The development and validation of a method using high-resolution mass spectrometry (HRMS) for the qualitative detection of antiretroviral agents in human blood

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Abstract

Background—Antiretroviral drugs are used for the treatment and prevention of HIV infection. Non-adherence to antiretroviral drug regimens can compromise their clinical efficacy and lead to emergence of drug-resistant HIV. Clinical trials evaluating antiretroviral regimens for HIV treatment and prevention can also be compromised by poor adherence and non-disclosed off-study antiretroviral drug use. This report describes the development and validation of a high throughput, qualitative method for the identification of antiretroviral drugs using high-resolution mass spectrometry (HRMS) for the retrospective assessment of off-study antiretroviral drug use and the determination of potential antiretroviral therapy (ART) non-compliance.

Methods—Serum standards were prepared that contained 15 antiretroviral drugs: 9 protease inhibitors (PIs), 4 nucleotide/nucleoside reverse transcriptase inhibitors (NRTIs), and 2 nonnucleoside/nucleotide reverse transcriptase inhibitors (NNRTIs). Analytical separation was achieved on a Hypersil Gold PFP (100 × 3 mm) column and the eluent was analyzed using the Thermo Exactive Orbitrap mass spectrometer (Exactive-MS) operated in full scan mode. Limit of identification, signal intensity precision, retention time analysis, selectivity, and carryover studies were conducted. Concordance with liquid chromatographic-tandem mass spectrometric (LC-MS/MS) methods was evaluated using remnant plasma samples from a clinical trial.

Results—The limit of identification ranged from 5-10 ng/ml for 14 drugs (9 PIs, 1 NNRTI, 4 NRTIs) and was 150 ng/ml for 1 NNRTI. Precision studies with high and low control mixtures revealed signal intensity coefficients of variation of 3.0-27.5%. The Exactive-MS method was

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selective for the compounds of interest. Overall, concordance ranged from 89.1%-100% for the screening of antiretroviral drugs in clinical plasma specimens as compared to LC-MS/MS methods.

Conclusion—Using the Exactive-MS, we developed and validated a highly selective, robust method for the multiplexed detection of 15 antiretroviral drugs.

Keywords

antiretroviral; clinical trials; high-resolution mass spectrometry; Exactive-MS; validation

1.0 Introduction

An estimated 34 million people are living with human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) worldwide, with approximately 2.5 million new infections occurring annually [1]. Numerous antiretroviral drugs are approved for HIV treatment by the United States Food and Drug Administration (FDA) in single drug and multi-drug formulations [2]. The most widely used classes of antiretroviral drugs are nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) [3,4]. Antiretroviral drugs were first introduced in the late-1980s; however, subsequent research demonstrated that potent, multi-drug regimens are needed for durable suppression of viral replication and clinical benefit [3]. Most antiretroviral treatment regimens include two NRTIs with an NNRTI or PI [4,5]. Newer classes of antiretroviral drugs include entry inhibitors, fusion inhibitors, and integrase inhibitors [6,7].

While antiretroviral drugs are effective for treatment of HIV infection, non-adherence to treatment regimens can lead to treatment failure and emergence of drug-resistant HIV strains [8,9]. Adherence counseling is an important component of HIV treatment. However, the complexity of treatment regimens, adverse events, and other factors can reduce adherence [9,10]. Antiretroviral drugs are also used for HIV prevention in a variety of settings, including prevention of mother-to-child transmission, pre-exposure prophylaxis in HIV-uninfected individuals (PrEP), and "treatment for prevention" in HIV-infected individuals [11-13]. A variety of approaches have been used for adherence monitoring in clinical trial settings (e.g., self-report, pill counts, electronic monitoring, monitoring pharmacy refills); however, each of these approaches has limitations [14,15]. Furthermore, some participants in clinical trials may not disclose that they are using antiretroviral drugs provided outside of the study [16,17]. For example, in the HIV Prevention Trials Network (HPTN) 052 study [18], some HIV-infected participants who reported that they were antiretroviral drug naïve at study enrollment had antiretroviral drugs detected in their blood that indicated that they were on antiretroviral treatment [17].

The limitations of self-report and other approaches used for adherence monitoring highlight the need for rapid, high-throughput methods that can be used for the direct detection of antiretroviral drugs in clinical and research settings. Several methods are available for the quantification of a single antiretroviral agent in plasma. The majority of these approaches involve liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analysis in

selected reaction monitoring (SRM) mode. However, since antiretroviral regimens almost always include a combination of drugs, and since study participants may access a variety of antiretroviral medications outside of clinical trials, laboratory methods are needed that can detect a large number of antiretroviral drugs, ideally in a single analytical run. To address this need, sensitive methods have been developed for simultaneous quantification of 12-18 drugs (including PIs, NRTIs, and NNRTIs) in a single chromatographic run using LC-MS/MS techniques [19-21]. However, those methods may not include newer antiretroviral drugs [19,20]. Further, while SRM-based quantitative analysis is useful for pharmacokinetic studies, qualitative, multiplexed methods may be more suitable for evaluation of non-compliance or off-study use of antiretroviral drugs, similar to qualitative methods used for monitoring pain management regimens, as well as drugs of abuse. With existing challenges to antiretroviral therapy (ART) adherence, including healthcare systems and socioeconomic factors, qualitative identification of drugs to assess compliance is warranted [22]. Therapeutic drug monitoring of antiretroviral classes is dependent on inter-individual pharmacokinetic variability, as well as the identification of a range that discriminates a therapeutic drug concentration from a toxic concentration. Because the treatment of HIV infection requires multi-drug regimens that may act antagonistically or synergistically, and several collections may be needed to understand the pharmacokinetic parameters of the drug, routine quantitative monitoring of antiretroviral drugs is not commonly performed [23].

Many methods currently available for drug monitoring use a triple quadrupole mass analyzer operated in SRM mode. In this report, we exploit high-resolution mass spectrometry (HRMS) for qualitative drug detection; HRMS has previously been pursued for the identification of other compounds in biological matrices [24-28]. An advantage of this approach is that instruments used for HRMS may be operated in a full scan mode, in which all ions are detected and analyzed by the mass spectrometer. A major benefit of using HRMS for antiretroviral drug detection is that sample data may be retrospectively analyzed for analytes that were not part of the initial screening assessment (e.g., other antiretroviral drugs, substances of abuse); this increases the flexibility of data collection and review. Current analyzers that offer high resolution accurate mass determination include time of flight (TOF) instruments, as well as instruments using Orbitrap technology. The Orbitrap consists of an outer barrel-like electrode and a co-axial central electrode; ions within the Orbitrap oscillate around this central electrode [29]. Mass to charge ratios are measured from the frequency of harmonic ion oscillations around this central electrode along the axis of the electrostatic field [29, 30]. The frequency of these oscillations are detected and transformed into mass spectra via Fourier-transformation [29]. The Orbitrap offers high mass accuracy set at thresholds of $\pm$ 5 ppm of theoretical mass values [29]. Currently, the Thermo Exactive mass spectrometer (Exactive-MS) uses the Orbitrap for HRMS; the Exactive-MS also contains a higher-energy collision cell, in which trapped ions may be subjected to high-energy collision-induced dissociation potentials, generating fragment ions [31]. Fragmentation may also occur at the ionization source through the application of increased voltage. The work described in this report uses the Exactive-MS for the development, validation and application of a method for the retrospective, simultaneous, qualitative detection of 15 antiretroviral agents used for HIV treatment and prevention.

2.0 Experimental

2.1 Chemicals

The antiretroviral agents monitored by this method include PIs [amprenavir (APV), atazanavir (ATV), darunavir (DRV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV)]; NRTIs [emtricitabine (FTC), lamivudine (3TC), tenofovir (TFV) and zidovudine (ZDV)]; and NNRTIs [efavirenz (EFV) and nevirapine (NVP)]. These drugs were obtained as stock powder solutions through the AIDS Research and Reference Reagent Program (Pathogenesis and Basic Research Branch, Division of AIDS, NIAID, NIH; https://www.aidsreagent.org/index.cfm). Stock solutions were reconstituted in methanol or water (TFV and ZDV), depending on solubility, to master stock concentrations of 1 or 2 mg/ml. To ensure proper sample preparation and Exactive-MS analysis, an external reference molecule (morphine-D3, Toronto Research Chemicals, North York, ON) was included. Drug-free human serum was acquired from BioRad Laboratories (Irvine, CA). Formic acid was from Sigma-Aldrich. Drug-free human K2 EDTA plasma was acquired from Bioreclamation. Acetonitrile, acetone and methanol (all Optima-grade), and high performance liquid chromatography (HPLC)-grade water were from Fisher Scientific. HPLC-grade isopropanol was rom JT Baker.

2.2 Preparation of Reagents and Standards

To identify the specific retention times and fragmentation patterns for each analyte, working stock solutions were prepared from master stocks to final concentrations of 100 μg/ml and 10 μg/ml in 1:1 methanol:water. For each analyte, diluted stocks (1,000, 500, 250, 150, 100, 50, 25, 10 and 5 ng/ml) were prepared by spiking human serum with appropriate volumes from working solutions. After determining analyte-specific retention times and confirmation of fragment ions, when applicable, the 15 antiretroviral agents were combined from master stock solutions and prepared to the aforementioned final concentrations by spiking human serum or K2 EDTA plasma with appropriate volumes from combined working solutions. A standard mixture of morphine-d3 was prepared in acetonitrile at a final concentration of 125 ng/ml. Sample preparation involved combining 100 μl of spiked serum or subject specimen with 300 μl of acetonitrile containing morphine-d3, an easily-ionized species that was used to assess processing and analytical integrity of a specimen. The mixture was then briefly vortexed and centrifuged at $12,000 \times g$ for 5 min at room temperature. Entire supernatants were evaporated to dryness using a Biotage SPE Dry 96 well plate dryer with the application of constant airflow, and subsequently reconstituted in 150 μl water; 30 μl of reconstituted samples were subjected to chromatographic separation.

2.3 Instrument and Acquisition Parameters

The liquid chromatography system consisted of an Aria TLX1 system (Thermo Fisher Scientific) equipped with a CTC HTC PAL Autosampler with a sample stack maintained at 4°C and 2 Transcend pumps. The TLX1 chromatography system was also configured with two 6-port switching valves controlled by the Aria OS software (Thermo Fisher). The autosampler was programmed to inject 30 μl of sample into the TLX1 system. Analytical separation was achieved using the Thermo Scientific Hypersil Gold PFP, 100×3 mm column, with a 3 μm particle size (Thermo Fisher). Mobile phase A consisted of water with

0.1% acetic acid, while mobile phase B consisted of acetonitrile with 0.1% acetic acid. The chromatographic run began with 30 sec of mobile phase A, followed by a 60 sec ramp to 10% mobile phase B. This slow ramp facilitated the elution of water-soluble analytes. The chromatographic separation continued with a step to 15% mobile phase B followed by a ramp to 95% mobile phase B over 600 seconds. Following the elution of all analytes, the column was washed for 60 sec with a 2:2:1 ratio of isopropanol:acetonitrile:acetone. The column was then re-equilibrated for 180 sec with mobile phase A. The total analytical run time for this method is 16.0 minutes and occurs at a flow rate of 500 μl/minute.

Detection of antiretroviral agents was performed using the Exactive-MS (Thermo Fisher) with a heated electrospray-ionization source in positive ionization mode and full scan mode. The Exactive-MS method included two scan events in positive polarity: one full scan event with ultra-high resolution (100000 @ 1Hz) and an additional scan event with in-source collision-induced dissociation (SCID) at 45eV with enhanced resolution (25000 @ 4Hz). All scan events were programmed for 100 msec maximum injection time and balanced automatic gain control (AGC) intensity targets. Additionally, instrument parameters were optimized, including sheath gas flow rate (20), discharge current (5 μA), capillary temperature (250°C), capillary voltage (10 V), tube lens voltage (140 V), skimmer voltage (12 V) and vaporizer temperature (250°C), through the analysis of an extracted 500 ng/ml ARV mixture prepared in serum. Since this method includes a variety of structurally dissimilar compounds, the mass spectrometer parameters identified as optimal were based on the highest signal intensity and fragment identification for analytes of interest. The aforementioned parameters were optimal for protease inhibitors, as well as several NRTIs and NNRTIs, including 3TC and FTC (NRTIs), and NVP (NNRTI). Antiretroviral molecular structures, precursor ion m/z ([M+H$^+$]), and monitored fragments, when applicable, are described in Table 1.

2.4 Data Evaluation

Data analysis included automated review using the ToxID 2.1.1 and Xcalibur 2.2 programs (Thermo Fisher). Using the ToxID 2.1.1 data review software package, antiretroviral drug detection was evaluated in an automated manner, based on the presence of exact mass (± 5 ppm from theoretical mass), retention time (± 0.4 min from control standards), and the presence of fragments (with the exception of EFV, in which no fragment ions were observed). The presence of fragments was based on the identification of a fragment ion with an exact mass (±5 ppm from theoretical mass) as well as the identification of the fragment at the same retention time as the precursor drug. It should be noted that while signal intensity was not used as a specific acceptance criteria, identified fragments yielded signal intensities approximately one order of magnitude lower than the intensity observed for the precursor compound. Signal intensity thresholds were based on validation metrics, including limit of identification and carryover studies.

Fragment prediction was determined using the MassFrontier 7.0 (Thermo Fisher) prediction utility, which determines fragmentation based on theoretical models, as well as fragments based on a software-provided library containing known published fragmentation data. While alternative approaches, including the direct infusion of a neat mixture of an ARV agent,

could also be used to empirically select for fragment ions, prediction software was employed to identify species that result from fragmentation at susceptible chemical bonds and particular structural moieties. This approach avoids the inclusion of fragments based on potential adducts or fragments formed through secondary decompositions involving randomized rearrangements, which do not denote specific structural moieties, thereby reducing the reporting of falsely positive results. Predicted fragments were confirmed via spectral deconvolution, in which the ion spectra from the higher energy in-source CID scan event were evaluated for the presence of predicted fragment ions.

Potential positives for EFV and ZDV, which did not consistently elicit fragments, were manually confirmed using the XCalibur 2.2 software. The presence of the analyte at the correct retention time ($\pm$ 0.4 min) was assessed using post-processing mass filters with a $\pm$ 5 ppm threshold from theoretical mass. This manual review was performed for EFV because no fragment ion was identified, and for ZDV because fragmentation was not observed at concentrations <50 ng/ml.

3.0 Method Validation

The validation metrics assessed for this qualitative method included: analyte signal and retention time within-run precision, limit of identification, carryover, selectivity, and method concordance studies.

3.1 Precision

Within-run precision was evaluated for each analyte when standards were run five times within a single run as: (1) a high control mixture containing 1000 ng/ml of all analytes, and (2) a low control mixture containing 400 ng/ml of EFV and 50 ng/ml of the remaining 14 analytes. Mean signal intensities and retention times, as well as standard deviations (SD) and coefficients of variation (% CV), were assessed at each level. Within-run precision studies were also conducted for the morphine-d3 external reference molecule.

3.2 Limit of Identification and Carryover Analysis

For limit of identification (sensitivity) studies, antiretroviral agents were prepared at the aforementioned concentrations in drug-free human serum. The limit of identification was based on the lowest concentration at which a precursor ion was observed using a $\pm$ 5 ppm threshold from theoretical mass, retention time, and the presence of fragments. Notable exceptions for fragment identification include EFV at all analyzed concentrations, and ZDV at concentrations <50 ng/ml. The limit of identification was further established by comparing the signal intensity threshold of the lowest concentration that met the aforementioned criteria with a post-injection serum blank following an extracted specimen containing a 2.5 μg/ml mixture of the 15 antiretroviral agents.

Further, to illustrate comparability between plasma and serum matrices, ARV mixtures were spiked into drug-free human plasma to yield final concentrations of 1,000, 500, 250, 150, 100, 50, 25, 10 and 5 ng/ml of each antiretroviral drug. Samples were extracted as previously described and analyzed in parallel with serum-spiked specimens at the aforementioned concentrations. Samples were analyzed in triplicate and observed mean

analyte peak areas, as well as SDs and %CVs were calculated across each level. Further, a % difference was determined by calculating the difference between the plasma-spiked mixture and the serum-spiked mixture, divided by the serum-spiked mixture, and multiplied by 100.

3.3 Selectivity

To assess assay selectivity, five specimens free of antiretroviral agents were extracted as previously described. Results were reviewed using both the automated ToxID 2.1.1 and manual Xcalibur 2.2 software modules. Additionally, a 250 ng/ml ARV mixture was spiked into a series of solutions containing varying levels of bilirubin, hemoglobin, and lipids. Samples were extracted in triplicate and analyzed using the Exactive-MS method. A significant interference was determined as a decrease in peak area for an ARV drug >50% as compared to analyte peak area spiked into a solution with no interferent present. Sample indeces were determined on a cobas® series analyzer (Roche Diagnostics).

3.4 Method Comparison

Sixty-four plasma specimens were obtained from the HIV Trials Prevention Network (HPTN) 052 study [18]. These specimens were previously analyzed by the Clinical Pharmacology Analytical Laboratory (Division of Clinical Pharmacology, Johns Hopkins University School of Medicine) [17]. The previous analysis involved 3 LC-MS/MS methods (3 separate assays) using a triple quadrupole mass analyzer. One LC-MS/MS method identified the following analytes (PIs and NNRTIs) with a cutoff of 100 ng/ml: APV, ATV, DRV, EFV, IDV, LPV, NFV, RTV, SQV and TPV; a second LC-MS/MS method detected the NRTI 3TC and the NNRTI NVP, with a limit of identification of 20 ng/ml; a third LC-MS/MS method detected the NRTIs FTC, TFV, and ZDV with a limit of identification of 5 ng/ml. In this study, these plasma specimens were analyzed in a blinded manner using the qualitative, Exactive-MS method described above. Overall concordance between the two approaches was determined as the number of specimens that showed agreement based on the limits of identification for the Exactive-MS and the LC-MS/MS methods.

4.0 Results and Discussion

4.1 Full-Scan Ion Acquisition

In this work, the Exactive-MS was used for the qualitative monitoring of 15 antiretroviral agents. The described method was developed to retrospectively test blood specimens for off-study use of antiretroviral drugs in a clinical study, and may be applied to assess ART non-compliance. The Exactive-MS was operated in full scan mode, with two positive scan events. A representative total ion chromatogram of antiretroviral agents spiked into drug-free human serum is shown in Fig. 1. Since all ions are recorded using this approach, the Exactive-MS allows for the retrospective detection of analytes of interest. This non-targeted ion collection offers enhanced flexibility for post-hoc data analysis and specimen querying. HRMS has been used in the analysis and detection of synthetic cannabinoids, as well as organic pollutants and drugs of abuse [25-28,32,33,34]. By extension, study specimens analyzed for the presence of antiretroviral agents could also be retrospectively queried for other compounds (e.g., substances of abuse). The developed method was designed for the rapid and robust identification of antiretroviral drugs in a qualitative manner. Targeted

quantitative analyses could be performed using SRM-based LC-MS/MS methods with the inclusion of an isotopically-labeled internal standard or structural analog.

4.2 Analyte Identification Criteria

Antiretroviral agent detection in the raw data form was based on expected retention time, mass accuracy, and the presence of at least one fragment ion, with the exceptions of EFV and ZDV concentrations <50 ng/ml. Analyte detection was based on strict mass accuracy ($\pm$ 5 ppm) and retention time ($\pm$ 0.4 min) tolerance thresholds. These tolerance thresholds have been previously described and implemented in the qualitative monitoring of large panels of molecules using HRMS [25, 35]. The retention time and mass accuracy thresholds provide high selectivity in antiretroviral identification and reduce the probability of reporting false positives. An external reference molecule (morphine-d3) was added during initial protein precipitation, to monitor sample and analytical quality. This approach has been utilized in previously reported Exactive-MS methods [36,37].

Retention time windows were determined via the analysis of each antiretroviral agent individually on the Exactive-MS. Using the Xcalibur 2.2 software, retention time and mass filters were applied to view ion spectra and chromatograms, respectively. Fragment ions for the 15 ARVs, with the exception of EFV, were determined using the fragment prediction module of MassFrontier version 7.0; fragment ions were confirmed via spectral deconvolution (described in the methods section, Table 1). For each protonated molecular species, [M+H$^+$], a mass filter was applied with a mass accuracy tolerance of ($\pm$ 5 ppm) from theoretical mass. Using parameters offered by the Xcalibur 2.2 software module, ion spectra were reviewed to identify fragment ions across the scanning range of the precursor peak. Fragment ion generation was achieved via the incorporation of an enhanced resolution scan event performed during in-source collision-induced dissociation (SCID). The fragment ions used for antiretroviral identification are consistent with fragment ions observed in the literature.

An example of ion spectra and mass filtering for the PI, RTV, ritonavir (RTV) and the NRTI, 3TC, are depicted in Fig. 2 and Fig. 3, respectively. Using the aforementioned thresholds, ion spectral analysis for RTV ($CH_{37}H_{48}N_6O_5S_2$) depicts the [M+H$^+$] protonated species at 8.73 minutes (Fig. 2A). Analysis for abundant fragment ions at that retention time in the 45 eV SCID scanning event depicts two well-characterized fragment ions of RTV (Fig. 2B) [38]. Applying the appropriate mass filters to the total ion chromatogram indicates the presence of the precursor RTV molecule at 8.73 minutes, as well as the two fragment peaks (Fig. 2C). It should be noted that the configurations of these fragments is tentative, and based on the predicted cleavage of structural moieties; however, the aforementioned fragment ions, at m/z 268 and m/z 296, have been previously used for both RTV identification as well as quantification [38]. This approach is also depicted for the NRTI 3TC (Fig. 3A-3C) [39,40]. While many LC-MS/MS methods indicate a 3TC fragment at m/z 112, our mass spectrometric methods indicate the highest abundance ion was detected at m/z 95 across tested concentrations [40,41].

4.3 Limit of Identification Analysis

Following the establishment of retention time thresholds and fragment ion identification, the Exactive-MS method was evaluated for selectivity, limit of identification, precision and carryover. Selectivity was assessed through the analysis of 5 antiretroviral-negative remnant serum samples. No antiretroviral drugs were detected in the 5 specimens (data not shown), indicating the selectivity of the high resolution accurate mass thresholds and retention time windows for the qualitative detection of antiretroviral agents.

Limit of identification (sensitivity) analysis was determined as the lowest concentration for each analyte at which the previously described identification criteria were met: ± 5 ppm accuracy from theoretical mass, retention time ± 0.4 minutes, and identification of at least one fragment ion. The limits of identification for the protease inhibitors APV, ATV, IDV, LPV, NFV, RTV, SQV, TPV, the NRTI 3TC and the NNRTI NVP were 5 ng/ml. The limits of identification for the NRTIs FTC, TFV and ZDV and the PI DRV were 10 ng/ml. Lastly, the limit of identification for the NNRTI EFV was 150 ng/ml. All antiretroviral agents were detected in positive ionization mode. Due to the structural disparities between antiretroviral compound classes, analytical separation and analyte ionization was optimized for the majority of analytes. Consequently, TFV fragmentation was not observed <10 ng/ml and ZDV fragmentation was not observed <50 ng/ml. In addition to studies in serum, comparison studies were performed in drug-free plasma. There were no significant changes observed with regard to the limit of identification of the analytes across the aforementioned tested concentrations (data not shown). A comparison of coefficients of variation for serum and plasma-prepared samples, as well as the percent difference between matrices is demonstrated for the NRTI FTC in Supplemental Table 1. Based on these data, although serum prepared solutions were analyzed for validation metrics, plasma samples may be evaluated using the described method.

Traditional mass spectrometric methods identify the NNRTI EFV in negative ionization mode, largely due to the presence of a highly electronegative CF_3 constituent (Table 1) [42]. However, consistent with previously published observations, analyte detection in negative ionization mode significantly impacted overall peak shape and sensitivity of the NRTI TFV [43]. Notably, negative ionization mode significantly compromised the generation of the well-characterized TFV fragment ion, which lacks a phosphomethoxy group, and has a mass to charge ratio of m/z 176.0923 [43]. Therefore, the described Exactive-MS method was operated in positive ionization mode. A driving factor in the rationale to optimize the method for TFV is because TFV is commonly used for HIV treatment [5,44] and co-formulated TFV plus FTC (marketed as Truvada®, Gilead Sciences) has been shown to be effective for PrEP in some settings [13]. Further, pharmacokinetic analyses of these drugs indicate a five to ten-fold increase in the steady state concentration of EFV compared to TFV when administered at fixed doses [45,46]. Thus, a 150 ng/ml cutoff was deemed acceptable for the qualitative monitoring of EFV.

4.4 Precision and Carryover Studies

Precision studies were performed on both high (1000 ng/ml all compounds) and low (400 ng/ml EFV and 50 ng/ml all other drugs) mixtures of antiretroviral agents spiked in human

serum (Table 2). For signal intensity, % CVs ranged from 3.0-20.6% for the high control mixture and from 3.0-27.5% for the low control mixture. Across both low and high control mixtures, very little deviation was observed from expected retention times, with a maximum % CV of 2.6%. A full analysis of signal intensity and retention time precision is described in Table 2. It is notable that the highest variability in signal intensity was observed at 50 ng/ml of ZDV, ranging from 2456-5059 in raw signal values. However, due to the high mass accuracy thresholds, there was negligible noise, facilitating the qualitative identification of an antiretroviral drug at lower analyte concentrations. Isotopically-labeled morphine was added to each specimen during the sample preparation step at a concentration of 125 ng/ml. Across 10 injections, the signal intensity and retention time % CVs were 2% and 1%, respectively (data not shown). Upon characterization of retention time and signal thresholds, the identification of all analytes, with the exceptions of the NRTI ZDV and the NNRTI EFV, was conducted using automated peak review via the ToxID 2.1.1 software module (Supplemental Table 2 and Supplemental Fig. 1).

Carryover analysis was performed by preparing a mixture containing 2.5 μg/ml of the antiretroviral samples spiked in drug-free human serum and analyzing a post-injection unspiked specimen. Using the automated peak detection module, a post-injection blank did not identify any of the antiretroviral agents with the previously described signal intensity settings (Supplemental Table 2 and data not shown). Further, the potential impact of excess hemoglobin, lipids, or bilirubin on analyte ionization and detection was assessed. Evaluation of analyte peak areas were performed in solutions containing varying levels of the aforementioned interferents. None of the monitored ARVs were significantly impacted by increased hemolysis or icterus up to indeces of 200 and 30 units, respectively. However, several protease inhibitors, including ATV, IDV, LPV, NFV, RTV, SQV and TPV were impacted by increased lipemia. Modest lipemia (L-index greater than 150 units) resulted in decreased analyte peak area for LPV, NFV, RTV and SQV, while gross lipemia (L-index greater than 300 units) impacted the detection of ATV, IDV and TPV (data not shown). These data support the benefit of assessing specimen quality prior to analysis.

4.5 Concordance Analysis

The Exactive-MS method described above was used to screen for the presence of antiretroviral agents in human plasma samples from the HPTN 052 study, a Phase III randomized trial to assess treatment strategies for preventing the sexual transmission of HIV in serodiscordant couples [17,18]. Plasma specimens from individuals who were reportedly antiretroviral drug naïve and were not on study-administered treatment were previously identified using LC-MS/MS methods [18]. The purpose of that study was to detect undisclosed off-study antiretroviral drug use by study participants. To detect all of the antiretroviral agents included in that sample panel, three separate LC-MS/MS methods were required (see Method Comparison section of Experimental). A subset of the HPTN 052 specimens (n=64) was analyzed using the combined, qualitative, Exactive-MS method described in this report.

Of the 15 antiretroviral agents monitored in the Exactive-MS method, 7 PIs (APV, DRV, IDV, LPV, NFV, SQV and TPV) were not detected in any of the samples by any of the

methods (data not shown); the aforementioned drugs were not commonly used in the countries at the time of the study. It should be noted that samples used for analysis reflect remnant specimens from a large international trial, which includes 13 study sites across 11 counties, and are reflective of a heterogeneous population. Acquiring a sufficient sample set for the additional PIs was not feasible for the method concordance using the HPTN 052 sample cohort. The percent agreement, which was used as a measure of concordance, ranged from 89.1% (for zidovudine) to 100% (ATV, FTC, EFV; Table 3). It should be noted that while the Exactive-MS method was compared to LC-MS/MS methods, the LC-MS/MS methods are still qualitative, and do not include isotopically-labeled standards for all of the monitored compounds. While the triple quadrupole mass analyzers were operated in SRM mode, the selection of fragment ions and overall identification criteria may be disparate from the Exactive-MS identification conditions. Consequently, we were unable to provide sensitivity and specificity metrics to the described Exactive-MS method, and thus report overall agreement with another qualitative LCMS/MS approach for ARV identification.

Of note, for analytes in which there was not 100% agreement, this may be explained by the differing limits of identification for specific ARVs. For example, one specimen screened positive for NVP and one sample screened positive for RTV by Exactive-MS while both samples were negative for the respective drugs by LC-MS/MS, which is consistent with the lower limits of identification of the Exactive-MS method for NVP and RTV detection. Further, the agreement for ZDV identification was 89.1%, which was the lowest of the 8 analytes detected by either method. While the limit of identification of ZDV by Exactive-MS is 10 ng/ml, a fragment ion is not observed at concentrations <50 ng/ml. The LC-MS/MS method has a limit of identification of 5 ng/ml and includes a structural analog of ZDV (azidouridine), resulting in increased selectivity [17]. The increased selectivity and more sensitive limit of identification criteria for ZDV by the LC-MS/MS method likely explains the additional identification of ZDV in clinical plasma specimens.

Although several disparities between the Exactive-MS and LC-MS/MS methods could be explained by the limits of identification of the respective methods, there were two samples in which a lack of concordance could not be explained by assay limits of identification. Specifically, 2 specimens were screened as positive for 3TC and NVP by the LC-MS/MS methods, but not by Exactive-MS. This could be the result of a false positive by LC-MS/MS, or the inability of the samples to meet all acceptance criteria for compound identification. Upon further review of the specimens in question, it was noted that there was only a limited amount of sample available for testing, so a question of freeze-thaw stability and sample integrity may also explain the discrepancy, as the samples were analyzed by Exactive-MS several months after LC-MS/MS analysis. However, during this time, samples were maintained at -80°C for long-term storage.

While the Exactive-MS method offers the identification of a diverse set of compounds with high mass accuracy (±5 ppm difference from theoretical mass), and can operate in full-scan mode, the method has limitations. Technically, Exactive-MS is associated with slower scan speeds as compared to triple quadrupole mass spectrometers, making it more difficult to acquire ions in different scan modes [47]. Also, the Exactive-MS acquires all ions, and because it is not operated in SRM mode, and all ions are collected, strict adherence to

identification criteria, including both precursor and fragment retention time, and mass accuracy, becomes paramount.

In clinical and research settings, self-report of adherence to antiretroviral regimens and off-study antiretroviral use may be unreliable. Other methods for adherence monitoring also have limitations [14, 15]. Direct detection of antiretroviral drugs in clinical samples or research specimens may provide a more accurate picture of antiretroviral drug use. Since routine monitoring of antiretroviral drugs via mass spectrometry is not performed to assess adherence, defined cut-off concentrations for adherence are not known. However, pharmacokinetic analyses show circulating plasma concentrations of antiretrovirals are dependent upon drug interactions as well as specific therapeutic dosing formulations and regimens. Further, defined definitions for adherence would require knowledge of time of dose administration as well as time of collection, parameters which are not typically known.

5.0 Conclusions

This manuscript describes the development, validation and application of a robust, selective Exactive-MS method for the qualitative monitoring of 15 antiretroviral agents in human plasma. The method requires minimal sample pre-treatment, and based on the high mass accuracy of the Exactive-MS, is selective and specific in the identification of a variety of PIs, NRTIs and NNRTIs. The method also shows agreement LC-MS/MS qualitative assays. Positive identification of an antiretroviral drug by the described Exactive-MS method could be reflex-tested for identified drugs using quantitative SRM-based LC-MS/MS methods, which would include the presence of an isotopically-labeled internal standard or structural analog. A qualitative method for the identification of antiretroviral drugs facilitates the monitoring of overall adherence to a therapeutic regimen, as well as potential off-study use. Quantitative determination of a target drugs occurs to assess pharmacokinetic parameters, including drug efficacy. This method could be used to monitor drug adherence in clinical and research settings, and also to detect off-study antiretroviral drug use in clinical trials. A major advantage of HRMS is that all ions are collected and monitored in a non-targeted fashion, facilitating retrospective analysis and post-hoc data querying. Post-targeted data analysis may have significant benefits in monitoring off-study antiretroviral drug use, as well as re-querying data for other substances. Thus, the Exactive-MS method offers robust and highly selective identification approaches for a diverse array of small molecules in a non-targeted way.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: amprenavir, atazanavir, darunavir, efavirenz, emtricitabine, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, tenofovir, tipranavir and zidovudine.

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Highlights

- We use high-resolution mass spectrometry for antiretroviral drug identification.

- We show 96.7% concordance between HRMS and reference methods.

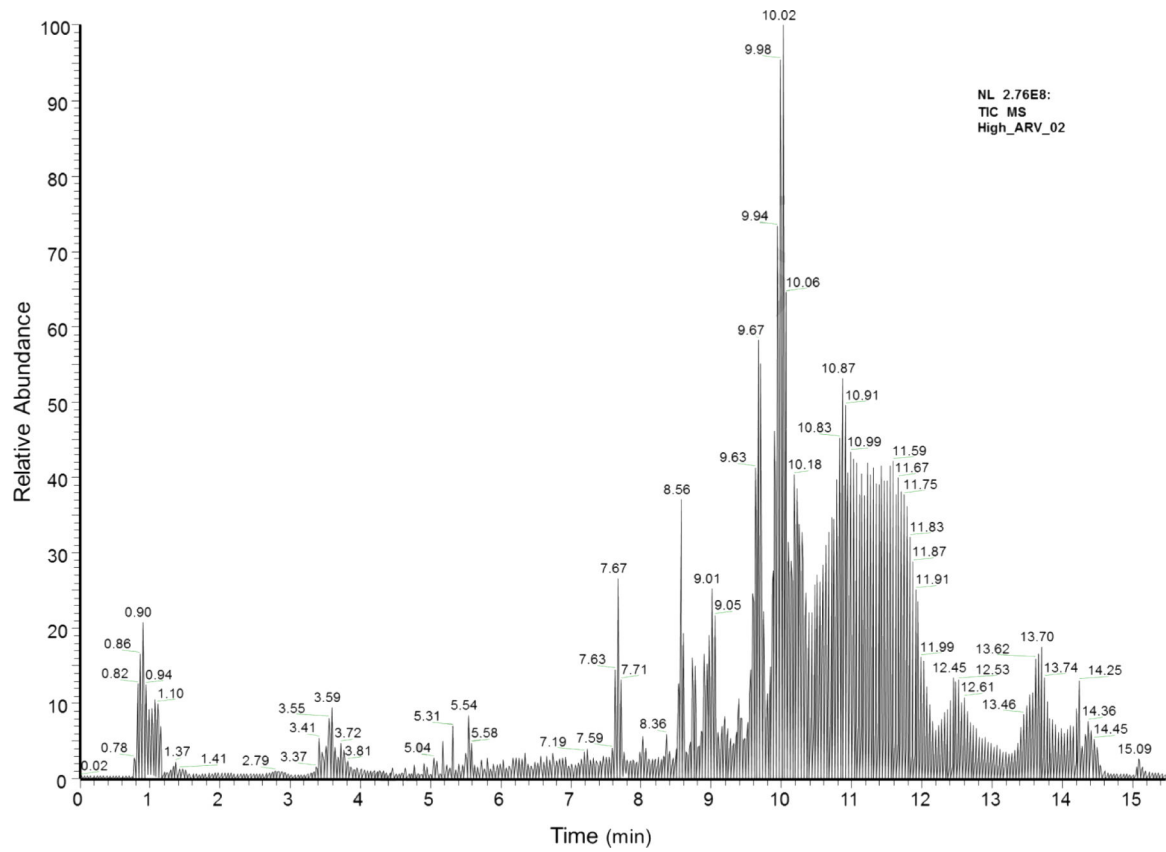- The Exactive-MS method has limits of identification of 5-10 ng/ml for most drugs.

Fig. 1.
Total ion chromatogram from the Exactive-MS method collected in positive ionization mode. Representative chromatogram of 1000 ng/ml antiretroviral mixture spiked into drug-free human serum.
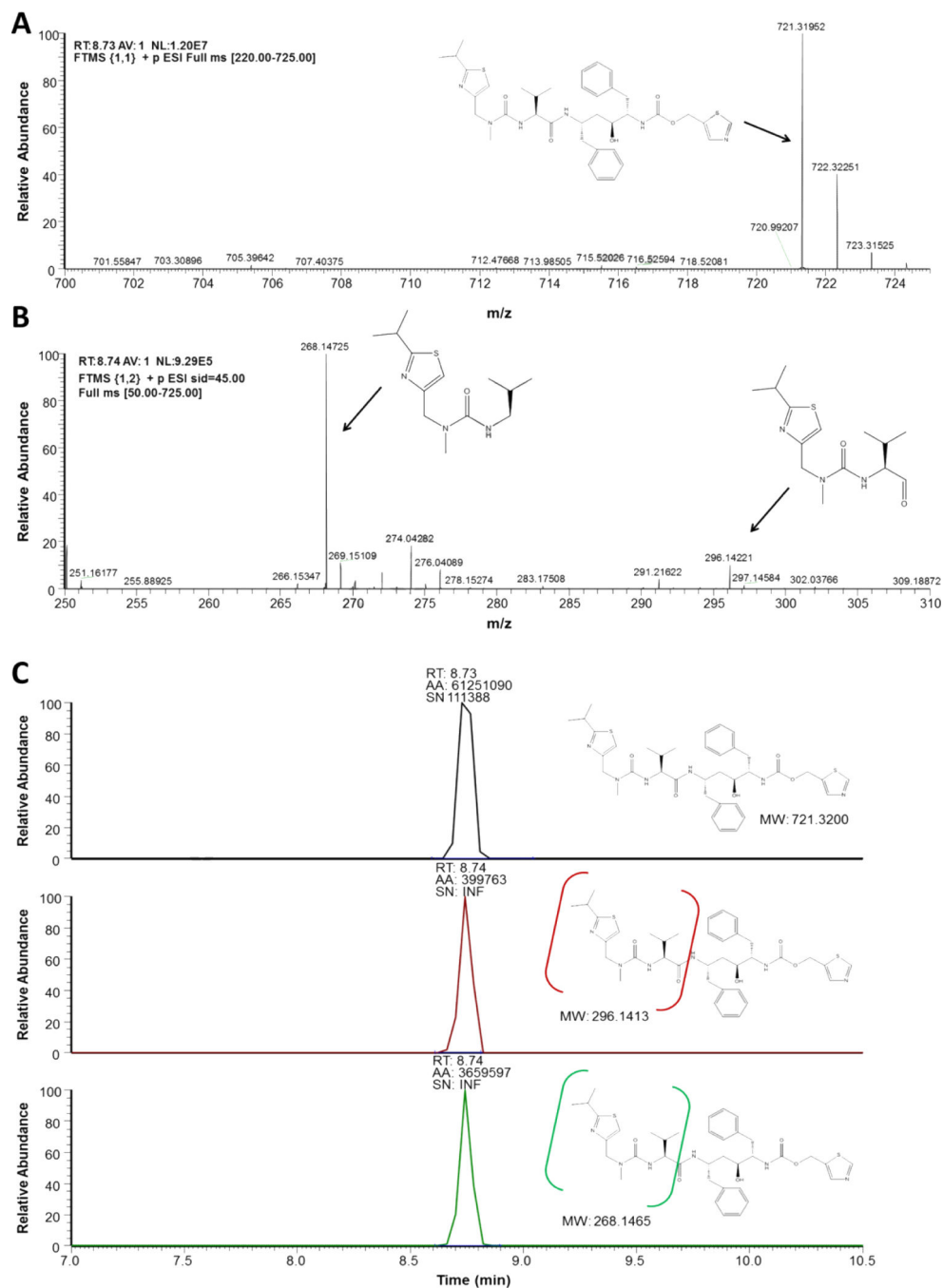
Fig. 2.
Ion spectra of ritonavir generated by Exactive-MS (A) with the inclusion of an in-source collision-induced dissociation scan event for fragment production and detection (B) . Based on the *m/z* fragment ions monitored, we postulate that the *m/z* 268 and *m/z* 296 fragment ions were formed through the cleavage of a keto-isopropyl linkage and an amide bond, respectively. Retention time determination through the application of a mass filter with ± 5 ppm discrimination from theoretical mass of precursor and fragment ions (C).
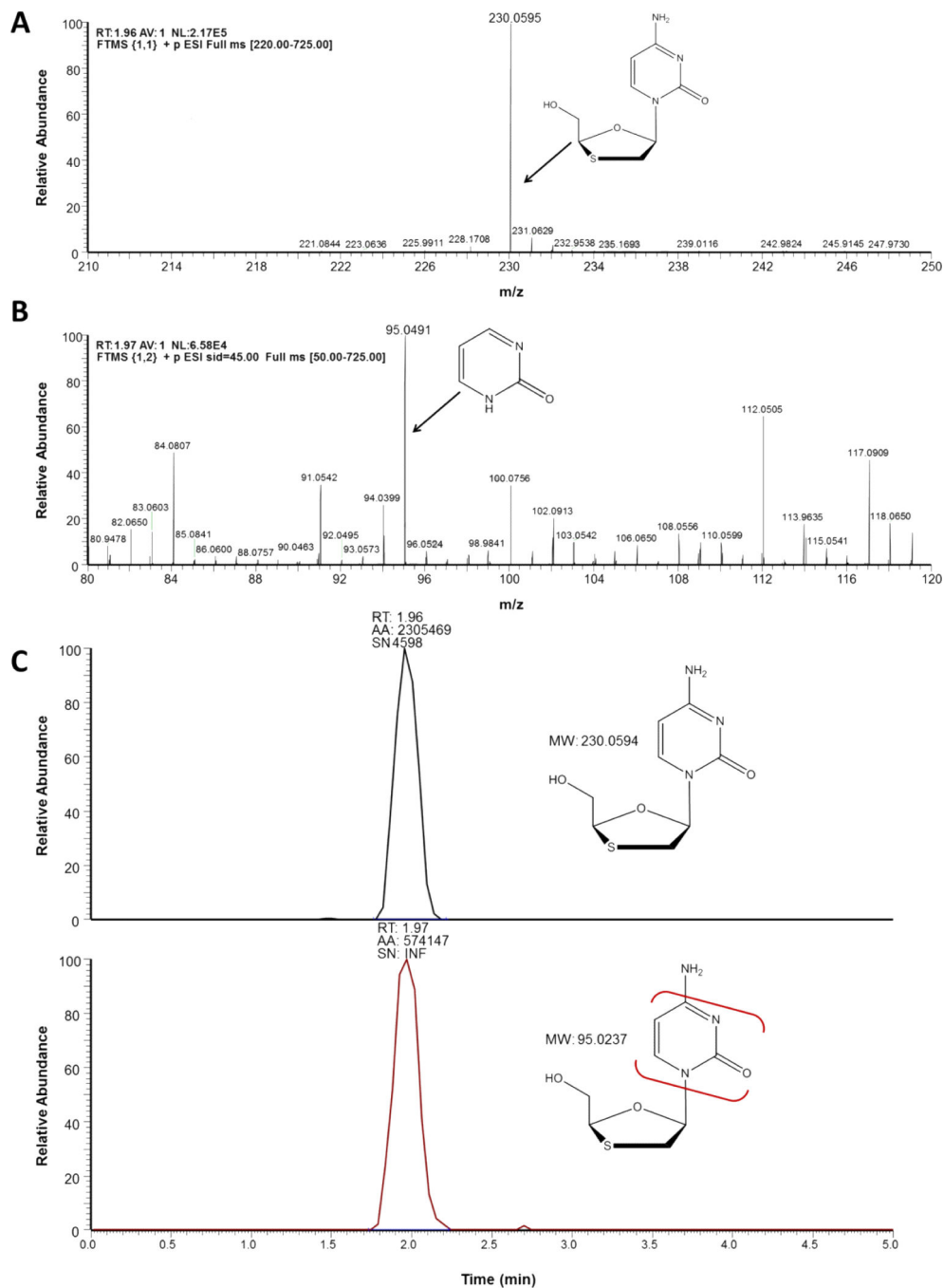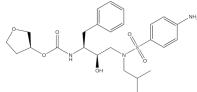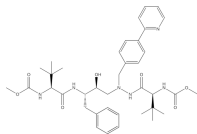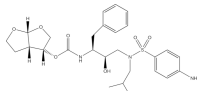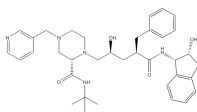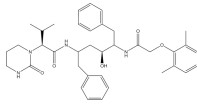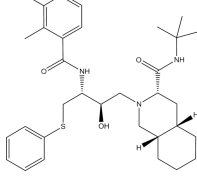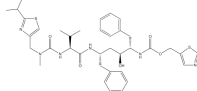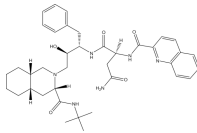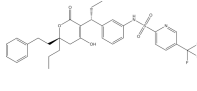
Fig. 3.
Ion spectra of lamivudine generated by Exactive-MS (A) with the inclusion of an in-source collision-induced dissociation scan event for fragment production and detection (B) .The 95 *m/z* fragment ion was likely formed through the loss of the oxathiol moiety and subsequent loss of a NH$_3$ constituent. Retention time determination through the application of a mass filter with ± 5 ppm discrimination from theoretical mass of precursor and fragment ions (C).

Table 1

Antiretroviral agents monitored in Exactive-MS method.

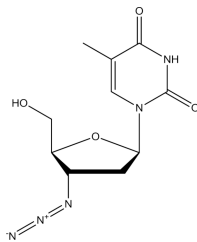| Antiretroviral Agent	Structure	Molecualar Formula	[M+H]$^+$ (*m/z*)	Fragment Ions
Protease Inhibitors				
Amprenavir (APV)		$C_{25}H_{35}N_3O_6S$	506.2319	245.1637
Atazanavir (ATV)		$C_{38}H_{52}N_6O_7$	705.3970	335.1948 168.0801
Darunavir (DRV)		$C_{27}H_{37}N_3O_7S$	548.2425	392.1989 69.0335
Indinavir (IDV)		$C_{36}H_{47}N_5O_4$	614.3701	513.2847 465.2845 421.2339
Lopinavir (LPV)		$C_{37}H_{48}N_4O_5$	629.3697	447.2621 183.1121 155.1172
Nelfinavir (NFV)		$C_{32}H_{45}N_3O_4S$	568.3204	467.2342 330.1142
Ritonavir (RTV)		$C_{37}H_{48}N_6O_5S_2$	721.3200	296.1413 268.1465
Saquinavir (SQV)		$C_{38}H_{50}N_6O_5$	671.3915	570.3052 433.1853
Tipranavir		$C_{31}H_{33}F_3N_2O_5S$	603.2135	585.1999 375.2175 333.1707
Nucleotide/Nucleoside Reverse Transcriptase Inibitors				
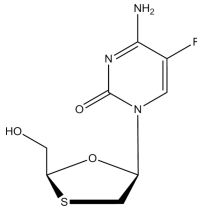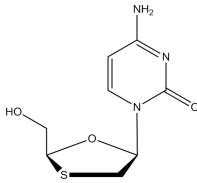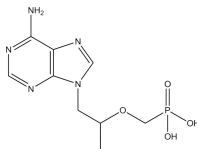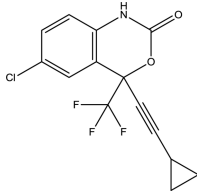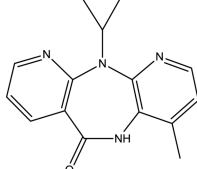
Antiretroviral Agent	Structure	Molecualar Formula	[M+H]$^+$ (m/z)	Fragment Ions
Emtricitabine (FTC)		$C_8H_{10}FN_3O_3S$	248.0500	130.0406
Lamivudine (3TC)		$C_8H_{11}N_3O_3S$	230.0594	95.0237
Tenofovir (TFV)		$C_9H_{14}N_5O_4P$	288.0856	176.0923
Zidovudine (ZDV)		$C_{10}H_{13}N_5O_4$	268.1040	127.0497
Non-nucleoside Reverse Transcriptase Inhibitors				
Efavirenz (EFV)		$C_{14}H_9ClF_3NO_2$	316.0347	N/A
Nevirapine (NVP)		$C_{15}H_{14}N_4O$	267.1240	227.0924 226.0837 107.0599

Table 2

Within-run precision of antiretroviral agent signal intensity and retention time.

Analyte	High ARV control mixture			Low ARV control mixture			High and Low ng/ml ARV control mixture		
	Mean Signal Intensity (NL) (n=5)	SD	%CV	Mean Signal Intensity (NL) (n=5)	SD	%CV	Retention Time (min) (n=10)	SD	%CV
Protease Inhibitors									
Amprenavir	6124463	183537	3.0	542972	26390	4.9	7.67	5.3E-03	0.07
Atazanavir	45121330	1625272	3.6	4192407	125739	3.0	8.56	5.7E-03	0.07
Darunavir	596270	57653	9.7	55117	7994	14.5	7.67	5.3E-03	0.07
Indinavir	28150923	1947717	6.9	2541560	310821	12.2	9.16	9.3E-02	1.01
Lopinavir	4612419	347425	7.5	358328	36614	10.2	8.89	1.3E-02	0.14
Nelfinavir	24949052	3455300	13.8	1139909	129655	11.4	10.40	8.3E-02	0.80
Ritonavir	13136967	1013771	7.7	1330593	113751	8.5	8.73	2.1E-02	0.24
Saquinavir	11302342	1382993	12.2	414473	77056	18.6	10.74	8.1E-02	0.76
Tipranavir	2114155	123453	5.8	127775	28046	21.9	9.66	1.3E-02	0.13
Nucleotide/Nucleoside Reverse Transcriptase Inhibitors									
Emtricitabine	395381	11898	3.0	50474	1767	3.5	3.38	1.9E-02	0.57
Lamivudine	240402	10299	4.3	3020	291	9.6	1.98	2.3E-02	1.18
Tenofovir	27743	2107	7.6	1111	71	6.4	2.42	6.4E-02	2.64
Zidovudine	36033	4599	12.8	4055	1117	27.5	5.53	5.2E-03	0.09
Non-nucleoside Reverse Transcriptase Inhibitors									
Efavirenz	7274	1497	20.6	12422	1472	11.9	8.57	3.1E-02	0.37
Nevirapine	5964711	355374	6.0	1467214	69109	4.7	5.54	1.0E-02	0.19

Table 3

Concordance of results obtained using the Exactive-MS method and LC-MS/MS methods for analysis of plasma samples from a clinical trial.

Analyte	LOI by LC-MS/MS (ng/ml)	LOI by Exactive-MS (ng/ml)	Positive by LC-MS/MS Only (n)	Positive by Exactive-MS Only (n)	Positive by Exactive-MS and LC-MS/MS (n)	Negative by Exactive-MS and LC-MS/MS (n)	Concordance (Percent Agreement, %)
Protease Inhibitors (PIs)							
Ataznavir	100	5	0	0	5	59	100
Ritonavir	100	5	0	1	2	61	98.4
Nucleotide/Nucleoside Reverse Transcriptase Inhibitors (NRTIs)							
Emtricitabine	5	10	0	0	11	53	100
Lamivudine	20	5	2	0	26	36	96.8
Tenofovir	5	10	0	1	9	54	98.4
Zidovudine	5	10^b	6	1	8	49	89.1
Non-Nucleotide/Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)							
Efavirenz	100	150	0	0	13	51	100
Nevirapine	20	5	2	1	15	46	95.3

Analyte	LOI by LC-MS/MS (ng/ml)	LOI by Exactive-MS (ng/ml)	Positive by LC-MS/MS Only (n)	Positive by Exactive-MS Only (n)	Positive by Exactive-MS and LC-MS/MS (n)	Negative by Exactive-MS and LC-MS/MS (n)	Concordance (Percent Agreement, %)
Protease Inhibitors (PIs)							
Atazanavir	100	5	0	0	5	59	100
Ritonavir	100	5	0	1	2	61	98.4
Nucleotide/Nucleoside Reverse Transcriptase Inhibitors (NRTIs)							
Emtricitabine	5	10	0	0	11	53	100
Lamivudine	20	5	2	0	26	36	96.8
Tenofovir	5	10	0	1	9	54	98.4
Zidovudine	5	10[b]	6	1	8	49	89.1
Non-Nucleotide/Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)							
Efavirenz	100	150	0	0	13	51	100
Nevirapine	20	5	2	1	15	46	95.3

a. The LC-MS/MS method included an isotopically-labeled internal standard for zidovudine and monitors fragments at a limit of identification of 5 ng/ml. The full scan-HRMS does not monitor fragments < 50 ng/ml.

a. The LC-MS/MS method included an isotopically-labeled internal standard for zidovudine and monitors fragments at a limit of identification of 5 ng/ml. The full scan-HRMS does not monitor fragments <50 ng/ml.

[b] The LC-MS/MS method included an isotopically-labeled internal standard for zidovudine and monitors fragments at a limit of identification of 5 ng/ml. The full scan-HRMS does not monitor fragments < 50 ng/ml.