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A fast and reliable reversed phase high performance liquid chromatography method for simultaneous determination of selected anti-retroviral and lumefantrine in human plasma

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ABSTRACT

A fast and reliable high performance liquid chromatography (HPLC) method with UV diode array detection for simultaneous quantitative analysis of the anti-retroviral drugs, nevirapine (NVP) and efavirenz (EFV) and the anti-malarial, lumefantrine (LUM) in human plasma has been developed and validated. The sample preparation consisted of a plasma protein precipitation with 0.5% acetic acid acetonitrile solution containing the internal standard halofantrine (HALO) prior the LC-analysis. Chromatographic separation was carried out on a Acclaim Polar Advantage C_{16}, column (150 mm × 4.6 mm, particle size, 3 μm) using a gradient of mobile phase made of 0.01% TFA in 0.1 M ammonium acetate (solvent A) and 0.1% TFA in acetonitrile (solvent B). The separation of NVP, EFV, LUM and HALO was achieved within 17 min at a flow rate of 1.0 mL/min and detections were initially performed at three wavelengths, 275 nm (NVP), 255 nm (EFV), and 300 nm (LUM). The method selectivity was demonstrated in six different human plasma batches. In addition, several concomitant drugs were analyzed under our experimental conditions and none of them co-eluted with EFV, NVP and LUM. This demonstrated that our method is highly selective. Calibration graphs plotted with seven concentrations in duplicate for each compound were linear between the selected ranges with a regression coefficient (R^2) greater than 0.998. Absolute extraction recovery for NVP, EFV and LUM were 99%, 98.6 and 102%, respectively. Inter- and intra-day coefficients of variation for LUM, EFV and NVP were ≤10%. The lower limits of quantification were 0.125 μg/mL for LUM and 0.250 μg/mL for both EFV and NVP. Intra- and inter-assay relative standard deviation values were found to be less than 15% at the concentrations examined (0.125–10.0 μg/mL for LUM and 0.250–15.0 μg/mL for both EFV and NVP). The present method was successfully implemented in Tanzania and only one wavelength (255 nm) was used to measure samples of patients receiving either NVP or EFV in combination with LUM. The concentration found in human plasma samples for all three compounds were within the calibration range. This makes our method particularly applicable and useful to resource-limited settings.

1. Introduction

Malaria and HIV/AIDS are common infections in "developing countries" that together causes more than 4 million deaths a year [1]. In malaria endemic areas, patients on anti-retroviral medications are likely to be co-administered anti-malarial drugs, thus, increasing the likelihood of drug-drug interactions (DDIs). In highly active antiretroviral therapy, a combination usually containing at least two nucleoside reverse transcriptase inhibitors and one non-nucleoside reverse transcriptase inhibitors is employed. Artemisinin-based combination treatment is currently the treatment of choice for uncomplicated acute malaria in most malaria endemic countries; a common combination is that of artemether-LUM. LUM NVP and EFV (Fig. 1) are both substrates of CYP3A4, which creates a potential of DDIs *in vivo* [2–4]. Common DDIs *in vivo* is either the induction or inhibition of the metabolic pathway of the victim drug [5]. An inhibition or induction of a major drug elimination pathway may result in drug toxicity or in serious adverse drug reactions and sub-therapeutic plasma level of the victim drugs, respectively [5,6]. Pharmacokinetic studies help to

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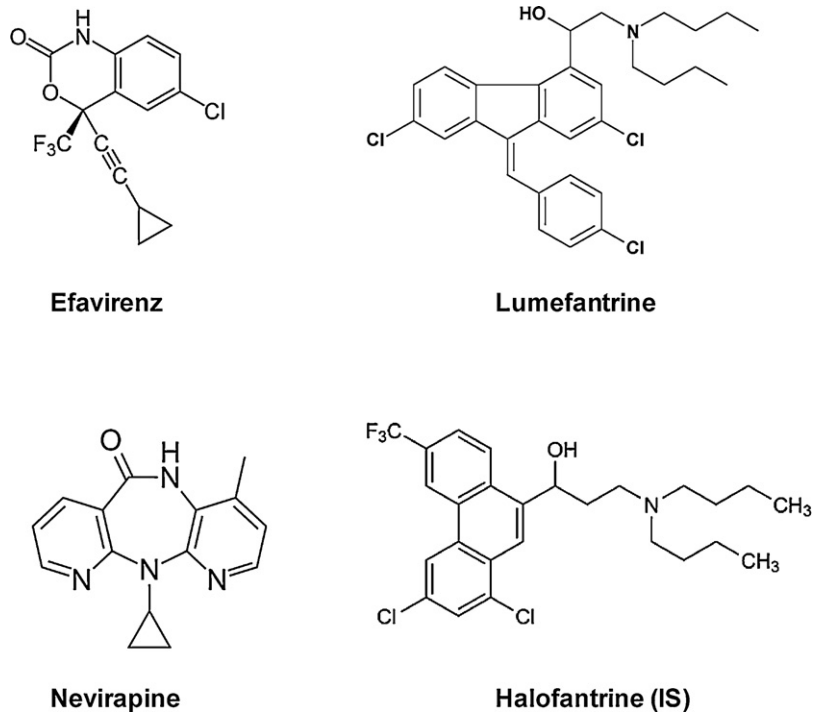

Fig. 1. Compounds used in this study.

identify those factors that cause changes in the dose–concentration relationship. In addition, anti-retroviral drugs such as NVP and EFV have shown hepatoxicity and the concomitant administration with some anti-malaria drugs could potentiate this effect [1]. Hence the determination of these drugs in biological fluids is of great importance for both pharmacokinetic and toxicity studies. To be able to quantify the amount of these drugs in plasma, in resource constrained countries a sensitive, precise, accurate and cost-effective analytical method is required. Numerous analytical methods such as high performance liquid chromatography (HPLC) with UV detection, ion pairing agent, gas chromatography, high throughput assay, mass spectrometry detection and immunoassay, for determination of LUM, NVP and EFV separately have been reported [7–18]. Also several methods have been proposed for the simultaneous determination of EFV and NVP or with other anti retroviral drugs in human plasma or individual LUM with its metabolites [19–25]. However, to our knowledge to-date, there are no published analytical methods dealing with the simultaneous determination of LUM, EFV and NVP in human plasma.

Additionally, in most of the reported methods, sample pretreatments are based on the use of solid phase extraction, protein precipitation followed by solid phase extraction, liquid–liquid extraction procedures and sample drying to dryness using nitrogen gas stream and reconstitute with compatible media [7,9–15,18,20,21,25]. The high cost associated with these procedures will make the aforementioned methods unaffordable in most countries with limited analytical chemistry resources and facilities.

The present study aimed to develop, a simple, cost-effective based HPLC-UV method for simultaneous quantitative analysis of EFV, LUM and NVP in human plasma. A method for simultaneous analysis of several compounds will offer an obvious advantage over the single methods as less samples volume and solvent can be used. This is highly desirable is laboratory with limited resources.

2. Experimental

2.1. Chemical and reagent

LUM was obtained from Novartis Institute of Bioanalytical Research Base. EFV was purchased from Alsachim (France). NVP was purchased from USP (Rockville). Zidovudine was obtained from Biotrend AG (Switzerland). HALO (IS), acetonitrile (HPLC-grade), Trifluoroacetic Acid (TFA), Acetic Acid, Ammonium Acetate, Pyrimethamine, Trimethoprim Lamivudine, Sulfamethoxazole, Fluconazole, Quinine sulfate and stavudine were purchased from Sigma–Aldrich Chemie GmbH (Germany). Methanol (HPLC-grade) was obtained from Merck (Germany). Tenofovir was obtained from Desamo-(China). Emtricitabine was given as a gift from Muhimbili University Research and Development Laboratory (Tanzania). Amodiaquine was given as a gift by Prof. Lars L Gustafsson from Karolinska Institutet (Sweden). All other chemicals and solvents used in this study were of analytical grade.

2.2. Instrumentation

The HPLC system consisted of 1100 quaternary pump, 1100 series auto-sampler, an UV-VIS diode array detector (DAD) and 1100 series degasser from Agilent Germany. System management and data acquisition were performed by the Agilent Chemstation software.

2.3. Chromatographic conditions

The analytical column used was an Acclaim Polar Advantage C_{16}, 3 μm particle size, length 150 mm, ID 4.6 mm (Dionex, CA, USA). The mobile phase consisted of 0.01% TFA in 0.1 M ammonium acetate (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient was set at 0–2 min (50% B), 9–15 min (98% B) and 15.5–20 min (50% B).

LUM was monitored at 300 nm, EFV at 255 nm and NVP at 275 nm, at a flow rate of 1 mL/min.

2.4. Preparation of stock and standard solutions

Stock solutions containing 1.25 mg/mL of LUM and 1 mg/mL of NVP, EFV and IS were prepared. LUM and IS were both dissolved in methanol–acetic acid (100/2, v/v) whereas, NVP and EFV were dissolved in methanol. All solutions were stored at −20 °C. Methanol–water (1/1, v/v) was used to prepare the standard solutions. The working solutions were freshly prepared and added to the human plasma blank to obtain 7 standard calibration curves in a range of 0.250–15.0 μg/mL (EFV and NVP) and 0.125–10.0 μg/mL (LUM). Four levels of Quality control (QC) samples were prepared independently but in a similar way at 0.250, 0.750, 5.00 and 12.5 μg/mL (EFV and NVP) and 0.125, 0.500, 3.20 and 7.50 μg/mL (LUM). A solution of acetonitrile containing 0.5% acetic acid was used to dilute the IS to achieve a final concentration of 1 μg/mL.

2.5. Extraction procedure

A 100 μL aliquot of human plasma spiked with respective drugs was mixed with 200 μL of 0.5% acetic acid acetonitrile solution containing the IS, the sample was vortexed for 40 s, and the samples were allowed to equilibrate under gentle agitation at room temperature for 25 min, centrifuged for 10 min at 20,817 × *g* (4 °C). Then, 75 μL of the upper layer was transferred into a new glass vial and 25 μL was injected into the LC-UV system for analysis.

3. Method validation

This method was validated according to some principle of FDA guideline for validation bioanalytical assay [26].

3.1. Selectivity

To determine whether the endogenous matrix constituents co-elute with the peak of interest of NVP, EFV and LUM, six different blanks of human plasma were analyzed at three different wavelengths (255, 275 and 300 nm) and also at only one wavelength (255 nm). Additionally, human blank plasma spiked with IS, and each compound at the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were analyze. Potential co-medications: trimethoprim, sulfamethoxazole, lamivudine, zidovudine, pyrimethamine, fluconazole, emtricabine, tenofovir, quinine sulfate, amodiaquine and stavudine were tested for co-elution by analyzing them in solution and by spiking them into human blank plasma and were processed according to the mentioned procedures above.

3.2. Accuracy, precision and linearity

Precision (expressed as percent relative standard deviation, %CV) and accuracy (expressed as percent error, %bias) were calculated for the four QCs at 0.125, 0.500, 3.20 and 7.50 μg/mL for LUM and 0.250, 0.750, 5.00 and 12.5 μg/mL for EFV and NVP. At least five replicates of each QC point were analyzed every day to determine the intra-day accuracy and precision. This process was repeated over 4 days in order to determine the inter-day accuracy and precision. Over the 4 days of validation, the standard curve was evaluated by duplicate analysis of seven spiked human blank plasma samples with analytes. The calibration curve for all the analytes were plotted using peak area ratio (peak area of analytes/peak area of IS) against the nominal concentration of the analytes.

3.3. Recovery

The recovery of EFV, NVP and LUM was determined by comparing the peak area of the QC samples in the low, mid and high with the unprocessed samples in triplicate for 3 days.

3.4. Stability

Stability was evaluated at different time and procedures. The samples were assayed at QC-low and QC-high in triplicate. The unprocessed samples were kept at room temperature for 24 h and were subjected to 3 freeze thaw cycles at or below −20 °C. Stability of the samples in auto-sampler over 24 h pending to analysis and the stability of the stock solution after being stored at −20 °C for 2.5 months were also evaluated.

3.5. Analysis of plasma samples from patients

Samples from patients recruited in one of our study were also analyzed using our method. The patients were undergoing antiretroviral therapy containing either EFV or NVP based triple therapy and were also taking an anti-malarial drug containing a combination of artemether and LUM.

4. Results and discussion

4.1. Optimization of the chromatographic condition

The aim of the present work was to develop a simple and cost-effective HPLC-UV method for the simultaneous analysis of NVP, EFV and LUM, three compounds with very different physicochemical properties. In this respect, the column choice has a great influence on compounds separation which is essential for the success of the method in complex matrix such as plasma. Consequently, a compromise between the hydrophilic and lipophilic characters of the column stationary phases and mobile phase has to be found. During the method development, four end capped reversed-phase columns: the C_{18} Gemini (150 mm × 4.6 mm ID particles size 5 μm); Xterra MS (2.1 mm × 150 mm × 2.1 mm ID particle size 5 μm), ACE-CN (150 mm × 4.6 mm ID particles size 3 μm); and C_{16} Dionex Acclaim polar advantage (150 mm × 4.6 mm ID particles size 3 μm) were tested with different mobile phase compositions. Using the Gemini column with a mobile phase made of 0.1 M ammonium acetate buffer and acetonitrile, NVP, EFV and LUM eluted after 2, 18 and 30 min, respectively with broad peak shapes. Acidification of ammonium acetate buffer and acetonitrile with 0.01 and 0.1% TFA respectively decreased the retention time of LUM (12 min) and EFV (9 min) with an improved peak shape. However, NVP co-eluted with endogenous substances in the plasma. We used 0.1% TFA (in the organic mobile phase) instead of phosphate buffer to maintain an acidic condition in order to minimize peak tailing for LUM and its IS as it was previously demonstrated by Huang et al. [9]. With the XTerra column, LUM and EFV were baseline separated in plasma with good peak shape with the use of 0.1 M ammonium acetate in 0.01% TFA and 0.1% TFA in acetonitrile as mobile phase. Under these conditions the retention times of EFV and LUM were 9 and 13 min, respectively. However, NVP was not resolved from the plasma background. With the use of ACE column under the acidified mobile phase conditions, NVP, LUM and EFV were chromatographically separated and resolved from plasma endogenous compounds but the peak shapes for these three compounds were very broad and this makes their integrations quite difficult. The optimal chromatographic conditions for all the 3 analytes and HALO (IS) were obtained on the Dionex C_{16} column, with a mobile phase made of a 0.01% TFA in 0.1 M ammonium acetate buffer and 0.1% TFA in acetonitrile at a flow rate of 1 mL/min. Under the final chromatographic

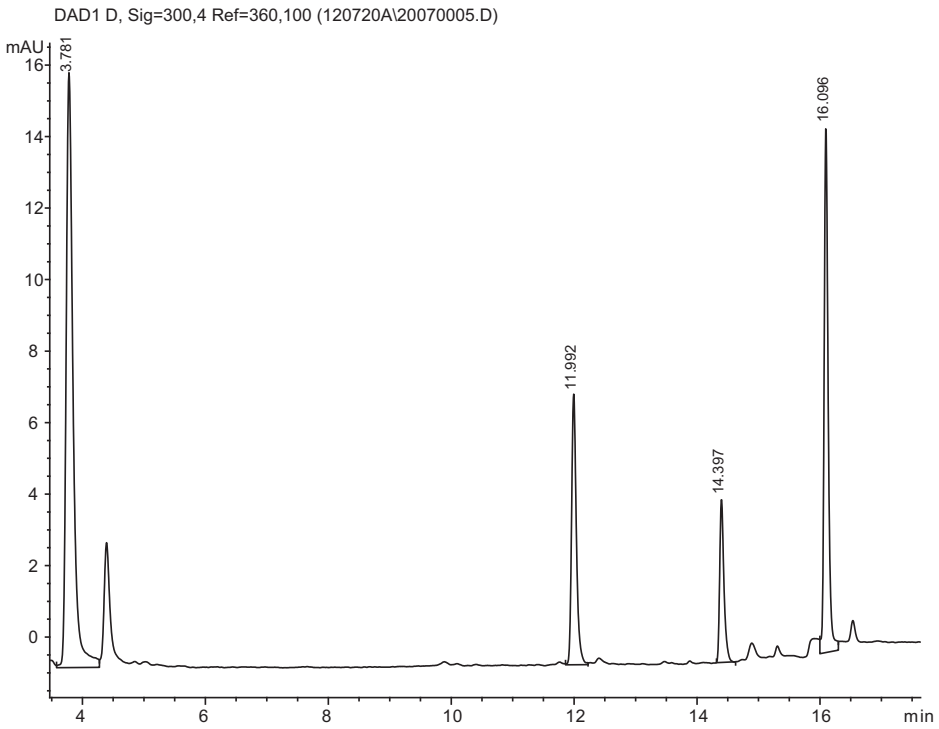

Fig. 2. A representative chromatogram in plasma of NVP, EFV, LUM and the IS from the final method.

conditions, the total run in this assay was 20 min and the retention times for NVP, EFV, IS and LUM were 3.8, 11.9, 14.4 and 16.0 min, respectively. A representative chromatogram of NVP, EFV, LUM and IS from extracted plasma obtained with the final method is showed in Fig. 2.

4.2. Sample preparation

Due to the fact that, LUM, EFV and NVP have different physicochemical properties, it was necessary to find the right method for sample pre-treatment. The use of the standard two-phase liquid–liquid extraction method was not considered for this development, as the extraction procedure is time consuming and may require large amount of organic solvents. Solid phase extraction (SPE) has been used for the extraction of EFV, NVP or LUM from plasma. Since our goal was to develop a cost-effective method that can be implemented in facilities with limited resources, we did not consider such an approach within the frame of this work. Protein precipitation (PPT) is a commonly used procedure for the extraction of anti-malarial or anti-retroviral drugs and related compounds prior the LC-UV analysis because of its simplicity. Several PPT procedures using various reagents including zinc sulphate, perchloric acid, methanol, acetonitrile were tested. We found that acetonitrile containing 0.5% acetic acid was the most efficient reagent as a better recovery was obtained. Furthermore, acetonitrile was compatible for a direct injection onto the HPLC-UV system. This simplifies our method by avoiding sample drying and reconstitution with compatible solvent prior HPLC-UV analysis.

4.3. Peak identification and selectivity

The UV absorption spectra and resultant λ_{max} of LUM, EFV and NVP were found to be quite different (Fig. 3). Hence three monitoring wavelengths were used throughout the run in order to maximize response in human plasma. The λ_{max} wavelength for EFV was 246 nm. However, it was chosen to monitor EFV at 255 nm in human plasma in order to optimize the method selectivity and the signal-to-noise ratio in plasma. NVP and LUM were both monitored at 275 and 300 nm, respectively. The specificity of the method in human plasma was demonstrated by the retention characteristics, UV spectra and by comparing the peak purity with the standard of NVP, EFV and LUM. No interfering peaks were observed in the extract from six different lots of plasma (Fig. 4). Furthermore, as can be seen in Table 1, 11 potential concomitant drugs were analyzed under our experimental conditions and none of them co-eluted with EFV, NVP and LUM. This demonstrated that our method is highly selective.

4.4. Sensitivity

The limit of quantification (LOQ) of the method was 0.125 μg/mL for LUM and 0.250 μg/mL for EFV and NVP when using a human plasma extract volume of 100 μL. Representative LC-UV chromatograms of blank plasma sample spiked with NVP, EFV and LUM at the LOQ are depicted in Fig. 4, Fig. 5 and Fig. 6, respectively. As can be seen, the signals of the three compounds at the LOQ were above the noise level. Also the IS did not co-elute with the three analyzed compounds.

4.5. Linearity

The calibration curve was plotted using peak area ratio and the concentration of analytes without weighing and was fitted by linear least square regression. The calibration concentrations were

Table 1
Summary of the calibration parameters for LUM, EFV and NVP.

Compounds	Slope	Intercept	R^2	Range (μg/mL)	Wavelength (nm)
LUM	0.021	0.324	0.999	0.125–10.0	300
EFV	0.008	0.216	0.999	0.250–15.0	255
NVP	0.034	0.478	0.998	0.250–15.0	275

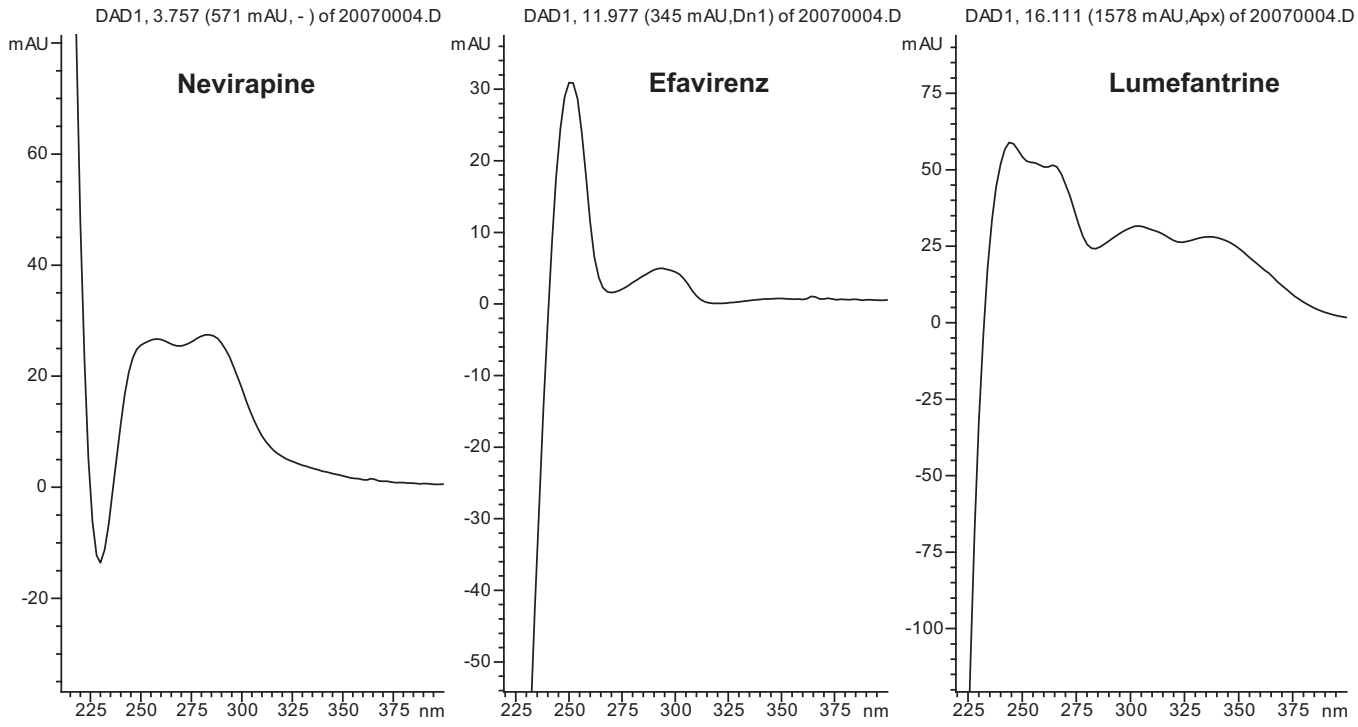

Fig. 3. UV–vis spectra of the different compounds used in the present study.

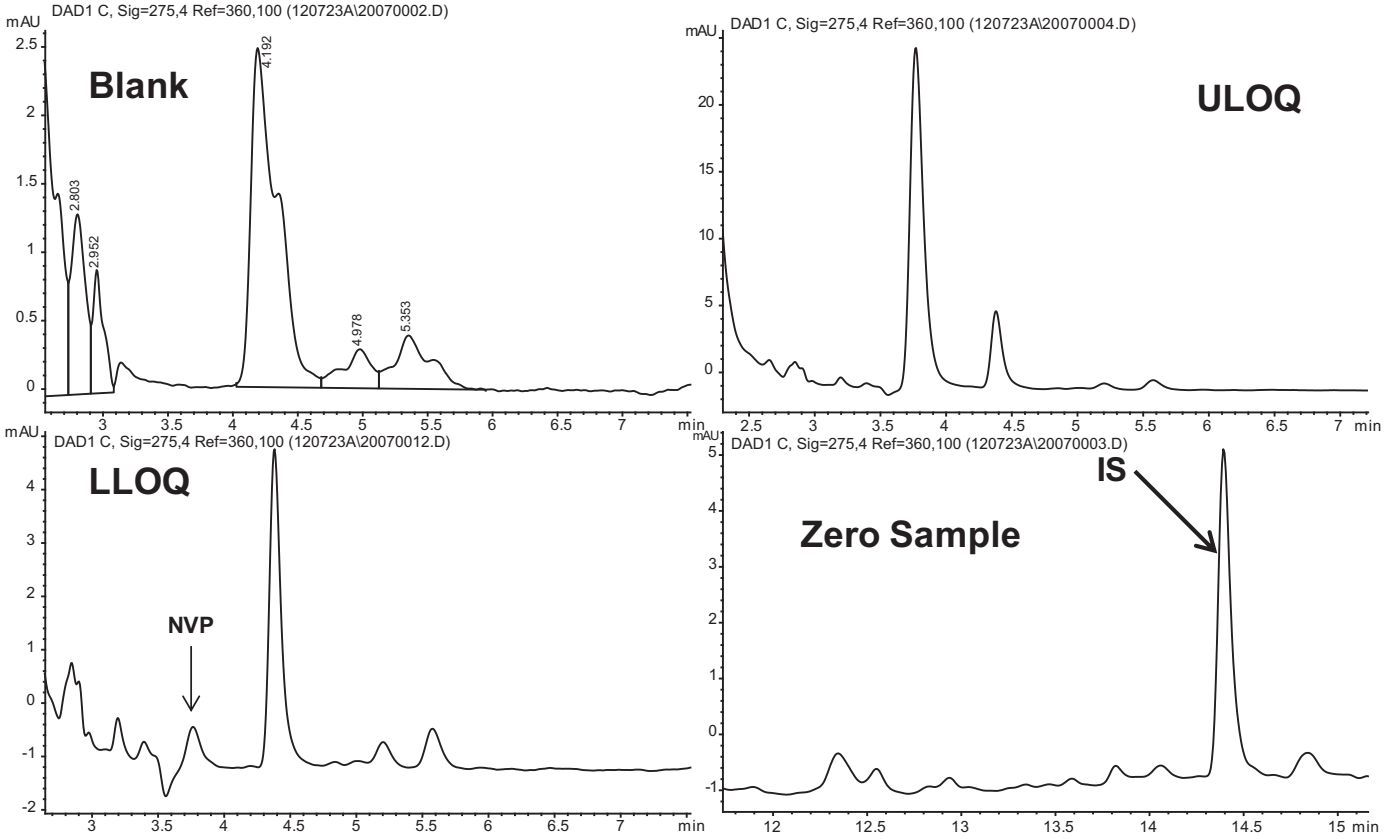

Fig. 4. Examples of LC-UV chromatograms obtained at 275 nm of blank extracted plasma and that of NVP spiked in human plasma at the LLOQ and the ULOQ. The zero sample corresponds to the extracted plasma spiked with the IS only. The retention time of NVP and IS are 3.8 and 14.4 min, respectively.

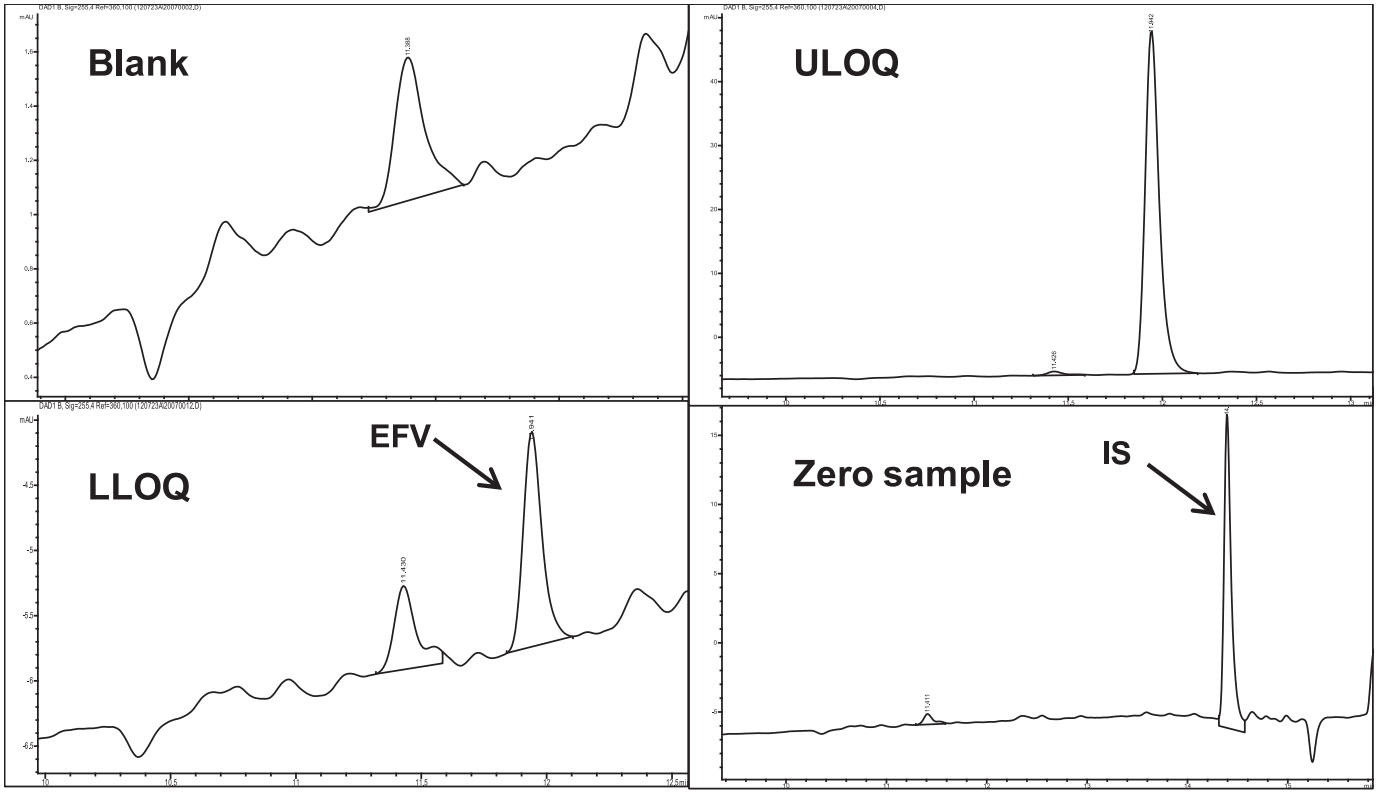

Fig. 5. Examples of LC-UV chromatograms obtained at 255 nm of blank extracted plasma and that of EFV spiked in human plasma at the LLOQ and the ULOQ. The zero sample corresponds to the extracted plasma spiked with the IS only. The retention time of NVP and IS are 11.9 and 14.4 min, respectively.

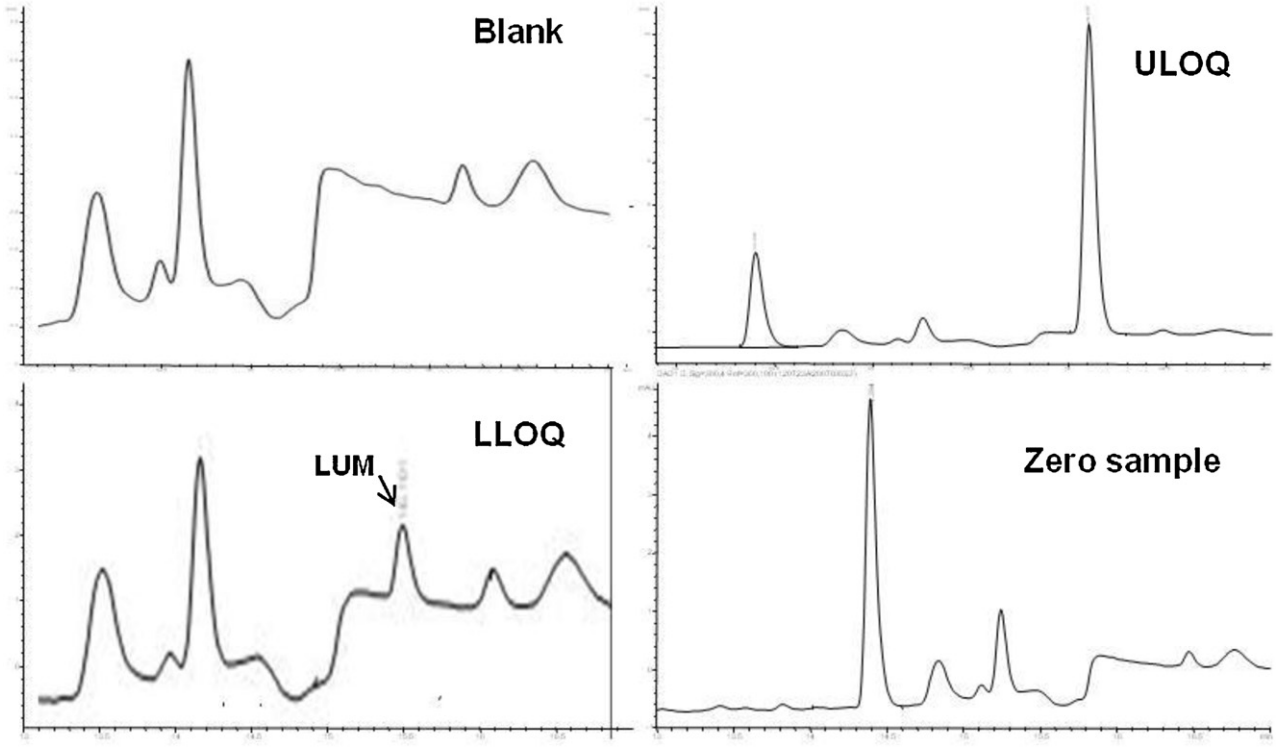

Fig. 6. Examples of LC-UV chromatograms obtained at 300 nm of blank extracted plasma and that of LUM spiked in human plasma at the LLOQ and the ULOQ. The zero sample corresponds to the extracted plasma spiked with the IS only. The retention time of NVP and IS are 16.0 and 14.4 min, respectively. The arrow in the blank chromatogram indicates the retention time of LUM.

Table 2
Precision and accuracy data of LUM, EFV and NVP in human plasma.

Quality control	Measured concentration of LUM (μg/mL)			Measured concentration of EFV (μg/mL)			Measured concentration of NVP (μg/mL)		
	Mean	CV (%)	Bias (%)	Mean	CV (%)	Bias (%)	Mean	CV (%)	Bias (%)
Intra-day precision and accuracy (5 replicates at each concentration)									
LLOQ	0.124	1.5	−0.8	0.24	0.9	−4.0	0.25	0.6	0.0
QC-Low	0.49	2.4	0.0	0.76	3.2	−1.3	0.78	0.9	−4.0
QC-Mid	3.10	0.2	−3.7	5.03	0.7	−0.6	5.05	1.5	−1.0
QC-High	7.58	0.4	−1.3	12.42	0.5	0.6	12.49	0.7	−0.1
Inter-day accuracy and precision (20 replicates at each concentration)									
LLOQ	0.129	1.6	−3.2	0.25	1.6	0.0	0.26	2.2	−4.0
QC-Low	0.48	2.1	0.0	0.73	2.3	−2.7	0.73	2.3	−2.7
QC-Mid	3.38	0.9	−3.6	5.14	0.5	−2.8	5.08	1.4	−1.6
QC-High	7.78	1.1	−3.7	13.06	0.6	−4.5	12.77	1.4	−2.2

Table 3
Stability study results for LUM, EFV and NVP in human plasma ($n = 3$).

Quality control	Stability study	LUM			EFV			NVP		
		Mean conc. (μg/mL)	Bias (%)	CV (%)	Mean conc. (μg/mL)	Bias (%)	CV (%)	Conc. (μg/mL)	Bias (%)	CV (%)
QC-Low	24 h at room temperature	0.47	−5.7	0.3	0.74	−1.5	1.0	0.72	−5.3	4.1
	24 h in auto sampler	0.49	−1.7	2.9	0.72	−4.1	0.3	0.68	−6.7	7.0
	Three freeze–thaw cycles (−20 °C)	0.48	−4.2	2.2	0.74	−1.5	1.3	0.71	−4.0	5.5
QC-High	24 h at room temperature	7.5	0.4	2.4	13.28	6.3	1.7	13.07	4.0	3.0
	24 h in auto sampler	7.6	1.6	1.7	13.40	7.2	1.3	12.98	3.8	1.3
	Three freeze–thaw cycles (−20 °C)	7.8	4.1	0.6	13.12	4.9	1.6	12.58	0.7	1.4
STOCK	2.5 months at −20 °C	10.62	6.2	0.1	10.48	4.8	0.1	11.71	8.4	0.04

back calculated from the calibration response (Table 2). The mean of the regression coefficients were >0.998 (Table 2) for all the three compounds under the analytical range used.

4.6. Accuracy and precision

Table 3 shows the summary of the precision and accuracy of the validated method. The intra and inter-day precision and accuracy for the QC samples for all the analytes met the FDA guideline requirements [26]. EFV intra and inter bias were between −4.5 and 0.0%; the precision was between 0.5 and 3.2%. NVP intra and inter-day bias were between −4.0 and 0.0%, precision between 0.6 and 2.3%. LUM intra and inter-day bias were between −3.6 and 0.0%, and precision between −3.7 and 0.0%.

4.7. Stability and recovery

The bench-top stability at room temperature over 24 h, sample in auto sampler pending to analysis over 24 h and 3 cycles freeze thaw stability studies for, NVP and LUM in human plasma was evaluated. The analytes were evaluated in triplicate using QCs at 0.500 and 7.50 μg/mL for LUM and 0.750 and 12.5 μg/mL for EFV and NVP. The measured concentrations of the analytes in these QCs were comparable to the nominal values, with accuracy ranging from −5.7 to 4.1% for LUM, −4.1 to 7.2% for EFV, −6.7 to 4.0% for NVP (Table 4). These results indicate that our analytes were stable under the tested conditions. Long-term stability studies for LUM, EFV and NVP were not assessed during this validation as some stability data have been already reported. Khalil et al. [10] showed that LUM is stable for at least 9 months when stored at −80 °C. Kappelhoff et al. [20] have reported 36 months stability for NVP and EFV when stored at −20 °C.

The recovery was determined in spiked plasma samples ($n = 3$) at three concentrations: 0.5, 3.2, and 7.5 μg/mL for LUM and 0.75, 5 and 12 μg/mL for NVP and EFV by dividing the peak area of the analytes sample spiked before extraction by the peak area of an equal concentration of analyte sample in the same matrix spiked after extraction. The mean recovery was 104%, 98.6% and 99% for LUM, EFV and NVP, respectively (Table 5).

4.8. Method transfer and clinical application

The present method was implemented in Tanzania. The HPLC system, the binary pumps and the injection loop volume (50 μL instead of 900 μL as the one used during the development and validation at Novartis) were different from those used during the method cross-validation. As a matter of fact, the HPLC system and the pumps used in Tanzania were from a 1050 series. In addition, the UV-detection was performed at a compromised wavelength as the detector used was not a diode-array. Based on the UV spectra displayed in Fig. 3, the wavelength 255 nm was chosen as its represents one UV-absorption peak for each tested compound. The specificity of the method in human plasma

Table 4
Recovery data obtained at two differents concentration ($n = 3$) for LUM, EFV and NVP.

	Conc (μg/mL)	Recovery (%)	Bias (%)	CV %
LUM				
QC-Low	0.5	103	3.0	1.1
QC-Mid	3.2	103	3.0	1.1
QC-High	7.5	101	1.0	0.7
Mean		102		
EFV				
QC-Low	0.75	98	−1.9	2.1
QC-Mid	5	98.7	−1.3	0.7
QC-High	12.5	99	−1.3	0.5
Mean		98.6		
NVP				
QC-Low	0.75	104	4.4	1.3
QC-Mid	5	98	−2.2	0.5
QC-High	12.5	95	−5.1	0.7
Mean		99.0		

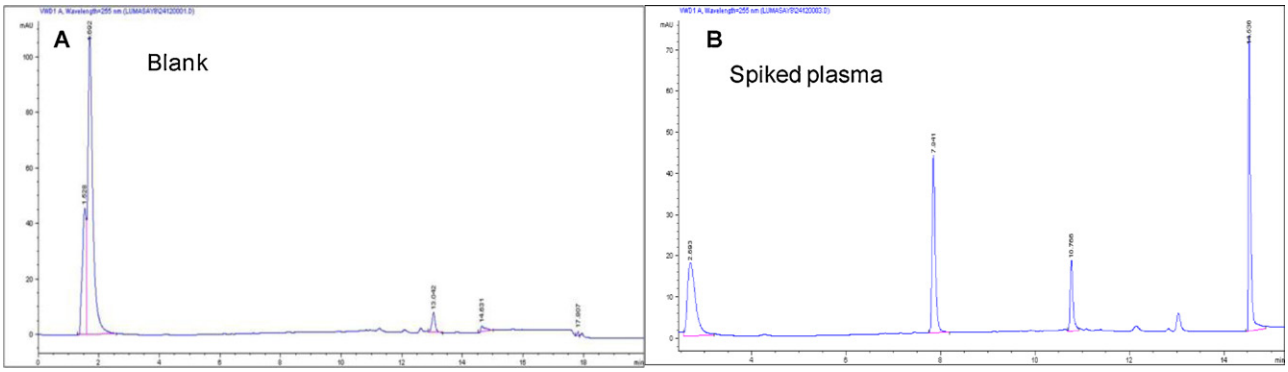

Fig. 7. Examples of LC-UV chromatograms obtained at 255 nm of (A) blank extracted plasma and that of (B) NVP, EFV, HALO and LUM spiked at 10 μg/mL (NVP, EFV and LUM) and 1 μg/mL (HALO) in human plasma. The retention times of NVP, EFV, HALO and LUM were 2.7, 7.9, 10.8 and 14.5 min, respectively.

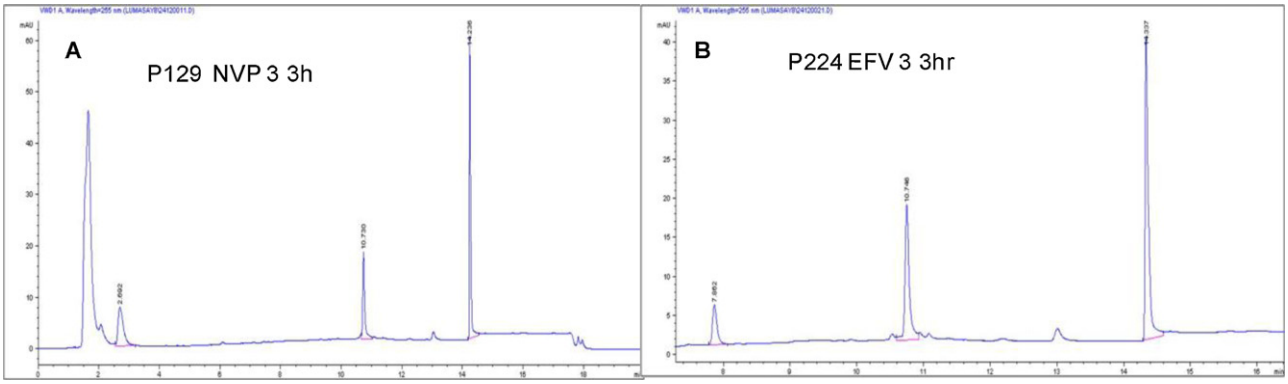

Fig. 8. Examples of LC-UV chromatograms obtained at 255 nm from patients receiving (A) NVP and LUM and (B) EFV and LUM.

at 255 nm was demonstrated by the retention characteristics, UV spectra and by comparing the peak purity with the standard of NVP, EFV and LUM. No interfering peaks were observed at the retention time of each compound in the extract from different lots of plasma. An example of extracted blank and spiked human plasma chromatograms are given in Fig. 7A and B. Furthermore as can be seen in Table 1, 11 potential concomitant drugs (zidovudine, pyrimethamine, trimethoprim lamivudine, sulfamethoxazole, fluconazole, emtricitabine, tenofovir, quinine sulphate, amodiaquine, and stavudine) were analyzed under our experimental conditionsand none of them co-eluted with EFV, NVP and LUM. This demonstrated that our method is highly selective. The difference in the equipment and also setting such as injection loop could explain shift in the retention times of NVP, EFV, HALO and LUM observed during the method implementation. Since the UV-absorption of LUM and NVP at $\lambda = 255$ nm were higher than those obtained at 275 and 300 nm monitoring the compound at 255 nm will not affect the method sensitivity and validation data already discussed such as precision, accuracy stability and recovery. Hence $\lambda = 255$ nm appears suitable for monitoring the human plasma samples.

Our method was used to analyse samples from an on-going study. For this clinical study, HIV/AIDS patients co infected with malaria were on day 0 given an oral dose of Coartem (artemether 80 mg and LUM 480 mg), after the patients had been given a standardized fat meal and 250 mL UHT milk (6% fat). Patients were asked to come back to the Hospital on day 3 and the last dose was taken at the hospital and administered as above. These patients had

Table 5
Retention times of the co-medicated drugs and that of NVP, EFV, IS and LUM under our experimental conditions.

Compounds	Retention time (min)
Sulfamethoxazole	19.8
Fluconazole	1.8
Pyrimethamine	4.7
Lamivudine	2.07
Zidovudine	2.7
Trimethoprim	ND
Emtricitabine	5.08
Tenofovir	1.9
Quinine sulphate	2.01
Amodiaquine	3.17
Stavudine	2.03
NVP	2.8
EFV	7.9
IS	10.2
LUM	14.3

ND: not detectable under our experimental conditions.

Table 6
Concentration of LUM from two patients P129 on NVP and P124 on EFV.

Patient code	Time (h)	Concentration (ng/mL) LUM	NVP CONC (ng/mL)
P129	0	0	7401 post dose
	52 hr	6252	
	55	6702	
	168	704	
	336	139	
Patient code	Time (h)	Concentration (ng/mL) LUM	EFV CONC (ng/mL)
P124	0	0	2620
	41	6045	
	3	6252	
	168	739	
	336	178	

also been taking NVP 200 mg twice a day and EFV 600 mg once at night including 2 nucleoside reverse transcriptase inhibitors for the past 2 months before they were treated with an anti-malarial drug (Coartem). Blood samples were collected on day 0 pre-dose, day 3 pre-dose then 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, 7, 10, 14 and 21 days post-dose and plasma was obtained thereafter and stored at −80 °C until analysis. Chromatograms showing patient plasma sample at day 3 post-treatment is presented in Fig. 8A and B. In all tested plasma samples NVP, EFV and LUM concentrations were above the LLOQ. Thus our method was suitable to monitor EFV, NVP and LUM in the plasma of patients enrolled in the clinical study. Table 6 summarizes the concentration of LUM from two patients P124 on EFV and P129 on NVP. The studied patients were HIV infected and had been on anti retroviral drugs for >2 months.

5. Conclusions

A simple and cost-effective method was successfully developed and validated for simultaneous analysis of LUM, EFV and NVP in human plasma. The assay fulfilled the criteria for accuracy and precision for the determination of these drugs, which have very different physico-chemical properties. The easiest and fast sample preparation (protein precipitation) and the use of a small aliquot of plasma and less reagents and the possibility of achieving maximum detection with a single detection wavelength (255 nm) makes this assay method highly suitable for measurement of drug concentrations in various PK and/or clinical studies, especially in countries with limited facilities and resources.

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