Use of micellar liquid chromatography to analyze darunavir, ritonavir, emtricitabine, and tenofovir in plasma

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Abstract

Danuravir, ritonavir, emtricitabine and tenofovir are together prescribed against AIDS, as a HAART regimen. Micellar liquid chromatography has been applied to determine these four antiretroviral drugs in plasma. The sample preparation is shortened to the dilution of the sample in a micellar solution, filtration and injection. Clean-up steps are avoided, due to the solubilization of plasma matrix in micellar media. The drugs were analyzed in < 20 min using a mobile phase of 0.06 M SDS - 2.5 % 1-pentanol - pH 7 running under isocratic mode through a C18 column at 1 mL min^{-1} at room temperature. Absorbance wavelength detection was set at 214 nm. The method was successfully validated following the ICH Harmonized Tripartite Guideline in terms of: selectivity, limit of detection (0.080 - 0.110 μg mL^{-1}), limit of quantification (0.240 - 0.270 μg mL^{-1}), linearity between 0.25 and 25 μg mL^{-1} (r^2 > 0.995), accuracy (89.3 - 103.2 %), precision (<8.2 %) and robustness (<7.5 %). Real plasma sample from patients taking this therapy were analyzed. This is the first paper showing the simultaneous detection of these four drugs. Therefore, the methodology was proven useful for the routine analysis of these samples in a laboratory Hospital for clinical purposes.

Keywords: Immunodeficiency; Micellar; Plasma; Therapy; Validation

1. Introduction

HAART (highly active antiretroviral therapy) was introduced in 1996 to treat patients with acquired immunodeficiency syndrome (AIDS) with strong clinical results [1]. It consists in the prescription of a set (named HAART regimen) of antiretroviral (ARV) which prevents the activity of human immunodeficiency virus 1 (HIV-1). Generally, a HAART regimen combines three or four different ARV [2]. These ARV mixtures reduce the amount of active virus, even until it is undetectable by current blood testing techniques [3]. However, it does not reach a definitive cure of the disease, so that the treatment has to be lifelong, complicating the compliance and favoring the appearance of drug resistance mutations. Moreover, the pharmacokinetics of the drugs is sometimes unpredictable, and they show many side effects [4]. Continuously, clinicians propose new combinations looking for higher therapeutic activity and less adverse effects for a specific patient than previous regimen [5].

Recently, a new HAART combination, containing darunavir (Prezista ®), ritonavir (Norvir ®), tenofovir and emtricitabine (Truvada ®), has been introduced in the Spanish market for naïve-patients, those do not have previously taken other HAART regimen. The structures and main physico-chemical characteristics can be seen in Table 1. The initially prescribed dose, be taken one time per day, is: one tablet of Prezista ® + Norvir ® (400 mg darunavir + 100 mg ritonavir) and one tablet of Truvada ® (300 mg tenofivir + 200 mg emtricitabine). Darunavir and ritonavir are protease inhibitors (PI). They act by blocking HIV aspartyl protease which the virus needs to cleave the HIV polyprotein into its functional fragments, preventing the completion of the reproductive cycle of the virus. Tenofovir and emtricitabine are nucleotide and nucleoside reverse transcriptase inhibitor (NtRTIs/NRTIs), respectively. These drugs work by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA, and foiling the generation of new virus [6,7].

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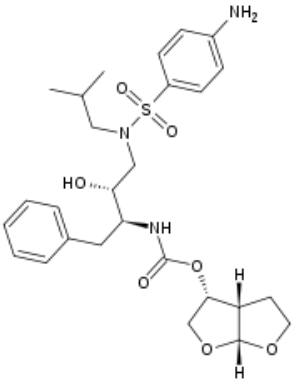
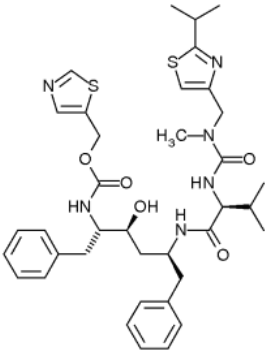
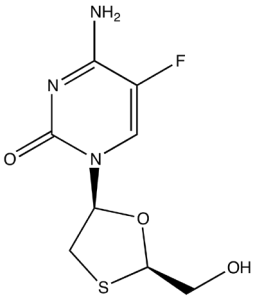
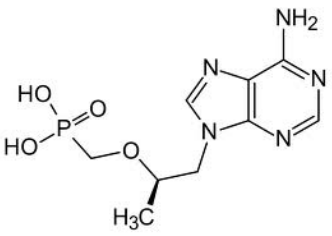
76 **Table 1**. Structure, pKa and log Po/w of the studied antiretroviral.

Compound	Structure	pKa (protonated/neutral)	Charge at pH = 7	log*Po/w*
Darunavir		(Darunavir+H^+) 2.10	0	1.80
Ritonavir		(Ritonavir-H^+) 2.84	0	3.90
Emtricitabine		(Emtricitabine-H^+) 2.65	0	-1.4
Tenofovir		3.75	-1	1.25

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The simultaneous quantification of darunavir, ritonavir, emtricitabine and tenofovir in blood from a patient treated with this HAART regimen can be useful to study their biological behavior and to explain therapeutic and adverse effects, including failure [8]. This information would be useful to personalize the prescription timeline of the HAART regimen for each patient, improving the probability of success [9]. The quantification of ARV can also be performed to check the compliance level with the treatment, which must be above 95 % [10], and to detect sub-therapeutic levels of ARV in blood, that may provoke the virus to develop resistance [11]. However, at our knowledge, a method to simultaneously determine these four drugs in blood has not been previously issued.

Reverse-phase high performance liquid chromatography (RP-HPLC), using mixtures of organic solvent and water as mobile phases, is the preferred technique for the analysis antiretroviral in plasma [12]. HPLC coupled to mass spectrometry (MS) has been used to detect ritonavir, darunavir [13], tenofovir [13,14] and emtricitabine [14,15]. However, LC-MS has serious limitations, such as high cost and expensive maintenance. Emtricitabine can be analyzed using HPLC coupled with fluorescence detection (HPLC-FLD) [16], but the other studied drugs are not fluorescent. Several methods based on HPLC coupled with UV-Visible absorbance (HPLC-UV) have been developed to measure the concentration of darunavir [17], ritonavir mixed with other ARV [18-20] and darunavir/ritonavir mixtures [21,22]. HPLC-UV has been also applied to simultaneously determine ARV mixtures containing emtricitabine [20] and tenofovir [23], and to quantify a combination of emtricitabine and tenofovir [24]. These chromatographic methods involve tedious and time-consuming cleanup steps as liquid/liquid [19,21,22] solid/liquid [17,20,23,24] extraction and deproteneization by precipitation [18], to avoid the introduction of harmful compounds in the chromatographic system.

Micellar liquid chromatography using sodium dodecyl sulfate (SDS) as surfactant is an excellent choice as analytical method when dealing with plasma analysis [25]. Proteins are denatured and, together with other hydrophobic compounds, solubilized in micellar media, and eluted at the front of the chromatogram. Therefore, plasma samples can be directly injected, after simple dilution and filtration, into the chromatographic system without risk of

precipitation [26]. SDS monomers modify the nature of the stationary phase, and the apolar inner core of the micelle can interact with analytes, introducing a new environment in the retention mechanism. A chemometric approach provides a simulation of the chromatographic behavior of the ARV from the data obtained in few mobile phases following an experimental design, expediting the optimization of the mobile phase composition [27]. The use of MLC also reduces the cost and the environmental impact of the analysis, as micellar mobile phases usually contain non-pollutant inorganic reagents and organic solvents only up to 12.5 % [28]. MLC-based methods have been developed to analyze antiretroviral mixtures in plasma, including ritonavir [29] and tenofovir [30].

The aim of the work was to develop an analytical procedure based on MLC using SDS as surfactant for the simultaneous quantification of four antiretroviral forming a HAART regimen (darunavir, ritonavir, emtricitabine and tenofovir) in plasma. The method should be able to resolve the mixture of the four drugs in low analysis time with high sensitivity, reliable, simple, inexpensive and environmentally-friendly. The method was validated following the requirements a validation guide proposed by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use: ICH Harmonized Tripartite Guideline. The evaluated parameters were: selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness [31]. The results can be used to study the pharmacokinetics of these drugs in AIDS patients taking this regimen, and evaluate the compliance with the treatment.

2. Materials and methods

2.1 Chemical and reagents

The drug standards (purity > 99.5 %) were: darunavir (Janssen Pharmaceuticals Titusville, NJ, USA), ritonavir (Abbot Laboratories, North Chicago, IL, USA), emtricitabine (Gilead Sciences, Foster City, CA, USA) and tenofovir (Bristol Myers Squibb, New York,

NY, USA). The main physicochemical parameters (pKa; log Po/w) of the studied ARV are: darunavir (2.1; 1.80), ritonavir (2.84; 3.90), emtricitabine (2.65; -1.4) and tenofovir (3.75; 1.25). The structures can be found in [6].

SDS (purity > 99%) was purchased from Merck (Darmstadt, Germany). HCl, NaOH, $NaH_2PO_4.H_2O$ (reagent quality), methanol and 1-pentanol (HPLC grade) were supplied by Scharlab (Barcelona, Spain). An ultrapure water device (Millipore S.A.S., Molsheim, France) was used to generate ultrapure water from deionized water. Ultrapure water was used for all aqueous solutions.

2.2 Instrumentation

Solids were weighted using a Mettler-Toledo analytical balance (Greifensee, Switerland). The pH measurements were performed using a GLP 22 potentiometer (Crison, Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. An ultrasonic bath (model Ultrasons-H, Selecta, Abrera, Spain) used to dissolve the standards and mobile phases.

The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA). It was equipped with an isocratic pump, a degasser, an autosampler and UV-Visible diode array detector (DAD). The signal was acquired by a personal computer connected to the chromatograph by means of an Agilent Chemstation version B.01.01. Chromatograms were treated using Michrom software (Marcel Dekker, New York, NY, USA) to extract the chromatographic parameters: retention time (t_R), retention factor (k), dead time (t_0), efficiency (N), asymmetry (B/A) and peak area (A) [30,32].

All solutions and mobile phases were filtered before injection in the chromatographic system, through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA).

163 *2.3 Standard solutions and mobile phase preparation*

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165 Antiretroviral standard stock solutions were prepared by weighting the appropriate
166 amount to obtain 100 μg mL^{-1}, solving it in few mL of methanol and diluting with water up to
167 10 mL. Working solutions were prepared by dilution of the stock solution in water. The
168 standard solutions were kept at 4ºC in amber flask. No degradation was detected after 3
169 months of use.

170 Mobile phases were prepared by dissolving the appropriate amount of SDS and
171 sodium dihydrogenphosphate in water. The pH was adjusted to the desired value by adding
172 drops of HCl and NaOH solutions, and the suitable volume of 1-pentanol was added. At last,
173 ultrapure water was added to reach the final volume, ultrasonicated and filtered.

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175 *2.4 Sample treatment*

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177 Blood samples were provided for Hospital La Plana of Vila-real (after consent by the
178 patients) and collected from AIDS patients taking the studied HAART regimen and healthy
179 volunteers, as control samples. The samples were collected with DB SST tubes (SBD
180 Vacutainer Systems, Plymouth, UK), centrifuged at 3000 rpm for 10 min (3000 g) to obtain
181 the non-cellular fraction. Obtained plasma was frozen and stored at -20ºC. Samples were
182 thawed one day before the analysis and kept at +4ºC in amber flasks.

183 One mL of plasma was introduced in a vial and filled up to 5 mL with a 0.05 M SDS
184 solution at pH 7 (1/5 dilution). Then, these samples were then injected into the
185 chromatographic system without another treatment than filtration.

186 Optimization and validation of the method were performed using spiked plasma blank
187 (drug-free) samples from healthy volunteers. The spiking was performed by adding the
188 adequate volume of the antiretroviral working standard solution prior to filling. ARV
189 concentration taken for calculations were those the equivalent in the original plasma sample
190 (considering the dilution factor), instead of the concentration of the injected aliquot.

2.5 Chromatographic conditions

The analysis were performed using a mobile phase of 0.060 M SDS - 2.5 % 1-pentanol fixed at pH 7 with 0.01 M phosphate buffer, running at 1 mL min^{-1} under isocratic mode through a Kromasil C18 (Scharlab) column (150 x 4.6 mm; particle size 5 µm: pore size 100 Å, working pH range 1.5 - 7.5) at room temperature. The injection volume was 20 µL and detection absorbance wavelength was set at 214 nm. The special care applied to chromatographic instrumentation when dealing micellar mobile phases are detailed in [33]. The needle was cleaned by injecting water between two injections. Under these conditions, the column was a life span of nearly 1000 injections.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

Several chromatographic conditions (stationary phase, surfactant, pH, injection volume, temperature) were taken from previously published papers [29,30]. The detection conditions and the composition of the mobile phase were optimized for the analysis of a mixture of darunavir, ritonavir, emtricitabine and tenofovir in plasma.

3.1.1 Optimization of the absorbance wavelength detection

The optimum absorbance wavelength detection for ritonavir [29] and tenofovir [30] in micellar medium is 214 nm. Darunavir [17,21] and emtricitabine [20,24] show absorbance at < 280 nm in hydroorganic solvents. However, the new organization of the analytes in micellar media can provoke the shifting of the maximal absorption wavelength and the change of the absorbance intensity. Thus, UV-Visible spectra of darunavir and emtricitabine were taken by analyzing a plasma sample spiked with 5 µg mL^{-1} of these two drugs, using the

optimal micellar mobile phase. As a strong absorbance was found at 214 nm, this value was selected for the analysis. Therefore, the whole chromatogram was registered using the same wavelength detection.

3.1.2 Optimization of the micellar mobile phase composition

The composition of the mobile phase was optimized focusing on the separation of the peaks corresponding to the four studied ARV. Ritonavir [29] and tenofovir [30] are strongly retained on C18 columns, and need a mobile phase with high elution ability. Thus, the study was restricted to aqueous micellar mobile phase with 1-pentanol.

The optimal SDS and 1-pentanol concentration were simultaneously optimized using an interpretative strategy. A mixture of 5 μg mL^{-1} of darunavir, ritonavir, emtricitabine and tenofovir were analyzed by five mobile phases containing the following SDS (M)/1-pentanol (%) values: 0.05/2; 0.05/6; 0.10/4; 0.15/2 and 0.15/6. In all the tested mobile phase, the protein band elutes at < 2.5 min and does not overlap with any drug. Indicative parameters of the chromatographic behavior, as *k*; t_R; N; B/A and t_0, were measured for each ARV and mobile phase using Michrom software [32].

These data were processed using a statistical model based on chemometrics. It is able to relate the chromatographic behavior of the analytes with the composition of the mobile phase. Equation 6.1 provides the relationship between retention factor and SDS/1-pentanol concentration [34]:

$$k = \frac{K'_{AS}/(1 + K_{AD}\varphi)}{1 + [M](K_{AM}(1 + K_{MD}\varphi)/(1 + K_{AD}\varphi))} \quad (6.1)$$

where [M] is the SDS concentration (M) and φ is the amount of 1-pentanol (%). The meaning of the constants can be found in [33].

The peak shape, which depends on N and B/A, is modeled through the equation 6.2, which provides the predicted h(t) (absorbance signal provided by the detector) at each elution time (t) [34]:

$$h(t) = H_0 e^{-0.5\left(\frac{t - t_R}{s_0 + s_1(t - t_R) + s_2(t - t_R)^2 + \ldots}\right)^2} \qquad (6.2)$$

s_i are constants depending on t_R; N and B/A. They remain ideally invariant for each ARV and mobile phase. H_0 (peak height) depends on the concentration of the ARV.

The data obtained in the tested mobile phases (k; N and B/A) are used by the Michrom software as "calibration levels" to calculate the constant parameters of the two equations. Once known, the statistical model allows the prediction of the chromatographic conditions of the analytes and the drawing of simulated chromatograms, at intermediate values of SDS/1-pentanol. The global resolution (R) is considered as the resolution (r_{ij}) of the least resolved peak pair, calculated by the valley-peak criterion.

The selected mobile phase was 0.060 M SDS - 2.5 % 1-pentanol at pH 7. It provides the maximal global resolution (R = 0.9998) in the minimum analysis time. A plasma sample spiked with a mixture of darunavir, ritonavir, emtricitabine and tenofovir at 5 μg mL^{-1} was analyzed using the optimal conditions (Figure 1B). The experimental chromatographic parameters (t_R; N; B/A) were: darunavir (8.2 min; 3958 theoretical plates; 0.97), ritonavir (18.4; 3201; 1.07), emtricitabine (3.6; 4682; 1.11) and tenofovir (5.5; 4501; 1.20). The errors of the predicted retention times were < 5 %.

The possibility to simultaneously analyze these four drugs using a mobile phase running under isocratic mode is highly interesting. This reduces the total analysis time because the stabilization time between two successive injections is not needed, facilitating the analysis of a large amount of samples. Moreover, the optimized micellar mobile phase uses a less amount of toxic organic solvent, than typically employed in hydroorganic mobile phases.

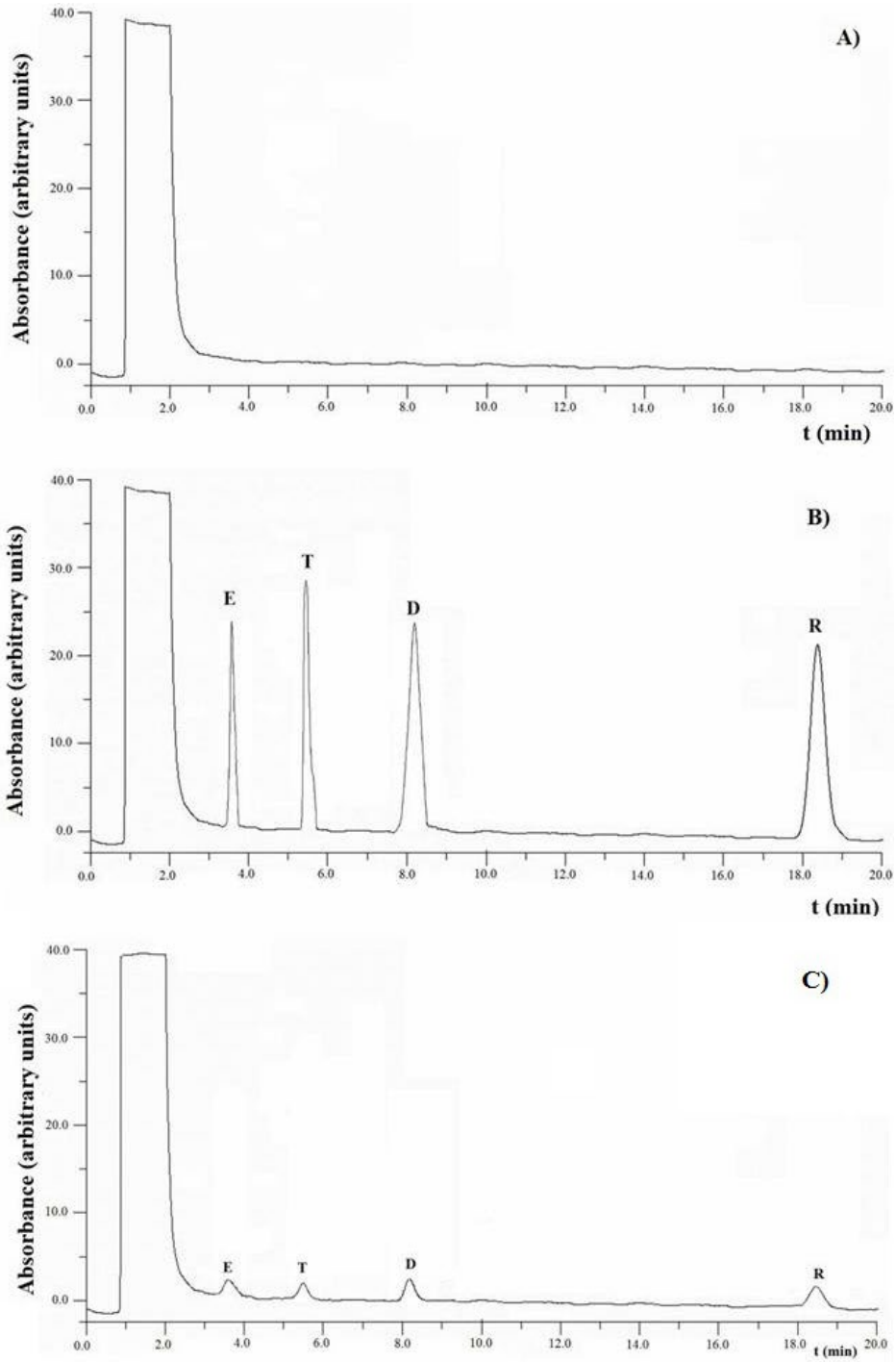

Fig. 1. Chromatogram by analysis of a plasma sample: A) blank, B) spiked with 5 μg mL^{-1} of darunavir (D), ritonavir (R), emtricitabine (E) and tenofovir (T) and C) spiked at the LOQ level for each ARV.

3.2. Method validation

The validation was performed following the requirements of the ICH Harmonized Tripartite Guideline, especially devoted to the analysis of drugs in any kind of samples worldwide [31]. The evaluated parameters were: specificity, calibration range, linearity, sensitivity (LOD and LOQ), inter- and intraday accuracy, repeatability, intermediate precision and robustness. The method was validated using spiked blank plasma samples, collected from healthy volunteers and originally free of ARV.

3.2.1 Specificity

Ten different blank plasma samples (from five males and five females) were analyzed using the optimized conditions (Figure 1A). In all cases, the chromatograms only show a high front and totally eluted at < 2.5 min, corresponding to the protein band. No peaks were detected at higher retention times. As the four studied ARV elutes at > 2.5 min, there are no potential interfering substances. The obtained chromatograms are clean, despite the complexity of plasma and the absence of cleanup procedure. The proteins and the endogenous compounds of plasma strongly interacts with the micelles in the mobile phase, provoking their fast elution. This result is habitual when analyzing plasma with MLC, expediting the analysis of drugs in plasma [30].

The blank plasma samples were spiked with 5 μg mL^{-1} of darunavir, ritonavir, emtricitabine and tenofovir, and analyzed. Results are shown in Figure 1B. The analytes are eluted enough separate between them and the protein band, and no coeluting endogenous compounds were detected, thus avoiding overlapping. Therefore, the ARV can be unambiguously identified and their area measured without error.

The possible interference of other drugs also administered to HAART patients was studied by analyzing a 5 μg mL^{-1} solution of each one (log Po/w, [6]): acebutolol (1.71), N-acetylprocainamide (0.99 [35]), acetaminophen (0.46), acetylsalycilic acid (1.19), amiodarone (7.57), amoxicillin (0.87), ampicillin (1.35), atenolol (0.16), azithromycin (4.02),

bromazepam (2.54), caffeine (-0.07), captopril (1.02), carbamazepine (2.1), ciprofloxacin (-0.57), clonazepam (2.76), chloramphenicol (1.14), cocaine (2.30), codeine (1.19), corticosterone (1.99 [35]), desipramine (4.90), dexamethasone (1.83), diazepam (2.82), diltiazem (2.8), doxycycline (-0.72), ephedrine (1.00), flunitrazepam (2.2), furosemide (2.03), gentamicin (-1.6), hydrocortisone (1.79), imipramine (4.80), levofloxacin (2.1), lidocaine (2.44), loratadine (4.8), medazepam (4.43 [35]), methotrexate (-1.85), nicotine (1.17), nifedipine (2.49), norfloxacin (-0.47), oxazepam (2.01), phenylephrine (-0.69), propranolol (3.48), quinidine (3.44), sulphametoxazole (0.79), sulpfthiazole (0.88), tetracycline (-1.30), theophylline (-0.02) and valproic acid (2.75) [29]. None of them eluted near the studied ARV under the optimized conditions.

3.2.2 Carry over effect

In order to evaluate the carry over effect, a plasma sample spiked with 25 μg mL^{-1} of darunavir, ritonavir, emtricitabine and tenofovir was analyzed under the optimal conditions. There concentration is over the expected in plasma patient. Immediately after, a blank plasma sample was analyzed, and no peak was observed near the retention time of the studied ARV. Therefore, there is not significant carry over effect at ARV concentrations under 25 μg mL^{-1}.

3.2.3 Calibration and sensitivity

Nine increasing concentrations ranging from 0.25 to 25 μg mL^{-1} for each ARV were analyzed by triplicate to construct a calibration curve. All the analyses were performed the same day. The slope, y-intercept and determination coefficient (r^2) were calculated by correlating the chromatographic areas (average value of the three replicates) with the corresponding concentration by least-square linear regression.

330 The regression curve for each ARV was calculated as the average of five calibration
331 curves built over a 2-months period, by preparing all the spiked samples each time. The
332 results are shown in Table 2.

333 The limit of detection is the minimal ARV concentration producing a response
334 significantly different from the noise of the baseline. It is calculated by the 3.3s criteria: 3.3
335 times the standard deviation of the signal obtained by analysis of blank samples, divided by
336 the sensitivity (slope of the calibration curve). The limit of quantification (LOQ) is the lowest
337 concentration of ARV that can be quantified with acceptable accuracy and precision, and was
338 taking following the 10s criteria. Results are presented in Table 2. A chromatogram of a
339 blank plasma sample spiked with the LOQ for each ARV is shown in Figure 1C. The analytes
340 can be detected enough above the noise level.

341 Excellent linearity was found in the studied interval, and the sensitivity was enough to
342 detect the ARV in plasma from patients taking this HAART for pharmacokinetics purposes.

343

344 **Table 2.** Calibration curve parameters and sensitivity ([ARV] in μg mL^{-1})

Compound	Slope	Y-intercept	r^2	LOD	LOQ)
Darunavir	3.8 ± 0.2	0.4 ± 0.8	0.9996	0.090	0.250
Ritonavir	4.6 ± 0.3	- 0.7 ± 0.9	0.9991	0.080	0.240
Entricitabine	2.41 ± 0.05	0.3 ± 0.5	0.998	0.110	0.270
Tenofovir	2.88 ± 0.03	-0.5 ± 0.2	0.995	0.100	0.260

345 n = 5

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347 *3.2.4 Accuracy and precision*

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349 Intraday accuracy and repeatability was measured for darunavir, ritonavir,
350 emtricitabine and tenofovir at 0.5; 2.5 and 5 μg mL^{-1}. The solutions were different that those
351 prepared for the calibration studies. The analyses were 6-fold performed within the same day.
352 The accuracy was the closeness between the average value of ARV concentrations provided

by the method and the true spiked value (recovery), whereas the repeatability was calculated as the relative standard deviation (RSD) of the signal. Interday accuracy and intermediate precision were determined as the average values of five intraday measurements taken over a 3-months period. Results are shown in Table 3. Adequate recoveries (89.3 - 103.2 %) and high precision (<8.2 %) of the signal were found, thus indicating the reliability of the ARV concentrations in plasma obtained by the analytical method.

Table 3. Intra- and inter-day accuracy and precision.

		Intra-day[a]		Inter-day[b]	
Antiretroviral	[ARV] (μg mL^{-1})	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Darunavir	0.5	93.6	8.2	96.8	6.0
	2.5	102.5	4.6	100.5	4.2
	5	98.4	2.9	97.7	1.3
Ritonavir	0.5	89.3	5.7	93.5	6.5
	2.5	94.6	3.3	97.4	5.0
	5	103.2	4.0	99.4	3.5
Emtricitabine	0.5	92.4	7.0	91.2	6.3
	2.5	96.2	6.2	95.6	5.2
	5	97.5	4.0	98.4	2.4
Tenofovir	0.25	101.1	7.2	98.9	5.0
	2.5	94.8	4.8	96.5	3.9
	5	98.2	1.1	100.6	2.5

[a]n=6; [b]n = 5

363 *3.2.5 Robustness*

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365 The variability of the elution strength and sensitivity was studied at slight variations
366 of the following chromatographic conditions (range): SDS concentration (0.055 - 0.065 M),
367 1-pentanol amount (2.4 - 2.6 %), pH (6.9 - 7.1) and flow rate (0.95 - 1.05 mL min^{-1}). Thus, a
368 plasma sample spiked with 5 μg mL^{-1} of each ARV was analyzed (n = 3), at three levels:
369 minimum, optimum and maximum value for each condition. The relative standard deviation
370 of the three obtained values for retention time and peak area was calculated.

371 As shown in Table 4, the possible experimental oscillations of the chromatographic
372 conditions would have a minimal effect in the retention time (<7.5 %) and peak area (<5.4
373 %).

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375 *3.2.6 Stability*

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377 For stability study purpose, a blank sample plasma was spiked with 5 μg mL^{-1} of
378 each ARV and kept in a fridge (-20ºC in absence of light). Each day, this sample was thawed,
379 analyzed and put another time in the fridge, during a 3-months period. No significant
380 diminution of the peak area was detected for the analytes in this period. As the normal
381 storage time in hospitals is about ten days, the analytical method does not show degradation
382 problems.

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Table 4. Evaluation of the robustness of the MLC method.

Compound	Parameter (Studied range)	Retention time (RSD, %)	Peak area (RSD, %)
Darunavir	SDS (0.055 - 0.065 M)	3.5	2.6
	1-pentanol (2.4 - 2.6 %)	4.8	3.0
	pH (6.9 - 7.1)	2.3	1.9
	Flow rate (0.95 - 1.05 mL min^{-1})	5.6	3.2
Ritonavir	SDS	5.2	2.8
	1-pentanol	6.2	5.4
	pH	4.5	3.1
	Flow-rate	7.5	1.5
Emtricitabine	SDS	3.5	4.7
	1-pentanol	6.2	1.8
	pH	2.6	4.2
	Flow-rate	5.9	2.5
Tenofovir	SDS	4.8	3.4
	1-pentanol	5.7	2.8
	pH	3.8	1.9
	Flow rate	5.8	3.1

3.3. Analysis of plasma samples of AIDS patients

Several plasma samples from AIDS patients whose the doctors have prescribed this HAART regimen were studied to measure the concentration of darunavir, ritonavir, emtricitabine and tenofovir. The samples were taken 6 hours after the ingestion of the tablets. For confidentiality reasons, no more information about this can be provided.

In order to assure the quality of the results, blank sample plasma was analyzed at the beginning of the analytical run and each 5 analysis. The quality control samples (blank

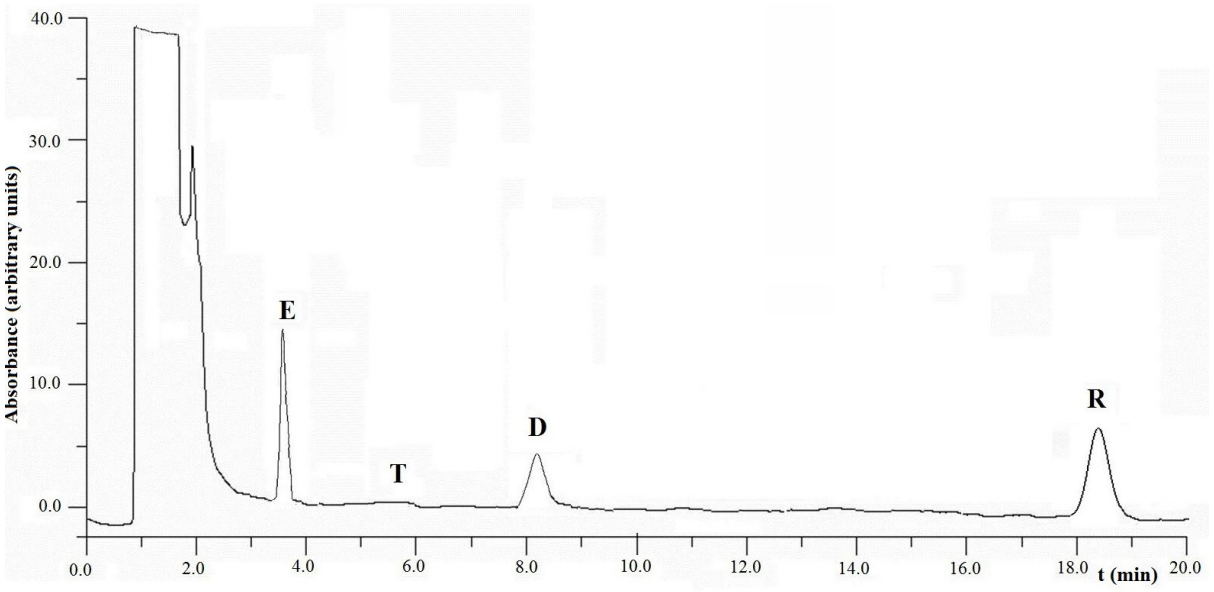
401 sample spiked with 1 and 5 μg mL^{-1} of ARV) were analyzed at the end of the run. The
402 acceptance criteria were: blank runs, no detection of ARV; and quality control (QC) samples,
403 a bias and variability < 15 %. Results are shown in Table 5 and meet the acceptance criteria.
404 According to the obtained values, the patients effectively take their medications.

405 The chromatogram obtained by the analysis of the plasma sample from the patient P1
406 can be seen in Figure 2. In all cases, the four ARV were clearly detected and quantified
407 without interferences. No other peaks were detected in the chromatogram. The
408 chromatograms obtained by the analysis of the other patients showed similar shape.

409

410 **Table 5.** Quantification of the antiretroviral (μg mL^{-1}) in plasma of HIV patients (n = 3)

Patient	Darunavir	Ritonavir	Emtricitabine	Tenofovir
P1	0.65 ± 0.04	1.78 ± 0.08	3.1 ±0.05	under LOD
P2	1.05 ± 0.09	0.85 ± 0.04	5.1 ± 0.4	0.45 ± 0.05
P3	0.85 ± 0.04	3.14 ± 0.9	0.75 ± 0.06	under LOD
P4	4.2 ± 0.3	3.7 ± 0.4	6.7 ± 0.5	0.82 ± 0.06
P5	3.4 ± 0.2	2.8 ± 0.4	0.95 ± 0.08	under LOD
P6	3.8 ± 0.3	3.0 ±0.4	1.8 ± 0.2	0.56 ± 0.04
P7	2.4 ± 0.2	4.5 ± 0.5	3.7 ± 0.3	1.05 ± 0.09
P8	5.1 ± 0.3	1.7 ± 0.3	4.2 ± 0.7	under LOD
QC 1 μg mL^{-1}	0.94 ± 0.21	1.01 ± 0.10	0.95 ± 0.09	0.98 ± 0.15
QC 5 μg mL^{-1}	4.8 ± 0.2	4.7 ± 0.3	5.0 ± 0.3	4.8 ± 0.4



411

412 **Figure 2.** Chromatogram corresponding to P1, obtained using the optimal analysis conditions (Marks are as in
413 Fig. 1).

414

415

416 **4. Concluding remarks**

417

418 MLC has been proven as a valuable methodology to analyze four ARV forming a
419 HAART regimen (darunavir, ritonavir, emtricitabine and tenofovir) in plasma of AIDS
420 patients. This is the first paper showing the determination of these four antiretroviral in
421 plasma. The drug mixture is effectively resolved with good peak shape using an isocratic-
422 running mobile phase in less than 20 min. The use of an interpretative strategy based on a
423 chemometric tool has allowed the optimization of SDS and 1-pentanol amount by testing
424 only five the mobile phase.

425 The main advantage of this technique is the shortening and simplification of the
426 preparation of the sample, despite the complexity of the matrix. The method was successfully
427 validated following the ICH Harmonized Tripartite Guideline, assessing the reliability of the
428 quantitative data. The method can be considered environmental friendly, as it uses low
429 amounts of toxic organic solvents and biodegradable reagents. Moreover, the analysis can be

performed at a low cost, because it only needs inexpensive reagents and instrumentation, the reduction of the global analysis time and the possibility to successively analyze a high amount of samples. All these characteristics make it highly attractive in routine analysis of this HAART regimen in plasma from AIDS-patients for clinical purposes.

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6. Conflict of interest disclosure

The authors declare that they do not hold any financial/commercial conflict of interest.

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