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Development of an HPLC–UV assay method for the simultaneous quantification of nine antiretroviral agents in the plasma of HIV-infected patients☆

Nitin Charbe[a,*], Sara Baldelli[a], Valeria Cozzi[a], Simone Castoldi[a], Dario Cattaneo[a,b,c], Emilio Clementi[b,c]

[a] Unit of Clinical Pharmacology, Department of Biomedical and Clinical Sciences, L. Sacco University Hospital, Università di Milano, 20157 Milan, Italy
[b] Clinical Pharmacology Unit, CNR Institute of Neuroscience, Department of Biomedical and Clinical Sciences, L. Sacco University Hospital, Università di Milano, 20157 Milan, Italy
[c] Scientific Institute IRCCS Eugenio Medea, Bosisio Parini, Italy

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ABSTRACT

A new method using high-performance liquid chromatography coupled with ultra violet detection (HPLC–UV) was developed and validated for the simultaneous quantification of atazanavir, dolutegravir, darunavir, efavirenz, etravirine lopinavir, raltegravir, rilpivirine and tipranavir in human plasma. For the first time we reported here the development and validation of an HPLC–UV assay to quantify the frequently administered 9 antiretroviral compounds including dolutegravir and rilpivirine. A simple solid phase extraction procedure was applied to 500 μL aliquots of plasma. The chromatographic separation of the drugs and internal standard (quinoxaline) was achieved with a gradient of acetonitrile and sodium acetate buffer on a C_{18} reverse-phase analytical column with a 25 min analytical run time. Calibration curves were optimised according to the therapeutic range of drug concentrations in patients, and the coefficient of determination (r^2) was higher than 0.99 for all analytes. Mean intraday and interday precisions (RSD) for all compounds were less than 15.0%, and the mean accuracy (% deviation from nominal concentration) was also found to be less than 15.0%. Extraction recovery range was between 80% and 120% for all drugs analysed. The solid phase extraction and HPLC–UV method enable a specific, sensitive, and reliable simultaneous determination of nine antiretroviral agents in plasma. Good extraction efficiency and low limit of HPLC–UV quantification make this method suitable for use in clinical trials and therapeutic drug monitoring.

1. Introduction

The use of combination antiretroviral therapy (HAART) has improved markedly the survival of HIV-1 infected patients such that the life expectancy of HIV-infected patients aged 20 years was projected to increase from 36.1 years to 49.4 years, according to the various multinational cohort studies [1]. Despite marked increases in life expectancy, mortality rates among HIV-infected persons remain 3–15 times higher than those seen in the general population [2]. Although some of the excess mortality observed among HIV-infected persons can be directly attributed to illnesses that occurred as a consequence of immunodeficiency, more than half of the deaths observed in recent years among HAART-experienced HIV-infected patients were attributable to non-infectious comorbidities [3,4].

Prolonged exposure to antiretroviral therapy along with aging may increase the risk of developing metabolic complications and cardiovascular diseases among HIV-infected patients. Several studies have provided evidence that comorbidities such as diabetes mellitus [5–7], hypertension [5,8], coronary artery disease [5,6], hyperlipidemia [5,7], renal disease [9] and reduced bone mineral density [10] happen more frequently among HIV-infected elderly patients than in HIV-uninfected controls. In addition to HAART, medications for management of metabolic and other complications further increase the pill burden and potential for drug-drug interactions in the elderly patients [11]. Further treatments may also be required for other indications including hepatitis co-infections, psychiatric illnesses, oncology diagnoses and solid-organ transplantations. Finally, patients may also take vitamins, food supplements, complementary/alternative medicine

(CAM), or recreational agents on a regular or occasional basis. These factors further enhance the drug-drug interaction possibilities.

Negative consequences of drug interactions include viral breakthrough and development of resistance, sub-optimal disease/symptom management, drug toxicity and possible non-adherence. Because of the poly-pharmacy treatment in HIV, it is advisable to have simultaneous methods for fast and friendly quantification of antiretroviral drugs in different matrices.

Many methods for the simultaneous quantification of various antiretroviral drugs using high-performance liquid chromatography with UV detection (HPLC–UV) have been published [12–19]. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been used to quantify dolutegravir (DTG) and rilpivirine (RPV) in plasma samples [20–26]. However, higher cost of the instruments and their maintenance, as well as the technical assistance limited the use of LC–MS in clinical laboratories of hospitals. HPLC–UV represents a cheaper option and is easier to adapt to the hospital setting. As far as we know, no HPLC–UV methods for the simultaneous quantification of atazanavir (ATV), DTG, darunavir (DRV), efavirenz (EFV), etravirine (ETV), RPV, raltegravir (RGV), lopinavir (LPV), and tipranavir (TPV) in human plasma have been published to date.

The aim of our study was to develop and validate an HPLC–UV method for the simultaneous quantification of the new drugs, DTG and RPV, together with 7 frequently used antiretroviral drugs in human plasma samples.

2. Experimental

2.1. Chemicals and reagents

ATV, DTG, EFV, ETV, LPV, and RGV standard powders were purchased from Spectra 2000 (Rome, Italy); DRV and RPV were kindly provided by Janssen Cilag (Beerse, Belgium); TPV was donated by Boehringer Ingelheim Pharmaceuticals; and quinoxaline (QX), used as internal standard (IS), was purchased from Sigma (Milan, Italy). Acetonitrile and methanol (Sigma) were of HPLC grade. Deionised water used in all aqueous solutions was obtained from a Milli-Q water purification system (Millipore, Milan, Italy).

Stock solutions with a concentration of 1 mg/mL were prepared for ATV, DRV EFV, ETV, LPV, RGV, and TPV in methanol. Stock solutions with a concentration of 1 mg/mL were prepared for DTG in methanol/dimethylsulfoxide (98:2, v/v) and for RPV in methanol/dimethylsulfoxide (90:10, v/v). A working solution of IS was made with QX (7.5 mg/mL) in methanol and stored at 4 °C until use.

2.2. Calibration curve and quality control samples

Calibration samples (500 µL) were prepared through mixing appropriate volumes of analytes working solutions with plasma from healthy volunteers to achieve six different concentrations as shown in Table 1. Quality control samples (QCs) (500 µL) were prepared by adding a determined volume of stock solution to blank plasma to obtain three concentration levels (low, medium and high) within the linear concentration (Table 1). The QC samples were stored frozen at −20 °C until analysis.

Two calibration curves of 6 standard (STD) points were made due to the coelution of DTG with RGV and EFV with LPV. Calibration curve A was made with DTG, DRV, ATV, ETV, RPV, TPV and EFV and calibration curve B was made with RPV, ETV, RGV, DRV, ATV, LPV and TPV. Calibration ranges, from STD 6 to STD 1, and QC concentrations for all drugs are listed in Table 1.

2.3. HPLC–UV apparatus and conditions

The chromatographic system consisted of an Alliance e2695 Separation Module equipped with an online degasser and an automatic injector maintained at 10 °C as well as a 2998 photodiode array detector, set at 260 nm for the detection of ATV, DRV, DTG, EFV, LPV, RGV and TPV and at 305 nm for ETV and RPV. Data were collected and processed using Empower software for HPLC system (Waters, Milan, Italy).

Separations were performed on an XBridge C_{18} (4.6 mm×150 mm, 3.5 µm; Waters) column equipped with a Sentry (4.6 mm×10 mm; Waters) guard column. Both columns were maintained at 35 °C. The mobile phase consisted of acetonitrile (solvent A) and 50 mM acetate buffer at pH 4.5 (solvent B) delivered at a flow rate of 1 mL/min. A gradient elution was applied: 40% of solvent A was maintained for 9 min, then raised in 7 min to 70%. A re-equilibration step was included and the total run time was 25 min.

2.4. Sample preparation

Patients receiving standard doses of different antiretroviral drugs underwent blood sampling after obtaining their informed consent for the measurement of plasma drug concentrations. 5 mL blood samples were collected in EDTA tubes and plasma was obtained after centrifugation at 3000 rpm for 10 min at 4 °C (Sigma 3–16PK). Plasma samples were then underwent heat inactivation procedure for HIV (35 min at 58 °C). To avoid thawing cycles, each patient plasma sample was aliquoted into micro tubes of 2 mL and stored at −20 °C until analysis. On the day of analysis an aliquot of 500 µL of plasma samples was pipetted into labelled disposable polypropylene eppendorf tubes and 50 µL of IS solution was added. The tubes were vortex-mixed for 30 s, and then 500 µL of 50 mM acetate buffer at pH 4.5 was added, for protein precipitation. The tubes were vortex-mixed for further 60 s and centrifuged at 10,000g for 10 min at 4 °C.

Table 1.
Retention time, UV wavelengths, calibration points and QC concentrations.

Analyte	Retention time (min)	Wavelengths (nm)	Calibration points (ng/mL)						QC (ng/mL)		
			STD1	STD2	STD3	STD4	STD5	STD6	Low	Medium	High
ATV	15.3	260	60	180	600	1800	6000	12,000	90	900	2000
DTG[a]	5.4	260	20	60	200	800	4000	8000	50	300	2000
DRV	9.6	260	150	300	900	3000	6000	15,000	600	1800	8000
EFV[a]	17.1	260	150	300	900	3000	6000	15,000	600	1800	8000
ETV	17.4	305	50	100	500	1000	2000	4000	200	1600	3000
LPV[b]	17.4	260	150	300	900	3000	6000	15,000	600	1800	8000
RGV[b]	5.6	260	40	120	400	1200	4800	9600	80	600	6000
RPV	13.8	305	20	60	200	400	600	2000	50	300	1000
TPV	18.7	260	500	1000	5000	10,000	20,000	40,000	2000	16,000	30,000
Internal standard	8.6	260	–	–	–	–	–	–	–	–	–

[a] DTG and EFV were only present in calibration curve A due to its co-elution with RGV and LPV, respectively.

[b] LPV and RGV were only present in calibration curve B due to its co-elution with EFV and DTG, respectively.
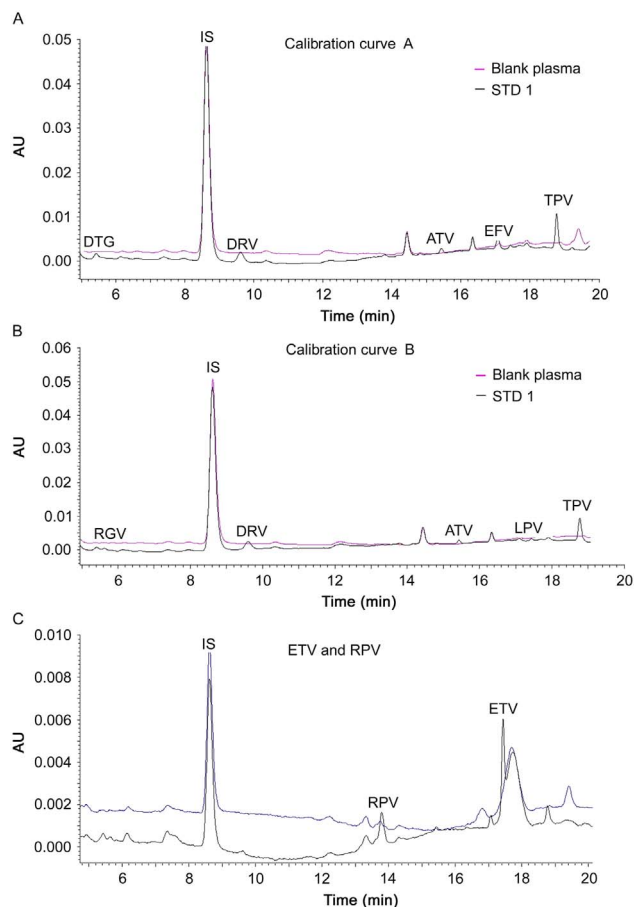
Fig. 1. Representative chromatograms of standard point 1 and blank plasma of (A) calibration curves A and (B) calibration curves B at 260 nm and (C) RPV and ETV at 305 nm.
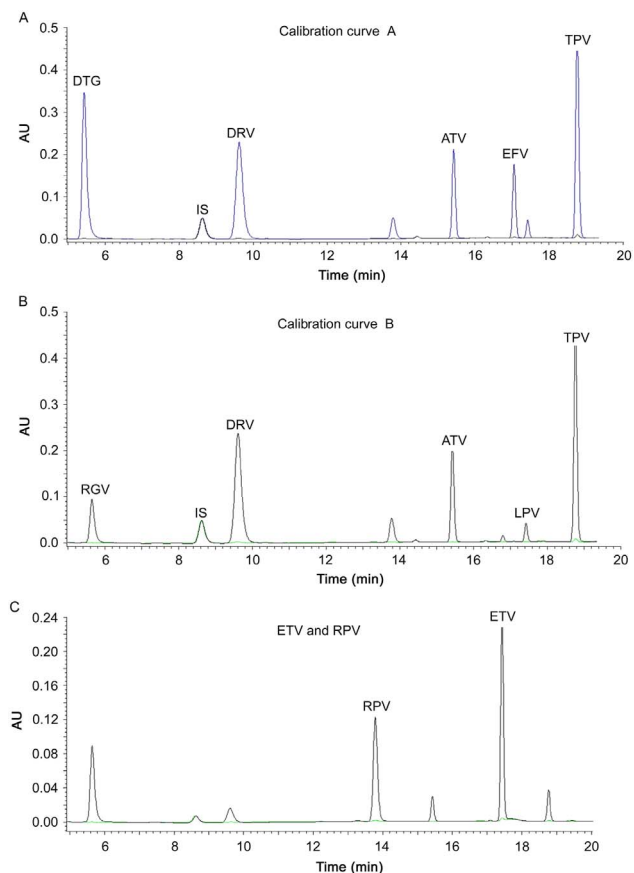

Fig. 2. Comparative chromatograms of standard point 6 and standard point 1 of (A) calibration curves A and (B) calibration curves B at 260 nm and (C) RPV and ETV at 305 nm.

Solid phase extraction (SPE) C18 cartridges were placed on a Vac Elut 20 Manifold (Agilent Technologies) and activated with 1 mL of methanol followed by 1 mL of HPLC solvent B before sample loading. Loading was carried out under gravity. The cartridges were then washed with 500 µL of HPLC solvent B, followed by 250 µL of HPLC grade water and then elution was carried out using 1 mL of methanol and acetonitrile solution (90:10, v/v). Eluted solution was collected into a polypropylene tube and dried at 50 °C in a model Speedvac centrifugal evaporator (Bioinstruments, Italy). The residue was re-suspended in 150 µL of $H_2O:CH_3CN$ (60:40, v/v), centrifuged, filtered and then transferred to polypropylene vials. 30 µL sample was then injected into the HPLC system.

2.5. Method validation

2.5.1. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of different blank plasma samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated by spiking blank plasma with them. To test potential concomitant medication or xenobiotic interference, plasma samples from 30 patients given different combinations of anti-HIV drugs (together with abacavir, lamivudine, tenofovir, ritonavir, amprenavir, zidovudine, nelfinavir, maraviroc), antibiotics (linezolid, vancomycin, gentamicin, rifampicin, levofloxacin), or antifungal agents (voriconazole, posaconazole) were used for the analysis.

2.5.2. Accuracy, precision, calibration, and limit of quantification

Intraday and interday accuracy and precision were determined by assaying 6 spiked plasma samples at 3 different concentrations (QCs) for all drugs. Accuracy was calculated as the percent deviation from the nominal concentration. Interday and intraday precisions were expressed as the relative standard deviation (RSD) at each QC concentration. Each calibration curve was obtained using 6 calibration points, the ranges of which are listed in Table 1. Calibration curves were constructed by linear least-squares regression ($1/x^2$ weighting) of peak height ratios (analyte/IS) versus nominal concentrations. The method has a good linearity if the coefficient of regression (r^2) calculated as mean of 10 curves was ≥ 0.99 [27]. The calibration curves for estimating all the drugs concentrations in unknown samples consisted of six concentrations of plasma samples. These samples were prepared in every analysis together with a blank plasma sample.

The within-day and between-day coefficient of variation (CV) and the accuracy of the method were assessed by calculating daily and overall CVs and bias values for QC (five replicates at each concentration per analytical run) that were assayed in 5 separate analytical runs. The assay was considered acceptable if CV at each concentration was < 15% for both within-and between-day variability and the deviation of the mean from the true value was within ± 15% [27]. The lowest identifiable peak that yielded a signal to noise ratio of 10:1 with a reproducible concentration (imprecision of 20% and accuracy of 80%–120% for each analyte) was accepted as limit of quantification (LOQ) and was set as the first calibration curve point (Table 1).

2.5.3. Recovery

Recovery from plasma, using the extraction procedures, was assessed by comparing the peak height ratio obtained from multiple analyses of spiked plasma samples (QCs) with the peak height ratio from direct injections of the same amount of all analytes and IS. The

Table 2.
Intraday and interday precision study.

Analyte	Level	Intraday			Interday		
		Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)	Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)
ATV	LOQ	60.3 ± 7.1	11.7	100.5	63.3 ± 6.8	10.7	105.5
	LQC	90.9 ± 6.7	7.3	101.0	99.7 ± 6.6	6.7	110.8
	MQC	875 ± 65	7.4	97.2	964 ± 57.4	5.9	107.1
	HQC	2170 ± 281	12.9	108.5	1815 ± 79	4.3	90.7
DTG	LOQ	19.9 ± 1.6	8.1	99.6	19.9 ± 0.3	1.5	99.8
	LQC	46.7 ± 2.9	6.1	93.5	53.7 ± 6.8	12.7	107.4
	MQC	337 ± 21	6.4	112.4	340 ± 16	4.7	113.5
	HQC	2216 ± 201	9.1	110.7	2182 ± 253	11.6	109.1
DRV	LOQ	155 ± 12	7.5	103.5	149 ± 15	10.1	99.4
	LQC	625 ± 77	12.3	104.2	611 ± 15	2.4	101.8
	MQC	1887 ± 68	3.6	104.8	1949 ± 52.6	2.7	108.3
	HQC	8626 ± 531	6.1	107.8	8374 ± 829	9.9	104.7
ETV	LOQ	51.7 ± 5.7	11.0	103.4	50.1 ± 5.7	11.3	100.2
	LQC	195 ± 25	13.0	97.0	216 ± 31	14.3	108.2
	MQC	1648 ± 120	7.3	103.0	1538 ± 178	11.5	96.1
	HQC	4283 ± 306	7.1	107.1	4162 ± 298	7.1	104.0
EFV	LOQ	153 ± 13	8.7	102.1	148 ± 12	8.1	98.9
	LQC	638 ± 50	7.8	106.3	603 ± 53	8.8	99.43
	MQC	1835 ± 186	10.2	101.9	1850 ± 125	6.7	102.8
	HQC	8177 ± 787	9.72	102.2	8032 ± 346	4.3	99.6
RGV	LOQ	41.9 ± 5.0	12.0	104.8	42.1 ± 4.75	11.2	105.3
	LQC	81.9 ± 7.9	9.7	102.4	85.9 ± 8.2	9.6	107.4
	MQC	620 ± 51	8.3	103.4	567 ± 18	3.2	94.5
	HQC	6098 ± 663	10.9	101.6	6121 ± 254	4.1	102.0
RPV	LOQ	19.8 ± 0.9	4.6	99.2	19.7 ± 0.3	1.4	98.5
	LQC	43.5 ± 0.8	1.8	87.1	44.9 ± 4.6	12.3	89.7
	MQC	264 ± 6.1	2.3	88.0	273 ± 30	11.1	91.0
	HQC	935 ± 32	3.5	93.5	882 ± 88	10.0	88.2
LPV	LOQ	146 ± 16	10.6	97.3	151 ± 12	7.7	100.4
	LQC	620 ± 65	10.5	103.4	570 ± 73	12.9	95.0
	MQC	1828 ± 127	7.0	101.6	1841 ± 110	6.0	102.3
	HQC	8600 ± 585	6.8	107.5	8039 ± 1049	13.1	99.51
TPV	LOQ	529 ± 50	9.4	105.8	517 ± 59	11.4	103.3
	LQC	2030 ± 225	10.0	101.5	2040 ± 148	7.3	102.0
	MQC	13,962 ± 849	6.1	87.3	14,989 ± 2023	13.5	93.7
	HQC	30,761 ± 1981	6.4	102.5	30,102 ± 1469	4.8	100.3

LQC: low quality control; MQC:medium quality control; HQC: high quality control.

assay was accepted if recovery exceeded 80%.

2.5.4. Stability

Drug stability in plasma samples was studied as per the FDA guidelines [27]. Stability studies evaluated the stability of all the analytes during sample collection and handling, after long-term (intended storage temperature) and short-term (bench-top, room temperature) storage, after going through freeze and thaw cycles and the analytical process. The freeze-thaw stability was determined after three freeze-thaw cycles of freezing at −60 °C for 24 h and then thawing completely at room temperature. The stability of extracted samples at 20 °C in the autosampler was evaluated up to 24 h after extraction. The long-term stability was evaluated using QC samples that were stored at −60 °C for at least 3 months.

2.6. Patients samples analysis

In order to ascertain accuracy when measuring clinical samples, our laboratory participated in an external quality assurance programme (KKGT, Radbound University Medical Centre, Nijmegen, The Netherlands) [28]. The developed method has been routinely used for the therapeutic drug monitoring (TDM) of ATV, DRV, DTG, ETV, EFV, LPV, RGV, RPV and TPV and for the optimisation of HAART therapy for HIV-infected patients.

3. Results

Retention time of all the analytes are shown in Table 1. Retention time of DTG and EFV was found to be close to that of RGV and LPV, respectively. Furthermore, LPV and ETV even had the same retention time. For this reason, 2 different lots of the STD and the QCs were made. DTG, DRV, RPV, ATV, EFV, ETV and TPV were present in calibration curve A while RGV, DRV, RPV, ATV, LPV, ETV and TPV were present in calibration curve B. The use of two wavelengths for LPV and ETV permits to quantify the two analytes concomitantly in a precise way, the absorbance at the other wavelength is in fact negligible. Representative chromatograms of STD 1 and blank plasma of calibration curve A, calibration curve B at 260 nm, and ETV and RPV at 305 nm are shown in Fig. 1. The comparative chromatograms of STD 6 and STD 1 of calibration curves A and calibration curves B at 260 nm, and ETV and RPV at 305 nm are shown in Fig. 2. Mean regression coefficient of determination (r^2) of all calibration curves was better

Table 3.
Results of the stability studies.

Analytes	Levels	Freeze–thaw (3 cycles)			Autosampler (24 h)			Long term (90 days)		
		Calculated concentration (µg/mL) (mean ± SD)	REC[a] (%)	RSD[b] (%)	Calculated concentration (µg/mL) (mean ± SD)	REC[a] (%)	RSD[b] (%)	Calculated concentration (µg/mL) (mean ± SD)	REC[a] (%)	RSD[b] (%)
ATV	LQC	90.86 ± 2.5	100.9	2.7	90.3 ± 1.6	100.4	1.8	90.4 ± 1.7	98.1	1.8
	MQC	918.4 ± 44.9	102.0	4.8	914 ± 30.8	102.3	3.3	904.6 ± 38.2	102.3	4.2
	HQC	2095.8 ± 82.7	104.7	3.9	2059.4 ± 92.5	97.6	4.4	1995.8 ± 40.5	103.4	2.0
DTG	LQC	49.8 ± 1.8	99.7	3.7	50.83 ± 2.0	101.6	4.0	50.6 ± 2.2	101.2	4.5
	MQC	297.1 ± 12.2	99.0	4.1	298.82 ± 12.5	99.6	4.2	304.6 ± 14.8	101.5	4.8
	HQC	1979 ± 110.5	98.9	5.5	2042.8 ± 68.1	102.1	3.3	2067 ± 59.7	103.3	2.9
DRV	LQC	607.2 ± 23.6	101.2	3.8	608.7 ± 29.9	101.5	4.9	603.3 ± 29.8	100.5	4.9
	MQC	1819.1 ± 35.3	101.1	1.9	1821.8 ± 94.5	101.2	3.1	1820.6 ± 46.7	101.1	2.6
	HQC	8034.9 ± 144.5	100.4	1.8	8033.7 ± 162.8	100.4	2.0	8059.0 ± 93.5	100.7	1.1
ETV	LQC	195.6 ± 8.4	97.8	4.3	200.56 ± 9.3	100.2	4.6	195.2 ± 7.3	97.6	3.8
	MQC	1603.1 ± 75.3	100.1	4.7	1619.8 ± 52.0	101.2	3.2	1620.7 ± 42.9	101.2	2.6
	HQC	3005.3 ± 89.4	100.1	2.9	2011.4 ± 89.9	100.4	2.3	3009.4 ± 81.7	100.3	2.7
EFV	LQC	597.6 ± 27.6	99.6	4.6	609.8 ± 25.8	101.6	4.2	582.8 ± 25.3	97.1	4.3
	MQC	1824.9 ± 54.3	101.3	3.0	1835.2 ± 44.8	101.9	2.4	1794.6 ± 31.4	99.7	1.4
	HQC	7983.0 ± 111.8	99.7	1.4	7899.0 ± 611	98.7	0.8	7915.7 ± 115.8	98.9	1.4
RGV	LQC	81.2 ± 5.6	101.9	4.0	82.1 ± 3.2	102.5	3.9	83.4 ± 3.6	104.2	4.3
	MQC	585.4 ± 17.2	97.5	2.9	579.1 ± 12.0	96.5	2.1	585.5 ± 20.1	97.6	3.4
	HQC	5933.1 ± 81.6	98.8	1.4	5961 ± 96.4	99.1	1.6	5931.1 ± 95.4	98.8	1.6
RPV	LQC	51.66 ± 2.4	103.3	4.6	51.48 ± 2.0	102.9	3.9	50.7 ± 2.2	101.4	4.3
	MQC	290.1 ± 13.3	96.7	4.6	296.2 ± 14.2	98.7	4.8	298 ± 13.5	99.3	4.5
	HQC	984.9 ± 23.9	98.4	2.4	971.6 ± 26.5	97.1	2.7	1016.1 ± 37.1	101.6	3.6
LPV	LQC	575.8 ± 26.3	95.9	4.5	588.1 ± 21.5	98.0	3.7	597.4 ± 26.9	99.5	4.5
	MQC	1853.9 ± 51.6	102.9	2.7	1755.7 ± 35.1	97.5	2.0	1761.7 ± 37.5	97.8	2.1
	HQC	8076.6 ± 104.5	100.9	1.3	8122.6 ± 35.3	101.5	0.4	8063.0 ± 110.6	100.9	1.3
TPV	LQC	2049.7 ± 89.4	102.4	4.3	2074 ± 71.4	103.7	3.4	2038.7 ± 95.5	101.9	4.6
	MQC	16,031.6 ± 177.7	100.1	1.1	16,280.8 ± 415.7	101.7	2.5	16,171.0 ± 505.8	101.1	3.1
	HQC	30,650.1 ± 1205.3	102.1	3.9	30,048.7 ± 1096	100.2	3.6	30,862.4 ± 3.5	102.8	3.5

LQC=low quality control, MQC=medium quality control, HQC=high quality control.
[a] REC (%)=calculated concentration/spiked concentration×100%.
[b] RSD (%)=SD/calculated concentration×100%.

than 0.99. A linear through zero regression was chosen due to good linear response for all the drugs up to STD 6.

3.1. Specificity and selectivity

The assay did not show any significant interference with antiretroviral drugs or other concomitant drugs administered to the patients at therapeutic doses, excluding the overlapping of DTG with RGV, EFV and ETV with LPV (Figs. 1 and 2). Five drug-free plasma samples did not show any interfering peaks in the retention time windows, considering the specific wavelength for each drug.

3.2. Accuracy, precision, and limit of quantification

Method validation results are listed in Table 2 for all analytes. All observed data [intraday and interday precision as percent relative standard deviation (RSD%)] were below 15.0%, in line with the FDA guidelines [27]. A linear response was shown for all drugs up to STD 6 (Table 1). LOQ for each analyte is listed in Table 2.

3.3. Recovery

Multiple aliquots at each of the QC concentrations were assayed; and the mean recovery of all drugs ranged between 80% and 120%.

3.4. Stability

After three freeze-thaw cycles, the changes in the drug concentrations were less than ± 5%. The run-time stability study showed that extracted analytes were stable in the autosampler at 20 °C for up to 24 h (deviation within ± 5% compared with the result at 0 h). Long-term stability tests were in the acceptable range (Table 3).

3.5. Patients samples analysis

The developed method was applied for the determination of DRV plasma concentration in 371 samples, of which 7.8% were found to have levels less than the LOQ. The measured DRV trough concentrations ranged from 205 to 13,488 ng/mL, with an inter-individual CV of 70.6% (Fig. 3A). ATV plasma concentrations in 1022 samples were analysed, of which 13.5% were found to have concentration less than the LOQ. The measured ATV trough concentrations ranged from 60 to 6144 ng/mL, with an inter-individual CV of 96.8% (Fig. 3B). ETV plasma concentration was measured in 74 samples, out of which 8.1% samples were found to have concentration less than the LOQ. The measured ETV trough concentrations ranged from 53 to 3158 ng/mL with an inter-individual CV of 85.6% (Fig. 3C). LPV plasma concentration was measured in 209 samples out of which 16.7% samples were found to have concentration less than the LOQ. The measured LPV trough concentrations ranged from 174 to 26,125 ng/mL, with an
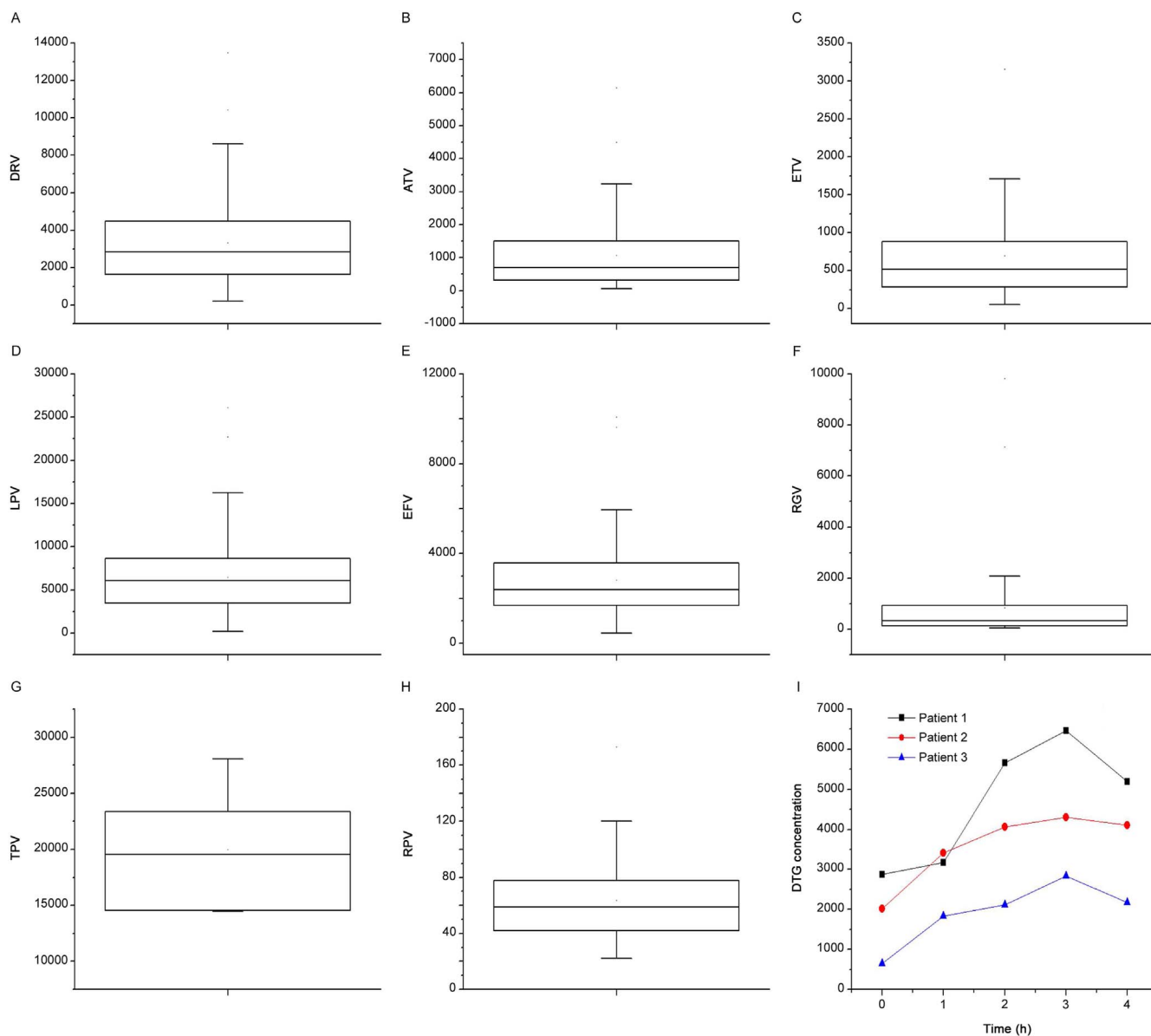
Fig. 3. Box-plot of (A) DRV ($n=342$), (B) ATV ($n=885$), (C) ETV ($n=68$), (D) LPV ($n=174$), (E) EFV ($n=181$), (F) RGV ($n=344$), (G) TPV ($n=5$), (H) RPV ($n=48$) and (I) DTG ($n=3$), where n=number of samples above LOQ.

inter-individual CV of 66.6% (Fig. 3D). EFV plasma concentration was measured in 207 samples, out of which 12.6% samples were found to have concentration less than the LOQ. The measured EFV trough concentrations ranged from 454 to 10,078 ng/mL, with an inter-individual CV of 65.5% (Fig. 3E). Out of 408 RGV samples measured, 17.1% samples were found to have concentrations less than the LOQ. The measured RGV trough concentrations ranged from 42 to 9814 ng/mL, with an inter-individual CV of 158.63% (Fig. 3F). Out of 12 TPV samples measured, 58.3% samples were found to have concentrations less than the LOQ. The measured TPV trough concentrations ranged from 14,450 to 28,065 ng/mL, with an inter-individual CV of 29.3% (Fig. 3G). The measured RPV trough concentrations ranged from 22 to 173 ng/mL, with an inter-individual CV of 48.0% (Fig. 3H) in 48 samples and no samples were found below the LOQ level. The measured DTG trough concentrations ranged from 649 to 2878 ng/mL with an inter-individual variability of 60.8% in 3 samples. The same distribution was also observed for the other sampling time points: DTG concentrations at 1, 2, 3 and 4 h after the morning drug intake ranged

from 1835 to 3420 ng/mL (CV, 30.4%), from 2112 to 5666 ng/mL (CV, 45.1%), from 2836 to 6462 ng/mL (CV, 40.2%) and from 2167 to 5195 ng/mL (CV, 40.1%), respectively (Fig. 3I).

4. Discussion

As discussed in the introduction, several HPLC–UV methods are available in the literature for the simultaneous quantification of antiretroviral drugs in human plasma. However, for the first time we reported here the development and validation of an HPLC–UV assay to quantify the 9 antiretroviral compounds including DTG and RPV with accuracy and precision. This bio-analytical method is now being successfully applied to measure antiretroviral plasma concentrations for therapeutic purposes.

Under our laboratory conditions, all drugs appeared to be stable when subjected to the freeze/thaw cycles, in comparison with freshly prepared controls. Moreover, long-term stability data suggested that all analytes remained sufficiently stable under our current storage condi-

tions (−60 ºC). The stability of extracted samples at room temperature (20 ºC) or within the autosampler was found to be stable over the period of analysis. This HPLC−UV SPE assay had a short run time of 25 min per cycle with simple gradient, making it suitable for a high-throughput TDM, whereby large numbers of samples were processed quickly and efficiently. This is of high value in a clinical setting where laboratory analyses have to be optimised based on the time-efficiency and reliability.

Emergence of drug-resistant HIV-1 strains and problems of long-term tolerability of some anti-HIV compounds can jeopardise efficacy and acceptability of existing anti-HIV medications. DTG and RPV are among the most recent and promising anti-HIV molecules. Both drugs have been shown to be highly effective in multi-treated patients and found to be well tolerated. DTG and the P450(CYP)3A4 inhibitor, RPV, have the high potential for the interactions with other antiretroviral agents, notably with protein inhibitors and maraviroc [29,30]. RGV and ETV also have a high potential for drug interactions, being substrates and inducers of CYP3A4 [31,32]. Patients administered with DTG and/or RPV are also given other antiretroviral compounds; therefore, the measurement of plasma concentrations of the different drugs in the same antiretroviral regimen is necessary. Our method is of particular clinical value as it allows a quick and reliable assessment of the plasma level of these drugs, although the clinical usefulness of TDM of DTG and RPV and their interactions with other co-administered drugs still need to be investigated further.

Our assay method included widely prescribed protein inhibitors, non-nucleoside reverse-transcriptase inhibitors and integrase inhibitors [33]. The choice for the limits of their ranges was based on the highest values reported in the clinical reports and pharmacokinetic studies. Reliability, costs, ruggedness, sensitivity and reproducibility are key points of measurement of drug plasma concentrations. Our assay, relying on SPE coupled with a high-sensitive Waters UV detector, was simple and less expensive in terms of consumables and instrumentation when compared with other methods developed on LC−MS or LC−MS/MS.

The relative accuracy of all QCs at three different concentration levels and intraday and interday precision study support both the accuracy and precision of our procedure. The choice of a specific wavelength for each drug (Table 1) was based on the need for adequate sensitivity and high specificity. An example is ETV and RPV for which quantification at 305 nm was chosen to ensure the absence of interferences and better sensitivity. The absence of interfering peaks, excluding the overlapping of DTG with RGV and EFV, ETV with LPV, allows accurate measurement of drugs plasma concentrations. The co-elution of DTG and RGV, occurring with our method because of the similar retention time, is not an issue as both drugs belong to the same class and hence are highly unlikely to be prescribed together. Co-elution of LPV and EFV can be tackled by running two different assays or by analysing either one of them in a single assay.

5. Conclusion

The HPLC−UV method described here allows an accurate and reproducible simultaneous quantification of nine antiretroviral agents in plasma. Good extraction efficiency and low limit of quantification make this a suitable method for use in clinical trials and for TDM. This method has been successfully applied to our routine TDM and pharmacokinetics studies in HIV-infected patients.

Acknowledgments

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