Development of a Novel Method for Determination of 11 Antiretroviral Drugs Concentrations in Human Plasma by Ultra-Performance Liquid Chromatography-Photodiode Array Detection Method

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ABSTRACT

Objective: To develop ultra-performance liquid chromatography-photodiode array detection (UPLC-PDA) method that is fully validated for measuring 11 antiretroviral drugs concentrations in human plasma.

Methods: Chromatographic separation was performed on an ACQUITY UPLC™ BEH Shield RP, 1.7 µm (100 mm x 2.1 mm I.D.) and used acetronitrile with 0.1% formic acid in Milli-Q water as a mobile phase. ACQUITY UPLC® Photodiode Array (PDA) Detector was performed at 210, 240, and 260 nm.

Results: This method demonstrated a good separation result for plasma levels of 11 antiretroviral drugs within 16 minutes. The lower limit of quantification (LLOQ) was 0.25 µg/mL for maraviroc, 0.5 µg/mL for lopinavir, and 20 ng/mL for the remaining 9 antiretroviral drugs. This method was fully validated in terms of selectivity, accuracy, precision, and stability. The standard curves are in the expected ranges of drug concentration in the patients' plasma with good linearity (r2>0.995). Recoveries of extraction ranged from 72.27- 110.80% with repeatability.

Conclusion: A novel, sensitive, accurate, and reproducible UPLC/PDA method for quantification of 11 currently-prescribed antiretroviral drugs concentrations in human plasma was successfully developed and fully validated according to USFDA guidelines.

Keywords: UPLC/PDA; antiretroviral; therapeutic drug monitoring (Siriraj Med J 2018;70: 238-246)

INTRODUCTION

Antiretroviral (ARV) drugs that are commonly used to treat human immunodeficiency virus (HIV) include: protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), integrase inhibitors (INSIs), and entry and fusion inhibitors (EIs). The use of three or more antiretroviral medicines is referred to as highly active antiretroviral therapy or HAART. Presently, HAART is the standard treatment for HIV infection.[1]

Failure of treatment with antiretroviral drugs in HIV patients has remained unacceptably high. Low concentrations of ARVs are evaluated as drug administration's failure. High concentrations of ARVs also increase the risk of drug toxicity.[2] Recommended standard dosages of multi-drug ARVs in patients with immune deficiencies who have some degree of liver and/or kidney impairment may be dangerously higher than the general regimen. Therapeutic drug monitoring (TDM) can identify and prevent excessively abnormal ARV concentration. This can increase clinical response and decrease their overdose's toxic side effect. The target therapeutic concentrations in the leading ARVs are, as follows: atazanavir >200 ng/mL;

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lopinavir >1,000 ng/mL; ritonavir >2,100 ng/mL; efavirenze >1,000 ng/mL; nevirapine >3,500 ng/mL; maraviroc >50 ng/mL; darunavir >3,300 ng/mL; etravirine >300 ng/mL; raltegravir >65 ng/mL; elvitegravir >200 ng/mL; and, dolutegravir >40ng/mL.[2-4]

Many methods for the simultaneous quantification of ARVs have been developed. The most commonly used techniques are liquid chromatography-tandem mass spectrometry (LC-MS/MS)[5-11] and reversed-phase HPLC with UV detection.[12-21] The LC-MS/MS tandem method is not only highly sensitive and highly specific, but it also has a very short runtime, because complete separation of all antiretroviral's chromatogram is not necessary. However, the limitation of LC-MS/MS analysis is not only requiring a more expensive instrument than other techniques, but also susceptibility to their matrix effects. Moreover, HPLC methods with UV detection have a very long analytic time to complete the resolution of all chromatographic peaks. High sensitivity, high specificity, and short runtime are ideal performance parameters of therapeutic drug monitoring (TDM). Then, Ultra-performance liquid chromatography (UPLC) may have an opportunity in this TDM because of its advantages of cheaper method and can detect broader separated chromatograms. Although other methods for the simultaneous quantification of protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) using UPLC with photo diode array detection have been published[22,23], neither of those studies included the quantification of integrase inhibitors (INSIs), and entry and fusion inhibitors (EIs).

The aim of this study was to develop the ultra-performance liquid chromatography-photodiode array detection (UPLC-PDA) method which has been fully validated for measuring 11 antiretroviral concentrations in human plasma. We anticipate that this method will have clinical value for monitoring the levels of these drugs in patients who received several antiretroviral drugs' administration.

MATERIALS AND METHODS

Chemicals and Materials

Atazanavir, etravirine, raltegravir, elvitegravir, and dolutegravir were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Maraviroc, lopinavir, ritonavir, darunavir, efavirenze, nevirapine, and tenofovir (internal standard, IS) were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany). HPLC grade acetonitrile and methanol were purchased from Labscan Ltd. (Bangkok, Thailand). Water used for experimentation was produced by Milli-Q® water purification system (EMD Millipore, Billerica, MA, USA). Any other chemicals used were of analytical grade. Drug-free human plasma was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital. The protocol for this study was approved by the Siriraj Institutional Review Board (Si 655/2015), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Instruments and Analytical Conditions

Acquity™ Ultra Performance Liquid Chromatography (Waters Corporation, Milford, MA, USA) was used for the separation module. Chromatographic separation was performed on an ACQUITY UPLC™ BEH Shield RP (1.7 µm, 100 mm x 2.1 mm I.D.) (Waters Corporation, Milford, MA, USA). To achieve an optimum result, the mobile phase was performed with a flow rate of 0.45 mL/min and a column temperature of 40°C. The gradient programmed for the mobile phase was optimized using acetronitrile and 0.1% formic acid in water, as shown in Table 1. ACQUITY UPLC® Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA) was operated at wavelengths between 200-380 nm and quantitation was performed at 210 nm for maraviroc and lopinavir; 240 nm for nevirapine, raltegravir, atazanavir, ritonavir, efavirenze, and etravirine; and, 260 nm for dolutegravir, darunavir, and elvitegravir. The autosampler was conditioned at 8°C and the injection volume was 10 µL. Empower 2 software (Waters Corporation, Milford, MA, USA) was used for data management.

Preparations of Standard and Quality Control Samples

Standard stock solutions of both analytes and IS were prepared in dimethyl sulfoxide at a concentration of 400 µg/mL. Stock solutions were diluted with a mixture of methanol and water (50:50, v/v) to obtain working solution at eight concentration levels. Calibration standards were prepared by spiking working solutions with drug-free human plasma to the final concentration, which ranged from 0.25-2.50 µg/mL for maraviroc, 0.5-20.0 µg/mL for lopinavir, and 20-2,000 ng/mL for the other ARVs. Quality control (QC) samples were prepared separately in the same way to create low, medium, and high controls at 0.75, 1.20, and 2.20 µg/mL for maraviroc; at 1.50, 8.00, and 18.00 µg/mL for lopinavir; and at 60, 450, and 950 ng/mL for the other ARVs. The IS working solution was prepared by diluting the stock solutions in a mixture of methanol and water (50:50, v/v) to a final concentration of 50 µg/mL. All standard stock solutions, working solutions, and QC samples were stored at -20°C until use.

TABLE 1. Gradient programmed for the mobile phase: Solvent A (Acetonitrile) and Solvent B (0.1% formic acid in water).

Time (min)	Flow (mL/min)	% Solvent A	% Solvent B
0.0	0.45	20	80
1.0	0.45	30	70
3.0	0.45	35	65
4.0	0.45	32	68
9.0	0.45	45	55
12.0	0.45	40	60
14.0	0.45	65	35
16.0	0.45	20	80

Sample Preparations

For solid phase extraction (SPE), OASIS HLB: Hydrophilic-Lipophilic-Balanced reversed-phase sorbent 30 mg 1 mL (Waters Corporation, Milford, MA, USA) was used for sample preparation. SPE HLB cartridges were initially conditioned with 1 mL of methanol and equilibrated with 1 mL of Milli-Q water before use. Twenty µL of IS (50 µg/mL) was added into 1.0 mL of plasma samples. Then, 1 mL of plasma samples were aspirated into the wetted preconditioned SPE HLB cartridges. The plasma components were then washed in two steps with wash solvent, as follows: step 1) 1 mL of ammonia solution and 5% methanol in water (2:98, v/v); and, step 2) 1 mL of ammonia solution and 50% methanol in water (2:98, v/v). ARVs were subsequently eluted from the dried columns using 0.5 mL of an eluting solution (acetic acid and methanol, 2:98, v/v). Three hundred µL of elute was then diluted with 200 mL of 0.1% formic acid before being injected into the UPLC system.

Bioanalytical method validation

The developed method was fully validated according to the Guidance for Industry, Bioanalytical Method Validation by the U.S. Food and Drug Administration (FDA).[24]

1) Selectivity and Sensitivity

Selectivity was examined using six sources of drug-free plasma, for which the result should not have interfering peaks at the retention times of the 11 ARVs and IS. Sensitivity at the lower limit of quantification (LLOQ) was also examined by dilution of standard compounds in plasma, which were extracted and then quantified at the lowest detectable concentration.

2) Intraday and Interday Precision and Accuracy

Intraday and interday precision and accuracy were examined by analysis of six replicates of LLOQ, LQC, MQC, and HQC for three consecutive days. Percentage of relative error (%RE) indicating accuracy and percentage of coefficient of variation (%CV) indicating precision were calculated. Both %RE and %CV should be within ±20% at LLOQ and ±15% at other concentrations.

3) Linearity and Calibration Curve

A calibration curve was represented by a linear regression model, y=mx+b and weighting by 1/x, where y was the ratio of peak area of analyte to the peak area of IS, x was the concentration at different levels, including 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL for MRV; 0.5, 2.0, 5.0, 10.0, 15.0, and 20.0 µg/mL for LPV; and, 20.0, 100.0, 500.0, 1,000.0, 1,500.0, and 2,000 ng/mL for all 9 other ARVs. All calibration ranges yielded linear relationships with coefficient of determination (r^2) that exceeded 0.995.

4) Extraction Efficiency

Extraction efficiency of the method was performed by comparing peak areas of extracted samples at three QC concentrations, with peak areas of non-extracted standard solutions at the same concentrations. Recovery of the analyte was not required to be 100%, but the extent of recovery of an analyte should be precise and reproducible.

5) Stability

Stability of analysis was performed by analyzing three replicates of plasma spiked with three QC concentrations under various conditions with freshly prepared samples. The first condition was freeze and thaw, in which plasma samples were frozen at -20°C and thawed at room temperature for three cycles before analysis. For

short-term stability test, plasma samples were stored at room temperature for 6 hours before analysis. For post-preparative stability test, vials of plasma samples were placed in the auto-sampler at 8°C for 10 hours before analysis. Lastly and for the long-term stability test, plasma samples were frozen at -20°C for 3 months before analysis. The acceptable percentage of variation in each condition must be within ±15%.

6) Matrix Effect

The matrix effect was determined by analysis of six replicates of QC samples at three different concentrations in the blank extracts and in the solution. The matrix factor (MF) was calculated as the ratio of the peak area between presence and absence of matrix. An average matrix value within the range of 0.8-1.2 indicates no matrix effect.

RESULTS

This method demonstrated a good separation result for 11 ARVs in 16 minutes as shown with PDA spectrum in Fig 1. The capacity factor (k′) was in the range of 1.832-34.628 and the resolution factor was in the range of 1.577-39.263 as summarized in Table 2. The specific wavelength selected for each drug was 210 nm for maraviroc and lopinavir; 240 nm for nevirapine, raltegravir, atazanavir, ritonavir, efavirenze, and etravirine; and, 260 nm for dolutegravir, darunavir, and elvitegravir. Wavelength selection was based on the requirement for high sensitivity and specificity. There were no interfering peaks for retention time of 11 ARVs in selectivity testing (Fig 2). The lower limit of quantification (LLOQ) was 0.25 µg/mL for maraviroc, 0.5 µg/mL for lopinavir, and 20 ng/mL for the remaining 9 ARVs. The accuracy and precision of the proposed method were acceptable, as summarized in Table 3. Method accuracies were in the range of 92.23-113.51%. Interday and intraday precision was also observed, with %CV in the range of 1.60-10.43%. This method also showed good linearity, with a coefficient of determination (r^2) greater than 0.996 (Table 4). Although extraction efficiency was in the range of 77.57-101.66%, the recovery of each ARV was consistent, precise, and reproducible with a %CV range of 1.61-9.27 (Table 4). Under the various conditions used for stability testing, the percentage of variation for each condition was within an acceptable range (Table 5). For matrix effect, a matrix factor (MF) between 0.92-1.19 indicated that other substances in the sample cannot significantly affect the accuracy and precision of the method.

DISCUSSION

These were only two previously published studies in ARVs analysis by UPLC-PDA method. Analysis of 8 ARVs took longer than 9.5 min in a study by Antunes, *et al.*[23] and 14 min for 10 ARVs in a study by Elens, *et al.*[22] Although the resolution factors between elvitegravir and lopinavir were lower than 2.0 (1.577), their elution peaks and analytic peaks were also completely separated. The LLOQ is defined as the lowest concentration that can be quantified below the suggested minimum target of trough concentrations in patients with HIV. In our method of detections, 10 ARVs were detected within accuracate time and in therapeutic recommended doses. The exception was Maraviroc, which needed higher concentrations 50 ng/mL for detection by UPLC method. However, these methods can measure peak concentrations when dosed 150 mg or more (recommended dose is 300 mg bid). The lower LLOQ values of ritonavir, nevirapine, atazanavir, and efavirenze indicate higher sensitivity than previously published UPLC-PDA methods.[22-23] As shown in Fig 1, Lopinavir and Maraviroc can absorb only at a wavelength of 210 nm. At 190 -210 nm, the analysis will be disturbed by the other substances' noise. It's better to avoid analysis below 210 nm due to low sensitivity of method. Therefore, the analytical concentration range of Lopinavir and Maraviroc are higher or different from the others. Solid phase extraction was optimized in order to obtain a more rapid and simple procedure for routine analysis than was previously published[19], due to no evaporating step that consumes about 45-60 minutes. Then, our UPLC method may have an opportunity in therapeutic monitoring in HIV patients who are treated with several antiretroviral drugs because it is a cheaper method and can detect broader separated chromatograms. The patients who have low plasma antiretroviral drugs level will result in administration failure and treatment failure. In the opposite side, the patients who have high plasma drugs level will need awareness of more adverse drug effects and toxicities.

CONCLUSION

A novel, sensitive, accurate, and reproducible UPLC/PDA method for quantification of 11 currently prescribed and monitored antiretroviral drug concentrations in human plasma was successfully developed and validated according to USFDA guidelines. The authors propose this method for therapeutic drug monitoring in HIV patients who are treated with several drugs regimen.

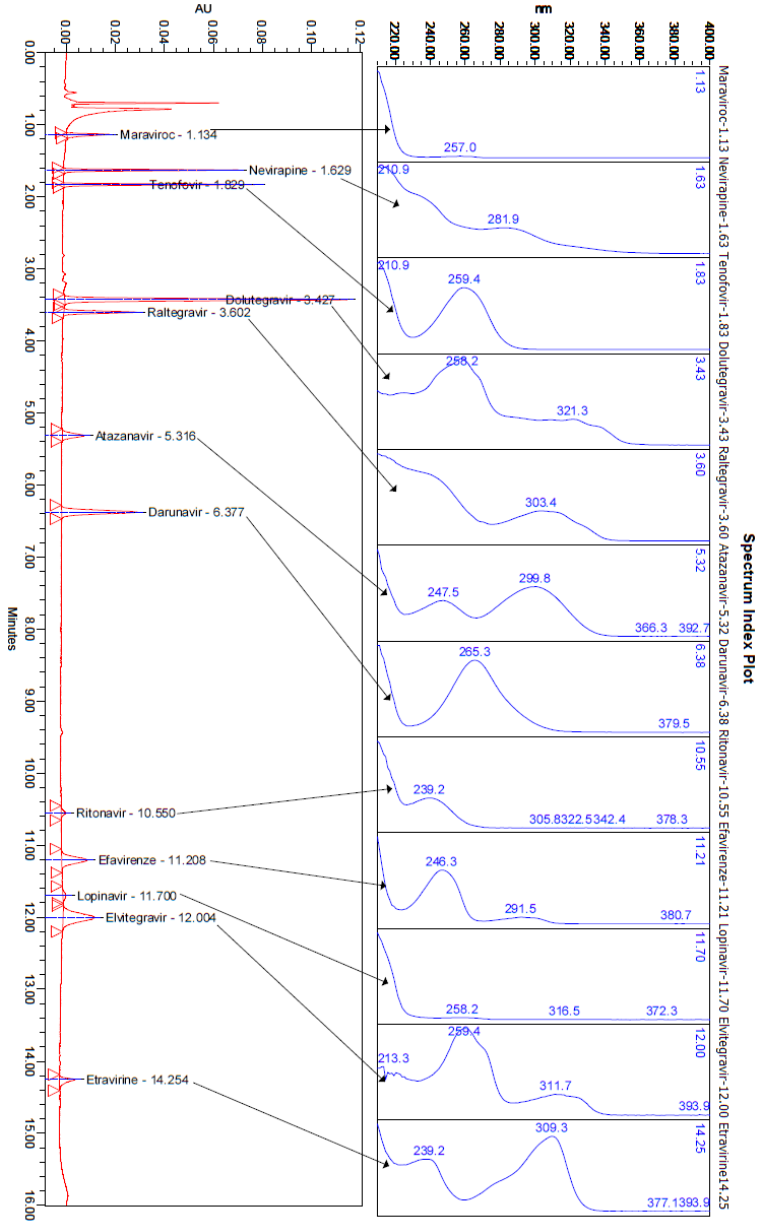

Fig 1. Chromatograms obtained during simultaneous separation of 11 ARVs on the Left panel. PDA Spectrum of each chromatogram has been show on the Right panel, which is the specific pattern of absorbance of each drug.

TABLE 2. Retention time, capacity factor (k′), and chromatogram resolution of ARVs.

Compound	Retention time	Capacity factor	Resolution
Maraviroc	1.134	1.832	-
Nevirapine	1.629	3.070	11.321
Tenofovir	1.829	3.570	6.080
Dolutegravir	3.427	7.566	35.372
Raltegravir	3.602	8.004	2.998
Atazanavir	5.316	12.287	20.173
Darunavir	6.377	14.940	10.309
Efavirenze	10.550	25.371	39.263
Ritonavir	11.208	27.014	4.671
Lopinavir	11.700	28.244	2.942
Elvitagravir	12.004	29.003	1.577
Etravirine	14.254	34.628	14.817

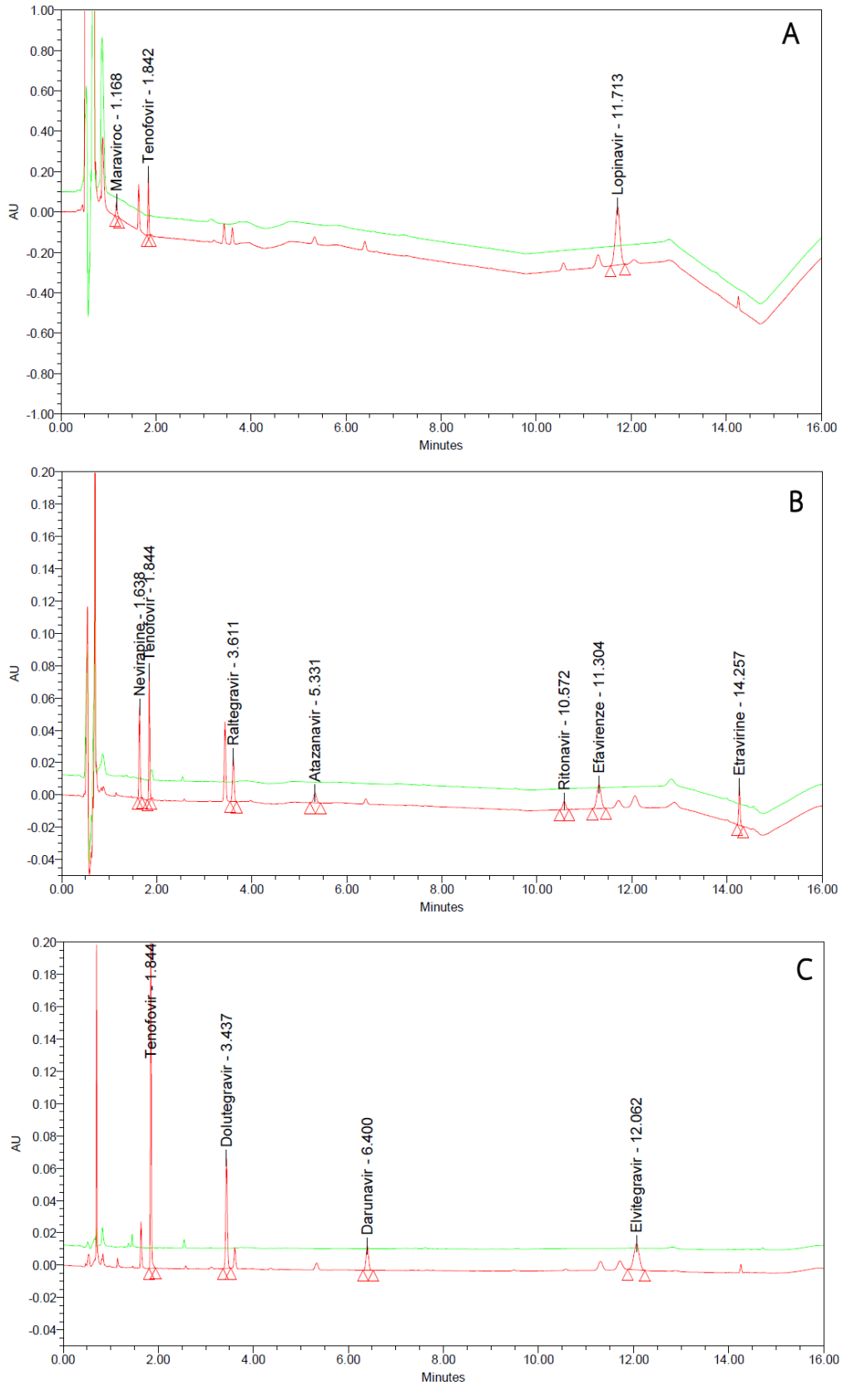

Fig 2. Chromatograms of blank plasma and blank plasma spiked with ARVs: (A) measurement at 210 nm; (B) measurement at 240 nm; and, (C) measurement at 260 nm.

TABLE 3. Accuracy and interday and intraday precision of ARVs

ARVs	LLOQ			LQC			MQC			HQC		
	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)	Accuracy (RE%) (n=18)	Precision (CV%) Intraday (n=6)	Precision (CV%) Interday (n=18)
Maraviroc	99.85	6.90	6.37	96.79	5.04	7.01	96.13	3.76	7.49	100.44	9.73	8.44
Nevirapine	104.11	6.88	5.15	102.43	6.61	7.19	100.44	9.57	7.98	96.24	3.95	3.84
Dolutegravir	102.38	8.20	6.48	94.83	5.08	5.03	96.48	4.82	7.85	103.59	6.80	5.89
Raltegravir	105.47	4.93	5.15	100.44	6.57	7.79	101.73	1.60	9.87	102.68	7.73	9.73
Atazanavir	113.51	6.14	9.49	100.75	7.04	7.68	97.08	5.35	6.66	97.40	3.42	8.30
Darunavir	101.39	2.14	3.26	92.23	5.08	5.89	97.22	4.87	5.22	102.05	2.84	5.89
Efavirenze	98.50	5.49	4.96	95.74	7.06	8.94	103.15	5.15	5.30	108.59	1.77	4.04
Ritonavir	112.98	5.77	7.37	98.57	6.86	6.98	94.76	5.56	6.13	94.91	4.73	5.00
Lopinavir	107.71	7.57	4.96	99.84	8.27	6.35	109.95	2.64	3.06	95.46	8.92	8.38
Elvitegravir	109.15	4.20	4.83	100.61	4.53	9.47	100.43	10.43	7.63	102.20	8.44	7.18
Etravirine	108.39	8.20	5.42	99.71	4.19	7.72	103.15	4.17	8.04	108.75	3.30	5.94

Abbreviations: n = number of replicates; LLOQ = lower limit of quantification; LQC = lower quality control; MQC = mid-quality control; HQC = high quality control

TABLE 4. Concentration range, linearity, recovery of extraction, and target concentrations of ARVs.

ARVs	Concentration range (ng/mL)	Coefficient of determination (r^2) (n=3)	Recovery of extraction (%RE) (n=6)	Target concentration (ng/mL)
Maraviroc	250-2,500	0.996932	89.38	>50
Nevirapine	20-1,000	0.998057	93.39	>3,500
Dolutegravir	20-1,000	0.997449	85.12	>40
Raltegravir	20-1,000	0.997997	83.52	>65
Atazanavir	20-1,000	0.997843	85.13	>200
Darunavir	20-1,000	0.997304	81.89	>3,300
Efavirenze	20-1,000	0.998031	101.66	>1000
Ritonavir	20-1,000	0.998474	95.60	>2,100
Lopinavir	500-20,000	0.996364	77.57	>1,000
Elvitegravir	20-1,000	0.997734	83.47	>200
Etravirine	20-1,000	0.996066	85.28	>300

Abbreviation: n = number of replicates

TABLE 5. Stability of ARVs.

ARVs	Variation (%) (n=3)			
	Freeze and thaw	Short-term	Long-term	Post-preparative
Maraviroc	1.42-9.58	1.46-7.82	2.20-10.77	2.04-5.02
Nevirapine	2.13-5.12	0.39-5.02	4.67-13.32	2.44-4.00
Dolutegravir	2.09-10.22	3.26-9.49	0.41-6.99	1.80-7.75
Raltegravir	1.15-11.92	1.69-10.38	3.66-9.97	0.17-4.75
Atazanavir	2.41-11.89	5.02-11.17	0.80-10.53	0.36-2.03
Darunavir	4.33-12.40	2.10-11.86	3.86-7.81	0.41-3.97
Efavirenze	2.85-9.15	1.31-8.23	2.77-6.86	1.29-5.32
Ritonavir	5.16-10.76	1.11-5.06	0.11-10.29	0.65-1.38
Lopinavir	0.06-13.44	2.32-3.79	0.25-8.34	3.38-8.06
Elvitegravir	1.07-2.38	1.33-6.04	1.86-3.31	0.98-4.00
Etravirine	3.60-14.02	2.06-5.62	2.61-12.09	0.17-6.75

Abbreviation: n = number of replicates

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