Cloud point extraction for analysis of antiretrovirals in human plasma by UFLC-ESI-MS/MS

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ABSTRACT

An analytical methodology based on cloud point extraction (CPE) coupled to Ultra-Fast Liquid Chromatography and electrospray tandem mass spectrometry (UFLC-MS/MS) was developed for analysis of Abacavir (ABC), Efavirenz (EFV), Lamivudine (3 TC) and Nelfinavir (NFV) in human plasma. It is the first time that CPE was used for extraction of antiretrovirals (ARV) from plasma. The effects of relevant physic-chemical variables on analytical response of each ARV, including pH, surfactant concentration, equilibration time and temperature, were study and optimized; as well as its coupling to UFLC-ESI-MS/MS. Under optimized conditions, the resulting methodology was as follows: a 500 μL aliquot of human plasma was diluted with 2 mL deionized water in a 10 mL centrifuge tube. A 500 μL aliquot Triton X-114 5% w/v was added and homogenized using a vortex stirrer. The resulting cloudy solution was kept at 65 °C for 20 min for promoting the condensation of surfactant micelles. Then it was centrifuged at $3000 \times g$ for 5 min for separation of the surfactant-rich phase. After discarding the aqueous supernatant, 400 μL ACN were added to the remaining surfactant rich phase and centrifuged in order to precipitate proteins and separate them. A 150 μL aliquot of the supernatant was transferred to 2 mL vial and further diluted with 400 μL deionized water. A 30 μL aliquot of the so-prepared solution was injected and analyzed into the UFLC-MS/MS. The method detection limits for ABC, EFV, 3 TC and NFV under optimized conditions were 31, 77, 57 and 21 ng mL^{-1}, respectively. The RSD% for the studied analytes were <15%, except at the LOQ, which were <19%. Recovery values ranged from 81 to 107%. The proposed methodology was successfully applied for the analysis of ABC, EFV, 3 TC and NFV in human plasma within the concentration range of 43–6816, 125–4992, 81–3248 and 49–7904 ng mL^{-1}, respectively. Under optimized working conditions the proposed analytical methodology meets standard requirements of international guidelines, which makes it suitable for pharmacokinetic studies of the four ARV, as well as for therapeutic monitoring of HIV patients.

1. Introduction

Abacavir (ABC), Efavirenz (EFV), Lamivudine (3 TC) and Nelfinavir (NFV) are antiretrovirals (ARV) drugs frequently prescribed to treat human immunodeficiency virus (HIV) infection [1]. Therapy based on antiretroviral combination is used for patient treatment infected with HIV. Accurate dose of ARV is essential in this kind of therapy for guaranteeing viral suppression, as well as to avoid patient intoxication [2]. Additionally, reports show that patients whose therapy is based on dose plasmatic ARV level, responded better to it than individuals who followed a standard and/or fixed regimen [3]. Due to the complexity presented by the management of patients infected with HIV, it seems clear that any way to monitor the pharmacokinetics of ARV therapy could substantially

improve the design of the therapy protocol. To assess plasmatic drug levels in ARV combination therapy it is important to count on an analytical methodology for analyzing multi-components in a single sample. Several methodologies were proposed for the analysis of ARV in plasma by using modern instrumental technique, including liquid chromatography coupled to electrospray tandem mass spectrometry detection (UFLC–MS/MS) [4–6]. As it is well known, despite the outstanding sensitivity and selectivity of UFLC-MS/MS, an exhaustive sample preparation is required for an appropriated multiple ARV analysis; especially when they present significant differences on their physicochemical properties. This fact, together with the sample volume limitation, the sample preparation step turns to be a bottle neck for multiple ARV analysis in biological samples for clinical studies [7].

Several sample preparation techniques have been reported for extraction and isolation of ARV from plasma before chromatographic analysis. Although the most commonly reported is liquid–liquid extraction (LLE) [8,9] and solid phase extraction (SPE) [10,11]; they have as disadvantages to be laborious, to require a large sample volume and to be time consuming. An additional disadvantage of LLE is the use of significant amount of volatile and toxic organic solvents. Moreover, due to the low concentration of ARV in plasma, large sample volumes are typically required to ensure their detectability. In recent years, with the developing interest in miniaturization in analytical chemistry for solvent and sample saving, some newer miniaturized approaches to LLE have been reported. Several different types of liquid-phase microextraction (LPME) have been developed, including single drop microextraction (SDME) [12], dispersive liquid–liquid microextraction (DLLME) [13] and ultrasound assisted emulsification microextraction (USAEME) [14]. An alternative to the microextraction with traditional organic solvents is the cloud point extraction technique (CPE) [14,15]. The micelles formation phenomenon is based on the aggregation of surfactants monomers under specific physicochemical conditions, which result dispersed into the sample bulk [16,17]. The resulting micelles provide a new phase within the sample bulk with regions of diverse polarities that enhance its potential for solubilizing solutes in a wide range of polarities. The solutes affinity depends on its nature and the surfactant structure. Hydrophobic solutes are solubilized in the inner micellar core, polar/charged analytes are believed to be solubilized in the polar region through a number of interactions (e.g. electrostatic, π-cation, hydrogen bonds, etc.), and amphiphilic solutes are incorporated to the micelles through both hydrophobic and polar interactions, forming mixed aggregates [15,16]. Thus, analytes can be in-situ extracted into the micellar phase and selectively separated from the liquid sample bulk [18]. CPE has been successfully applied for extraction of a wide range of analytes from biological [15,19] and environmental media, including estrogens, vitamin A, vitamin E [16], several kinds of proteins, as well as metal ions [20,21] prior to liquid chromatography (LC) analysis. In addition to the analytical advantages, standing out high separation efficiency, selective isolation and wide range of flexibility for coupling it to different analytical instrumentation [11,21], it is important to consider the operational advantages. In this sense CPE is low cost, simple to operate and environmentally friendly because uses alternative solvents such as surfactants, lowering the organic solvent consumption.

The aim of this work was to develop and validate a methodology based on CPE coupled to ultra-performance liquid chromatography and electrospray tandem mass spectrometry detection (UFLC-MS/MS) for analysis of Abacavir (ABC), Efavirenz (EFV), Lamivudine (3 TC) and Nelfinavir (NFV) in human plasma. Table 1 summarizes the relevant physicochemical information of the studied ARV of this work [23]. The effects of relevant physic-chemical variables on

analytical response of each ARV, including pH, surfactant concentration, equilibration time and temperature, were study and optimized; as well as its coupling to UFLC-ESI-MS/MS. The analytical performance of the proposed method was evaluated in terms of quantification limits (LOQ), repeatability, reproducibility, accuracy, recovery and linear working range. Moreover, the methodology was applied for the determination of ABC, EFV, 3 TC and NFV in human plasma samples of patients under treatment and its robustness was evaluated in terms of recovery factors (RF%).

2. Experimental

2.1. Chemicals and reagents

Abacavir Sulfate (USP, LOT F1L487, 99.4% w/w), Efavirenz (USP, LOT F0G376, 99.8% w/w), Lamivudine (USP, LOT H0I378, 99.7% w/w), Nelfinavir Mesylate (USP, LOT F0K050, 98.3% w/w) and Nelfinavir-d3 (Toronto Research Chemicals, LOT 4-SDJ-86-3, 98% w/w) were supplied by the pharmaceutical industry. Stock solutions (400 μg mL^{-1} of each ARV) were prepared by dissolving an appropriate amount of each compound in acetonitrile (ACN). Working solutions were prepared daily by dilution of the stock solutions with ACN. Instrument tuning parameters were optimized using 1000 ng mL^{-1} solutions of each compound prepared in mobile phase (10 mM ammonium acetate:ACN, 70:30 v/v). The Triton X-114 (AppliChem, Germany) was prepared in deionized water (DW-conductivity < 3 μS cm^{-1}) within the range of 2.5–10% (w/v) in DW. ACN and methanol (MeOH) were purchased from Carlo Erba, France and Ammonium acetate (NH$_4$Ac) was purchased from Sigma–Aldrich, Germany. All reagents were analytic grade or above.

2.2. Instrumentation

The assay was carried out by using an ultra-performance chromatography coupled to a triple quadrupole (UFLC–MS/MS) instrument, described as follows: UFLC Shimadzu (LC 20AD), automatic injector Shimadzu (SIL-20A XR), coupled to an electrospray ionization (ESI) source (Applied Biosystems, Ontario, Canada). Liquid chromatographic analysis was carried out on a Hypersil Gold C$_{18}$ column (100 mm × 2.1 mm i.d., 3 μm particle, Thermo Fisher Scientific, USA). Data were acquired and processed using Analyst software (version 1.5.1; ABSciex, Toronto, Canada).
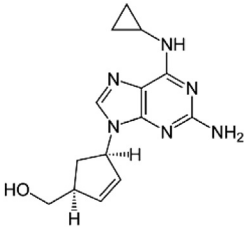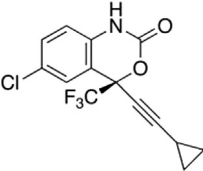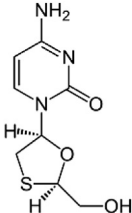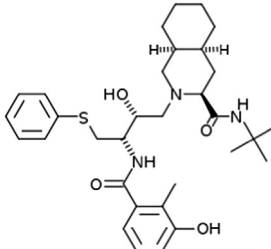
2.3. Sampling and sample conditioning

Samples were obtained from volunteers of DominguezLab, which is developing a program for determining drugs bioequivalence (Protocol number: PRO-BEQ-EFV-001-V.01). Sampling was carried out using a catheter system (BD Saf-T-Intima™, BD Vacutainer®) and syringes of 5 mL. The blood sample was collected into heparinized polypropylene 4 mL tubes (NAHEP PLH 13X75 4.0 PLBL GN, BD Vacutainer®, Broken Bow NE 68822 US) and centrifuged at 3000 rpm (1400 × g) for plasma separation. Aliquots of 1 mL were preserved in polypropylene 2 mL cryovials and frozen at −20 °C ± 5 °C until analysis.

2.4. Cloud point extraction

A 500 μL aliquot of human plasma was diluted with 2 mL DW in a 10 mL centrifuge tube. An aliquot of 500 μL Triton X-114 5% w/v was added and homogenized using a vortex stirrer. The resulting cloudy solution was kept at 65 °C for 20 min for promoting the condensation of surfactant micelles. Then it was centrifuged at 3000 × g for 5 min for separation of the surfactant-rich phase. After discarding the aqueous supernatant, 400 μL ACN were added to the

Table 1
Physicochemical information of ABC, EFV, 3 TC and NFV.

Analyte	ABC	EFV	3 TC	NFV
Chemical structure				
Chemical formula	$C_{14}H_{18}N_6O$	$C_{14}H_9ClF_3NO_2$	$C_8H_{11}N_3O_3S$	$C_{32}H_{45}N_3O_4S$
MW (g moL^{-1})	286.3	315.7	229.2	567.8
pK$_a$	5.0	10.2	4.3	6.0 and 11.1
Solubility	Solubility in H_2O 1.2 g L^{-1}, Soluble in methanol	Solubility in H_2O 9.2 mg L^{-1}, Soluble in methanol	Soluble in H_2O, Slightly soluble in methanol	Practically Insoluble in H_2O, Soluble in methanol

MW: Molecular weight.

remaining surfactant rich phase and centrifuged in order to precipitate proteins and separate them. A 150 μL aliquot of the supernatant was transferred to 2 mL vial and further diluted with 400 μL DW. A 30 μL aliquot of the so prepared solution was injected and analyzed into the UFLC-MS/MS.

3. Results and discussion

As it is well known, CPE efficiency is conditioned by physicochemical variables as well as the matrix nature of the sample and analytes [14,18]. Therefore, it is of interest to study the effect of the experimental physicochemical variables on the analytical response of the ARV and optimize it towards achieving high sensitivity and selectivity of the methodology.

Developing analytical method using synthetic solution and further application on real samples generally requires severe adjustments. This could lead to a new method different from the former one. The use of real matrix all along the process is a convenient choice for characterizing, studying and optimizing the analytical methodology that is been developed. In this sense, not only the signal enhancing due to the improvement of the extraction efficiency resulted significant, but also the matrix load minimization, which led to a satisfactory chromatographic analysis. These aspects were what finally determined the sensitivity and selectivity of the resulting analytical methodology. Thus, those particular factors of a real matrix, as well as all experimental variables that condition the analytical responses of the ARV were evaluated, including extraction pH, surfactant concentration, extraction time and temperature, salting out effect and the use of modifiers, including ACN, MeOH and acid, respectively. These studies were carried out by modifying one variable at the time keeping constant the remaining ones. A 500 μL aliquot of plasma, originally free of ARV, was spiked with antiretroviral standard-mix (750 ng mL^{-1} 3 TC and ABC, 300 ng mL^{-1} NFV and 150 ng mL^{-1} EFV in H_2O:MeOH 75:25 v/v) and 50 μL of Nelfinavir-d3 (Internal Standard, 2200 ng mL^{-1}) for carrying out the assays, which were done by triplicate. The chromatographic peak area was used to evaluate the impact of modified experimental conditions on the analytical response.

From Section 3.2–3.5 are presented the results and discussion of the relevant variables of this work. The remaining variables mentioned above, did not show significant effect on the analytical response of the studied ARV.

3.1. UFLC-MS/MS conditions

In the present work, the MS optimization was carried out using direct infusion of standard solutions of each ARV into de ESI source of the mass spectrometer at 20 μL min^{-1} using a built-in syringe pump. Base ion of each ARV was selected from full scan mode recorded within the range 50–600 m/z. In all cases [M+H]$^+$ mode were selected. Declustering Potential (DP), Entrance Potential (EP) and Collision Cell Entrance Potential (CEP) voltages were selected according to the sensitivity of precursor base ions, whereas Collision energy (CE) and Collision Cell Exit Potential (CXP) were chosen to give the maximum intensity of the obtained fragment base ion. The remaining operative conditions were as follows: 250 ms dwell time, 5500 V ion spray voltage, 20 psi nebulizer gas (GS1:N$_2$), 20 psi curtain gas, 7 psi collision gas (N$_2$), 20 psi drying gas (GS2:N$_2$), 500 °C drying gas temperature and 100 °C source temperature. The mass spectrometer was used in the multiple reaction monitoring (MRM) mode and the m/z transition for quantification collected in positive mode were 287.1 → 191.2, 316.0 → 168.2, 230.0 → 112.1, 568.4 → 330.1 and 571.4 → 333.1 for ABC, EFV, 3 TC, NFV and NFV-d3, respectively.

The chromatographic separation was carried out using a mobile phase of two components: A and B. A: 10 mM NH$_4$Ac:ACN (70:30 v/v) and B: 100% ACN. The flow rate was set at 0.25 mL min^{-1} and the column temperature was kept at 40 °C along the run. The injection volume was 30 μL and the run time was 4 min. The retention time for the target analytes were 1.30, 1.34, 1.61 and 2.06 min for ABC, 3 TC, NFV and EFV, respectively. Representative chromatograms of a standard mixture of the analytes are illustrated in Fig. 1.

3.2. Surfactant concentration

Triton X-114 is one of the nonionic surfactant extensively used for CPE [24]. It has been successfully applied for extracting a wide range of analytes from biological [15,19] and environmental samples, including estrogens, vitamin A, vitamin E [16], kinds of proteins, as well as metal ions [20,21]. Additional advantages include commercial availability with high purity, low toxicity and low cost, high density of the resulting surfactant reach phase facilitating the phase separation by centrifugation, and relatively low coacervating temperature [22].

Reports indicate that the amount of Triton X-114 not only affected the extraction efficiency but also the resulting surfactant-rich phase volume after phases separation [15]. The smaller the
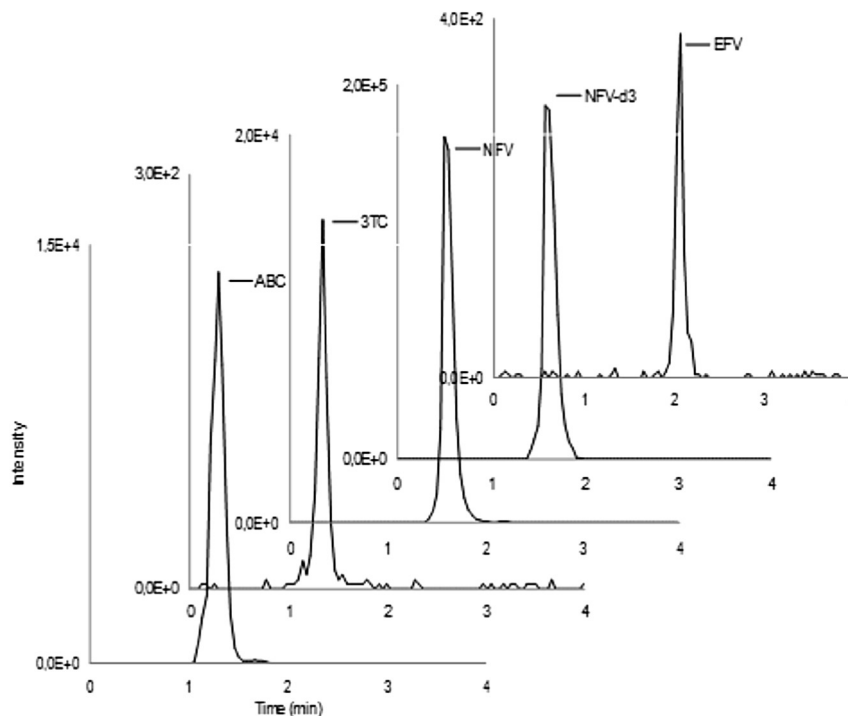
Fig. 1. Chromatograms of plasma sample spiked with 1.7 μg mL^{-1}, 1.2 μg mL^{-1}, 0.8 μg mL^{-1}and 1.9 μg mL^{-1} of ABC, EFV, 3 TC and NFV respectively.

initial surfactant concentration, the smaller is the resulting surfactant-rich phase volume leading to higher sensitivity of the methodology. However, it is well known that a small surfactant-rich phase volume (<20 μL) leads to operative difficulties to efficiently separate it from the supernatant, which affect on the accuracy and reproducibility of the methodology [25].

Considering those aspects, four Triton X-114 concentrations were assayed: 2.5%, 5.0%, 7.5% and 10.0% w/v. Higher concentrations of surfactant were no assayed since it could affect the spectrometer ion source performance, mainly by clog, which lead to signal loss. The remaining steps were carried out as described in section 2.4. Considering that different surfactant concentration would lead to different surfactant reach phase volumes, it was referred to the same final volume (ca. 200 μL) with DW for all surfactant concentrations assayed in order to achieve comparable results. From Fig. 2, it can be observe that the analytical response of ABC and 3 TC

increased as the surfactant concentration increases. For EFV, the highest analytical response was achieved for the smaller surfactant concentration assayed falling to ca. 30% at 10% w/v Triton X-114. NFV reported its highest relative response at 5% w/v Triton X-114 falling to ca. 75% at 10% w/v Triton X-114.

These results could be attributed to the analytes physical−chemical properties influencing the extraction efficiency of the CPE technique, and thus the analytical responses [26]. At the working pH 3 TC and ABC were in their molecular form, while EFV and NFV were in their ionic forms. They show different water solubility behavior (Table 1) coherent with their analytical responses. In this sense, it is expected to obtain the lowest responses for 3 TC and ABC since they are the most polar analytes and the surfactant is no-ionic, limiting thus the affinity between them and the micelles. The performance of EFV could be attributed to the fact that at the lowest surfactant concentration assayed, the extraction efficiency was the maximum. Higher concentration led to higher surfactant reach phase volume; therefore, concentration of the extracted analytes was lower due to a dilution effect. In this sense, NFV reported the highest analytical response at intermediate concentration, falling for dilution into the resulting coacervate phase when higher surfactant was used. The most polar analytes contain higher number of polar atoms into their structure. This leads to higher molecular effective radio due to the solvatation, reducing their interaction with micelles. Therefore, 3 TC and ABC require higher surfactant concentration to achieve a successful extraction and analytical response.

Based on these experiment results, 5% (w/v) Triton X-114 was adopted as working condition considering it as the best compromise concentration that lead to successful analytical response for the studied ARV. This fact is relevant for the development of an analytical methodology for a multi-component analysis.

3.3. Effect of pH

To the knowledge of the authors, CPE was not previously applied for ARV extraction in any matrix; therefore there was no evidence
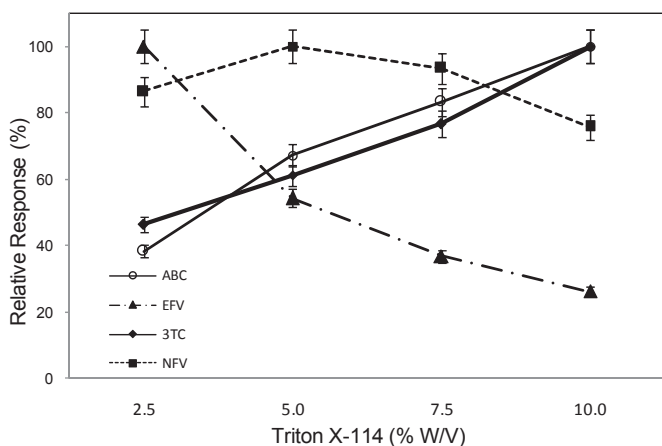

Fig. 2. Extraction effect of target ARV as a function of Triton X-114 concentration. Experimental conditions as described in Section 3.2.

of which could be the pH effect on analytical signal for this methodology. Analytical responses would be conditioned by CPE and instrumental efficiency. CPE is strongly conditioned by physicochemical properties of the analytes, sample matrix, as well as CPE particularities (surfactant type and concentration, matrix modifiers, etc). On the other hand, instrumental efficiency is also conditioned by sample matrix characteristic and analytes physicochemical properties, additionally to the particularities of the detection system. It is well know that plasma matrix contains dissolved proteins (6–8%) (i.e. albumins, globulins, and fibrinogen), glucose, clotting factors, electrolytes (Na^+, Ca^{2+}, Mg^{2+}, HCO_3^-, Cl^-, etc.), hormones and carbon dioxide [27,28] which could affect the extraction efficiency of the CPE technique [29]. Considering that the studied ARV are ionizable species according to their pK_a (Table 1), it was expected that extraction pH could affect their analytical responses as follows: the extraction pH may condition the ionizable equilibrium of the ARV, and thus it molecular form, which affect their affinity for micelles, and consequently their extraction efficiency. On the other hand, it is important to highlight that due to the nonionic character of the surfactant, it is not expected to see a pH effect on micelles [16,17]. Considering this information, the effect of pH on the analytical response of ARV was analyzed. As shown in Fig. 3, at pH 2.5 the lowest relative responses were obtained for all ARV (<50%). At this pH the analytes were in the molecular form, thus it was expected to have the highest affinity for nonionic micelles.

On the other hand, it was observed an inefficient phase separation evidenced by on small volume of the surfactant rich phase (<50 μL), which affected the extraction efficiency and consequently their analytical response.

At pH 5 the resulting coacervate phase volume was ca. 450 μL and showed turbid, which clear up after a protein precipitation step. At this pH, 3 TC and ABC prevailed in ionic form, while NFV and EFV prevailed in their nonionic form according to their pK_a. On this basis, it was expected to see lower relative responses for the ionic analytes since they would have lower affinity for the nonionic micelle, and therefore, lower extraction efficiency for these ARV. However, the analytical response of ARV reached their highest values at pH 5 (>95%), except for NFV (ca. 70%), and remained invariant only for EFV at higher pH values. These results could be due to the tradeoff between the effect of affinity of the analyte by the micelles and their efficiency in forming, resulting in an improvement of the analytical signal. NFV analytical responses reached the highest values (>95%) at pH 9 and remained invariant at pH 11.5. The analytical responses of 3 TC and ABC decreased from

pH 7 and pH 9, respectively. Phases separation was efficient from pH 7 ahead, obtaining a coacervate-phase volume of ca. 350 μL. Coacervate phase showed the highest dehydration and the smaller volume (ca. 300 μL) at pH of 11.5; resulting in a better phases separation performance and reducing of the loss of coacervate phase when the supernatant was discharged. The decrease in the analytical signal of ABC and 3 TC at pH 11.5 could be due to a signal suppression effect in the mass spectrometer because of a high matrix load in the solvent front. Considering the short retention time of ABC and 3 TC (1.30 and 1.34 min^{-1}, respectively) it was expected that their analytical signal were more affected by this phenomenon than for NFV and EFV, whose retention time were higher (1.61 and 2.06 min^{-1}, respectively).

Based on these results, pH 7.5 was adopted as working condition considering it as the best compromise pH that lead to successful analytical response for the studied ARV. Considering that human plasma pH is 7.4 [27], the extraction pH was not adjusted for CPE of ARV from plasma. This fact is relevant for the development of an analytical methodology for a multi-component analysis since it simplify the procedure.

3.4. Optimization of extraction temperature and time

Reported cloud point temperatures for TritonX-114 are within the range of 15–20 °C [19]. It refers to the temperature at which the surfactant coacervation results notorious in the extraction system, because of its turbid aspect. As it is well known, extraction temperatures above the cloud point, and/or the extended extraction time (>2 min), diminishes the water content of the resulting surfactant reach phase, and thus, its final volume [25]. This argument is based on the fact that as the extraction temperature increases, the hydrogen bonds are disrupted leading, thus, to dehydration of micelles [25]. On the other hand, it is important to take into account that the use of excessive high extraction temperature can reduce the CPE efficiency, because of the thermal stability of surfactant aggregates [30] as well as the analytes lability. Therefore, it was of interest to study the effect of the extraction temperature on the extraction system performance, as well as on the analytes analytical response along with the extraction time within the range 45–75 °C and 10–30 min, respectively. The extraction temperature assays were carried out considering a 20 min extraction time; while the time assays, were carried out at the optimum temperature resulting from the former. As the temperature increased from 45 to 65 °C a better surfactant-rich phase separation as well as an increased response of the analytes was observed. The results showed no significant change neither on the CPE performance nor on the analytical response of the studied ARV at extraction temperatures above 65 °C. Therefore, 65 °C were chosen for further assays. For the extraction time, it was observed that by increasing it, the relative response is subsequently increased, reaching the maximum analytical responses of the studied ARV at 20 min; after which, and remained invariant. Thus, in the following experiments, an extraction time of 20 min was selected for the extraction.

3.5. Analytical performance and method validation

The proposed analytical multi-component method was evaluated in terms of linearity, recovery, accuracy and precision intra- and inter-day, selectivity and sensitivity. Blank plasma was assessed in six different batches of plasma samples by analyzing blanks and spiked samples at LOQ levels. The calibration curves were made under optimized conditions following the proposed methodology, which was described above. Blank plasma were spiked with ARV standards achieving different concentration levels [31,32]. No significant chromatographic signals of endogenous ARV were
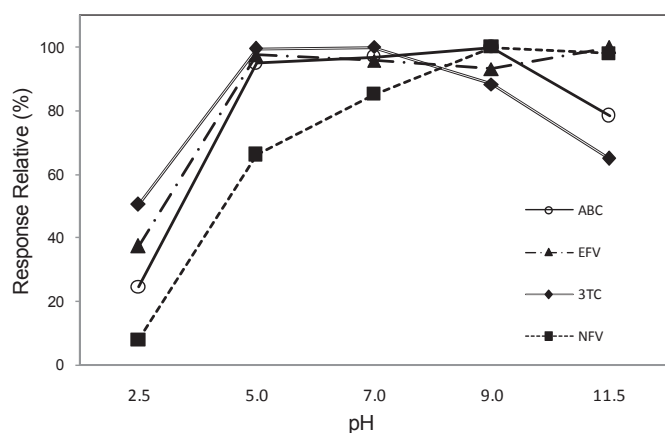

Fig. 3. Effect of pH on the analytical response of target ARV. The concentration of each antiretroviral was 0.5 μg mL^{-1}. Experimental conditions as described in Section 3.3.

Table 2
Analytical figures of merit of CPE-UFLC-ESI-MS/MS for analysis of ARV in human plasma.

Analyte		ABC			EFV			3 TC			NFV	
LOQ (CV%)		43 (6.9)			125 (19.0)			81 (0.1)			49 (5.3)	
Linear Range ng mL^{-1}		43–6816			125–4992			81–3248			49–7904	
Correlation coefficient		r = 0.99			r = 0.99			r = 0.99			r = 0.99	
	Concentration (ng mL^{-1})			Concentration (ng mL^{-1})			Concentration (ng mL^{-1})			Concentration (ng mL^{-1})		
	Added	Found	RE (%)	Added	Found	RE (%)	Added	Found	RE (%)	Added	Found	RE (%)
Accuracy intra-day	85	75	−12	125	104	−17	81	65	−20	99	91	−8
	1704	1808	6	1248	1279	3	812	819	1	1976	1986	1
	3408	3675	8	2496	2715	9	1624	1689	4	3952	4230	7
Accuracy inter-day	85	72	−15	125	111	−11	81	70	−13	99	93	−6
	1704	1669	−2	1248	1215	−3	812	898	11	1976	2034	3
	3408	3447	1	2496	2559	3	1624	1821	12	3952	4295	9

observed for any of the plasma batches at the target analytes retention times.

The analytical figures of merits were summarized in Table 2. The LOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision according to international requirements [31,32]. In this sense, the criteria for evaluating the accuracy were as follows: relative error (RE) value should be within 15% of the spiked value except at LOQ, where it should not deviate by more than 20%. The regression equations with a weighting factor of 1/x (where x = concentration) were judged to produce the best fit for the concentration-detector ratio response for all ARV in plasma [22,23]. The calibration curves showed a satisfactory linearity within the concentration range: 43–6816 ng mL^{-1}, 125–4992 ng mL^{-1}, 81–3248 ng mL^{-1} and 49–7904 ng mL^{-1} for ABC, AFV, 3 TC and NFV, respectively. These concentrations were chosen based on the expected pharmacokinetic studies of drugs containing these active values. Calibration data was fitted by using a 1/x curve resulting coefficients of correlation (r^2) > 0.99 for all analytes. The intra- and inter-day precision assay was evaluated over three replicates at three concentration levels. The precision was evaluated over the sample preparation step of the proposed methodology. The precision was determined at each concentration level, and did not exceed 15% of the percent relative standard deviation (%RSD) except for the LOQ, which it did not exceed 20% of the RSD [31,32]. The resulting RSDs values in the LOQ level ranged from 0.10 to 19%, and 1.0–15% for the remaining concentration level. The stability of ARV in human plasma after three freeze–thaw cycles, short-term room stability (6 h), post processing stability (12 h remain at the auto-sampler temperature) and long-term stability (119 days at −20 °C) were evaluated. The ARV analyzed were considered to be stable in human plasma or extracts when accuracy was within ±15% (Table 3). A

typical chromatogram of spiked plasma sample is shown in Fig. 1. The proposed methodology meets standards required by international guidelines for pharmacokinetic studies of drugs, as well as for therapeutic monitoring of HIV patients. CPE technique resulted efficient, quick and easy to carry out the sample preparation for ARV analysis in plasma by 4 min run UFLC-MS/MS.

4. Comparison of proposed CPE-UFLC-MS/MS with previously reported analytical methodologies

The proposed methodology, CPE-UFLC-MS/MS, developed for analyzing multiple ARV in plasma samples, presents several characteristics that makes it suitable for routine analysis. Table 4 summarizes analytical methodologies for analyzing ARV in plasma samples. The figures of merit satisfy the requirements of international guidelines so the analytical methodology is suitable for bioequivalence studies. The resulting analytical figures of merits (linear range, %RSD, recovery (%), and running time) are within the values reported for other methodologies. It can be successfully applied for analyzing four main ARV (ABC, EFV, 3 TC, and NFV) used for HIV treatments in a reasonable running time (4 min). The proposed technique, CPE, is suitable for batch preparations of samples feasible to be carried out in 25 min with simple equipment available in most of the laboratories. It does not include a solvent evaporation step, neither requires toxic solvents, regularly used for traditional techniques, like LLE and SPE.

5. Conclusion

This work presents a valuable contribution to analytical chemistry field as well as those related disciplines including pharmacology, biochemistry and medicine, principally. A new application

Table 3
Stability of the ARV in plasma samples at different conditions.

Stability		Room temperature (6.0 h)			Autosampler sample (12 h)			Freeze and thaw (cycle 3)			Long-term (119 days)		
		Found	RE %	%RSD	Found	RE %	%RSD	Found	RE %	%RSD	Found	RE %	%RSD
ABC	Low (72 ng mL^{-1})	82	112.9	8.0	82	113.6	5.2	84	115.3	8.0	81	111.8	9.6
	Middle (3367 ng mL^{-1})	3210	95.3	2.3	3684	109.4	4.9	3851	114.4	3.0	3659	108.7	3.6
	High (6266 ng mL^{-1})	6046	96.5	2.4	6264	100.0	2.7	6545	104.5	2.3	6349	101.3	5.9
EFV	Low (226 ng mL^{-1})	237	104.8	4.8	239	105.6	0.7	219	97.1	0.8	247	109.4	1.5
	Middle (2259 ng mL^{-1})	2322	102.8	2.0	2431	107.6	1.9	2345	103.8	3.7	2306	102.1	2.3
	High (4518 ng mL^{-1})	4807	106.4	3.5	4545	100.6	0.4	4839	107.1	4.8	4694	103.9	2.8
3 TC	Low (78 ng mL^{-1})	79	100.8	9.2	87	111.6	4.3	83	106.0	4.8	80	102.8	5.8
	Middle (1756 ng mL^{-1})	1715	97.7	5.1	1777	101.2	5.0	1726	98.3	5.8	1640	93.4	7.0
	High (3267 ng mL^{-1})	3053	93.4	2.7	3211	98.3	1.5	3055	93.5	3.0	2963	90.7	7.5
NFV	Low (102 ng mL^{-1})	104	101.6	4.2	110	107.7	4.3	114	111.4	4.5	109	107.3	6.4
	Middle (3250 ng mL^{-1})	3402	104.7	3.4	3400	104.6	1.0	3337	102.7	3.1	3417	105.1	2.2
	High (6500 ng mL^{-1})	6324	97.3	5.6	6740	103.7	3.6	6452	99.3	4.5	6216	95.6	6.7

Table 4
Analytical methodologies for analyzing ARV in plasma samples.

Instrumental technique	Analyte	Extraction technique	Sample size (µL)	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	Reproducibilty[a] (RSD %)	Recoveries (%)	Running time (min)
HPLC-UV [2]	ABC	LLE	1000	200 − 10,000	n.a.	<8.0	66	n.a.
	NFV						51	
	EFV						54	
LC-MS/MS [5]	ABC	LLE	100	30 − 9318	n.a.	<10.8	87	2
HPLC-UV [11]	ABC	SPE	800	10 − 10000	n.a.	<7.6	>97	40
	3 TC					<9.1	>90	
LC-MS/MS [33]	EFV	SPE	100	20 − 2000	n.a.	<8.4	54	4
LC-MS/MS [34]	3 TC	SPE	100	1 −3000	n.a.	<8.3	94	9
LC-MS/MS [35]	ABC	SPE	200	100 − 7000	1	<7.6	59	2
	3 TC			80 − 5000	1	<9.1	105	
UFLC-MS/MS[b]	ABC	CPE	500	43 − 6816	31	<9.2	90	4
	EFV			125 − 4992	77	<13.7	105	
	3 TC			81 − 3248	57	<14.7	82	
	NFV			49 − 7904	21	<6.4	98	

n.a.: not available.
[a] The values of intra-and inter-day precision were compared.
[b] Proposed methodology.

of the CPE for the analysis of ARV in human plasma by UFLC-MS/MS was proposed for direct application in real studies. The CPE-UFLC-MS/MS was developed on real matrices, which allowed a comprehensive characterization and optimization of the procedure with a feasible validation according to international standards. The proposed analytical methodology was developed and applied for analyzing of ABC, EFV, 3 TC and NFV in human plasma. Under optimized working conditions the proposed analytical methodology meets standards requirements of international guidelines, which makes it suitable for pharmacokinetic studies of four ARV, as well as for therapeutic monitoring of HIV patients. Additionally to its analytical performance, it is important to point out that the proposed sample preparation technique has an environmentally friendly character that makes it suitable for current green chemistry trends.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ancr.2015.08.002

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