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Validation of a sensitive LC/MS/MS method for the determination of zidovudine and lamivudine in human plasma

Joseph E. Rower, Brandon Klein, Lane R. Bushman, and Peter L. Anderson

University of Colorado Denver, School of Pharmacy, Department of Pharmaceutical Sciences, Aurora, Colorado

Abstract

A sensitive LC/MS/MS assay for determining zidovudine (ZDV) and lamivudine (3TC) in human plasma was validated to support antiretroviral pharmacology research programs. After addition of stable labeled isotopic zidovudine (ZDV-IS) and lamivudine (3TC-IS) as internal standard, a solid phase extraction was performed with an Oasis HLB 1cc cartridge, with recoveries of 92.3% for ZDV and 93.9% for 3TC. A Phenomenex Synergi Hydro-RP (2.0 × 150 mm) reversed phase analytical column was utilized for chromatographic separation. Mobile phase consisted of an aqueous solution of 15% acetonitrile and 0.1% acetic acid. Detection was accomplished by ESI/MS/MS in the positive ion mode, monitoring 268/127 and 271/130, and 230/112 and 233/115 transitions, for ZDV and ZDV-IS and 3TC and 3TC-IS, respectively. The method was linear from 1 to 3000 ng/mL with a minimum quantifiable limit of 1 ng/mL when 100 µL of plasma were analyzed. Validations results demonstrated high accuracy ($\leq 8.3\%$ deviation) and high precision ($\leq 10\%$ CV) for the quality control samples. The method was also shown to be specific and reproducible. The value of the high sensitivity was demonstrated by quantitation of approximately 100 existing samples that had ZDV below the limit of quantitation using a previously validated, less sensitive HPLC-UV method utilized in the laboratory.

Keywords

lamivudine; zidovudine; human plasma; LC/MS/MS

1. Introduction

Zidovudine (ZDV) and lamivudine (3TC) are nucleoside analog reverse transcriptase inhibitors (NRTIs) with a long history of use in human immunodeficiency virus (HIV)-infected persons (Broder, 2010). The combination of ZDV/3TC is commonly used in diverse patient populations and treatment settings such as in HIV-infected pregnant women, infants and children, and in resource-poor areas. In order to utilize these two drugs to their highest potential, it is important to fully understand their *in vivo* pharmacokinetic profiles. As NRTIs, the active moiety for ZDV and 3TC are the triphosphate anabolites formed intracellularly. Nevertheless, it is still necessary to examine the concentrations of the drug in plasma in order to understand their entire pharmacologic continuum.

High sensitivity is an important consideration when developing an assay for ZDV, as the 1-hour plasma half-life (Burger et al., 1994) leads to extremely low ZDV concentrations well

Corresponding Author: Peter L. Anderson, Pharm.D., 12700 E 19th Avenue, P15-C238, Aurora, Colorado, 80045; Phone: (303) 724-6128; Fax: (303) 724-6135; Peter.Anderson@ucdenver.edu.

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before the end of the dosing interval. Given that ZDV is dosed every 12 hours, drug concentrations are typically below the limit of quantitation (BLQ) of most assays for more than half the dose interval. This situation prevents an assessment of whether ZDV exhibits a long terminal half-life, which may inform the existence of slowly equilibrating tissues.

Previously published methods have been validated for these drugs with variable sensitivities, ranging from 0.5 to 50 ng/mL when extracted from 50 to 500 μ L of matrix (human plasma, human serum, rat plasma) assayed. (Jung et al., 2007), (Compain et al., 2005), (Fromentin et al., 2009), (Kenney et al., 2000), (Alnouti et al., 2005), (Estrela et al., 2004), (Le Saux et al., 2008), (Li et al., 2010), (Mudigonda et al., 2008). Most methods used higher sample volumes or were not as sensitive as the method described below.

This method replaces the laboratory's previously validated, less sensitive HPLC-UV method (Kakuda et al., 2001), in support of clinical research. As a result of the historical clinical knowledge of ZDV and its associated toxicities, it is still the drug of choice in pregnancy and for newborn infants to prevent vertical transmission. As infant blood samples are typically of lower volumes than adult samples, it is prudent to have assays capable of high sensitivity determinations from low volumes to facilitate studies investigating infant prophylaxis with ZDV. As such, the current method has a reportable linear range of 1 ng/mL (LLOQ) to 3000 ng/mL (ULOQ) for both analytes when a relatively low volume (100 μ L) of human plasma is assayed, which improves the previous assay's sensitivity 25 fold.

We performed a rigorous validation, which included a correlation to the previous UV method, incurred sample analysis (Rocci et al., 2007), and extensive stability experiments. The combined use of ZDV and 3TC is common in resource poor areas. Stability of the analytes is of concern in studies which must transport samples drawn in remote areas back to laboratories capable of determining analyte concentrations. As such, the described method extensively tested the stability of the analytes. Stability tests included four freeze/thaw cycles at two common storage conditions (-20°C and -80°C), ambient temperature storage for up to 10 days, and freezer storage of human plasma samples for up to 10 years, which met or exceeded the FDA Guidance for Industry recommended tests. (Viswanathan et al., 2007).

Finally, the assay utilizes a stable labeled isotopic internal standard for each analyte, which limits deleterious matrix effects and helps increase MS based method accuracy and precision. The use of stable labeled isotopes of the analyte as an internal standard is recommended for bioanalytical assays to increase assay precision and limit variable recovery between analyte and the internal standard (Viswanathan et al., 2007). Stable labeled isotopic internal standards have been successfully used in plasma assays of other NRTIs, for example, in an assay to determine concentrations of tenofovir and emtricitabine (Delahunty et al., 2009). Also, assays in matrices other than human plasma have demonstrated the utility of stable labeled isotopic internal standards for determination of ZDV and 3TC, such as the assay described by Pereira et al in seminal plasma (Pereira et al., 2000). To our knowledge, this is the first article to present a method using isotopic internal standards to determine ZDV and 3TC concentrations in human plasma.

The following was developed to be highly sensitive, selective, accurate, and precise method for simultaneously determining concentrations of ZDV and 3TC from a low volume of human plasma.

2. Experimental

2.1 Chemicals and Reagents

ZDV (>99.9% pure) and 3TC (>99.9% pure) were obtained through the NIH AIDS Research and Reference Reagent Program (Bethesda, MD). The chemical structures of ZDV (MW 267) and 3TC (MW 229) are shown in figure 1. Isotopic ZDV [$2-^{13}\text{C}$; $1,3-^{15}\text{N}$, ZDV-IS] was purchased from Moravek Biochemicals, Inc. (Brea, CA), while isotopic 3TC [$2-^{13}\text{C}$; $1,3-^{15}\text{N}$, 3TC-IS] was purchased from Martrex, Inc. (Minnetonka, MN), both for use as internal standards. HPLC grade methanol, acetonitrile, and glacial acetic acid were acquired from Fisher Scientific (Fairlawn, NJ). Ultrapure (UP) water was prepared in house from deionized water with a Barnstead Nanopure System (Thermo Fisher Scientific, Waltham, MA). Human EDTA anti-coagulant plasma was obtained from the Biological Specialty Corporation (Colmar, PA).

2.2 LC/MS/MS Instrumentation and Conditions

The HPLC system utilized a Surveyor LC autosampler and LC Pump (Thermo Finnigan, San Jose, CA). A Synergi Hydro-RP 80A, 2.0×150 mm, 4 micron particle size, (Phenomenex, Torrance, CA) analytical column was used for chromatographic separations. The mobile phase consisted of 0.1% acetic acid in a 15:85 acetonitrile/water (v/v) solution, delivered at a flow rate of 0.200 mL/min. At the end of each analytical run, the column was washed with a 50:50 acetonitrile/water (v/v) mobile phase. The analytical column was maintained at 35°C , and extracted samples kept at 15°C while inside the autosampler. The autosampler needle was washed with a filtered 10:90 methanol/water (v/v) solution between injections. A TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) was used in ESI positive polarity mode. All analytes and internal standards were detected using optimized parameters in MS/MS single reaction monitoring (SRM) mode. Precursor/product transitions of 268/127 and 271/130 (collision energy 19V) were monitored for ZDV and ZDV-IS respectively, while transitions of 230/112 and 233/115 (collision energy 17V) were monitored for 3TC and 3TC-IS. Data acquisition, processing, and storage were performed using Xcalibur software, version 1.3 (Thermo Finnigan, San Jose, CA). Calculations were based on peak area ratios of analyte to internal standard. Concentrations are interpolated from a linear least squares regression calibration curve based on $1/\text{concentration}^2$ weighting for both analytes.

2.3 Preparation of Calibration Standards, Internal Standard, and Quality Controls

ZDV and 3TC preparation stocks (1 mg/mL) were prepared for both standard and QC preparation from separately weighed reference standards. Standard preparation stocks were combined to prepare working standard stocks at concentrations appropriate for the calibration curve described in the following section. A 500 ng/mL solution combining both ZDV-IS and 3TC-IS was used as the working internal standard stock solution. All stock solutions were prepared in water and stored at 4°C . ZDV/3TC combined quality controls (QC) of 5 ng/mL (QC1), 15 ng/mL (QC2), 250 ng/mL (QC3), and 2500 ng/mL (QC4) were prepared in plasma from the separately prepared QC stock solutions of ZDV and 3TC. The QCs were stored at -80°C . A 1 ng/mL QC (LLOQ) used to validate the lower limit of quantification was prepared in five replicates on each of three days for validation.

2.4 Sample Preparation

Plasma ZDV/3TC standards were prepared in 13×100 mm test tubes on a daily basis by spiking the appropriate working standard stock solution into 100 μL of blank plasma, resulting in plasma ZDV/3TC calibration concentrations of 1, 5, 10, 50, 100, 500, 1000, 2000, and 3000 ng/mL. Unknowns and QC plasma samples (100 μL) were added to test

tubes, followed by addition of 20 μ L of working internal standard stock and 200 μ L of water to each sample. Samples were then applied to a pre-conditioned Oasis HLB 1cc SPE extraction cartridge, washed with 1mL water, and eluted with 0.5 mL of methanol. The SPE extraction mirrors that used previously in the laboratory, and demonstrated increased process efficiency as compared to protein precipitation methods during assay development.

Sample eluents were then dried under nitrogen, reconstituted with 100 μ L of water, and transferred to labeled vials containing 150 μ L low volume inserts (Waters Corporation, Milford, MA). A 10 μ L aliquot was injected. The retention time for 3TC and 3TC-IS was approximately 2.5 minutes, while ZDV and ZDV-IS were retained for approximately 6.5 minutes.

2.5 Method Validation

Validation of the method included an evaluation of the following characteristics: assay accuracy and precision, calibration curve performance, recovery and matrix effects, dilution accuracy and precision, analyte stability, and assay specificity/selectivity. These validation experiments followed the FDA Guidance for Bioanalytical Method Validation (Viswanathan et al., 2007). The method was also used to demonstrate the ability to accurately and precisely reanalyze samples incurred by the laboratory, both by correlation to the previous HPLC-UV and by reanalysis of samples already run by this HPLC-MS/MS method (Rocci et al., 2007).

2.5.1 Accuracy and Precision—Intra- and inter-day accuracy and precision were determined by the performance of the LLOQ (1 ng/mL) and four concentrations of QCs (QC1=5 ng/mL; QC2=15 ng/mL; QC3=250 ng/mL; and QC4=2500 ng/mL). The LLOQ was run in five replicates on three days; all other QC levels were run in five replicates on six separate days during validation. Accuracy was evaluated and reported by calculating the percent deviation (%dev) from the nominal concentration. Precision was determined by calculating the coefficient of variation (%CV) of replicates within one sample run (intra-day) and between sample runs (inter-day). Adequate accuracy and precision was defined as $\leq 15\%$, except for the LLOQ, where it was $\leq 20\%$.

Calibration curve performance was assessed by evaluating accuracy and precision of back-calculated standards and evaluating the slope, intercept, and coefficient of determination (r^2) of the weighted $1/\text{concentration}^2$ regression lines. At least six non-zero standards were required for a valid calibration curve where $\pm 20\%$ from the nominal value was acceptable at the LLOQ and $\pm 15\%$ from the nominal value accepted at all other concentrations. If a calibrator did not meet these criteria, it was dropped from the calibration curve and the curve recalculated.

2.5.2 Matrix Effects—Validation included an assessment of matrix effects on the quantitation of both analytes. To determine if endogenous compounds in plasma suppressed or enhanced analyte ionization during detection, potential matrix effects were tested following the method of Matuszewski et al. (Matuszewski et al., 2003). Three sets of samples (set 1, set 2, and set 3) were prepared containing ZDV/3TC standards of 10, 100, and 1000 ng/mL in five replicates, with each replicate in sets 2 and 3 using a different plasma lot. The set 1 samples (neat samples), consisted of analyte and internal standard added to water for a total volume of 100 μ L. In set 2 (post extract spikes), blank plasma samples were extracted, and then spiked with analyte and internal standard. For set 3 samples, the analyte and internal standard were spiked into plasma and then extracted as described above. A comparison of set 1 and set 2 samples yielded a measure of observed matrix effects. A comparison of set 2 and 3 demonstrated analyte recovery from the

extraction process. The difference between set 1 and set 3 samples described the overall efficiency of the analytical process. The effect of different plasma lots on the assay were determined by comparing the regression line slopes and peak area ratios for each different lot of plasma, as well as by examining the precision of the analyte and internal standard areas and ratios for each sample set and plasma lot. In addition, heparinized plasma, sodium citrate anticoagulant plasma, and serum were tested to determine assay performance in the presence of matrices other than EDTA anti coagulated plasma.

2.5.3 Dilutional Accuracy and Precision—In order to determine the accuracy and precision (n=3) of measuring ZDV/3TC in diluted samples, a QC was prepared at a concentration of 10000 ng/mL, then diluted to 2000 ng/mL (5x) and to 1000 ng/mL (10x) with blank plasma. These samples were allowed a difference of $\pm 15\%$ from the expected value to be acceptable.

2.5.4 Stability—The stability of both analytes in EDTA plasma was tested by subjecting QCs to different test conditions. Freeze/thaw stability of the QCs was tested in triplicate with QC2 (15 ng/mL) and QC4 (2500 ng/mL) after four freeze/thaw cycles. The samples were allowed to thaw completely and remained at room temperature for at least one hour. The samples were returned to freezer storage conditions (both -20°C and -80°C) for at least 24 hours prior to removal for the next freeze/thaw cycle. The stability of ZDV and 3TC in plasma at room temperature was tested for thawed QC samples (QC2 and QC4 in triplicate) maintained at room temperature for 10 days prior to extraction and analysis. Triplicate extracted QC2 and QC4 were retained in the autosampler at 15°C for nine days prior to re-analysis to determine stability of analytes in an extracted sample. Long term plasma stability was determined by analyzing a 100, 500, and 1000 ng/mL plasma sample that had been prepared 10 years previously and stored at both -20°C and -80°C conditions over this time period. The samples were considered to be stable at a given condition if the mean values obtained from the treated QCs were within $\pm 10\%$ of the mean values of the untreated or reference QC samples that were run within the same analytical run.

The stability of ZDV and 3TC aqueous stock solutions were analyzed by comparing the signal response of freshly prepared 1 mg/mL stock solutions with the signal response from the stock solutions prepared 4.5 months earlier. Furthermore, these solutions were compared to the 10000 ng/mL working standard stock solution to ensure that working standard stocks were stable throughout the refrigeration/thaw cycles between analytical runs. Stability analysis was carried out on a Waters HPLC-UV system (Waters Corporation, Milford, MA) and compared peak heights of the various solutions.

2.5.5 Specificity and Selectivity—Specificity and selectivity was tested with an experiment monitoring for possible interferences from concomitant medications by spiking high concentration stock solutions from the nucleoside, non-nucleoside, and protease inhibitor assay sets within the laboratory (10 $\mu\text{g/mL}$) into a QC3 (250 ng/mL) sample. Antiretroviral drugs included in these sets were: indinavir, amprenavir, nelfinavir, M8 (a nelfinavir metabolite), saquinavir, atazanavir, ritonavir, lopinavir, delavirdine, efavirenz, nevirapine, didanosine, emtricitabine, tenofovir, stavudine, and abacavir. In addition, other common drugs such as naproxen, ibuprofen, theophylline, and sulfamethoxazole were spiked into the QC3 sample prior to extraction and analyzed in triplicate. A difference of 10% from reference QCs in the same analytical run was allowed to show assay selectivity. Further evaluation of assay specificity and selectivity included: the absence of signal from multiple (n=6) sources of blank plasma, specificity of the signal in the ZDV, 3TC, ZDV-IS, and 3TC-IS SRM channels (i.e. channel cross talk), and assessment of carryover. Six different lots of blank plasma samples were extracted and analyzed for signal in any of the four monitored SRM channels. Channel cross-talk was assessed by analyzing the response

of an extracted sample containing only internal standard (no analyte) and of an extracted sample containing only a high ZDV/3TC (3000 ng/mL) concentration (no internal standard) for the absence of signal in the ZDV and 3TC channel or the ZDV-IS and 3TC-IS channel, respectively. Carryover was determined by evaluating the signal from an extracted blank plasma sample following injection of an extracted ULOQ sample (3000ng/mL). In all cases, a signal of less than 10% of that of the LLOQ was allowed to accept the assay as specific and selective.

2.5.6 Sample Reanalysis—Validation also tested the assay's ability to reproduce data from other modes of detection and from previous analytical runs. Samples (n=27) previously analyzed by the laboratory's HPLC-UV method were correlated to results for the same samples determined by the above method. Incurred samples (n=19) were reanalyzed at the conclusion of running clinical samples (described below), and compared to the initial analysis (Rocci et al., 2007). A difference of 20% between the two analyses was allowed to accept the assay as yielding equivalent results to previous analytical methods.

2.5.7 Proficiency Testing—Blinded external proficiency testing quality control samples (n=18, provided by DAIDS and the Clinical Pharmacology Quality Assurance (CPQA) program) from four different proficiency testing rounds were analyzed and compared to final target concentrations.

2.6 Clinical Application

The method was used to analyze plasma samples containing ZDV and 3TC in both HIV-negative and HIV infected patients. The samples arose from a protocol that was approved by the Colorado Multiple Institutional Review Board, and all subjects gave written informed consent. Subjects were administered 300 mg ZDV and 150 mg 3TC twice daily, and provided blood samples on several study visits. Blood was drawn into an EDTA anti-coagulant tube at various time points (depending on the study day), then centrifuged, with the supernatant plasma stored at -80°C until analysis. Plasma concentrations of both analytes were initially analyzed using the laboratory's previously validated HPLC-UV method for ZDV and 3TC, which had an LLOQ of 25 ng/mL. Because multiple samples were below limit of quantitation (BLQ) with this HPLC-UV assay, these same BLQ samples (n=115) were reanalyzed with this LC/MS/MS method. Furthermore, utilization of the LC/MS/MS method shortened the analytical run time from 38 to 9 minutes.

3. Results and discussion

3.1 Chromatography

Isocratic chromatography was optimized so that 3TC was retained on the column while ZDV eluted rapidly and was baseline separated from its glucuronide metabolite. A longer than usual column (150mm) and isocratic mobile phase was used to generate reproducible chromatography under these constraints. Representative LC/MS/MS chromatograms are shown in figure 2. Figure 2A shows a blank plasma sample; Fig. 2B shows an extracted LLOQ (1 ng/mL) sample; and Fig. 2C shows a typical extracted patient sample. In each figure, analytes are depicted in the top window, with the internal standard for each in the bottom window. 3TC elutes first, at approximately 2.5 min, followed by ZDV at 6.5 min. The peak at 5.65 min in the patient chromatogram represents the ZDV 5' glucuronide (gZDV) metabolite, which was found in all clinical samples. This indicates that the ZDV glucuronide metabolite present in subject sample fragments to ZDV within the ESI source, and must be successfully separated from the ZDV peak to ensure accurate determination. The gZDV peak was not observed in the non-clinical specimens (i.e. spiked standards or QCs), as expected.

3.2 Method Validation

3.2.1 Accuracy and Precision—Inter- and intra-assay precision and accuracy were determined by analyzing the LLOQ and four QC levels as described above. For both inter- and intra-assay data the mean percent deviation (as a measure of accuracy) for either analyte at any QC concentration level was no more than $\pm 5.2\%$, while the %CV (as a measure of precision) was no more than 6.4%. The analysis of an LLOQ of 1 ng/mL resulted in percent deviations of less than $\pm 3.3\%$ and a %CV of no more than 10% for either analyte, validating the use of a 1 ng/mL LLOQ for the assay (see Table 1 for ZDV and Table 2 for 3TC). The precision and accuracy for all of the four tested QC levels and the LLOQ were within the acceptable range of $\pm 15\%$.

A $1/\text{concentration}^2$ weighting was used to fit a linear least squares regression calibration curve to the response versus concentration data. Good linearity in the range of 1 to 3000 ng/mL was achieved, with typical r^2 values between 0.9994 and 0.9999 for ZDV and 0.9984 and 0.9996 for 3TC, with consistent slopes, during the six validation runs. The LLOQ of the assay was 1 ng/mL, which was 25-fold lower than our previous method, and more sensitive than most other published methods utilizing the same volume of human plasma.

3.2.2 Matrix Effects—The described extraction protocol yielded a high recovery, ranging from 91.7 to 93.3% (mean 92.3%) for ZDV and 90.7 to 99.4% (mean 93.9%) for 3TC. The matrix effect was consistent over the concentration range, with a mean 5% suppression for ZDV and ZDV-IS, and 2% suppression for 3TC and 3TC-IS. Equivalent matrix effects for both the analyte and the internal standard are a result of using a stable labeled isotopic internal standard for each analyte (Viswanathan et al., 2007). The overall process efficiency was 89.1% for ZDV and 92.0% for 3TC. Finally, the %CV between the different plasma lots used was less than 1.9% for both analytes, indicating that different plasma sources do not adversely affect quantitation. Finally, testing of matrices other than EDTA anti coagulant plasma or serum demonstrated a percent deviation from nominal of less than 5.2% for either analyte, showing that quantitation was not affected by serum versus plasma or the anti-coagulant used during blood draw.

3.2.3 Dilutional Accuracy and Precision—Samples could also be accurately and precisely diluted to five and ten times their original concentrations with accuracy and precision within $\pm 6\%$ of expected concentrations.

3.2.4 Stability—Stability was demonstrated under a wide variety of conditions. QC samples were stable in plasma through at least four freeze/thaw cycles at both -20°C and -80°C , and when kept at room temperature for 10 days. These results are significant in light of the varying conditions samples may encounter during transportation. Extracted samples were also stable when maintained at 15°C in the autosampler for at least nine days. The analytes were stable in plasma for at least 10 years (when stored at -20°C or -80°C), as the percent deviation for ZDV was within $\pm 1.0\%$ and within $\pm 6.1\%$ for 3TC. The stock and working aqueous solutions of both analytes were shown to be stable for at least four months when stored at 4°C , and in spite of numerous refrigerated/thaw cycles. For each stability test, treated and reference samples were within $\pm 10\%$ of each other and precision ($n=3$) within 15%.

3.2.5 Specificity and Selectivity—Assay selectivity was demonstrated in a number of ways. Each analytical run included a blank with no internal standard and also a blank with internal standard. The blank with internal standard showed that the IS did not contribute to analyte response ($\ll 10\%$ LLOQ signal for ZDV or 3TC), while the blank without internal standard showed that no signal came from the sample matrix. Furthermore, the extraction of

a sample spiked with the highest concentration ZDV/3TC standard (3000 ng/mL), but without internal standard, was examined and no signal for either the ZDV-IS or 3TC-IS SRM channels was observed (no cross-talk of ZDV or 3TC into the ZDV-IS or 3TC-IS SRM channel). Analysis of six different sources of blank EDTA plasma did not show an analyte or internal standard peak, indicating that the source of plasma does not contribute to analyte or internal standard signal. No interferences were observed when examining the effect of multiple possible concomitant medications. The mean percent difference of these samples was -1.4% and -2.3% from nominal for ZDV and 3TC, respectively; precision, as %CV, was less than 1.9% for both analytes.

3.2.6 Sample Reanalysis—The patient samples ($n=27$) previously analyzed by HPLC-UV and subsequently analyzed by this method were within $\pm 16.7\%$ for either analyte. The correlation between the two methods is shown in figures 3 (ZDV) and 4 (3TC). The correlation slopes are 1.0099 and 1.0464 for ZDV and 3TC respectively, with R^2 values of 0.9960 and 0.9897, respectively. This data demonstrates that data generated by this method are reproducible despite differing modes of detection. Method reproducibility was also demonstrated by reanalyzing incurred samples ($n=19$) following the FDA Guidance for Industry (Rocci et al., 2007). No sample differed by more than $\pm 8.1\%$ from the original analysis for either analyte (table 3). The incurred sample analysis and the correlation between the MS/MS and UV methods indicate that both drugs are stable within human plasma from subjects taking the medication, and that the potential conversion of metabolite to drug after sampling is negligible.

3.2.7 Proficiency Testing—All ZDV/3TC proficiency samples passed acceptance criteria, as none varied by more than 20% from the target concentrations.

3.3 Clinical Application

Plasma samples from both HIV negative and HIV positive subjects were analyzed for ZDV and 3TC concentrations as described above. Using a previously validated HPLC-UV assay with a LLOQ of 25 ng/mL, 115 ZDV samples were determined to be BLQ. Using this new LC/MS/MS method, all samples had measurable ZDV with a median concentration of 15.6 ng/mL and a range of 3.5 to 24.7 ng/mL (3TC median for these samples was 252 ng/mL, with a range of 104 to 1265 ng/mL). Two samples from the baseline visit (before drug) had undetectable ZDV and 3TC, further demonstrating assay selectivity. Thus, the advantage of high sensitivity was demonstrated with clinical research samples.

4. Conclusions

A highly sensitive, simple, efficient, and reliable method for determining ZDV/3TC concentrations in human plasma was validated. Assay sensitivity was better than most previously published methods while utilizing a relatively small plasma volume (100 μ L), and much better than the laboratory's previously validated HPLC-UV method. The method was rigorously validated, and included extensive evaluations of analyte stability, incurred sample analysis, and a demonstration that mode of detection (UV vs. MS/MS) does not influence method results. The use of stable labeled isotopic internal standards for both analytes provided results that were free from significant matrix effects. The extraction procedure yielded consistently high recoveries, resulting in a high process efficiency for the assay. The validated method was selective for the desired analytes, and reproducible both within runs and between days. The analytes were shown to be stable for all test conditions, including an extended time (10 days) at room temperature, four freeze/thaw cycles, and 10 years in freezer storage. The isocratic liquid chromatography conditions allowed for relatively short analytical run times, increasing sample throughput. Finally, the assay was successfully

applied to clinical research samples, and the value of the greatly improved the sensitivity compared to a previous less sensitive UV methodology was realized.

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Abbreviations

CV	coefficient of variation
ZDV	zidovudine
3TC	lamivudine
LC/MS/MS	liquid chromatography-tandem mass spectrometry
LLOQ	lower limit of quantitation
BLQ	below limit of quantitation
ULOQ	upper limit of quantitation

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Figure 1.
Chemical structure of (A) zidovudine (MW 267) and (B) lamivudine (MW 229).

Figure 2.

Representative LC/MS/MS chromatograms. (A) blank plasma sample; (B) blank plasma sample spiked with ZDV/3TC at the LLOQ (1.0 ng/mL) and internal standard; and (C) a subject unknown sample. The top chromatogram is the analyte while the bottom chromatogram is the internal standard; 3TC elutes first and is in the left chromatograms, followed by ZDV in the right chromatograms. The unknown subject sample (C) shows a peak retained at 5.65 minutes, which may be due to the presence of an ZDV 5' glucuronide metabolite formed *in vivo*, which is separated from ZDV chromatographically and degraded in the MS/MS ESI source.

Figure 3.

Graph showing the correlation of ZDV samples (n=27) analyzed by HPLC-UV (x axis) and LC/MS/MS (y axis). Square points indicate samples (n=9/27) that were reported as below limit of quantitation by the UV method.

Figure 4. Graph showing the correlation of 3TC samples (n=27) analyzed by HPLC-UV (x axis) and LC/MS/MS (y axis).

Table 1

Summary statistics for ZDV QCs used for accuracy and precision determination during validation.

Table 1	ZDV Interassay Statistics					
nominal conc.	1	5	15	250	2500	
mean	1.03	5.04	14.2	244	2438	
SD	0.10	0.28	0.67	15.7	109	
%CV	10	5.6	4.7	6.4	4.5	
%dev	3.3	0.7	-5.2	-2.3	-2.5	
n	15	30	30	30	30	

Table 2

Summary statistics for 3TC QCs used for accuracy and precision determination during validation.

Table 2	3TC Interassay Statistics						
nominal conc.	1	5	15	250	250	2500	
mean	1.02	5.21	14.4	247	2550		
SD	0.08	0.16	0.65	13.7	109		
%CV	8.3	3.1	4.5	5.5	4.3		
%dev	1.5	4.2	-4	-1.4	2		
n	15	30	30	30	30		

Table 3

Incurred sample analysis data.

Sample #	Incurred Sample Analysis							
	Reanalysis		Original		% Diff		Reanalysis	
	3TC		3TC		3TC		ZDV	% Diff
1	337		332		1.77		59.2	4.92
2	209		206		1.61		38.9	3.31
3	718		743		-3.40		52.0	-6.53
4	1049		1066		-1.62		336	-4.19
5	220		223		-1.31		20.1	-3.39
6	289		296		-2.53		24.5	-2.23
7	347		354		-1.99		23.7	-2.86
8	630		685		-7.96		101	-8.11
9	577		579		-0.39		29.2	0.10
10	1032		1067		-3.33		116	-5.57
11	530		540		-1.84		86.8	-6.49
12	901		897		0.44		47.6	-0.78
13	897		886		1.23		45.4	-1.49
14	335		336		-0.33		15.0	-3.39
15	458		465		-1.67		53.9	-0.06
16	706		694		1.76		33.3	-0.66
17	155		154		0.62		12.7	-4.87
18	127		130		-2.52		14.6	-4.86
19	600		595		0.79		275	-2.02