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Development and validation of LC–ESI-MS method for sensitive, accurate and rapid determination of UC-781 in New Zealand white rabbit plasma

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ABSTRACT

The highly potent non-nucleoside reverse transcriptase inhibitor UC-781 is under development as a potential microbicide to prevent sexual transmission of human immunodeficiency virus type 1 (HIV-1). A sensitive and reproducible liquid chromatography–mass spectrometric method has been developed and validated for the quantification of the drug in New Zealand white rabbit plasma after liquid–liquid extraction procedure. The method was validated over the range of 1–500 ng mL^{-1}. Average recoveries of the extraction method were high and consistent: 72%. The method is accurate with average accuracies over three QC (n = 30) concentrations ranging from 99.9% to 106.1%, and precise (within-day and between-day precision measures ranging from 2.2% to 9.9% and 6.50% to 9.0%, respectively). Plasma from other three species proved that extraction method did no affect analyte and internal standard stability. Due to its critical and consequential use, this assay could be readily used for investigational or clinical monitoring of plasma concentrations for low concentration as 1 ng mL^{-1} without interference.

1. Introduction

Animal models are critical tools for the preclinical evaluation of drugs [1]. Utilizing animal models in HIV-prevention research is necessary to study the effect of local administered microbicides to prevent vinous transmission. Among several classes of HIV inhibitors many drugs belongs to non-nucleoside reverse transcriptase inhibitors (NNRTIs). The thiocarboxanilides ((N-[4-chloro-3-(3-methyl-2-butenyloxy) phenyl]-2-methyl-3-furancarbotthioamide) or UC-781 (Fig. 1a) ranked among the most potent NNRTI's [2–5]. Currently, UC-781 or is subjected to heavy investigation as a prevention therapy [6]. It is under development as a potential microbicide to prevent sexual transmission of human immunodeficiency virus type 1 (HIV-1). Two gel formulations of UC-781 (0.1% and 1.0%) were evaluated in a range of preclinical safety assessments, including systemic absorption analysis following topical application in the pig-tailed macaque models for vaginally and rectally applied topical microbicides [7].

In many studies for using ARV's as gel with different concentrations, microbicide proves to be highly effective in preventing HIV-1 transmission [8]. Accurate evaluation of drug concentration in biological fluids is necessary to study PK of systemic absorption of UC-781 in vaginal or rectal application [9].

The primary objective of this work is to elaborate a novel extraction method for UC-781 from New Zealand white rabbit plasma. The method is comparing SPE and liquid–liquid extraction. The final method relies initially on protein precipitation followed by liquid–liquid extraction. The second objective is to develop and validate bioanalytical method for evaluating UC-781 in plasma. Plasma form three species are subjected for confirmation of the developed extraction method. These three species are under investigation for testing topical UC-781 gel. This paper describes in details the extraction method and full validated high performance liquid chromatography assay with electrospray ionization mass spectrometry (LC–ESI-MS).

2. Materials and methods

2.1. Chemicals

UC781 (purity 98.7%) was supplied by Regis Technologies, Inc. (Morton Grove, IL, USA). F2951 (purity 99.2%) used as internal standard was supplied by Chemtura Technology Center (Guelph, Ontario, Canada). Tetrahydrofuran (purity 99.9%) was purchased from Aldrich (St. Louis, MO, USA). HPLC grade of reagent and

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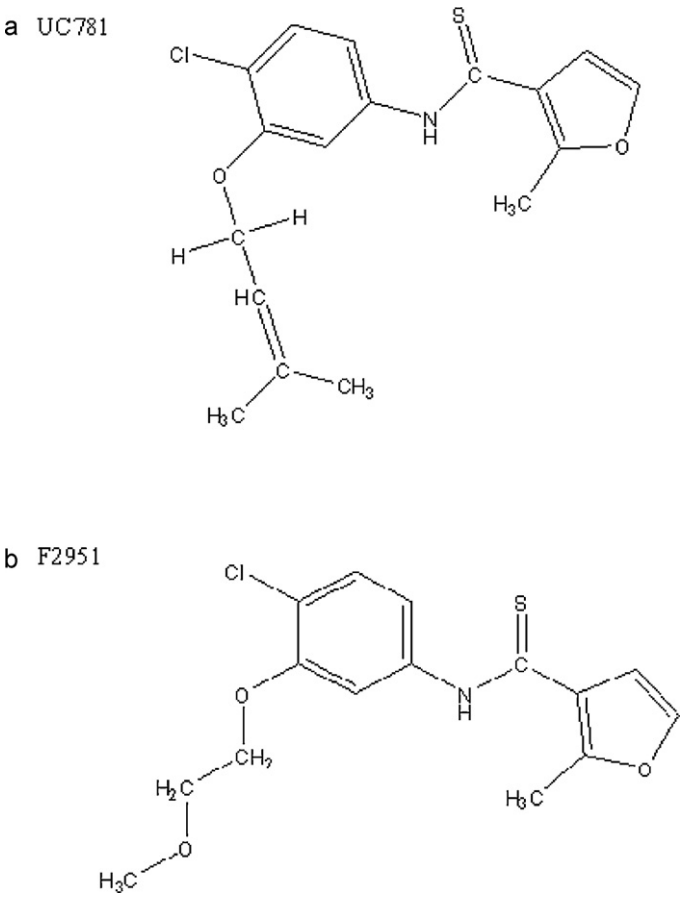

Fig. 1. (a) Chemical structure of UC-781 and (b) chemical structure of F295 the method internal standard.

chemicals were purchased from Fisher Scientific (Norcross, GA, USA). Purified compressed nitrogen gas was obtained from National Welders Supply (Charlotte, NC, USA). Drug-free pooled white New Zealand rabbit plasma was obtained from whole blood anticoagulated with potassium EDTA (Bioreclamation Inc., Westbury, NY, USA).

2.2. Equipments

An eppendorf 5415D centrifuge (Eppendorf AG, Hamburg, Germany) used for centrifugation during the sample prep. A high-performance liquid chromatography (HPLC) system is consisting of an Agilent Technologies (Wilmington, DE, USA) HP1100 binary pump, degasser, thermostatic auto sampler. HPLC system connected to 1100 series mass spectrometer. The ionization mode was positive electro-spray ionization for analytical compounds. Data analysis was accomplished by using HP ChemStation software (Version A.09.03) run on a Dell computer (operated by Windows 2000 professional).

2.3. Preparation of standards

Amount of 5.066 mg UC-781 (molecular weight 335.82) with purity of 98.7% powder was accurately weighed and dissolved in 5 mL methanol to make as 1 mg mL^{-1}. The master stock solution was prepared by diluting stock 1 ng mL^{-1} to 50 μg mL^{-1} in 50% HPLC grade methanol in HPLC grade water. This 50 μg mL^{-1} master solution was used to prepare six working solutions in methanol/HPLC-grade water (1:1) at concentrations of 10–5000 ng mL^{-1}.

Plasma calibration samples at 1, 5, 10, 50, 100, and 500 ng mL^{-1} of UC-781 were prepared by using a 1:10 dilution of the respective working solutions to blank plasma. From another 50 μg mL^{-1} working stock solution, concentrations of 30, 300 and 3000 ng mL^{-1} were prepared in methanol/HPLC-grade water (1:1). Plasma quality control samples at 3, 30, 300 ng mL^{-1} were prepared using a 1:10 dilution of their respective working solutions to blank plasma. This plasma was devoid of any drugs, and obtained from whole New Zealand rabbit blood anticoagulated with potassium EDTA (Bioreclamation Inc., Westbury, NY, USA).

Solutions of potential drugs of interference (primarily protease inhibitors and other non-nucleoside reverse transcriptase inhibitors) were prepared from 1 mg mL^{-1} of each drug pure slandered. These solutions were prepared in 100% methanol to a final concentration of 1 mg mL^{-1}, and diluted with mobile phase before injection onto the HPLC system at concentrations of 10 μg mL^{-1}. Metabolites were not considered.

2.4. Internal standard (IS) preparation

Amount of F2951 powder (5.187 mg, purity 99.2%) was dissolved in 5 mL methanol to achieve a final concentration of 1 mg mL^{-1} (stock solution). From this solution, an aliquot was diluted in HPLC grade acetonitrile to a final concentration of 20 ng mL^{-1} (working solution).

2.5. Sample pre-treatment

Prior to extraction, the protein in plasma sample was precipitated by adding 100 μL internal standard made in acetonitrile into 100 μL aliquot of blank, calibrators, and QCs. The solutions were mixed by vortex-mixing for 30 s, and then centrifuged for 5 min at room temperature at 13,000 rpm. Supernatant was transferred into clean 2 mL conical eppendorf centrifuge tube.

2.6. Liquid–liquid extraction method

Exactly 1.6 mL of 100% hexane added to each tube with supernatant. All tubes were caped immediately and vortex-mixed for 10 min. Tubes were transferred into dry ice/acetone bath for about 2 min. Immediately, the organic layer was decanted into a clean labeled centrifuge tube, and then evaporated to dryness under a nitrogen stream at 30 °C. Finally, the residue was reconstituted with 50 μL of 50/50 methanol/water. The resulting solutions were carefully vortex-mixed for 30 s and centrifuged at 13,000 × *g* for 10 min. The supernatants were transferred to 200 μL HPLC micro-vials (Agilent Technologies) and 10 μL was injected for LC–MS analysis.

2.7. SPE extraction method

In summary, solid phase extraction column (1 mL, 100 mg BOND ELUTE-C18 Varian, Harbor City, CA, USA) was placed on a vacuum elution manifold (20-SPE system, waters, Milford, MA, USA). Samples without protein precipitation were loaded to the conditioned columns. After washing SPE cartridges, the eluent was collected and evaporated to dryness. The remaining residue was reconstituted and prepared for LC–MS analysis as in the above liquid–liquid extraction procedure.

2.8. High performance liquid chromatographic conditions

The chromatographic separation was performed with gradient elution. An Allure C-18 (100 mm × 2.1 mm, 3.0 μm particle size, Restek, Bellefonte, PA, USA) analytical column, with an Allure C-18 (10 mm × 2.1 mm, 5.0 μm particle size, Restek) guard column was

Table 1a
Accuracy and precision of UC-781 at LLQ and ULQ.

UC-781 concentration (ng mL^{-1})	Accuracy (%)	Inter-day precision (%), $n = 30$
1 (LLQ)	102.0	5.0
500 (ULQ)	102.5	3.7

used for separation. The two mobile phase components were as follows. Mobile phase (A): 10 mM ammonium formate in water, and Mobile phase (B): LC–MS grade methanol containing 0.01% THF. A linear gradient was programmed as 80% mobile phase B to 100% B over the first 5 min, followed by half minute at 100% of mobile phase B, then go back to 80% B at 6 min, with re-equilibration over the final 4 min. The analysis was performed at 30 °C, with a mobile phase flow rate of 0.3 mL min^{-1}.

2.9. Mass spectrometric conditions

Mass spectral analysis was performed on an Agilent quadruple 1100 mass spectrometer, fitted with electrospray ionization (ESI) source and operated in the positive ionization mode. The vaporizer operated at 300 °C; the nebulizer gas pressure was set to 40 psig and the capillary voltage set to 3000 V. The IS and UC-781 were detected by their positive ion (m/z 326.0, and 336.1 respectively) with single ion monitoring (SIM) mode.

3. Assessment of performance characteristics

3.1. Linearity

Calibration standards were prepared and analyzed in duplicate in six independent runs. Daily standard curves were constructed by using the ratio of the observed peak area for each analyte to the internal standard peak area. An equal weighted regression was used to assess linearity; deviation of the mean calculated concentrations over five runs were required to be within 15% of the nominal concentrations for the non-zero calibration standards.

3.2. Specificity, selectivity and matrix effect

Two different rabbit plasma lots were compared in three levels. Interference from endogenous compounds was investigated by analysis of two lots of pooled rabbit blank plasma at 3 concentration levels. In LC–MS analysis co-eluted compounds form the matrix may affect the detection of the analyte of interest. Matrix effect on the LC–ESI-MS sensitivity was evaluated based on calculated matrix factor [10]. Matrix factor calculated as a ratio of peak response (peak area) in presence of matrix ion to the peak response in the absence of matrix. The method's low, medium and high QC's (3, 30 and 300 ng mL^{-1}) samples prepared in plasma and out of plasma were used in triplets. In plasma UC-781 spiked into pooled plasma made of six deferent lots before extraction.

3.3. Accuracy and precision

Accuracy and intra- and inter-day precision of the method were determined by assaying six replicates of each of the spiked QC samples in five separate analytical runs. Samples included a low QC with a concentration three times the LOQ [11,12], a medium QC and a high QC ranges. Accuracy was measured as the percentage of deviation from the nominal concentrations. All intra- and inter-day precision should be within a coefficient of variation (CV%) of 15% or less.

3.4. Recovery

Recovery is represented as % extraction efficiency. Extraction efficiency is calculated by dividing the area response of three pre-spiked QC levels (low, medium, and high) by the area response of extracted blank plasma that is post-spiked with the same three QC concentrations.

3.5. Limits of quantitation

The lower limit of quantitation (LLQ) was defined as the concentration for which both the relative standard deviation (RSD) and the percent deviation from the nominal concentration were less than 20%. The upper limit of quantitation (ULQ) defined as the concentration for which both the relative standard deviation and the percent deviation from the nominal concentration were less than 15%.

3.6. Stability

HIV-infected samples are routinely heated at 58 °C to inactivate the virus prior to handling. Heat deactivation studies were performed to verify the stability of UC-781 in rabbit plasma under these conditions. The stability during sample handling was also verified by subjecting samples to three freeze–thaw cycles, and storage for 2 days in the refrigerator at 4 °C prior to analyses. An additional stability test was performed to verify the stability of the drugs in the autosampler tubes while waiting for HPLC analysis. The samples were left at room temperature for 6 h prior to analysis. Quality controls (QC) samples at two concentrations (30 and 300 ng mL^{-1}) were utilized in the stability test. Long term stability planed for 1 month, 6 months, 1 year and 2 years. Stock solution stability was performed suing master stock solution (50 μg mL^{-1}) diluted 1:10 in 50% methanol:water solution.

3.7. Partial validation

Sample dilution was used to evaluate the applicability of this method in case only small animal samples are available. Spiked sample volumes of 50, 20 and 10 μL were diluted in drug free plasma for investigation of 1:1, 1:4 and 1:9 dilutions. Table 3 illustrates the dilution factor.

Table 1b
Method validation accuracy and precision.

UC-781	Intra-assay ($n = 6$)			Inter-assay ($n = 30$)		
Concentration (ng mL^{-1})	Back calculation (ng mL^{-1})	Accuracy (%)	Precision CV%	Back calculation (ng mL^{-1})	Accuracy (%)	Precision CV%
3	2.9	96.4	8.0	3.0	99.9	8.0
30	32.4	108.0	9.9	31.1	103.6	9.0
300	315.9	105.3	2.2	318.3	106.1	6.5

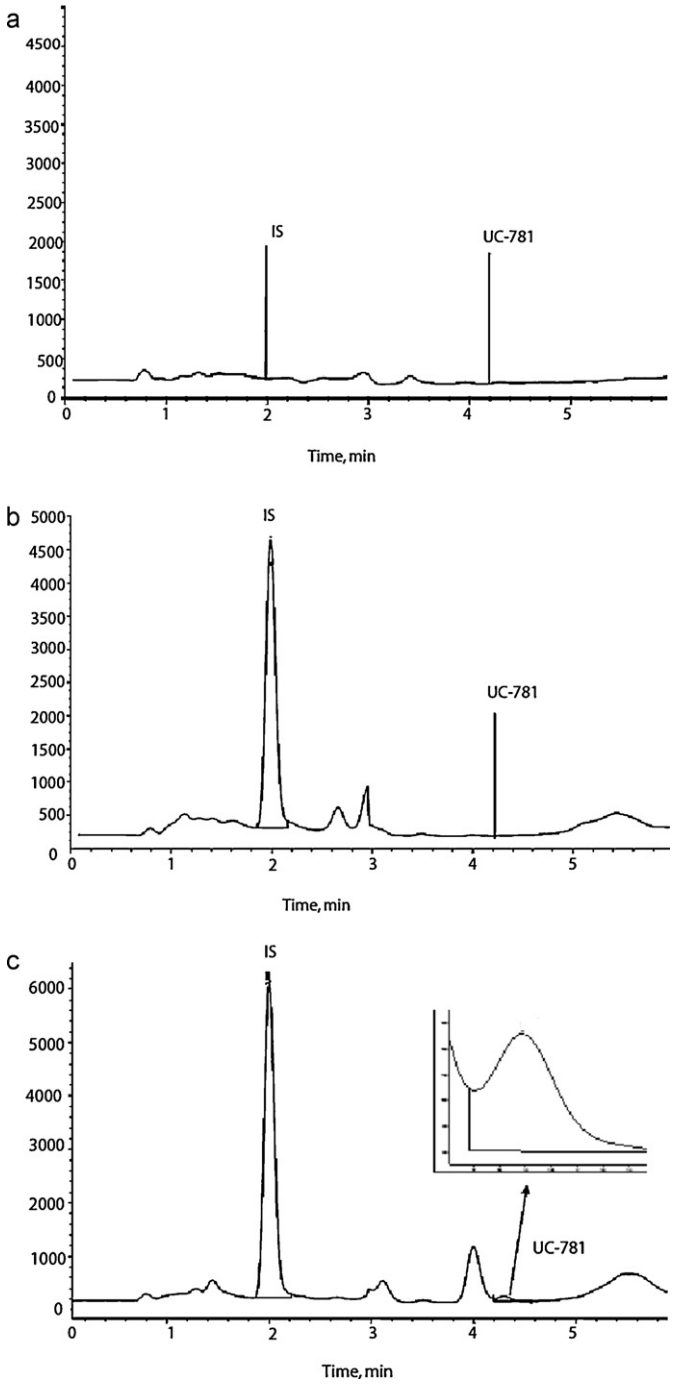

Fig. 2. (a) Chromatogram of extracted blank of rabbit plasma, (b) chromatogram of extracted blank of rabbit plasma with F2951 (IS) and (c) chromatogram of extracted (1 ng mL^{-1}) of rabbit plasma.

4. Results

4.1. Chromatographic separation, selectivity and matrix effect

Approximate retention times for UC-781 and IS were 1.98 and 4.34 min, respectively, following a 5 min run time plus a 4 min re-equilibration time. Fig. 2a–c illustrates chromatograms of extracted blank plasma, blank plasma with internal standard and the low limit of quantification, respectively. Overall, no endogenous substances interference was detected.

When the matrix effect of the method or the matrix factor (MF) is equal to one, there is no matrix effect. Positive MF values demonstrate ion enhancement, while negative values represent ion suppression. The MF values ($n = 3$) for rabbit plasma showed no significant ion suppression effect for low, medium and high QC with 0.91, 0.95, 1.05 respectively with 0.97 average and 0.07 STDEV.

4.2. Linearity and limit of quantification

The peak area UC-781:IS ratio for calibration standards were proportional to the concentration of drug in plasma over the range tested. The linear regression data for the calibration curves of the method ($n = 6$) consistently demonstrated coefficient of determination $\geq$0.998. The method was linear from 1 to 500 ng mL^{-1}. The low limit of quantification of UC-781 is 1 ng mL^{-1} (Fig. 2c) with low percent deviation and high precision as shown in Table 1a. Linearity was also tested without the internal standard to determine the direct proportionality of UC-781 peak area with there corresponding concentrations. The regression coefficient (r^2) of all calibration curves was $\geq$0.999.

4.3. Accuracy, precision

Values in Table 1b support the validity of this method in rabbit plasma (Table 1b). All observed data (intera-day and inter-day precision [CV]) were at or below 15%, and in accordance with the FDA guidelines [12]. Chromatograms of the three QC concentrations were illustrated in Fig. 3a (3 ng mL^{-1}), Fig. 3b (30 ng mL^{-1}), and Fig. 3c (300 ng mL^{-1}). The UC-781 represented as a percent deviation form the nominal concentrations for both within-day and between-day analysis. The method precision for UC-781 was always within $\leq$9.9% for both within-day and between-day analysis. Throughout the concentration range of the control samples, the intra-day precision was always lower than 9.9%. Overall, the mean inter-day precision was 9.0%, with mean RSDs ranging from 2.2% to 9.9%.

4.4. Extraction efficiency (recovery %)

The extraction efficiencies of UC-781 and IS from plasma using the described liquid–liquid extraction method were calculated using the ratio of the analyte's concentration in blood plasma to the identical concentration of the analyte prepared in mobile phase without extraction. Fig. 4 illustrates the extraction comparison between SPE and four other liquid–liquid conditions. The absolute recovery of the analyte from rabbit plasma using the (hexane) liquid–liquid extraction procedure was investigated. This extraction method reliably eliminated interfering material from plasma, with high recovery of 71–73% for three (QC) concentration levels. One peak consistently eluted in proximity to the analyte peak; however, this peak did not interfere with the data accuracy and precision.

4.5. Stability

Stability of UC-781 under various conditions is shown in Table 2. Under all conditions tested, it proved to be stable. All results were within the acceptance criteria of $\pm$15% deviation from the nominal concentration.

4.6. Partial volumes accuracy and precision

Sample dilution accuracy and precision are represented as the percentage of deviation from measured concentration of each sample before dilution. Table 3 lists the accuracy and precision of diluted samples which obtained from varying dilutions.

Table 2
Stability study data under four different conditions.

Stability condition	Medium QC (30 ng mL^{-1}) Mean (n = 3)	Medium QC (300 ng mL^{-1}) Mean (n = 3)	% Deference of treated vs. control medium QC	% Deference of treated vs. control high QC
Heat deactivation	27.3	302.0	−00.58	−02.91
Room temperature	26.1	275.9	−05.05	−11.30
Three cycle F/T	25.8	291.5	−06.21	−06.31
Refrigerator	26.2	276.1	−04.64	−11.25
Autosampler	30.9	325.6	12.48	04.61
1 month	34.7	353.0	07.32	06.17

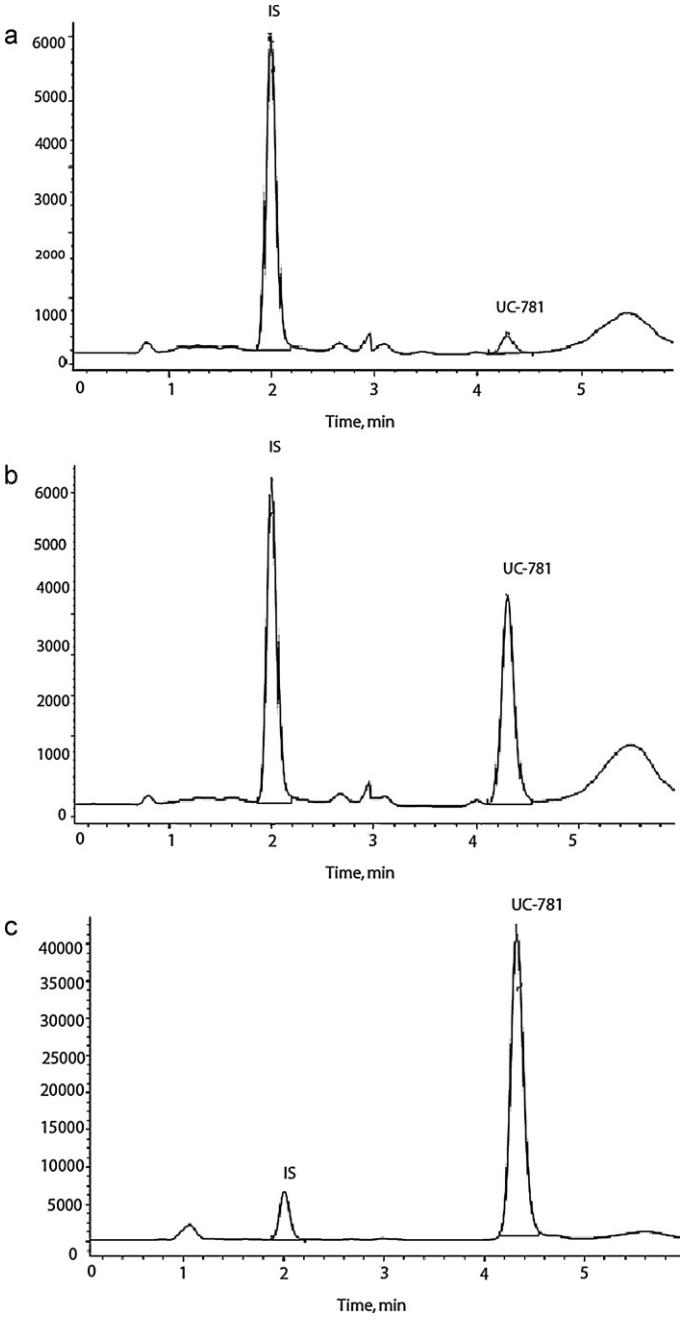

Fig. 3. (a) Chromatogram of extracted low QC (3 ng mL^{-1}) of rabbit plasma, (b) chromatogram of extracted low QC (30 ng mL^{-1}) of rabbit plasma and (c) chromatogram of extracted low QC (300 ng mL^{-1}) of rabbit plasma.

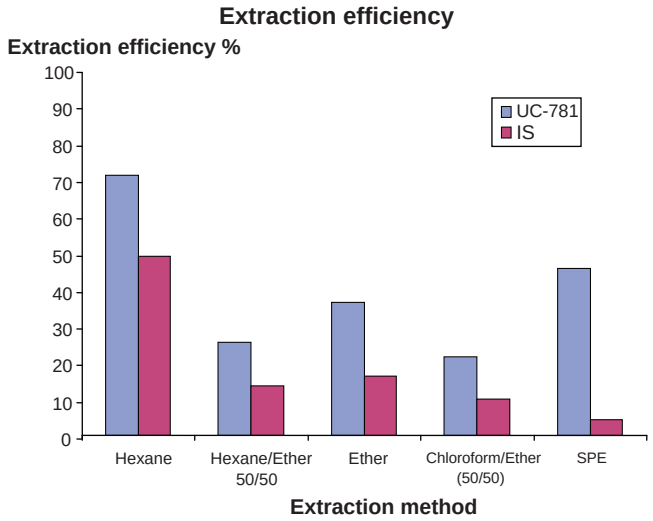

Fig. 4. Extraction of UC-781 and method internal standard using SPE and liquid–liquid extraction (100% hexane, 100% ether, 50/50 hexane/ether and 50/50 chloroform/ether.

5. Discussion

The non-nucleoside reverse transcriptase inhibitor UC-781 is under development as a potential microbicide to prevent sexual transmission of human immunodeficiency virus type 1 (HIV-1). Several gel formulations of UC-781 had been evaluated in a range of preclinical safety assessments, including systemic absorption analysis following topical application in animal models. High levels of UC-781 were detectable in the female genital tract up to 6 h after product exposure [13]. The expected drug level in plasma is very low; therefore, a high sensitive and accurate assay for evaluating UC-781 in plasma is extremely important.

There are many factors that limit the oral bioavailability of UC-781. Some of these factors are; low aqueous solubility and low

Table 3
Accuracy and precision of partial volume validation.

UC-781 dilution factor	High QC (300 ng mL^{-1})			Middle QC (30 ng mL^{-1})		
	1:1	1:4	1:9	1:1	1:4	1:9
1	145.7	58.5	27.1	15.1	6.0	2.8
2	146.4	57.0	29.0	14.3	6.0	3.0
3	134.6	55.0	29.6	14.5	5.6	3.2
Mean	142.2	56.8	28.5	14.6	5.9	3.0
SD	6.6	1.8	1.3	0.4	0.2	0.2
%CV	4.7	3.1	4.7	2.7	3.9	6.6
%Dev	−5.2	−5.3	−4.9	−2.4	−2.5	−0.3
n	3	3	3	3	3	3

dissociation rate [14]. Due to its poor aqueous solubility [15], UC-781 extraction from plasma is a challenging task. In this work we investigated the use of SPE procedure with most possible optimized conditions vs. liquid–liquid extraction in several forms. It was necessary to find an efficient extraction procedure for extracting the drug agent from plasma in order to achieve accurate, specific and precise measurements.

As an active research field in chemical analysis, sample preparation technique is the key step in any analytical procedure, especially in clinical pharmacology [16]. We found SPE yields poor base line, outmost interference and low extraction efficiency, for the analyte and internal standard. Liquid–liquid extraction procedure under four conditions was better in base line and less interference. Hexane 100% was the best liquid–liquid extraction condition in terms of higher extractability and less interference as shown in Fig. 4. We noticed also, that; ether, ether/hexane 50/50 and chloroform/ether 50/50 produce some degradation. Hexane did not seem to cause any degradation through the extraction process. Next we focused on optimizing hexane extraction procedure conditions to be selective with the highest possible recovery. Testing the method with partial volume validation samples and plasma from other species (monkey and human) was done as an extra confirmation of the accuracy and precision of the method.

Thus, it was critical to keep UC-781 in 50/50 methanol/HPLC-water or higher methanol content before spiking the matrix to create the working solutions. In addition, using the same amount of organic reconstitution solution (1:1 methanol:HPLC-water) was necessary to ensure complete dissolution of UC-781 before injection onto the LC–MS system. Consistent results were obtained by using this solution, along with mechanical vortex-mixing. We have noticed that the time of mixing is an important factor for transferring analyte and internal standard into the extraction solvent. Our experiment for testing several mixing times indicated that 10 min were optimal for transferring the highest percentage of drug and internal standard to the upper organic layer.

The chromatographic conditions of this method were optimized for a short 5 min run time. The gradient conditions are simple and applicable to ordinary binary pump system. The re-equilibration time is excellent in cleaning the column of all endogenous plasma components from each injection. The analytical column chosen for this method was based on its power to retain the analyte and internal standard with a perfect peak shape. Allure (C18) from Restek provided sharp peak than any other column tested, which increased the assay sensitivity. Furthermore, we achieved a good resolution for the analyte and internal standard peaks from other endogenous plasma interferences. The method sensitivity was achieved not only by the optimal LC–MS conditions but also the excellent sample clean-up method. The data demonstrate that F2951 was a suitable choice as an IS for the method. The IS peak was well separated from the assay analyte, ionized positively under the same conditions as UC-781, and most importantly, extracted reliably with good recovery.

Because of the possibility of the drug degradation, we have spent more efforts to investigate the drug stability under all assay conditions in plasma as well as the stability of the drug in the stock solution. With no exception, and under all conditions tested UC-781 proved to be stable. As listed in Table 2, all the six tested conditions % of deviation from the nominal concentration was always $\leq$12.5. Since UC-781 will be also investigated for use in human and monkey, we have also tested this method to human and monkey plasma. We run 5 days partial validation for both of those species, all accuracy and precision met the required criteria, and both were extracted with recovery reached 70%.

6. Conclusion

Our developed and validated LC–MS bioanalytical method after a rugged extraction procedure provides a sensitive and an accurate means for measuring UC-781 in rabbit plasma. This method was optimized and proved to be quick, accurate, specific, and highly reproducible. It demonstrates good linearity, precision and accuracy within a wide concentration range (1–500 ng mL^{-1}). The liquid–liquid extraction procedure proves to be an excellent sample treatment for such sensitive compound. This method is highly recommended for similar poor water soluble and unstable compounds. The high extraction efficiency for the analyte was essential for increasing the method specificity and sensitivity.

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