviroc and its internal standard were detected in selected reaction monitoring (SRM) mode. Due to the high signal observed for the maraviroc parent ion (m/z 514.5), the ^{13}C isotope of maraviroc (m/z 515.5) was monitored at the Q1 quadrupole. The most abundant product ions for maraviroc and its internal standard from collision-induced dissociation were observed at m/z 390.2 and m/z 389.1, respectively (Fig. 3). The ion spectrum depicted in Fig. 3 shows the structure of the parent ion, the ^{13}C isotope of maraviroc (m/z 515.5), as well as the suggested structure of the most abundantly monitored product ion at m/z 390.2.

4.2 Precision, Accuracy and Calibration Curve Analysis

Intra- and inter-assay precision and accuracy were assessed at the LLOQ (0.5 ng/ml) and at low (1.5 ng/ml), mid (50 ng/ml) and high (850 ng/ml) QC levels. Intra- and inter-assay precision studies ranged from 3.64% to 5.38% and 4.76% to 5.98%, respectively. Intra- and inter-assay accuracy, which is indicated as % DEV, ranged from 1.29% to 10.2% and 1.89% to 8.44%, respectively. These values are within the acceptable limits of variation and deviation recommended by the FDA Bioanalytical Guidelines, Guidance for Industry [23]. Accuracy and precision data are summarized in Table 2.

The analytical measuring range of this assay is 0.5 ng/ml to 1000 ng/ml. Standard curves were constructed using weighted ($1/x^2$) quadratic regression of calibrators. The peak area ratio of ^{13}C -maraviroc/ $^2\text{H}_6$ -maraviroc, as well as the standard curve intercept, and quadratic and linear coefficients, were used to determine maraviroc concentrations in quality control and unknown specimens. Average regression from three analytical runs was .996. The lower limit of quantitation was defined as the lowest calibrator with a % CV $\leq$ 20%. The lowest calibrator for this LC-MS/MS assay is 0.5 ng/ml; and the % CV of MVC for this calibrator (n=6) is 3.78%. Precision for remaining calibrators ranged from 3.36% to 6.60% (Table 3). Further, the accuracy of calibration standards, which was determined as the mean back-calculated calibrator concentrations for the three calibration curves, ranged from 94.4% to 105%.

4.3 Dilutional Integrity and Carryover Studies

As per FDA recommendations, studies were performed to ensure the dilutional integrity of specimens that may be sample limited or contained maraviroc levels above the upper limit of quantitation (ULOQ). Precision and accuracy of diluted samples ranged from 3.90 % to 7.05% and -11.7% to -0.80%, respectively. These data are within acceptable limits and indicate that samples above our highest calibrator may be diluted for analysis. Further, volume-limited specimens were analyzed by testing dilutions of mid and high QC levels. Precision ranged from 4.78% to 7.05% and -8.06% to 2.8%, respectively (data not shown).

Carryover analysis was determined through the described protocol of alternating injections containing high and low concentration standards. The means and calculated standard deviations of low-low specimens and high-low specimens were determined. Low-low specimens resulted in a mean of 7.52 ± 0.40 ng/ml and high-low specimens resulted in a mean of 8.19 ± 0.89 ng/ml. Based on these results, no carryover was observed when running a sample with a low concentration of maraviroc following a sample containing a high concentration of the CCR5 antagonist. Further, carryover was qualitatively assessed by running a blank specimen following the highest calibrator (1,000 ng/ml). Qualitative analysis showed that the post-injection blank intensity at the retention time at which maraviroc was identified was less than 20% the signal observed at the LLOQ (0.5 ng/ml; Supplemental Fig. 1).

4.4 Stability Studies

A variety of experiments were performed to assess the stability of maraviroc following three freeze-thaw cycles, for 10 d in separated plasma at room temperature, and for 72 h in a 1:1 mixture of acetonitrile and water, both containing 0.1% formic acid, at 4°C. Maraviroc was shown to be stable under all of the aforementioned conditions (Table 4). Acceptability was determined through the calculation of a percent difference in stability-stressed specimens to freshly prepared or tested specimens. Percent differences ranged from -7.21% to 9.76% for freeze-thaw stability, -5.86% to 8.59% for sample matrix stability, and -0.30% to 5.05% for injection matrix stability. Lastly, stock solution stability of maraviroc was determined by preparing fresh stock solutions and comparing results to stock solutions stored for 16 h at room temperature and 6 months at -20°C. When compared to a freshly prepared stock solution, the percent differences of a solution incubated at room temperature for 16 h and -70°C for 6 months were 4.04% and -9.12%, respectively.

4.5 Selectivity and Matrix Effects

No interfering peaks from endogenous compounds were observed at the retention times at which ^{13}C -maraviroc and $^2\text{H}_6$ -maraviroc elute (Fig. 4A). The selectivity of the maraviroc LC-MS/MS method was evaluated by analyzing five independent lots of human plasma, and no response was observed for any of the blank samples analyzed. Further, an overlay of chromatographic spectra showing a lot of blank human plasma and the same lot spiked with the maraviroc at the LLOQ (0.5 ng/ml) is depicted in Fig. 4B.

To assess the potential impact of endogenous compounds on ion suppression or enhancement, matrix effects studies were performed. Peak responses for both ^{13}C -maraviroc and $^2\text{H}_6$ -maraviroc from processed specimens (pre-extracted) were compared to both neat standard solutions spiked with drug-free plasma (post-extracted samples), as well as standards prepared in mobile phase (un-extracted samples). The peak areas for both ^{13}C -maraviroc and $^2\text{H}_6$ -maraviroc are summarized in Table 5. Average matrix effects (post-extracted samples v. un-extracted), recovery efficiency (pre-extracted v. post-extracted), and processing efficiency (pre-extracted v. un-extracted) for maraviroc and the deuterated internal standard were 237.3% and 238.7%, 74.9% and 73.9%, and 177.5% and 174.5%, respectively. Maraviroc displays an absolute ion enhancement; however, relative matrix effects reduced as the isotopically labeled internal standard is also subjected to the same

degree of ion enhancement. There is 3% difference between internal standard efficiency across matrices as compared to maraviroc.

4.6 Method Comparison

The LC-MS/MS method for maraviroc quantification described herein shows a strong correlation with a reference LC-MS/MS method used for maraviroc quantitation utilized by the University of Alabama-Birmingham. Although twelve samples were compared, two were blank samples containing no maraviroc; one additional sample fell below the lower limit of quantitation of the reference method, was quantified by the method described herein with a concentration of 0.96 ng/ml (Supplemental Table 1). The Spearman correlation coefficient for maraviroc was 0.98 and Deming regression analysis showed a slope of 0.948 and an intercept of 3.5 with an average bias of -12.7% (data not shown). Due to the sparseness of the method correlation data, a Deming regression analysis was performed. Further, the percent difference between observed results for analyzed specimens did not exceed 17.4% (Supplemental Table 1).

The described LC-MS/MS method was also applied to 10 de-identified remnant specimens from patients receiving maraviroc as part of an ART regimen. The range of observed concentrations ranged from 14.5 ng/ml – 605 ng/ml; the median concentration was 42.3 ng/ml. The average percent difference between the described method and the comparative LC-MS/MS assay was -8.76% (range: -20.2% - 2.4%) (data not shown). These data illustrate that the described bioanalytical method is sufficiently sensitive for the quantification of maraviroc in people using the CCR5 antagonist as an antiretroviral therapy. Additionally, the median concentration observed is below the minimum target trough concentrations for antiretroviral-experienced HIV-1 infected individuals receiving maraviroc as part of their therapy [16].

While reference material and proficiency testing challenges are not offered by accreditation programs such as the College of American Pathologists (CAP), the Clinical Pharmacology Quality Assurance (CPQA) Program offers a proficiency testing challenge for maraviroc. Of note, the CPQA provides not only a peer mean, but a target concentration. Using the described LC-MS/MS method, a recent proficiency testing challenge yielded percent deviation of $\pm 7.33\%$ from target concentrations.

5. Discussion

The data presented above demonstrate the development and validation of a robust LC-MS/MS method for the quantification of maraviroc in human plasma. The assay has an analytical measuring range of 0.5-1,000 ng/ml, with dilutional analysis tested three times the upper limit of quantitation, and has been successfully validated following the recommendations of the FDA Guidance for Industry, Bioanalytical Method Validation guidelines [23]. Notably, the described method has a superior lower limit of quantification (0.5 ng/ml) to many reported methods [26-31]. While previous studies indicate that LC-MS/MS methods have been utilized with a LLOQ of 0.1 ng/ml, the method referenced by these studies actually has a linear range of 0.5 to 500 ng/ml [12,27,28]. Additionally, the described method offers testing of samples in a 96-well format, with minimal sample

cleanup, making the method amenable for high-throughput testing of batched samples from clinical trials.

Maraviroc is a hydrophobic molecule, binding to the CCR5 receptor via hydrophobic interactions [29]. Maraviroc was approved by the FDA in 2007 as a combinatorial therapy for the management of drug-resistant HIV-1, and later as an initial therapy for treatment of the virus [7,8]. While maraviroc is not recommended for clinical therapeutic monitoring, plasma measurements are useful in population pharmacokinetic analyses in the support of clinical studies, especially when determining efficacy and optimal delivery and treatment modalities. Additionally, maraviroc is being assessed in phase II clinical trials as a pre-exposure prophylactic agent [30]. These studies require the development and rigorous validation of sensitive and selective LC-MS/MS methods for maraviroc quantification.

In addition to being assessed as an oral antiretroviral agent, maraviroc is also being pursued as a topical microbicide to prevent the local transmission of HIV-1 in vaginal and rectal cavities. Both humanized mouse and non-human primate models have explored the use of maraviroc as a topical microbicide in HIV-1 prevention; the results have been mixed [11,12,31]. Consequently, phase I clinical trials evaluating the safety and pharmacokinetics of an intravaginal ring containing the CCR5 antagonist and the NNRTI dapivirine, are ongoing [32]. This further illustrates the need for a sensitive and selective method for the quantification of systemic plasma concentrations of maraviroc.

There are several additional points of discussion with regards to the described method. First, standard curves were constructed using weighted quadratic regression of calibrators, with a set run at the beginning and end of each validation run. During the development of the assay, the use of quadratic regression function showed the best fit of the data for a method with this dynamic range. Also, the ^{13}C isotope of maraviroc was monitored due to the intense signal response observed with the $[\text{M}+\text{H}]^+$, as well as the reduction of carryover observed at higher maraviroc concentrations using the $[\text{M}+\text{H}]^+$ species (data not shown). Taking into account these factors led to the development of a sensitive method for maraviroc quantification. Further, although a small cohort of specimens were tested, the described method correlates well with another LC-MS/MS approach. The performance of the assay is also substantiated by a successful proficiency testing challenge administered by the CPQA. The CPQA is a quality assurance initiative to provide support to pharmacology laboratories conducting HIV/AIDS translational research, particularly in the area of specimen analysis for large clinical trials [33]. Although maraviroc concentrations are being assessed in several clinical studies, there is a sparseness of validation metrics described in the literature. The described method includes detailed carryover analysis, as well as participation in proficiency testing challenges and method comparisons.

It should be noted that this assay has been developed and validated for drug quantification in EDTA plasma. The primary difficulty in the development and validation of the method was to achieve sufficient assay sensitivity to detect maraviroc concentrations under a variety of administration routes. This was met through the careful evaluation of regression analysis of calibrators, as well as quantitative and qualitative assessment of carryover. While the described LC-MS/MS method may be amenable to quantification of maraviroc in other

specimen sources, pre-analytical and sample preparation considerations would require further development and validation studies. Further, linear ranges would need to be tailored to the specimen source based on literature observations.

6. Conclusions

A robust LC-MS/MS method for the quantification of maraviroc concentrations in plasma has been developed and validated. This method offers high-throughput analysis of samples to support large clinical trials, providing important pharmacokinetic information. Validation of the assay according to FDA recommendations, with additional correlation and carryover studies, indicates that the method is sensitive, accurate and reproducible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Development of a tandem mass spectrometric method for plasma maraviroc quantification
- Validation of LC-MS/MS assay following FDA guidelines
- Validation of a robust method with a measuring range of 0.5-1000 ng/ml

Fig. 1.
Structure of the CCR5 antagonist maraviroc ($C_{29}H_{41}F_2N_5O$). * denotes position of deuterium atoms for the isotopically-labeled 2H_6 -maraviroc internal standard.

Fig. 2A.

Chromatograms of (A) ^{13}C isotope of the maraviroc and (B) $^2\text{H}_6$ -maraviroc, with retention time demarcated at 0.53 min. cps-counts per scan.

Fig. 3. Product ion spectrum of ^{13}C -maraviroc at m/z 515/5 shows the most abundant maraviroc product ion at m/z 390.2.

Fig. 4.

Selectivity of ^{13}C -maraviroc and $^2\text{H}_6$ -maraviroc transitions. **(A)** Chromatograms of drug-free human plasma illustrated the extracted ion current of the maraviroc transition and the $^2\text{H}_6$ -maraviroc transition. **(B)** Overlay of maraviroc at the LLOQ (0.5 ng/ml) spiked into K2 EDTA plasma and blank plasma. The relative intensity is 10-times the background level of the blank plasma (upper panel). Extracted ion current for the $^2\text{H}_6$ -maraviroc transition (lower panel).

Table 1

UPLC schematic for detection of maraviroc in human EDTA plasma.

Step	Start Time (min)	Flow (ml/min)	%A ^a	%B ^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	5	95
6	3.80	1.0	80	20
7	5.00	1.0	80	20

^a H₂O, 0.1% formic acid^b Acetonitrile, 0.1% formic acid

Table 2

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

QC Level	Intra-Assay Precision and Accuracy ^a				Inter-Assay Precision and Accuracy ^b			
	Mean (ng/ml)	SD (ng/ml)	% CV	% DEV	Mean (ng/ml)	SD (ng/ml)	% CV	% DEV
LLOQ (0.5 ng/ml)	0.55	0.03	4.79	10.2	0.54	0.03	5.12	7.81
Low (1.5 ng/ml)	1.64	0.06	3.64	9.44	1.63	0.09	5.38	8.44
Mid (50 ng/ml)	53.7	2.71	5.04	7.37	52.6	3.14	5.98	5.23
High (850 ng/ml)	861	46.3	5.38	1.29	866	41	4.76	1.89

^a n=6 for each level of QC; representative data from a single analytical run^b n=18 for LLOQ, low, mid and high QC materials; complex precision and accuracy from 3 analytical runs.

Table 3

Accuracy (% Recovery) of Maraviroc Calibration Standards

Calculated concentration (ng/ml)	N	Observed mean (ng/ml)	% Recovery	SD	%CV
0.50	6	0.50	100	0.02	3.78
1.0	6	0.99	99	0.07	6.60
5.0	5	5.04	101	0.28	5.63
10.0	6	9.95	99.5	0.43	4.31
50.0	5	51.8	104	3.00	5.80
100	6	102.6	103	3.45	3.36
250	5	239	95.6	12.2	5.11
500	6	472	94.4	28.1	5.96
1000	5	1052	105	51.1	4.86

Table 4

Maraviroc 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 QC (1.5 ng/ml)	1.49	1.63	9.76	1.49	1.61	8.59	1.60	1.60	-0.30
Mid QC (50 ng/ml)	50.3	51.2	1.74	50.3	47.4	-5.86	53.7	54.1	0.75
High QC (850 ng/ml)	867	804	-7.21	867	824	-4.99	861	905	5.05

Table 5

Matrix Effects, recovery efficiency and processing efficiency of maraviroc and deuterated maraviroc internal standard ($^4\text{H}_6\text{-MVC}$).

QC Level	MVC Peak Area			$^4\text{H}_6\text{-MVC}$ Peak Area			Matrix Effects		Recovery Efficiency		Processing Efficiency	
	Un-extracted	Post-Extracted	Pre-Extracted	Un-extracted	Post-Extracted	Pre-Extracted	MVC	$^4\text{H}_6\text{-MVC}$	MVC	$^4\text{H}_6\text{-MVC}$	MVC	$^4\text{H}_6\text{-MVC}$
Low QC (1.5 ng/ml)	12155	32503	22763	583855	1470343	950216	267	252	70.0	64.6	187.3	162.7
Mid QC (50 ng/ml)	375574	987644	778055	543152	1430389	1040435	263	263	78.8	72.7	207.2	191.6
High QC (850 ng/ml)	5872457	10668085	8108363	489684	983225	829021	182	201	76.0	84.3	138.1	169.3