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A Reliable HPLC-UV Method for the Simultaneous Determination of Three Antiretroviral Drugs in Human Blood Plasma Applied to Therapeutic Drug Monitoring

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Acquired immunodeficiency syndrome is an infectious disease caused by human immunodeficiency virus (HIV). Pharmacological antiretroviral therapy, along with therapeutic monitoring of drug plasma concentrations, facilitates adequate and effective follow-up in each patient. Our main objective was to develop and validate a high-performance liquid chromatography with ultraviolet detector (HPLC-UV) bioanalytical method for the simultaneous quantification of plasma concentrations of three commonly used antiretrovirals in Colombia: efavirenz, lopinavir, and ritonavir. This method is intended for therapeutic drug monitoring in persons living with HIV locally or in developing countries. The method is selective, specific, accurate, precise, and stable over the concentration range of 250 and 20,000 ng mL^{-1} for the drugs. It was successfully applied to measure the plasma levels of efavirenz, lopinavir, and ritonavir in HIV patients, which ranged from 251.0 ± 10.4 to 7186.2 ± 10.2, 8188.2 ± 5.9 to 15,312.5 ± 6.6, and 246.1 ± 6.5 to 1189.0 ± 6.4 ng mL^{-1}, respectively.

Keywords: efavirenz, lopinavir, ritonavir, plasma concentration, bioanalytical method, HPLC-UV

Introduction

Acquired immunodeficiency syndrome (AIDS) is an infectious disease caused by human immunodeficiency virus (HIV), which is transmitted by the exchange of body fluids such as blood, semen, and vaginal secretions, especially during unprotected sexual contact with infected individuals. HIV attacks the immune system by destroying immune cells and obstructing the body defense mechanism against infections and certain types of cancer, leading the infected person to gradually decline to a state of immunodeficiency.[1]

HIV infection is currently considered one of the most serious public health problems worldwide. An estimated 39.0 million people were living with the virus in 2022, of which 29.8 million (76%) were accessing antiretroviral therapy, but only 71% were virally suppressed.[1,2] In Colombia, a total of 141,787 cases of HIV infection were reported up to January 2022, with 125,264 (88.4%) individuals on antiretroviral therapy and 77.4% achieving suppressed viral loads.[3]

Antiretroviral (ARV) drugs are a group of molecules used to treat infections caused by HIV. They are intended to decrease or interrupt viral replication by inhibiting the enzymes involved in this process (reverse transcriptase, protease, and integrase inhibitors), blocking enzyme activities (maturation and integrase strand transfer inhibitors), or preventing the entry of HIV into cells (C–C chemokine receptor type 5 (CCR5) antagonists, fusion and anchor inhibitors).[4,5] Generally, clinical guidelines for the management of HIV infection recommend using a treatment regimen that combines several ARVs. Recently, regimens based on the strand-transfer inhibitor dolutegravir

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have been suggested as the first-line treatment for most people with HIV.[6-8] However, alternative regimens based on non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as efavirenz (EFV), are more common in countries that are members of the World Health Organization (WHO), including Colombia.[3,9] In addition, EFV is highly recommended for once-daily regimens and for patients with tuberculosis coinfection who are taking rifapentine.[6]

Combined therapy with ARVs frequently includes protease inhibitors (PIs) boosted with ritonavir (RTV). A representative of this group is the lopinavir/ritonavir (LPV/RTV) combination, currently the only PI/RTV coformulation commercially available for HIV treatment.[10] LPV/RTV, along with nucleoside reverse transcriptase inhibitors, is used as a preferred PI-based regimen for infants aged from 14 days to < 4 weeks, a first-line alternative regimen in children, and a second-line regimen for adults and adolescents. In addition, RTV is also used to boost the PIs atazanavir or darunavir.[3,8,11]

In Colombia, EFV, RTV, and LPV are widely used, ranking as the third, fourth, and thirteenth most commonly used ARV drugs for people living with HIV covered by the health system in 2022, respectively, among the 29 of these drugs available in the country.[3]

ARV therapy enables HIV patients to improve survival by keeping them virally suppressed and reducing complications, thereby preventing transmission of the virus to other individuals. These benefits are possible if the treatment starts as soon as possible after the diagnosis, and the medications are taken consistently every day throughout the lifetime of the person.[12] However, prolonged use of ARVs might be challenging, and nonadherence is a frequent problem in HIV patients. Nonadherence is often associated with drug-related problems, adverse drug reactions, a history of nonadherence to treatment, and the use of psychoactive substances.[13] On the other hand, the practice of polypharmacy in patients living with HIV can lead to the development of pharmacokinetic interactions among the drugs used, either ARVs or with other concomitant treatments, which may significantly influence drug plasma levels, requiring dose adjustment or ARV change.[6,8,11] For these reasons, it is essential to implement strategies to optimize the medication regimen assigned to the patient in terms of efficacy and safety.

A valuable clinical tool for this purpose is therapeutic drug monitoring (TDM), which involves measuring *in vivo* post-dose drug concentrations once or over time and adjusting the doses of active ingredients to maintain these concentrations within a therapeutic range. Therefore, TDM is only feasible for drugs where an exposure-response relationship exists.[14,15] TDM has been used in people living with HIV and has shown a positive impact on managing patients with physiological changes (childhood, aging, or pregnancy), those suspected of drug-drug interactions or toxicity, and patients with questioned adherence.[14-18] Moreover, since EFV trough concentrations > 4000 ng mL^{-1} are associated with toxic central nervous system manifestations in children, it is recommended to measure the EFV trough plasma concentration when such symptoms appear, to decide between drug substitution or dose adjustment. Similarly, due to the high interindividual drug exposure variability in children, TDM is recommended to decide once-daily administration of LPV/RTV,[11] and measuring plasma concentrations of all three ARVs is also advised for adults with HIV taking anticonvulsants.[6]

Measurement of ARVs plasma concentration for the aforementioned applications requires validated bioanalytical methods that can selectively and reliably quantify the analytes of interest in the presence of any other substances from the matrix or other treatments used by the patients. Worldwide, the academic-scientific community has developed several bioanalytical methods to quantify EFV, LPV, and RTV, among other ARVs. Most of these methods use high-performance liquid chromatography coupled to mass detectors (HPLC-MS) or ultraviolet detectors (HPLC-UV) due to their sensitivity, suitability for making accurate quantitative determinations, and wide applicability.[19-30] However, the most sophisticated methods may be unfeasible for countries with limited resources. Therefore, institutions interested in implementing TDM should always develop and validate their methodologies according to local needs and capacities.

In this article, an HPLC-UV method for the simultaneous quantification of the concentration of three antiretrovirals commonly used in Colombia, EFV, LPV, and RTV, in human plasma, was developed and validated at the Bioavailability and Bioequivalence Laboratory of the Centro de Servicios Farmacéuticos Monitoreo de Fármacos (CESFAR), of the Universidad del Atlántico, located in the Caribbean Region of Colombia. It is intended for TDM in individuals living with HIV locally or in developing countries.

Experimental

Reagents

Analytical standards of EFV, LPV, RTV, and carbamazepine were obtained from USP (Rockville, MD, USA). Methyl *tert*-butyl ether (MTBE) was obtained from Macron Fine Chemicals (Batavia, IL, USA). HPLC-grade solvents, stationary phase, and the other reagents were

obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system from Millipore (Bedford, MA, USA).

Chromatographic conditions

The chromatographic analysis was performed on an HPLC Chromaster (Hitachi, Tokyo, Japan). The chromatographic conditions (mobile phase, flow, temperature, wavelength, and injection volume) were initially assessed based on the method described by Notari *et al.*,[24] using a reverse phase C18e monolithic chromatographic column. The initial system conditions were adjusted until satisfactory and reproducible results were obtained.

At the final conditions, system suitability criteria were established and verified at the beginning of each chromatographic run. This involved analyzing a minimum of five injections of a standard solution of EFV, LPV, and RTV at 10,000 ng mL^{-1}, along with the internal standard (IS), carbamazepine. The measured parameters included resolution, asymmetry (at 5 and 10% of the peak height), number of theoretical plates, and the relative standard deviation of the analytical signals of interest.

Preparation of stock solutions, working solutions, calibration curves, and quality control samples

Standard stock solutions of EFV, LPV, RTV, and IS were prepared at 1.0 mg mL^{-1} in methanol and refrigerated at 7 °C until use. Working solutions, containing a mixture of the ARVs and IS, were prepared before use by diluting from stock solutions. A mixture of 10 mmol L^{-1} pH 5.00 ± 0.05 phosphate buffer and acetonitrile (65:35) was used as a diluent. One working solution was prepared for each level of the calibration curve. Calibration curves were prepared by adding 40 µL of the corresponding working solution to blank human plasma (500 µL), resulting in a concentration range between 250-20,000 ng mL^{-1} for each ARV, with IS set at 1500 ng mL^{-1}. Quality control samples (QC) were similarly prepared at concentrations of 250 (lower limit of quantification, LLOQ), 1000 (low quality control, LQC), 10,000 (middle quality control, MQC), and 20,000 (high quality control, HQC) ng mL^{-1} using a fresh set of working solutions independent of those used for the calibration curve.

Drug extraction from human blood plasma

Two methods of drug extraction from blood plasma were tested, i.e., the protein precipitation method using acetonitrile (2:1 solvent to plasma), followed by the separation of the clear supernatant by centrifugation at 4200 × g and the liquid-liquid extraction method using MTBE (5:1 solvent to plasma), followed by the separation and air evaporation of the organic layer to obtain a dry residue. This residue was reconstituted with diluent to half the initial volume of plasma. Finally, the two methods were consecutively combined, with the pertinent modifications according to the results obtained in preliminary assays. The recovery of the method was evaluated as a measure of the efficiency of drug extraction from plasma by comparing the chromatographic signals corresponding to the analytes of interest (EFV, RTV, LPV, and IS) in QCs (LLOQ, LQC, and HQC) that underwent the extraction process against those obtained by analyzing equivalent samples prepared in diluent and not extracted, representing 100% recovery.

Method validation

Selectivity and specificity

Selectivity was evaluated according to the U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation,[31] by testing the methodology on six (6) different sources of blank plasma (without analytes), lipemic plasma (900 mg dL^{-1} triglyceride), and hemolyzed plasma. Chromatographic signals were compared with those from plasma containing the three ARVs at a concentration of 250 ng mL^{-1}, established in linearity as the low limit of quantification, plus the IS. This allowed to evaluate whether the matrix endogenous constituents coeluted with the analytes or the IS. Additionally, analytical interference with 22 drugs potentially used concomitantly with ARVs was assessed using 5000 ng mL^{-1} EFV, LPV, and RTV, 1500 ng mL^{-1} IS, and the highest plasma concentration from the literature for the other drugs.

Linearity

Linearity was evaluated on at least four calibration curves, each consisting of a blank sample corresponding to human plasma without analytes or IS, a zero sample consisting of plasma with IS, and seven concentration levels with the ARVs over a range from 250 to 20,000 ng mL^{-1}. A linear equation was defined to describe the relationship between the chromatographic areas of each analyte normalized with the IS and the nominal concentrations established for each level using least squares regression analysis with a weighting factor of $1/x^2$. Once the equation for the calibration curve for each ARV was obtained, the value of the relative error (E, in percentage) of each concentration level regarding the nominal concentration was calculated. The LLOQ was established as the lowest concentration with E and coefficient of variation (CV)

within the limit of 20%. Meanwhile, for the rest of the levels, a limit of 15% in those parameters was accepted.[31]

Accuracy and precision

Accuracy and precision were determined by analysis of five (5) replicates of the four QCs, quantified using the calibration curve obtained in linearity. Controls were analyzed within the same day (within run) and on different days (between runs). Accuracy was defined as E between the average of the calculated concentration and the nominal concentration for all, LLOQ, LQC, MQC, and HQC. Meanwhile, the CV was used to evaluate the precision of the method. The parameters for intra- and interday accuracy and precision had to comply with the criteria of 20% for the LLOQ and 15% for the other QCs.[31]

Stability

To verify the stability of the ARVs and IS in plasma and diluent, three replicates of LQC and HQC were analyzed fresh and after exposure to the following conditions:
(*i*) Freezing and thawing: three continuous cycles of 24 h of freezing at −35 °C and thawing at room temperature.
(*ii*) Short-term stability: freezing at −35 °C and thawing at room temperature for 24 h.
(*iii*) Stability in the autosampler: processed QCs were kept for 24 h in the HPLC autosampler module at 20 °C.
(*iv*) Long-term stability: freezing at −35 °C for at least one month.

The stability of stock standard solutions (1.0 mg mL^{-1}) was also tested after storage at 4 °C for up to 60 days. For all cases, analytes were considered stable when the final calculated concentrations were in the range from 80 to 120% of the initial value for LLOQ or from 85 to 115% for HQC.[31]

Application of the method

To evaluate the utility of the validated method, it was applied to quantify EFV, RTV, and LPV in 30 patients diagnosed with HIV/AIDS. At the time of sampling, these patients were being treated with LPV/RTV at doses of 400/100 mg twice daily (6 patients) or EFV at a dose of 600 mg once daily (24 patients) in AIDS-specialized medical centers from the Colombian Caribbean Coast region. Samples were drawn 12 h after the last dose of medication in heparinized tubes, centrifuged to obtain blood plasma, and subsequently stored at −35 °C until analysis.

Ethical considerations

The study was approved by the ethics committee of the health science division of the Universidad del Norte (Colombia, Act 104, 2013, 31 October 2013). Written informed consent was obtained from each participant, and the principles of the Declaration of Helsinki were followed throughout the study.

Statistical treatment

Regression analysis was performed using Statgraphics Centurion software version 17.1.12,[32] which included normality and homoscedasticity tests for the data, analysis of variance to evaluate the correlation between variables, a lack-of-fit test to evaluate the fit of the data to the model, and residual analysis by the Durbin-Watson test to confirm its non-correlation, all performed at a 95% confidence level. Other validation parameters were calculated using Microsoft Excel version 16.17.[33]

Results

Chromatographic conditions

A method employing a monolithic endcapped HPLC column and flow gradient was established, obtaining well-resolved peaks for the ARVs and the IS in 20 min. The criteria suggested by the Center for Drug Evaluation and Research (CDER) for the suitability of the system, corresponding to asymmetry (at 5 and 10% of the peak height) < 2.00, number of theoretical plates > 2000, and resolution > 2.00, were adopted.[34] A relative standard deviation (RSD) of up to 5.0% was accepted, considering the complexity of bioanalysis. The ARVs and the IS met all the criteria for all validation runs. The optimized chromatographic conditions and the resulting characteristic chromatogram are presented in Table 1 and Figure 1, respectively.

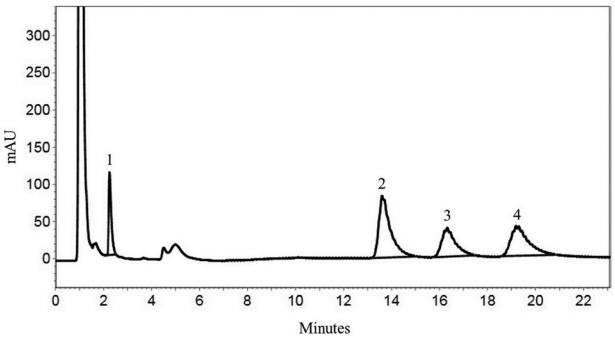

Figure 1. Representative chromatogram of efavirenz (EFV), lopinavir (LPV), ritonavir (RTV) and internal standard (IS) in human plasma. Sample at 20,000 ng mL^{-1}, analyzed by HPLC-UV with C18 monolithic column, and mobile phase with potassium dihydrogen phosphate 10 mmol L^{-1} pH 5.0: acetonitrile (65:35), detected at 205 nm. 1: IS, 2.2 min, 2: EFV, 13.6 min, 3: RTV, 16.3 min, 4: LPV, 19.3 min.

Table 1. Chromatographic conditions for the simultaneous determination of efevirenz, lopinavir, and ritonavir in human blood plasma

Instrumental parameter	Optimized conditions
Stationary phase	Chromolith® RP-18e, 10 × 4.6 mm (Merck, Darmstadt, Germany)
Mobile phase	potassium dihydrogen phosphate 10 mmol L^{-1} pH 5.0: acetonitrile (65:35)
Flow rate / (mL min^{-1})	1.5 (0-5 min), 3.0 (7-20 min), 3 min of re-equilibration
Detection wavelength / nm	205
Column temperature / °C	40
Injection volume / µL	100

Methodology for extracting ARVs and IS from human plasma

The protein precipitation method generated poor signals from analytes with recoveries less than 45%. On the other hand, the liquid-liquid extraction method was more efficient in recovery, but the presence of interference signals was evidenced. The combination of both methods solved this drawback and was, therefore, used as the extraction methodology, as follows. First, 1000 µL of acetonitrile were added to spiked plasma, vortexed for one minute, and centrifuged at 4200 × g for 10 min. Then, the supernatant was transferred to another tube and reserved. The protein residues from precipitation underwent liquid-liquid extraction by adding 500 µL of 10 mmol L^{-1} Tris buffer, adjusted to pH 10.00 ± 0.05, and 1 mL of MTBE. After stirring in a vortex for one minute and centrifugation for 15 min, the resulting organic phase was separated and combined with the previously reserved precipitation supernatant. The mixture was vortexed and centrifuged under the aforementioned conditions, and the last supernatant obtained was transferred to another tube and evaporated to dryness using an air stream. The final residue was resuspended in 500 µL of the diluent, centrifuged, and transferred to a vial for injection into the HPLC system.

The recovery with this extraction methodology ranged from 96.7 to 104.3% for EFV, from 87.9 to 96.8% for LPV, from 96.8 to 104.4% for RTV, and from 99.0 to 106.2% for the IS, the values being consistent among the concentration levels and reproducible throughout the study.

Validation parameters

Selectivity and specificity

The signals of the endogenous components of the different plasma sources tested did not show signals at retention times of the analytes or the IS, demonstrating the selectivity of the developed method. Similarly, most of the analyzed drugs potentially used concomitantly with the ARVs in the study did not present interference signals. Only naproxen eluted close to the IS (Figure 2b), a situation that should be considered during the clinical application of the method. The complete list of drugs with confirmed non-interference is presented in Table 2. It was noted that the method can detect atazanavir, as a resolved signal from the other ARVs analyzed (Figure 2c).

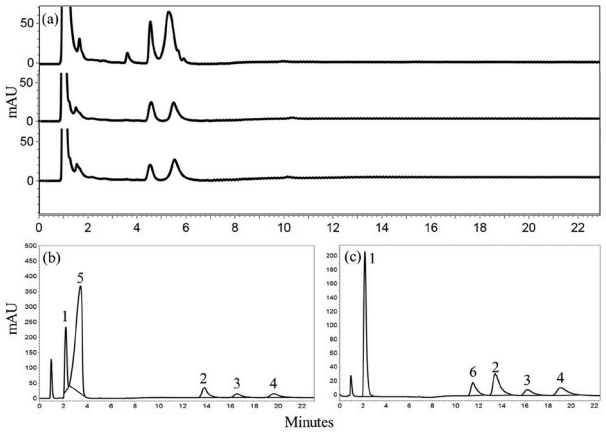

Figure 2. Selectivity and specificity of the method. (a) Chromatograms of blank plasma (bottom), lipemic plasma (middle) and hemolyzed plasma (top), without interferences. (b) Chromatogram showing the interference of naproxen with the internal standard (IS). 1: IS, 2.2 min, 2: efavirenz, 13.6 min, 3: ritonavir, 16.3 min, 4: lopinavir, 19.3 min, 5: naproxen, 3.3 min. (c) Chromatogram showing the non-interfering signal of atazanavir (6, 11.4 min).

Linearity

The values of slope (m), intercept (b), and coefficient of determination (r^2) for each analyte are presented in Table 3. For all curves, the analysis of variance showed *p*-values < 0.00001, indicating a statistically significant relationship between the variables, while the *p*-values for Durbin-Watson test were always > 0.05, confirming the absence of autocorrelation in the residuals. In addition, the values of r^2 were higher than 97%, and the *p*-values for the lack-of-fit test were > 0.05, providing evidence of a good fit for the linear model. The results obtained for E and CV of the concentration levels ≥ 500 ng mL^{-1} met the acceptance criteria (≤ 15%). For all analytes, the concentration level that met the criteria for the LLOQ (E and CV ≤ 20%) was 250 ng mL^{-1}, and the quantification ranges for all ARVs were established between 250-20,000 ng mL^{-1}.

Accuracy and precision

The method proved to be accurate and precise for the tests carried out under the same working conditions in a short time interval (within run) and in those developed in different analytical series (between runs), with E and CV ≤ 19% at the LLOQ and ≤ 15% at the other levels (Table 4).

Table 2. Names, retention times (t_R), and plasma concentrations of drugs evaluated for interferences in the HPLC-UV method developed for the plasma quantification of efavirenz (13.6 min), ritonavir (16.3 min), and lopinavir (19.3 min), using internal standard (2.2 min)

Drug	Retention time / min	Plasma concentration evaluated for interference / (ng mL^{-1})
Abacavir	nd	4700
Acetaminophe	nd	15,700
Ascorbic acid	nd	16,200
Valproic acid	nd	10,800
Atazanavir	11.4	4460
Atorvastatin	4.9	23
Clonazepam	nd	45
Clozapine	5.7	530
Emtricitabine	nd	2500
Enalapril	nd	310
Fluconazole	5.0	5430
Guaifenesin	7.1	1016
Ibuprofen	5.8	58,100
Lamivudine	nd	46,470
Loratadine	nd	74
Metoprolol	nd	233
Naproxen	3.6	63,400
Olanzapine	nd	30
Tenofovir	nd	326
Thiamine	5.0	195
Trimethoprim	1.4	1520
Zidovudine	nd	2810

nd: not detected.

Stability

EFV, LPV, RTV, and the IS were stable in human plasma and diluent under all the conditions evaluated, with recoveries ranging from 85.6 to 115% (Table 5). Stock standard solutions maintained high recovery after 60 days of storage in the refrigerator (4 °C), with values of 106.4 ± 1.8, 106.3 ± 2.5, 108.9 ± 11.0, and 108.6 ± 5.6% for EFV, LPV, RTV and IS, respectively.

Table 3. Calibration curves and linear regression parameters

Analyte	m	b	r^2
Efavirenz	0.00033	0.01115	98.9682
Lopinavir	0.00019	–0.00090	98.7128
Ritonavir	0.00012	0.01506	97.4133

m: slope; b: intercept; r^2: square correlation coefficient.

Application of the method

The method was successfully applied for the measurement of plasma concentrations of EFV, LPV, and RTV in 30 patients. The concentrations of EFV in the patients under treatment with 600 mg of this drug twice a day ranged from 251.0 ± 10.4 to 7186.2 ± 10.2 ng mL^{-1}. Meanwhile, patients under treatment with LPV/RTV at a dose of 400/100 mg twice daily presented concentrations ranging from 8188.2 ± 5.9 to 15,312.5 ± 6.6 and from 246.1 ± 6.5 to 1189.0 ± 6.4 ng mL^{-1}, respectively. Figure 3 shows representative chromatograms from two patients, one with an EFV concentration of 782.60 ± 10.21 ng mL^{-1} (Figure 3a), and another with LPV/RTV at 15,312.5 ± 6.6 and 922.56 ± 6.40 ng mL^{-1}, respectively (Figure 3b).

Discussion

A bioanalytical method was standardized and validated for quantifying the blood plasma concentrations of three ARVs frequently used in conventional therapy for the

Table 4. Accuracy and precision of the HPLC-UV method developed for the plasma quantification of efavirenz, ritonavir, and lopinavir

Concentration / (ng mL^{-1})	Analyte	Within-run		Between runs	
		Accuracy (E) / %	Precision (CV) / %	Accuracy (E) / %	Precision (CV) / %
250	EFV	6.2	13.9	11.6	4.9
	LPV	10.8	6.0	6.6	16.9
	RTV	12.2	19.2	18.6	10.8
1000	EFV	7.0	14.0	5.8	13.6
	LPV	6.4	10.0	12.4	8.5
	RTV	3.6	12.4	5.3	5.4
10,000	EFV	11.2	14.4	15.0	0.6
	LPV	14.9	13.1	11.9	4.9
	RTV	4.4	14.7	9.0	1.2
20,000	EFV	11.1	6.0	9.6	1.2
	LPV	14.3	5.7	9.9	2.3
	RTV	4.0	2.6	4.0	2.1

Data from five replicates for each concentration. EFV: efavirenz; LPV: lopinavir; RTV: ritonavir; E: relative error; CV: coefficient of variation.

Table 5. Stability of the analytes on diluent and human plasma

Concentration / (ng mL^{-1})	Analyte	Recovery at different conditions / %			
		Freezing and thawing	Short-term	Long-term	Autosampler
250	EFV	104.1 ± 4.1	96.3 ± 2.1	104.0 ± 9.4	99.2 ± 0.6
	LPV	86.8 ± 4.8	93.5 ± 2.4	104.5 ± 10.0	107.0 ± 9.9
	RTV	90.0 ± 3.3	89.5 ± 3.9	101.2 ± 13.5	95.9 ± 1.7
20,000	EFV	114.7 ± 1.9	115.0 ± 1.2	107.8 ± 10.3	89.0 ± 3.4
	LPV	85.6 ± 0.0	86.0 ± 0.4	102.5 ± 6.1	93.0 ± 2.5
	RTV	88.7 ± 3.5	86.6 ± 0.9	100.3 ± 2.1	94.1 ± 3.3

Recovery is the percentage of the concentration found at the end of the stability assay relative to the initial value. Data are presented as the mean ± SD of three replicates. EFV: efavirenz; LPV: lopinavir; RTV: ritonavir.

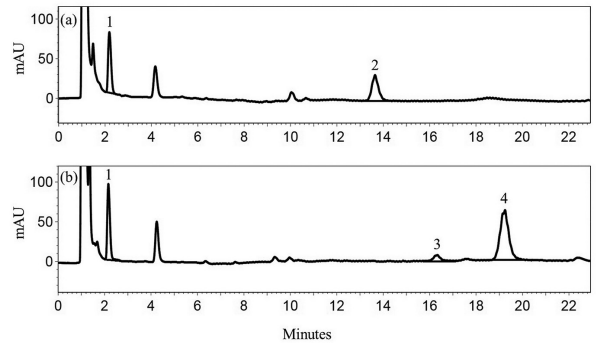

Figure 3. Chromatograms from patients. (a) Chromatogram from a patient treated with efavirenz (EFV) 600 mg once daily, concentration 782.60 ± 10.21 ng mL^{-1}. (b) Chromatogram from a patient treated with lopinavir/ritonavir (LPV/RTV) 400/100 mg twice daily, with concentrations of 15,312.5 ± 6.6 ng mL^{-1} for LPV and 922.56 ± 6.40 ng mL^{-1} for RTV, respectively. 1: internal standard, 2.2 min, 2: EFV, 13.6 min, 3: RTV, 16.3 min, 4: LPV, 19.3 min.

treatment of HIV in Colombia: EFV, LPV, and RTV. To our knowledge, this report marks the first instance of a method established and applied in the country for measuring plasma levels of any ARV.

The developed method combines two widely used techniques for extracting ARVs from blood plasma, i.e., protein precipitation (PP)[19,26,30] and liquid-liquid extraction (LLE),[20,21,25,29,35] resulting in improved drug recovery. PP was performed using acetonitrile, which reduces the polarity of the matrix, thereby lowering protein solubility and causing precipitation.[36] For the LLE, MTBE was employed, a solvent with relatively safe handling characteristics, since it does not cause any specific target organ toxicity. The combination of PP and LLE for ARVs extraction has been previously employed by Zhang *et al.*,[22] achieving lower recoveries (80%) and using solvents that may cause central nervous system toxicity, such as ethyl acetate.

The HPLC conditions established stand out from other reports by using a monolithic column. Unlike traditional packed columns, the main advantage of a monolithic column is its ability to operate at higher mobile phase flows with a relatively small loss in efficiency.[37] This characteristic results in lower retention times for the analytes of interest compared to the majority of reports from other authors quantifying the same ARVs using HPLC-UV,[21,24,25] including those by Rezk *et al.*,[28] and Keil *et al.*,[20] who also used flow gradients (Table S1, Supplementary Information (SI) section).

Consistent with other authors, the method employs a short UV detection wavelength, where most substances absorb UV light. This, coupled with the high need for polypharmacy among HIV patients, underscores the imperative to assess the ability of the method to differentiate analytes from signals of concomitant medication. In this report, specificity was evaluated for 22 other drugs, with only one interference identified, providing crucial information for the routine clinical use of the method. In contrast, other authors who used HPLC-UV for quantifying EFV, RTV, and LPV tested specificity for a smaller number of drugs,[20] and did not indicate the potentially concomitant drugs evaluated[24] or simply did not assess the specificity of the proposed methods[21,28,29] (Table S1).

Considering that the target plasma concentration of EFV and LPV is 1000 ng mL^{-1} and that of RTV, used as a booster of LPV, is 2100 ng mL^{-1},[17] the sensitivity of the proposed method is appropriate for the intended use and even better than that obtained by Rentsch,[27] who used solid phase extraction and mass detection. The concentration range obtained is consistent with the concentrations found in clinical studies focused on TDM, pharmacogenetics, or pharmacokinetics of those drugs, which ranged from approximately 250 to 18,000 ng mL^{-1} for EFV,[38,39] and from approximately 1700 to 37,000 ng mL^{-1} for LPV.[40,41] Meanwhile, some of the reported methods for plasma quantification of these ARVs, using UV or mass detection, fall short of covering the highest concentrations from such clinical studies for at least one of the drugs, making their clinical application difficult[19,22-29] (Table S2, SI section).

Concerning the most sensitive methods reported, several of them use technologies or materials that are

difficult to access, such as ultra-high performance liquid chromatography (UHPLC) coupled with triple quadrupole mass spectrometry,[19] the mobile phase modifier tetramethylammonium perchlorate (TMAP),[21] or the internal standard A-86093.[21,23,26,27,29] The use of more hazardous solvents for drug extraction, such as ethyl acetate and *n*-hexane, was also common in most sensitive methods.[20-22,29] In addition, some of those methods did not demonstrate specificity,[22,23,30] or did not use internal standards,[24] limiting their clinical application and the precision of the measures.[42]

Since the proposed method was tested and demonstrated its suitability in clinical settings, its maturity level as a technology was established by experts in the Technology Readiness Level 6 (TRL-6).[43] The usefulness of the method was validated by several potential users (physicians and pharmacists assisting HIV patients in Colombia), who recognized it as a tool to monitor medication adherence, highlighting the novelty of the technology in the local setting and the rapid turnaround time for results. Potential users also emphasized the need to quantify plasma levels of other ARVs, and our group will continue working on this line of research.

Conclusions

The developed and validated bioanalytical method for quantifying plasma concentrations of EFV, LPV, and RTV combines protein precipitation and liquid-liquid extraction. It includes the use of a monolithic column, allowing for efficient peak separation in 20 min. The method is selective for at least 21 potentially concomitant medications and demonstrates adequate linearity, precision, and accuracy across the concentration range from 250 to 20,000 ng mL^{-1}, making it suitable for clinical application.

Supplementary Information

Recompilations of liquid chromatography methods for quantifying plasma concentration of EFV, LPV, RTV (Table S1), and plasma level of the drugs, reported for different populations (Table S2), are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

David R. Jaspe Lentino was responsible for investigation, formal analysis; Eder J. Lara Mercado for investigation, formal analysis; Indira B. Pájaro Bolívar for methodology, supervision, writing original draft; Guillermo Cervantes Acosta for conceptualization, resources; Carlos Silvera Redondo for conceptualization, resources; Gina P. Domínguez Moré for methodology, validation, funding acquisition, project administration, writing-review and editing.

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