Development of a High Resolution Accurate Mass Method for the Multiplexed Monitoring of Antiretroviral Agents in Human Serum

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ABSTRACT

Background: There are currently several antiretroviral (ARV) drug classes used for the management of HIV infection, including drugs used for protease and reverse transcriptase inhibition. While drug therapy has largely been used for the management of HIV load and administered in cases of significantly decreased CD4+ cell counts, prevention studies have illustrated that the use of ARV therapies in an HIV positive individual decreases the chances of retroviral transmission. Development of a method including chromatographic separation for identification of the various classes of ARVs is challenging due to their variable chemical structures. The goal of this work is to qualitatively screen and confirm the presence of a panel of ARV drugs using a multiplexed approach.

Methods: The analytical method was developed to detect the following ARV agents: amprenavir, atazanavir, darunavir, efavirenz, emtricitabine, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, tenofovir, tipranavir, and zidovudine. 100 µl of ARV-spiked -standards in drug-free human serum (BioRad) were extracted in cold acetonitrile, evaporated to dryness and reconstituted in water. 10 µL of each treated sample was injected onto a liquid chromatography system equipped with Transcend pumps (Thermo Fisher Scientific) for analytical separation. The chromatographic run began with 60 sec of 100% water containing 10 mM ammonium acetate and 0.1% ammonium hydroxide followed by step to 100% B, methanol with 10 mM ammonium acetate and 0.1% ammonium hydroxide , at a flow rate of 500 µL/min. Analytes were eluted from a Hypersil Gold PFP 50 x 2.1 mm; 1.9 µm particle size UHPLC column (Thermo Fisher Scientific). Analytes were detected over a 5 min run using the Exactive Orbitrap mass analyzer (Thermo Fisher Scientific). The mass spectrometer method included one positive-mode and one negative-mode scan event with ultra-high resolution (100000 @ 1Hz) and one positive-mode scan event with in-source collision-induced dissociation (SCID) event with enhanced resolution (25000 @ 4Hz). All scan events were programmed for 100 msec maximum injection time and balanced ACG targets.

Results: The analytical method was found to have a limit of detection of ≤10 ng/ml for all ARV's. Positive identification was determined by exact mass analysis at 5 ppm discrimination, analyte retention time, and identification of fragments. These data indicate the ability to screen and subsequently confirm the presence of antiretroviral agents in a single specimen in a high-throughput, multiplexed format. In addition, using HRAM detection technology, in which full scan spectra are collected for all ions and fragments in a sample, data is amenable to retrospective data review for the presence of analytes not initially included in analysis. These may include pharmaceuticals, drugs-of-abuse or other analytes present in subject samples

Conclusions: This UHPLC-MS/MS method allows for the multiplexed qualitative detection and confirmation of ARV's in human serum. The Exactive Orbitrap allows for drug determination and MS-MS confirmation with high mass accuracy.

INTRODUCTION

There are numerous antiretroviral drugs (ARV's) currently used in management **of** the human immunodeficiency virus (HIV). ARV's largely elicit their therapeutic effects as protease inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs), and non-nucleoside reverse transcriptase inhibitors (NNRTIs). For maximum efficacy, many ARV's are administered combinatorially, showing increased anti-virological activity in the presence of HIV [1]. While ARV drug therapies have been used for management of HIV load, studies have also indicated the utility of ARV therapies in HIV prevention, both in pre-exposure prophylaxis studies, as well as studies monitoring seroconversion [2, 3]. However, in order to assess the effectiveness of ARV therapies in HIV prevention, monitoring adherence through serum ARV detection is essential [4].

OBJECTIVE

The objective of this study is to develop and validate a high resolution accurate mass spectrometry method for the qualitative detection of several common ARV's used in HIV management and prevention.

METHODS

Chromatographic Conditions

UHPLC System

Step	Start	Sec	Flow (ml/min)	Grad	A (%)	B (%)
1	0.00	60	0.50	Step	100	-
2	1.00	150	0.50	Step	-	100
3	3:30	120	0.50	Step	-	100
4	5:30	60	0.50	Step	100	-

Figure 1. Chromatographic and mass spectrometric conditions. Chromatographic gradient for analyte separation and elution. Mobile phases A and B are H_20 (A) or methanol (B) + 10 mM ammonium acetate, 0.1% ammonium hydroxide. A Hypersil Gold PFP (Thermo Scientific), 50 x 2.1 mm, 1.9 micron particle size column was used for chromatographic separation.

RESULTS

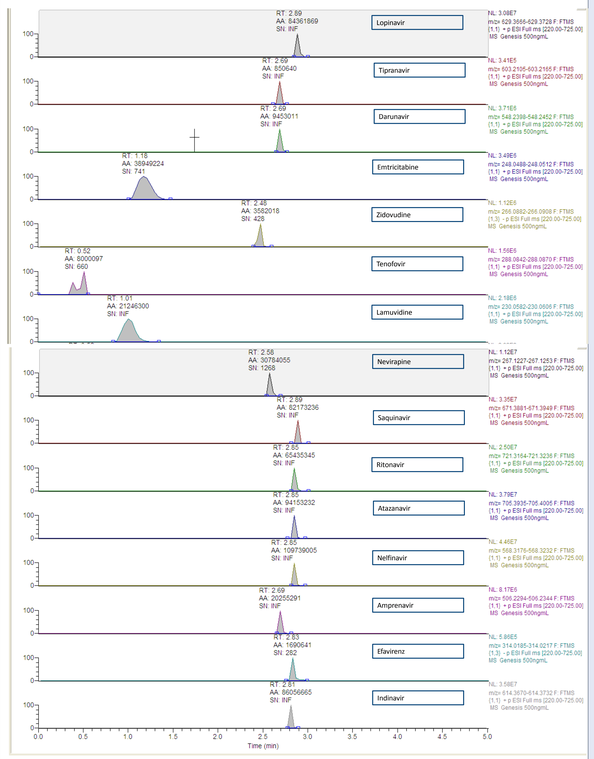

Figure 2. Summary of ARV's monitored in ARV panel. ARVs at a final concentration of 500 ng/ml were spiked into drug-free serum. Samples were extracted via a 3:1 cold acetonitrile:sample mixture, centrifuged, evaporated to dryness and reconstituted in water. ARV detection was determined by retention time and a mass discrimination of 5 ppm. Further, the presence of an ARV was confirmed via fragmentation.

Drug	Formula	m/z (M+H or M-H)	Retention Time (min)	Detection Limit (ng/ml)
Amprenavir	$C_{25}H_{35}N_3O_6S$	506.2319	2.69	10
Atazanavir	$C_{38}H_{52}N_6O_7$	705.397	2.8	5
Darunavir	$C_{27}H_{37}N_3O_7S$	548.2425	2.74	5
Efavirenz*	$C_{14}H_9ClF_3NO_2$	314.0201	2.78	5
Emtricitabine	$C_8H_{10}FN_3O_3S$	248.05	1.21	5
Indinavir	$C_{36}H_{47}N_5O_4$	614.3701	2.8	5
Lamivudine	$C_8H_{11}N_3O_3S$	230.0594	1.04	5
Lopinavir	$C_{37}H_{48}N_4O_5$	629.3697	2.88	5
Nelfinavir	$C_{32}H_{45}N_3O_4S$	568.3204	2.84	5
Nevirapine	$C_{15}H_{14}N_4O$	267.124	2.57	5
Ritonavir	$C_{37}H_{48}N_6O_5S_2$	721.32	2.84	5
Saquinavir	$C_{38}H_{50}N_6O_5$	671.3915	2.89	5
Tenofovir	$C_9H_{14}N_5O_4P$	286.0711	0.51	5
Tipranavir	$C_{31}H_{33}F_3N_2O_5S$	603.2135	2.61	10
Zidovudine*	$C_{10}H_{13}N_5O_4$	266.0895	2.43	5

Figure 3. Limit of identification analysis. The limit of identification of the ARV panel was determined by spiking ARV stock solutions (10 µg/ml) into drug free serum at final concentrations ranging from 5-500 ng/ml. Drug identification was determined based on accurate mass (discrimination of 5 ppm), presence of at least one fragment, and retention time. Carryover studies, which involved running three injections of 500 ng/ml ARV extractions followed by three blanks showed no appreciable carryover. Further, no matrix effects were observed when a comparison of signal, retention time and peak shape was compared between serum-spiked drugs and those prepared in 1:1 methanol: water. *Zidovudine and efavirenz were identified in negative mode.

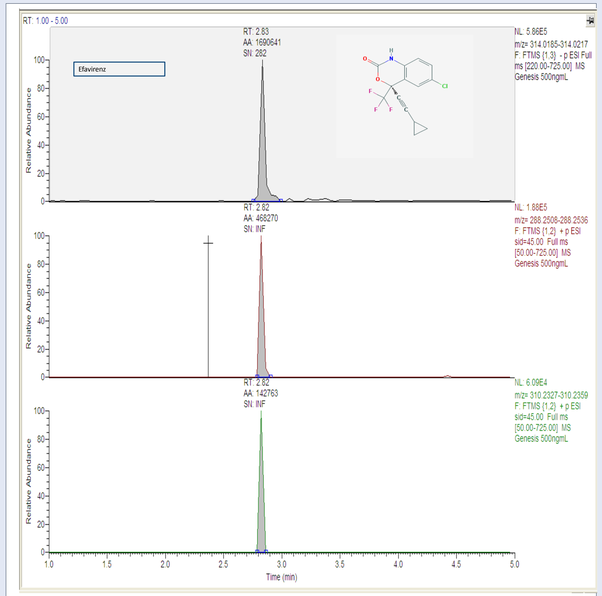

Figure 4. Efavirenz fragment analysis. Efavirenz parent molecule (M-H: 314.0201 m/z) was best determined in negative mode. Fragment analysis was not possible for efavirenz in negative mode, so positive-mode fragments were used for confirmation (288 and 310). A similar technique was used to identify and confirm the presence of zidovudine. Fragment confirmation was used for all ARV's.

SUMMARY & CONCLUSIONS

Development of a high resolution accurate mass LC-MS/MS method for simultaneous identification of 15 ARV's in a serum sample.

- Serum volume required: 100 µl
- Limit of identification: ≤ 10 ng/ml
- No matrix effects observed when compared to parent mass accuracy, fragmentation and retention time in drugs prepared in a 1:1 MeOH: H2O stock.
- Using a HRAM full scan technique to collect data for all ions in a scan range provides the opportunity for retrospective data review for analytes not included in original analysis.

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