CLINICAL EVALUATION OF A DRIED BLOOD SPOT ASSAY FOR ATAZANAVIR

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Running Title: Atazanavir Dried Blood Spot Assay

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ABSTRACT

Current procedures for obtaining and measuring plasma concentrations of HIV protease inhibitors (PI) are technically challenging. Dried blood spot (DBS) assays offer a way to overcome many of the obstacles. We sought to develop a DBS assay for quantitation of the PI atazanavir (ATV) and to compare this method with a previously validated plasma assay. We prospectively enrolled 48 patients with well controlled HIV disease who had been on ATV for at least 7 days. ATV was quantified from plasma using HPLC. A reversed-phase ultra high performance liquid chromatographic (UPLC) assay was utilized for DBS samples. The concentrations of ATV quantified in a DBS matrix showed very strong agreement with those measured in plasma ($R^2 = 0.988$). The mean difference in ATV concentrations between the two methods was -10.8% (95% CI, -7.65% to -13.95%) indicating the DBS method has a slight negative bias. 97.8% of the differences in concentrations between the two assays fell within +/- 2 standard deviations. ATV concentrations were lower in subjects who had detectable HIV RNA in plasma (mean 543 ng/mL) compared with those with HIV RNA < 50 copies/mL (mean 1582 ng/mL) (p=0.03, Wilcoxon rank-sum test). In conclusion, our study demonstrated that ATV quantitation in a DBS matrix is feasible and accurate. DBS use offers a convenient alternative for measuring plasma concentrations of ATV, and may have utility in monitoring of drug concentrations in clinical practice and in future studies.
INTRODUCTION

Presently approximately 33 million persons are living with human immunodeficiency virus (HIV) disease (35). Treatment options for HIV disease have expanded over the last 15 years particularly with the introduction of protease inhibitors (PI) as a component of combination antiretroviral therapy (ART). Use of these agents has been associated with significant decreases in morbidity and mortality (29, 13,16). Despite the efficacy of PIs, a substantial number of patients still experience virological failure (23). PIs show significant interindividual pharmacokinetic variability for identical dosing regimens (14, 33, 7,31). High PI concentrations have been associated with toxicity while subtherapeutic concentrations have been associated with virologic failure (32, 12, 28, 3, 9, 30, 31, 11, 2). These findings have led to interest in the use of therapeutic drug monitoring (TDM) which individualizes therapy to maximize outcomes and minimize toxicity (7, 1, 10). Currently the literature does not support and guidelines do not recommend routine use of TDM in HIV-infected adults (8,21).

Atazanavir (ATV) is an azapeptide PI approved for use in both treatment-naïve and treatment experienced patients (18). It has the advantage of being dosed once a day and can be used with or without ritonavir (RTV) although co-administration of RTV is preferred (8). The current techniques for quantitation of ATV (as well as all other PIs) are plasma or serum based analytical procedures. These procedures require specific processing of samples and specialized equipment. Measurement of plasma concentration requires the drawing of venous blood followed by immediate processing to obtain plasma and freezing of the sample. Specialized equipment used to measure drug concentrations is expensive and frozen samples typically are shipped to a centralized lab. These issues limit the ability to collect samples for quantitation of PI concentrations in both resource high and low areas.
Dried blood spot assays have been available for decades in neonatal screening for inborn errors in metabolism (15). The advantages of DBS techniques include the ease of acquisition, transport, and ability to be obtained in varied settings. Similarly, a DBS matrix for measurement of drug concentrations offers advantages over the conventional plasma matrix. The objectives of this work were to develop a DBS assay for quantitation of ATV concentrations, and to compare this method with a validated, externally quality-controlled HPLC method for ATV quantitation in plasma in patients on chronic, stable doses of an ATV-containing antiretroviral (ARV) regimen.

MATERIALS AND METHODS

Patients

Patients were recruited from the HIV Clinic of the University of Nebraska Medical Center from January to March 2009. Entry criteria included HIV infection; age greater than 19 years; receipt of ATV (with or without RTV) for at least 7 days prior; and HIV RNA <50 copies/mL for the last 90 days. Persons were excluded who had any intra-current illness that might interfere with the interpretation of the study. Demographic information and complete medication lists were obtained. Race and ethnicity data were from self-report. Patients were queried regarding the timing of their last 2 doses of ATV, and the number of missed doses in the last 7 days. The study was approved by the University of Nebraska Medical Center Institutional Review Board, and each participant gave informed consent.

Samples

At varied and random times after a reported dose of ATV, a single whole blood sample was obtained from each study participant in EDTA-containing tubes and processed to obtain plasma. Plasma specimens were frozen at -70°C. After sterile cleaning of the skin, single DBS
samples were obtained directly from the same patients via lancet puncture. Five 1 cm areas on filter paper (Protein Saver™ 903® Card, Whatman Inc, Piscataway, NJ), were saturated with whole blood, air-dried in the horizontal position and placed in sealed plastic bags for storage. All samples were stored until study enrollment was complete at which time both DBS and plasma samples were analyzed for ATV concentrations. All measurements of CD4 cell counts and HIV-1 RNA were performed as part of standard patient care. The detection limit of the HIV-1 RNA assay was 50 copies/mL.

Analytical Methods

The analysis of plasma ATV concentrations was performed using a previously developed and validated simultaneous reversed-phase high performance liquid chromatographic (HPLC) assay. The HPLC system included a Waters Alliance 2690 separations module with a Waters 2487 dual wavelength UV absorbance detector (Waters Corp., Milford, MA). Following the addition of internal standard (A86093, Abbott Laboratories, North Chicago, IL), a liquid-liquid extraction procedure using methyl tert-butyl ether at a basic pH was used to prepare the samples. The chromatographic separation of the compounds and the internal standard was accomplished on a YMC Octyl (C8) 120Å, 100 x 4.6 mm column with a 3 micron particle size (Waters Corp., Milford, MA). The mobile phase consisted of 54.7% 20mM acetate buffer, pH 4.9/45.3% acetonitrile, with an isocratic flow rate of 1 mL/min. Detection and quantitation of ATV occurred at 212 nm. The assay was linear in the range of 20 ng/mL to 20,000 ng/mL with a minimum quantifiable limit (LLOQ) of 20 ng/mL using 0.200 mL of human plasma. Inter- and intra-day accuracy and precision were within ±20% at the LLOQ and ±15% at all other concentrations. This assay has undergone assay validation according to FDA guidelines and is tested twice yearly by participation in an externally administered proficiency program. A series
of 5 blinded samples are tested for ATV concentrations and the results have to be within 20% of the target concentration on 3 of 5 in order to pass the round.

Our initial approach to development of a DBS method for ATV was to modify our HPLC plasma assay for the DBS matrix. We tested our standard curve and measured a series of quality controls and validation samples prepared by spiking whole blood. The QC concentrations were 75, 750 and 7500 ng/mL and the validation sample concentrations were 20 (LLOQ), 60, 1800 and 18000 ng/mL. The experiment was performed over five days with the standard curve, quality controls, and validation samples spiked daily. The overall HPLC back-calculated standard percent accuracy was $\leq 5.2\%$ for standards above the LLOQ and $3.0\%$ at the LLOQ. The percent coefficient of variation (CV) was $\leq 6.3\%$ above the LLOQ and $5.6\%$ at the LLOQ. The overall quality control variability was $<5\%$ for both percent CV and percent accuracy. The overall validation sample percent accuracy was $\leq 6.5\%$ above the LLOQ and $11.3\%$ at the LLOQ. The percent CV was $\leq 3.7\%$ above the LLOQ and $6.7\%$ at the LLOQ. These assay validation data indicated assay performance characteristics consistent with our plasma assay described above. However, a matrix stability issue was revealed with incubation of a series of spotted spiked DBS samples at 4°C, ambient temperature and 35°C at concentrations of 40, 1000 and 10000 ng/mL for intervals of 0, 6, 14 and 30 days. An immediate increase in concentration of the low concentration sample incubated at 35°C (+25%) was noted, which increased with time. DBS samples with the lowest ATV concentration were the most sensitive to change. The middle and high concentrations increased to a lesser, but still significant extent at the later time points. The samples at ambient and 4°C increased at a slower but notable rate. We were not able to isolate ATV from the interfering peak and transferred the assay to a reversed-phase ultra high performance liquid chromatography (UPLC) instrument.
The UPLC system included a Waters Acquity separations module with a tunable UV detector (Waters Corp., Milford, MA). The standard curve for the DBS assay was prepared by spiking 980 µL aliquots of purchased whole blood with 20 µL of ATV stock concentrations prepared in 50% methanol to final concentrations between 20 and 20,000 ng/mL. Using 80µL per spot, these aliquots were applied to the same type of filter paper as patient samples to produce five spots. Quality control stocks were prepared in 50% methanol from a separate weighing of ATV at three concentrations covering the range of the assay. These were spiked into whole blood in a similar manner as the standards and again applied to the filter paper as five spots. The five dried blood spots, whether standard curve, QC or patient samples, were punched out using a 5/8-inch tool, combined and transferred to a single culture tube and reconstituted with Type I water produced by a Millipore MilliQ Integral 3 purification system. Following the addition of internal standard, a liquid-liquid extraction procedure using methyl tert-butyl ether at basic pH was used to prepare the samples. The chromatographic separation of the compounds and the internal standard was accomplished using an Acquity UPLC HSS 100 x 2.1mm column with a 1.8 micron particle size (Waters Corp., Milford, MA). The mobile phase consisted of 41.0% 20mm sodium acetate buffer, pH 4.9/23.0% methanol/36.0% acetonitrile with an isocratic flow rate of 0.6 mL/min. Detection of the ATV peak occurred at a wavelength of 212 nm with a retention time of 3.3 min. The assay was linear in the range of 20 ng/mL to 20,000 ng/mL with a LLOQ of 20 ng/mL using five 5/8-inch punches. These conditions successfully isolated ATV from the interfering peak found with HPLC. A series of six different lots of whole blood matrix were spiked with low and high QC concentrations to test for potential matrix effects. In contrast with the matrix effects observed with the HPLC method, no apparent effects were noted with the UPLC method.
Statistics

We planned to obtain 50 paired samples from 50 subjects. This sample size was based upon guidance from the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), who recommend 40 patient samples to be run with both the new analytical procedure and the reference method (27). We chose to inflate this number to account for any subject having a concentration of ATV below the limit of quantitation (BLQ).

ATV dose, time of sample collection post dose, and plasma and DBS concentrations were compiled and summary statistics computed. The approach recommended by Bland and Altman was used for comparison of the two methods for quantitation of ATV, as well as linear regression (4). ATV concentrations that were BLQ were assigned a value of 5 ng/mL for method comparison purposes and for investigation of relationships between ATV concentrations and HIV-RNA. The Wilcoxon rank-sum test was used to compare ATV concentrations in subjects who had plasma HIV-RNA < 50 copies/mL with those who did not.

RESULTS

Patients

Forty-eight patients consented to participate in this study. The characteristics of the 48 patients enrolled are summarized in Table 1. Demographics were consistent with the population served at the clinic with 60% of patients being white and 81% male. 85% of patients had HIV RNA levels <50 copies/mL. Seven (15%) patients had detectable viral loads at the time blood samples were obtained for evaluation of ATV concentrations, despite the requirement for an undetectable viral load for the previous 90 days for entry into the study. Mean CD4+ cell count was 515 cells/mm³. Forty-three patients were taking 300 mg ATV plus RTV 100 mg daily (ATV300/r) and five were taking 400 mg ATV daily (ATV400). All but one individual was
taking other ARV medications, with the three most common combinations being
tenofavir/emtricitabine (24 patients); tenofavir and abacavir (10 patients); and
abacavir/lamivudine (five patients). All patients taking tenofavir were on RTV-boosted ATV.
No patients were taking acid blocking medications at the time of evaluation, although one patient
was on didanosine. Fifteen percent of patients reported missing at least one dose of ATV in the
last 7 days.

Atazanavir Concentrations

ATV concentrations measured in plasma (by HPLC) are shown in Table 2. Plasma
concentrations of ATV exhibited significant variability with patients on ATV400 (N=5) having a
mean concentration of 465 ng/mL (range <20 to 787 ng/mL) and those on ATV300/r (N=43)
having a mean concentration of 1629 ng/mL (range <20 to 7223 ng/mL). Three persons had
undetectable ATV concentrations, but only one of these three patients reported non-compliance
in the last seven days and then only admitted to missing a single dose of ATV.

Figure 1 shows the relationship between ATV concentrations quantified in plasma and
from DBS (R² = 0.988). Figure 2 shows a Bland Altman plot of the percent difference in ATV
concentrations (DBS concentration-plasma concentration) between the two methods, versus the
mean ATV concentration of the two methods. In this analysis, the mean percent difference in
ATV concentrations between the two methods was -10.8% (95% CI, -7.65% to -13.95%)
indicating the DBS method has a slight negative bias. 97.8% of the differences in concentrations
between the two assays fell within +/- 2 standard deviations. Figure 3 presents ATV plasma
concentrations in subjects who had HIV RNA < 50 copies/mL versus those who did not. ATV
concentrations were lower in subjects who had detectable HIV RNA in plasma compared with
those with HIV RNA < 50 copies/mL (p=0.03, Wilcoxon rank-sum test). The average ATV
concentrations in subjects with HIV RNA < 50 copies/mL was 1582 ng/mL (interquartile range, 197
593 ng/mL to 2156 ng/mL) compared with 543 ng/mL (interquartile range, 219 ng/mL to 713
ng/mL) in subjects with HIV RNA > 50 copies/mL. All patients with HIV RNA > 50 copies/mL
were on RTV boosted regimens. The average time that samples were collected after the last dose
of ATV was 16.3 hours in those with HIV RNA < 50 copies/mL and 15.6 hours in those with
detectable HIV RNA.

DISCUSSION

In this report we describe the development of a DBS assay to determine ATV
concentrations. The concentrations of ATV quantified in a DBS matrix measured by UPLC
showed very strong agreement with those measured in plasma by HPLC. The mean difference of
-10.8% with a 95% confidence interval of -7.65% to -13.95% indicate a negative bias of the DBS
method. This bias is sufficiently small to provide high confidence in an analytical result
measured via the DBS method and is suitable for clinical use. The minimal effective
concentration of ATV to prevent HIV viral replication is estimated to be 150 ng/mL (31).
Trough ATV concentrations average approximately 800 ng/mL with the ATV/r 300/100 once
daily regimen. Thus, the magnitude of separation among the usual trough, the threshold trough
and the 20 ng/mL LLOQ of the assay is wide enough that a -10% bias would not lead to a
different clinical interpretation. Use of this assay in formal pharmacokinetic studies would
simplify sample acquisition, and a correction factor of 10% to better correlate with
concentrations measured in plasma could be easily integrated. The source of the bias in the DBS
method is not known; potential sources of variation have been discussed in a recent review paper
(24).
To our knowledge, only two prior studies have been published describing the quantification of antiretroviral drug concentrations using a DBS assay (20,34). Koal and colleagues used simultaneous liquid chromatography/tandem mass spectrometry to evaluate plasma and DBS samples for lopinavir, ATV, RTV, saquinavir, and efavirenz concentrations in 70 patients. A summary correlation curve of all five ARV drugs tested showed a negative bias of approximately 15%, and a high correlation with plasma concentrations ($R^2 = 0.9772$). The use of UPLC in the present method allowed the resolution of the ATV peak from endogenous interfering peaks and reduced analysis time and solvent usage. Our DBS-UPLC assay using five 5/8-inch punches achieved the same lower limit of quantitation of 20 ng/mL as the plasma HPLC assay, which has been shown to be sufficient for pharmacokinetic studies in adults and children (33,19). Finally, UPLC instrumentation has a considerably lower acquisition cost than a triple quadruple mass spectrometer making it more readily available.

In the subjects who participated in this study, ATV concentrations were lower in those who had detectable levels of HIV RNA in plasma compared with those who had undetectable HIV RNA. The association between the lack of viral suppression and lower ATV concentrations, as found in our study, has been observed by others. Alexander and colleagues evaluated untimed plasma samples for ARV concentrations in 122 patients initiating ARV therapy and found a single “low” concentration within the first 6 weeks to be associated with more-rapid immunologic failure and failure to achieve virologic success in the first year (2). In an evaluation of 210 patients with HIV infection, Oette and colleagues noted that both adequate unscheduled drug concentrations and adequate trough concentrations of ARV were associated with virologic success at 12 weeks (28). Low ARV concentrations are often surrogate markers of poor medication adherence (33). In contrast, patients in our trial with non-suppressed HIV
RNA had not reported any lack of adherence in the last seven days. Interestingly, all six patients who admitted to non-adherence had completely suppressed HIV RNA levels despite one patient having completely undetectable ATV concentrations. ATV concentrations were generally lower in those who reported poor adherence.

There has been recent interest in the use of DBS assays for diagnosis and monitoring of patients with HIV infection, particularly in low income countries (25). DBS were first used to detect HIV infection in post-natal sero-surveillance studies (17). Their use in HIV has expanded to include nucleic acid detection, viral load determination, and drug resistance genotyping (36, 27, 5). DBS offers a number of advantages over conventional methods which involve venous puncture followed by rapid plasmid extraction and freezing for transport to a reference laboratory for further analysis. DBS are obtained without venous puncture or the use of trained phlebotomists, nor do they require on-site processing such as rapid centrifugation or a robust cold chain. DBS methodology also minimizes risk of potential occupational exposure to infected blood products, and after sample acquisition, DBS can be stored at room temperature for at least 7 days and transported via traditional postal means to reference laboratories (34). The collection and processing of blood samples for ARV concentrations in the setting of routine patient care is challenging regardless of the geography. DBS samples allow a bypass of this difficulty as they may even be obtained directly by patients and submitted via postal system for analysis at distant sites. A report describing blood samples self-obtained by patients and spotted onto filter paper every two weeks to monitor the antiepileptic agent lamotrigine during pregnancy is an illustration of the utility of a DBS assay (6).

The utility of DBS samples may enhance the ability to evaluate ARV pharmacokinetics at sites distant to reference laboratories, allowing for TDM at any site. While TDM is not
recommended for routine use it may be useful in managing significant drug-drug and drug-food
interactions, identifying non-adherence, evaluating patients with poor virologic response despite
good adherence, adjusting ARV dosing in patients with organ dysfunction, identifying patients
with adverse effects due to increased drug concentrations, maximizing therapeutic drug levels in
treatment-experience patients with resistant virus and reduced susceptibility to ARV, and dosing
guidance in children and pregnant women (8,22). Challenges for the implementation of TDM
include the confounding of non-adherence, the paucity of controlled trials demonstrating the
benefit of TDM, and data identifying the pharmacokinetic parameter and concentration threshold
that best predicts ARV response (1, 14).

In conclusion, our study demonstrates that ATV quantitation in a DBS matrix is both
feasible and accurate, and may have utility in both clinical practice and pharmacokinetic studies.
Further evaluation of this technology is warranted for other antiretroviral agents.
ACKNOWLEDGMENTS

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diagnosis and viral load monitoring at rural and remote healthcare facilities. AIDS.

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TABLE 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>No. of subjects (%)</td>
<td></td>
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<tr>
<td>Mean Age in years (range)</td>
<td>45 (23-64)</td>
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<tr>
<td>Race/Ethnicity</td>
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<tr>
<td>White</td>
<td>29 (60)</td>
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<tr>
<td>Black</td>
<td>9 (19)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>10 (21)</td>
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<tr>
<td>Male</td>
<td>39 (81)</td>
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<tr>
<td>CD4+ Lymphocyte count/mm³ Mean (range)</td>
<td>515 (79-1312)</td>
</tr>
<tr>
<td>&lt;200</td>
<td>5 (10)</td>
</tr>
<tr>
<td>201-350</td>
<td>8 (17)</td>
</tr>
<tr>
<td>≥351</td>
<td>35 (73)</td>
</tr>
<tr>
<td>HIV RNA level</td>
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<tr>
<td>&lt;50 copies/mL</td>
<td>41 (85%)</td>
</tr>
<tr>
<td>≥50 copies/mL</td>
<td>7 (15%)</td>
</tr>
<tr>
<td>≥1 missed dose of medication in last 5 days</td>
<td>6 (13%)</td>
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### TABLE 2. ATV Plasma Concentrations

<table>
<thead>
<tr>
<th>ATV Dose</th>
<th>Number of Patients</th>
<th>Average Time Post Dose in hours</th>
<th>ATV Plasma Concentrations in ng/mL</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>400 QD</td>
<td>5</td>
<td>13.06</td>
<td>465</td>
</tr>
<tr>
<td>300 QD</td>
<td>43</td>
<td>16.60</td>
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</table>

- SD = standard deviation
- QD = once daily
- BLQ = below the limit of quantitation
Figure 1. Atazanavir concentrations as measured in plasma and from dried blood spots (DBS). The solid line is the line of identity. The dashed line is the line of best fit \( y = 0.89x - 18.3; \) \( r^2 = 0.988 \).
The difference is the DBS concentration minus the plasma concentration. The mean atazanavir concentration is the mean of the values measured in plasma and from DBS. The mean difference is -10.8%. 97.8% of the differences are contained within two standard deviations of the mean difference as bracketed with the dashed lines. For illustration purposes only, one concentration at >7000 ng/mL is not shown.
FIGURE 3. ATV Concentrations by HIV RNA level

Figure 3. Atazanavir concentrations vs. HIV RNA in plasma.

Atazanavir concentrations in plasma vs. the level of HIV RNA in plasma at the time the atazanavir concentrations were obtained.