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Simultaneous determination of efavirenz, rifampicin and its metabolite desacetyl rifampicin levels in human plasma

Deirdre Fox[a,*], Robert O'Connor[b,c], Patrick Mallon[d], Gillian McMahon[a]

[a] School of Chemical Sciences, Dublin City University, Dublin 9, Ireland
[b] National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland
[c] School of Nursing, Dublin City University, Dublin 9, Ireland
[d] Infectious Diseases Outpatients Clinic, Mater Misericordiae University Hospital, Eccles Street, Dublin 7, Ireland

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ABSTRACT

A simple and rapid isocratic, high performance liquid chromatography (HPLC) assay employing solid phase extraction (SPE) for the simultaneous determination of the anti HIV drug, efavirenz, the anti-tuberculosis drug, rifampicin and the desacetyl metabolite of rifampicin in plasma from HIV/tuberculosis infected patients has been developed. Using a Zorbax SB-Phenyl reverse-phase analytical column with UV detection, good separation and detection of the drugs was attained within a 10 min run time. Intra- and inter-assay precision RSD values were found to be less than 15% at the concentrations examined (0.1–20 μg/mL). The LOQ was found to be 0.1 μg/mL for each agent and the assay was found to generate a linear response up to 20 μg/mL.

This low cost assay can accurately detect efavirenz and rifampicin concentrations within a clinically relevant concentration range using standard chromatography equipment, making it particularly applicable to resource-limited settings.

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1. Introduction

HIV infection is estimated to affect over 40 million people worldwide with infection rates increasing by nearly five million per year. Patients with HIV are prescribed antiretroviral agents [1]. There is a high incidence of tuberculosis (TB) in HIV-infected patients for which rifampicin (Rif) is the main drug of choice for treatment. However, concurrent treatment of HIV and TB is complicated by drug interactions between Rif and antiretrovirals [2]. Current guidelines recommend treatment of TB and HIV co-infection with antiretroviral combinations containing non-nucleoside reverse transcriptase inhibitors (NNRTI) [3]. Of the two NNRTI currently available, efavirenz (Efv) is preferred as it is dosed once daily and is less hepatotoxic than the alternative.

It has been hypothesised that Efv can alter the pharmacokinetics of Rif, in order to study whether the pharmacokinetics of Rif are adversely affected when a patient is also administered Efv, a clinical study termed the SPhEAR study (Study on the Pharmacokinetics of Efavirenz And Rifampicin) was developed which required an analytical method capable of extraction, separation and determination of Efv and Rif (and also Rif's metabolite desacetyl rifampicin (dRif)) simultaneously in plasma. See Fig. 1 for the structures of these analytes.

In order to routinely determine drug concentrations in plasma, it is necessary to have an accurate, precise and selective analytical method, requiring a small sample volume and if possible rapid sample processing. Methods for the individual analysis of Efv or Rif in biological fluids have been published (Supplemental Tables 1a and 1b). Reversed phase liquid chromatography has been used for the determination of Efv and Rif in tissues, plasma, serum and urine employing detection modes such as UV, fluorescence and MS (Supplemental Tables 1a and 1b). However, many of these methods suffer from limitations such as lack of sensitivity, lengthy and complex extraction procedures, expensive detectors (e.g. mass spectrometers), a requirement for large quantities of organic solvents and high sample volumes which can render them unsuitable for analysing large numbers of samples, especially in resource-limited settings. Recognising such challenges, Boffito et al. have reported the development of a method for the simultaneous determination of Efv and Rif [4] which exhibits comparable sensitivity with the method we outline herein. This assay has a lower limit of detection of 0.05 μg/mL for both Rif and Efv but requires gradient elution and hence suffers from a relatively long analysis time of 22 min. The authors also did not measure Rif's main metabolite, desacetyl rifampicin (dRif).

The circulating plasma concentrations of Rif and Efv, when co-administered to patients for the treatment of both HIV and TB

* Corresponding author. Tel.: +353 0863074522.
E-mail address: Deirdre.fox4@mail.dcu.ie (D. Fox).

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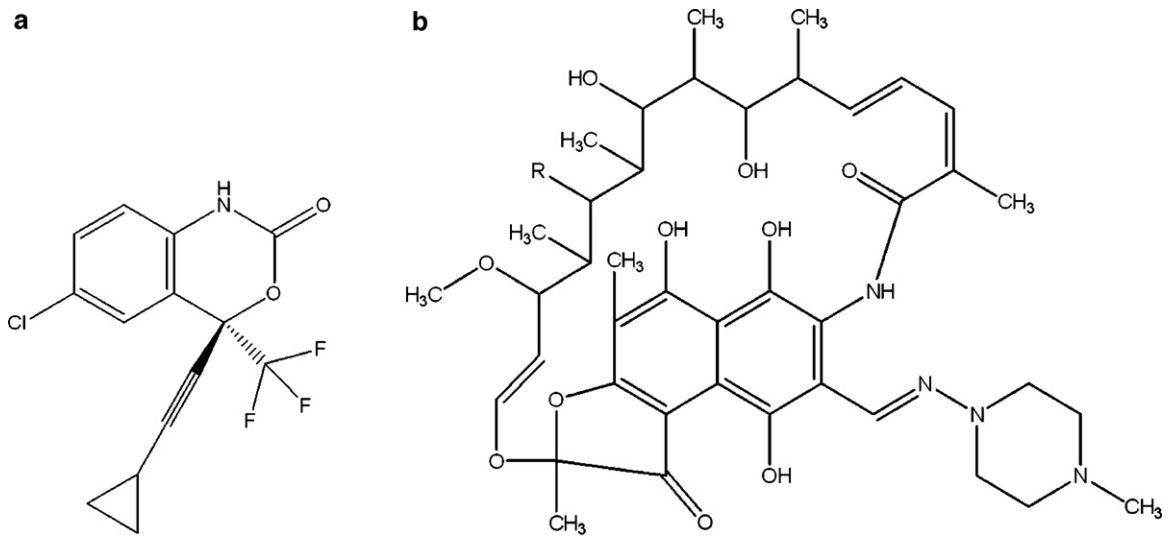

Fig. 1. Chemical structures of (a) efavirenz and (b) rifampicin (R=$COOCH_3$) and desacetyl rifampicin (R=OH).

infection, are high (1–20 μg/mL) and hence are well within the range that UV can detect. The UV detector is also both simple to use and cost effective (employing low price equipment which is cheap to maintain and run) [2,4–7]. Hence it is anticipated that adoption of such methodology in areas where there are high incidences of HIV infections, such as sub-Saharan Africa, Eastern Europe and Asia would be more easily achieved.

Various sample preparation techniques have been exploited for the separate determination of Efv and Rif such as solid phase extraction (SPE), liquid–liquid extraction (LLE) and protein precipitation (PP) (Supplemental Tables 1a and 1b). From Supplemental Tables 1a and 1b, it is evident that LLE is used most often for Efv and PP for Rif, each method offering good recoveries for each. All three approaches were evaluated in this work to see which was optimal. Following extensive testing, it was found that SPE gave the best recoveries for both drugs in one protocol.

The development and validation of a simple HPLC–UV method preceded by SPE clean-up for the determination of Efv, Rif and dRif in human plasma is outlined in this manuscript.

2. Experimental

2.1. Reagents and materials

HPLC grade acetonitrile (ACN), methanol (MeOH) and water were purchased from Sigma–Aldrich. Ammonium acetate, formic acid and acetic acid, all analytical grade, were obtained from Sigma–Aldrich. Drug-free sterile filtered human plasma (EDTA k3) was purchased from Europa Bioproducts, United Kingdom. Efavirenz (Efv), rifampicin (Rif) and rifampicin's metabolite, desacetyl rifampicin (dRif), were purchased from Sequoia Research Products Ltd., United Kingdom. Extraction cartridges and plates (SPE, LLE and PP) were purchased from Biotage, Sweden.

2.2. Equipment and HPLC assay conditions

The HPLC system consisted of a 1050 series quaternary pump, 1100 series autosampler, a diode array detector (DAD) and a 1200 series degasser, all from Agilent, United Kingdom. System management and data acquisition were performed by the Agilent Chemstation for LC 3D software. Other instrumentation employed included a pH meter and electronic mass balance both from a Mettler Toledo, USA and a Genevac EZ-2series personal evaporator from United Kingdom. The VacMaster-96 sample processing manifold was sourced from Biotage, Sweden.

The analytical column was an Agilent Zorbax SB Phenyl, 150 mm × 4.6 mm ID, with 5 μm particle size. The HPLC mobile phase consisted of ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v). A diode array UV detector was employed which monitored Efv at 246 nm and Rif and dRif at 334 nm. The overall runtime was 10 min and the flow rate was 0.8 mL/min at ambient temperature.

2.3. Preparation of standards

Stock standard solutions of Efv and Rif were prepared by weighing out the powders in a glovebox, dissolving them in ACN to 1 mg/mL concentration and storing them in amber vials at 2–4 °C in the dark. Working standards of Efv and Rif were prepared both in drug-free human plasma and in mobile phase (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)) to concentrations over the range 0.1–20 μg/mL (0.1, 0.5, 1, 5, 10 and 20 μg/mL in plasma which corresponded to 0.2, 1, 2, 10, 20 and 40 μg/mL in MP). This range of the standard curve was chosen to reflect the plasma concentrations expected in a typical 12 h pharmacokinetic profile post administration of both drugs Rif: [5] Efv: [2,7] and both: [4].

A 0.3 mL aliquot of each of the working standards was diluted 1:1 (v/v) with a 1% formic acid solution in water. The SPE Evolute Array Wells (25 mg ABN, 1 mL,) in the 96 well plate sample processing manifold were conditioned with 1 mL MeOH and then equilibrated with 1 mL of the 0.1% formic acid solution. A 0.6 mL aliquot of the diluted, acidified sample was loaded onto the well, washed with 20% MeOH in water and eluted with a water (1 mg/mL ascorbic acid)–ACN–MeOH mixture (25:50:25, v/v/v). The samples were evaporated to dryness and reconstituted with 250 μL HPLC mobile phase (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)). The overall clean-up process resulted in the diluted plasma sample being concentrated by a factor of four which equated to an overall enrichment factor of two for the neat plasma. The reconstituted sample was then transferred to an autosampler vial (with insert) and 50 μL was injected into the HPLC system for analysis.

2.4. Preparation of patient samples

Blood samples from two patients participating in the ethically approved SPhEAR project were obtained by informed consent in

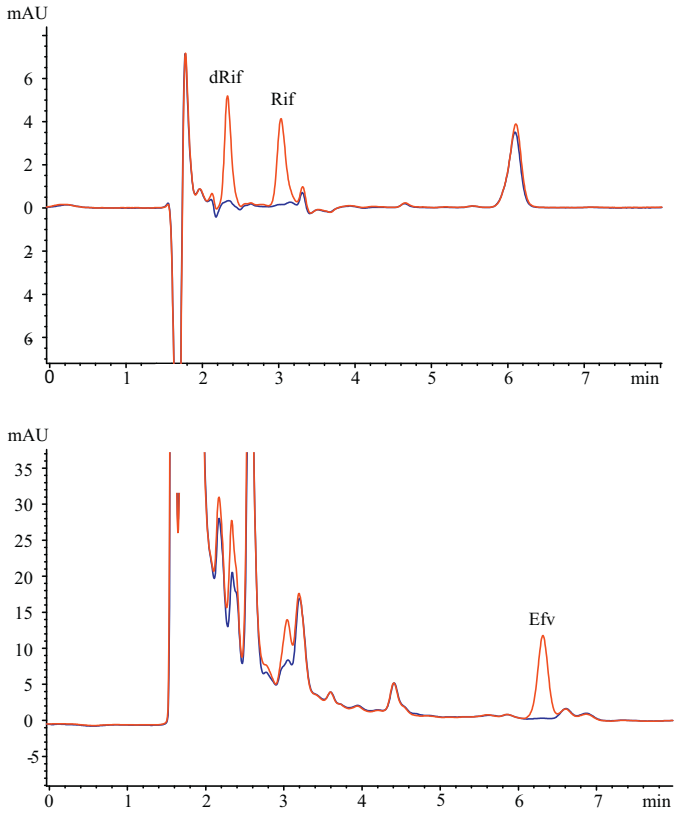

Fig. 2. Chromatograms of extracted drug-free plasma sample superimposed on extracted plasma spiked with 100 ng/mL dRif, Rif and Efv.

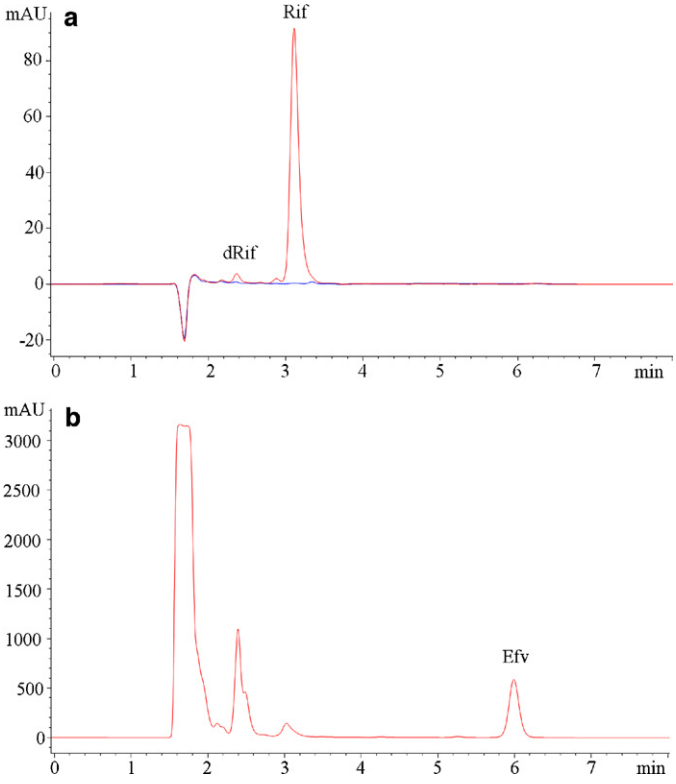

Fig. 3. (a) Overlaid chromatograms from 0 (blue) and 1 h (red) timepoints post-dosing in patient A being treated with rifampicin (Rif) and (b) chromatogram from 1 h timepoint post-dosing in patient B being treated with efavirenz (Efv). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the Infectious Disease Department at the Mater Hospital, Dublin, Ireland. The methodology of this study was approved by the Ethics committee of the Mater Misericordiae Hospital. Bloods samples were performed after at least three weeks on therapy to ensure steady state. Patients were administered a 600 mg dose of Rif. Samples were drawn at 8 different time points over 10 h. The first sample was taken fasting with a set meal provided for all study participants. Blood samples were taken in EDTA preservative which were then centrifuged in a chilled centrifuge and the plasma kept frozen at −80 °C until analysis. A 0.3 mL aliquot of each patient sample was extracted using the same procedure described above for the working standards. Samples were taken and prepared in duplicate.

2.5. Validation

The fundamental parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity and stability. Measurements for each analyte in the biological matrix were validated according to FDA guidelines [8].

2.5.1. Accuracy

In order to evaluate the accuracy of the method, six different concentrations of Efv, Rif and dRif (0.1, 0.5, 1, 5, 10 and 20 μg/mL) were prepared in plasma and analysed in sextuplicate on four consecutive days. The measured amounts were inserted into the equation of the calibration curves and treated as unknown concentrations. The calculated concentrations were compared with the nominal concentrations. Assay accuracy was expressed as % error, i.e. [the absolute difference between calculated concentration and spiked concentration]/nominal concentration × 100.

2.5.2. Precision

The precision of Efv, Rif and dRif standards were evaluated by analysing six different concentrations of Efv, Rif and dRif (0.1, 0.5, 1, 5, 10 and 20 μg/mL). The intra-day precision values were determined by processing each working standard concentration in octuplicate on the same day and calculating the relative standard deviation (RSD) values. The inter-day precision values were determined by processing each working standard concentration in sextuplicate for five consecutive days and calculating the RSD values. %RSD was expressed as [deviation from the mean]/mean concentration × 100.

2.5.3. Selectivity

For selectivity, six blank plasma samples from different sources were analysed and checked for peaks interfering with the detection of the analytes.

2.5.4. Sensitivity

Both the limits of quantitation (LOQ) and detection (LOD) were determined for the assay. The LOQ was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 10:1. LOD was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 3:1.

2.5.5. Recovery

Varying concentrations of Efv, Rif and dRif (1, 5 and 20 μg/mL) were prepared in drug-free human plasma and extracted using the SPE cartridges. The percentage of drug recovered from these plasma samples was determined by comparing the calculated concentrations following extraction and HPLC assay with the calculated concentrations from unextracted samples in MP of the same

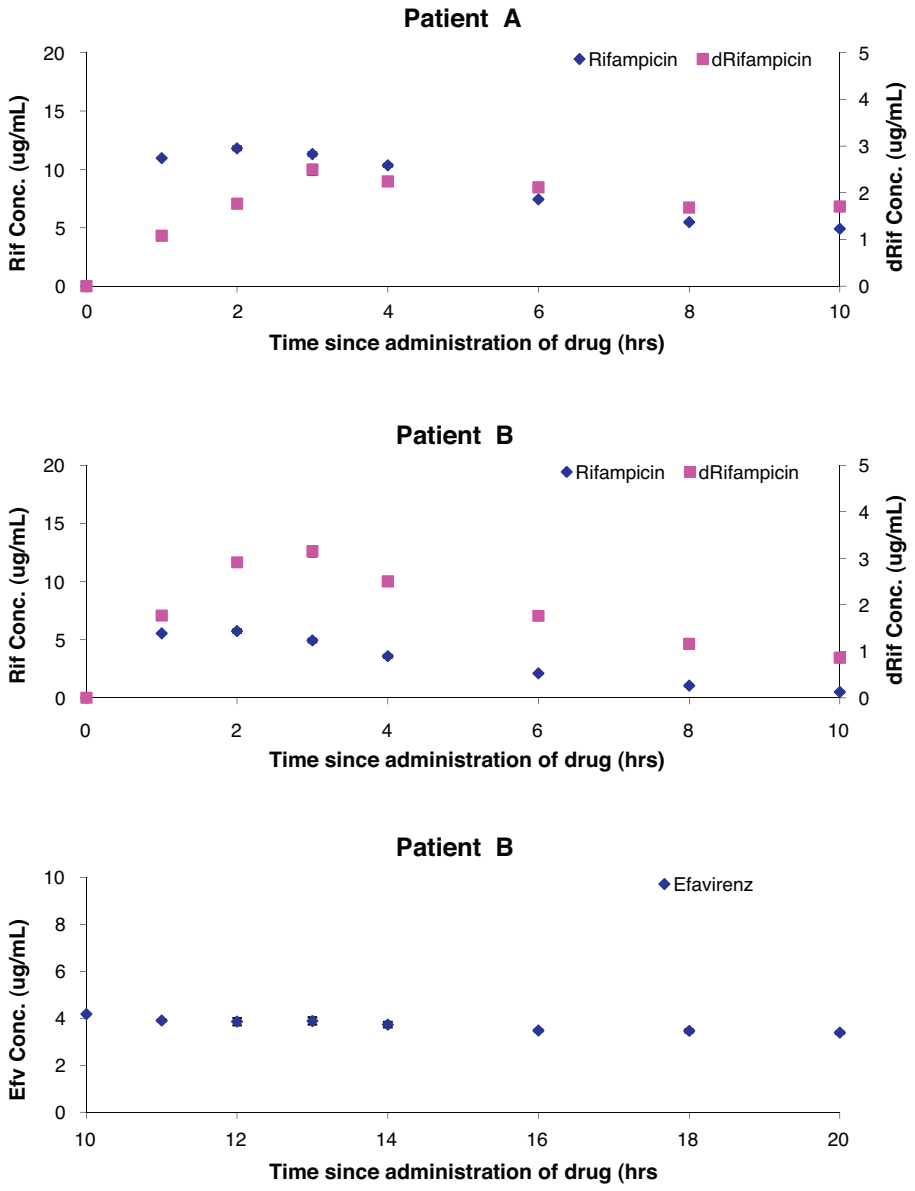

Fig. 4. Pharmacokinetic profiles of Rif, dRif and Efv from three patients in participating in the SPhEAR study.

concentration after HPLC assay. Recovery experiments were carried out on three consecutive days.

2.5.6. Stability

To examine relevant variables around storage and long term stability we determined the impact of freeze–thaw cycles on the stability of the drugs in the presence of plasma, fresh drug-free plasma samples were spiked with 0.5, 5 and 20 μg/mL of Efv, Rif and dRif. Samples underwent four freeze–thaw (−20 °C to room temperature) cycles. The drug concentrations were then determined in triplicate on three separate occasions and compared to plasma samples prepared to the same concentrations that were stored at −20 °C and only thawed once immediately prior to analysis. Long term stability was evaluated using plasma samples spiked with 0.5, 5 and 20 μg/mL of Efv, Rif and dRif after six months of freezing. This storage time was chosen as is does not exceed the time from when samples are first collected for the SPhEAR study to the time of last sample analysis. Finally, stock solution stability was evaluated by comparing the response obtained from standards prepared in mobile phase at three concentrations (0.5, 5 and 20 μg/mL) left at room temperature for 8 h to freshly prepared stock solutions.

3. Results and discussion

3.1. Method development

3.1.1. Extraction procedure

For this work, sample preparation was ultimately achieved using the VacMaster-96 sample processing manifold from Biotage. The manifold can support SPE, LLE and PP clean-up of samples in cartridges and/or well plates. All three extraction approaches were investigated in order to find one that could be optimised for extraction of both Efv and Rif quickly and efficiently. The three methods were:

- Supported liquid membrane (SLM) extraction, a scaled-down version of liquid–liquid extraction (LLE).
- Protein precipitation (PP).
- Solid phase extraction (SPE).

Table 1
A summary of the intra-day and inter-day precision and accuracy determinations.

Compound	Intra-day ($n=8$)			Inter-day ($n=6$)		
	Calculated conc. (μg/mL)	Accuracy (% error)	Precision % RSD	Calculated conc. (μg/mL)	Accuracy (% error)	Precision % RSD
dRif	0.1	−10.1	0.2	0.1	14.0	0.1
	0.5	−6.0	1.8	0.5	−4.3	3.2
	1.0	2.0	1.7	1.1	6.9	1.0
	5.1	1.4	7.2	5.0	−0.8	0.4
	9.9	−1.3	0.2	9.9	−0.2	0.3
	20.0	0.2	0.3	20.0	0.1	0.8
Rif	0.1	10.9	0.8	0.1	10.0	0.9
	0.5	1.7	3.3	0.5	3.5	1.5
	1.1	6.9	2.4	1.1	6.8	0.3
	4.9	−1.9	0.6	4.9	−1.4	0.3
	9.8	−1.6	0.5	9.8	−2.1	0.6
	20.1	0.5	0.2	20.1	0.6	0.3
Efv	0.1	−8.1	0.0	0.1	9.9	4.2
	0.5	5.8	0.8	0.5	1.0	0.9
	0.9	−4.7	1.6	0.9	−6.3	1.7
	5.0	0.2	0.2	5.1	0.9	0.8
	9.9	−0.8	0.1	10.0	0.1	0.5
	20.0	0.2	0.8	20.0	−0.0	0.2

Table 2
Summary of freeze–thaw stability findings for Rif, dRif and Efv ($n=3$).

Compound	Conc. (μg/mL)	Cycle 1% recovery	Cycle 2% recovery	Cycle 3% recovery	Cycle 4% recovery	Cycle 5% recovery
dRif	0.5	99.7	98.1	96.0	87.6	85.6
	5.0	100.7	100.8	96.8	89.5	90.3
	20	99.7	99.5	95.7	85.3	83.8
Rif	0.5	100.9	99.5	96.1	88.0	89.9
	5.0	99.9	102.0	94.3	90.4	88.4
	20	99.2	100.2	96.7	90.3	93.3
Efv	0.5	102.3	102.0	99.3	94.7	94.0
	5.0	100.6	103.5	100.3	92.9	94.8
	20	99.4	99.1	96.5	97.7	96.9

All three methods gave initial recoveries of greater than 64% for the extraction of both Efv and Rif individually at 10 μg/mL, but the extraction method which gave the best recoveries for both drugs combined was SPE.

Evolute ABN was the SPE cartridge chosen for this assay. This cartridge is recommended for the extraction of acidic, basic and neutral (ABN) analytes from biological fluids. Efv has a molecular weight of 316 g/mol [9] and a pKa of 10.2. Rif has a molecular weight of 823 g/mol and an acidic pKa of 1.7 and a basic pKa of 7.9 [6] (it is also very sensitive to light [10]). Hence, it was postulated that Efv, Rif and dRif – even with their different physiochemical properties – could be extracted by this one sorbent. This was borne out experimentally. There are safety implications working with material from HIV/TB infected patients. Some literature has described the use of, heat treatment to inactivate HIV in plasma samples (ranges between 56–60 °C and 30–60 min have been used) [4,11–14] but more recent research has proven that the process of heat inactivation significantly and adversely affects the stability of Rif [5]. When correct health and safety procedures are used, the heating process can be eliminated. It was therefore decided not to heat inactivate the samples used to validate this assay. The addition of ascorbic acid to the elution step of the SPE (1 mg/mL) has been used by some authors to prevent the oxidation of Rif to dRif [15,16]. In the course of this study, it was found that ascorbic acid had a significant impact in maintaining high recoveries for Rif and was hence employed in this assay.

3.1.2. Chromatographic procedure

The choice of column was challenging in this project due to the widely different polarities of the drugs of interest. Both C18 and C8 columns were found to be unsuitable due to the large difference in elution time between the Efv, Rif and dRif. Following trials of a number of columns, a phenyl phase was found to give the best chromatographic results in terms of good separation and fast runtime.

The selection of mobile phase composition and pH required extensive optimisation due to the sensitivity of Rif in particular to small changes in percentage of organic modifier and pH of the mobile phase. It was found necessary to use a ternary mixture of mobile phase containing water and two organic solvents – ACN and MeOH. A thorough pH study revealed that the water component of the mobile phase should be 20 mM ammonium acetate, adjusted to pH 4.75 with acetic acid for optimal analyte response and peak shape.

The UV absorption spectra and resultant λ_{max} of both drugs were found to be quite different and hence two monitoring wavelengths were used throughout the run in order to maximise response. The λ_{max} wavelength for Efv was 246 nm and for both Rif and dRif, 238 nm and 334 nm gave highest absorption. 334 nm was chosen as the monitoring wavelength for Rif and dRif as it is in a region of the UV spectrum that is typically more selective (nucleic acids and proteins absorb strongly up to 280 nm [17]) and also at 334 nm it is only slightly less sensitive than the maximum at 238 nm. Under the final chromatographic conditions, the drugs were well separated with retention times for Efv, Rif and dRif of 6.7, 3.1 and 2.7 min respectively.

3.2. Validation results

3.2.1. Linearity

The calibration curves for Efv, Rif and dRif over the concentration range 0.1–20.0 μg/mL exhibited good linearity with correlation coefficients (r^2) for all standard curves above 0.99.

3.2.2. Accuracy

The accuracies obtained at the three concentrations examined were all acceptable with % error values <10% (see Table 1).

3.2.3. Precision

Intra- and inter-assay precision RSD values were found to be less than 10% in all cases. See Table 1 for the summarised data.

3.2.4. Sensitivity

The limit of quantitation (LOQ) was 0.1 μg/mL for all three drugs. Extracted blank plasma samples did not yield any endogenous peaks at the retention times of the drug compounds – see Fig. 2. The LOD was found to be 0.075 μg/mL with % error and RSD values less than 15%.

3.2.5. Selectivity

The assay was found to be selective for all drugs analysed. No interfering peaks were observed in the extracts of the different blank plasma samples.

3.2.6. Recovery

As previously discussed, highest recoveries for the drugs in one extraction step were obtained with the SPE ABN well plates. Recoveries for plasma samples spiked with 1, 5 and 20 μg/mL of Efv were all ≥70%.

3.2.7. Stability

Having improved the short term stability of Rif by using ascorbic acid during pre sample treatment and extraction, the longer term stability of Efv, Rif and dRif in the presence of plasma was determined over four freeze–thaw cycles at concentrations of 0.5, 5 and 20 μg/mL as shown in Table 2. The data indicated that three freeze–thaw cycles had little significant impact on the Efv, Rif and dRif concentrations measured, which means that samples could be repeatedly thawed and re-analysed up to three times if required. However, it was noted that the drug quantiation showed increased evidence of instability in dRif and Rif on the fourth cycle of the freeze–thaw study which suggests that the drugs are less stable after further freeze–thaw cycles. Long term (6 months, −80 °C) storage at the three different concentrations showed no significant decline in responses obtained for the three drugs. The stability of the three stock solutions prepared in mobile phase was also evaluated. Standards (0.5, 5 and 20 μg/mL) left for 8 h at room temperature showed no decline in detector response at the three concentrations analysed which allowed for daily preparation of daily solutions.

3.3. Sample results

The blood samples analysed were obtained from two patients with patient A administered a 600 mg dose of Rif for the treatment of TB and Patient B administered both Rif and Efv for the simultaneous treatment of HIV and TB. Samples were drawn at eight different time points (0, 1, 2, 3, 4, 6, 8 and 10 h) over a 10 h period. See Fig. 3a for the chromatograms at 0 and 1 h post-dosing in patient A. Rif concentrations for both patients ranged from up to 15 μg/mL, over the full time period (with levels peaking 2–3 h post administration) and up to 4 μg/mL for dRif. The pharmacokinetic profiles for both drugs in each of the patients can be seen in Fig. 4. The results obtained agree with previous levels published [4,5].

Patient B (HIV positive) was administered Efv 10 h prior to T0 (treatment with Rif). The long half life of Efv generates a gradual decline in the blood concentration over time [2] and as expected, Efv concentrations showed a slight decrease over the same time-points with concentrations ranging from 3 to 5 μg/mL over this duration of time (see also Figs. 3b and 4). Again, results agree with previous levels published [4,7]. Examination of the Rif and dRif data from the two subjects shows that there are interpatient pharmacokinetic differences for this drug (as expected from published literature) and for example the same dose in patient B generated a lower circulating blood concentration of drug in this patient that patient A. A delay of approximately 1 h was also evident in the lag between peak Rif and corresponding dRif levels. Circulating levels of dRif represent a fraction (approximately 20–60% of the levels of Rif found).

4. Conclusions

In conclusion, a novel, validated protocol is described for the extraction, separation and quantitation of efavirenz, rifampicin and also rifampicin's main metabolite desacetyl rifampicin. The assay is accurate, precise and fast for the determination of these very different drugs in plasma. The easy sample preparation and fast separation makes this assay highly suitable for pharmacokinetic studies and therapeutic drug monitoring in patients with HIV only, TB only or both diseases being treated with Efv and Rif. This new protocol will be used in a SPhEAR project which will examine the effects of efavirenz medication on the pharmacokinetics of oral rifampicin in the treatment of tuberculosis in HIV infected patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.07.041.

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