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Determination of protein-unbound, active rifampicin in serum by ultrafiltration and Ultra Performance Liquid Chromatography with UV detection. A method suitable for standard and high doses of rifampicin

Eleonora W.J. van Ewijk-Beneken Kolmer, Marga J.A. Teulen, Erik C.A. van den Hombergh, Nielka E. van Erp, Lindsey H.M. te Brake, Rob E. Aarnoutse*

Department of Pharmacy & Radboud Institute for Health Sciences, Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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ABSTRACT

Rifampicin is the most important antibiotic in use for the treatment of tuberculosis (TB). Preclinical and clinical data suggest that higher doses of rifampicin, resulting in disproportionately higher systemic exposures to the drug, are more effective. Serum concentrations of rifampicin are the intermediary link between the dose administered and eventual response and only protein-unbound (free) rifampicin is pharmacologically active. The objective of this work was to develop an ultra performance liquid chromatography assay for protein-unbound rifampicin in serum with ultrafiltration, carried out at a sample temperature of 37 °C, suitable for measurement of concentrations achieved after currently used and higher doses of rifampicin.

Human serum was equilibrated at 37 °C and ultrafiltered at the same temperature in a Centrifree YM-30 ultrafiltration device, followed by dilution of the ultrafiltrate with methanol and ascorbic acid. Unbound rifampicin was analyzed using ultra performance liquid chromatography with a BEH C18 column, isocratic elution and ultra-violet (UV) detection. The run time was 5 min.

The assay was linear over the concentration range of 0.065–26 mg/L rifampicin in ultrafiltrate. Accuracies for measurement of rifampicin in ultrafiltrate were 97% and 102% at the higher and lower limits of quantitation. Accuracy of the ultrafiltration process cannot be established, as it is not possible to spike blank serum with known amounts of protein-unbound rifampicin. Within- and between-day precision of the method *including* ultrafiltration as well as *after* ultrafiltration were within prespecified limits (CV < 10%). Dilution of the ultrafiltrate with methanol and ascorbic acid kept rifampicin in solution and prevented it from degradation. Rifampicin loss during the ultrafiltration process and variation in analytical results when using two different batches of ultrafiltration devices were both limited. Processed ultrafiltrate samples were stable for 3 days in the autosampler.

The developed method can be applied in pharmacokinetic research, studying exposure-response relationships for rifampicin when administered at higher than currently used doses.

1. Introduction

Rifampicin is one of the pivotal drugs for the treatment of tuberculosis (TB), which is the leading infectious disease in terms of mortality worldwide [1]. A rifampicin dose of 10 mg/kg daily combined with pyrazinamide enabled shortening of the duration of TB treatment to six months decades ago [2]. This 10 mg/kg dose (often 450 or 600 mg, dependent on weight) was chosen in the 1960s and 1970s based on considerations related to pharmacokinetics, cost at the time, and presumed adverse effects [3]. Accumulating data in mice and humans suggest, however, that this 10 mg/kg daily dose is at the lower

end of the dose-response curve [4–9]. Our group has performed a dose escalating study in pulmonary TB patients showing that doses up to 35 mg/kg resulted in a more than proportional (ninefold) increase in exposure to rifampicin in plasma, which was safe and well tolerated in small groups [7]. This 35 mg/kg dose of rifampicin combined with isoniazid, pyrazinamide and ethambutol was able to reduce time to sputum culture conversion and may shorten pulmonary TB treatment [8]. A higher dose of rifampicin also reduced mortality in a small study among patients with TB meningitis, the most severe form of TB [9]. Clearly higher doses of rifampicin require more extensive follow-up research, including pharmacokinetic (PK) and pharmacokinetic-

* Corresponding author at: Radboud University Medical Center Department of Pharmacy, 864, P.O. BOX 9101, 6500 HB Nijmegen, The Netherlands.
E-mail address: Rob.Aarnoutse@radboudumc.nl (R.E. Aarnoutse).

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pharmacodynamic (PK-PD) evaluation.

In pharmacokinetic studies with rifampicin, measurement of the drug in plasma or serum is usually related to the total (protein-unbound plus bound) concentration of rifampicin. An equilibrium between total and protein-unbound concentrations is commonly assumed, yet protein-unbound (free) rather than total drug concentrations are preferably used in concentration-response evaluations [10], as only protein-unbound drugs are pharmacologically active and diffuse (or are being actively transported) into tissues and to the sites of action [11,12]. In a recent pharmacokinetic study with standard dose rifampicin we assessed a two-fold interindividual variation in the unbound rifampicin fraction [13]. This interindividual variability in the free fraction indeed shows that measurement of solely total concentrations could be misrepresentative of the relevant exposure. It is currently unknown what the free fraction of rifampicin is after administration of higher doses that result in much higher rifampicin exposures, possibly associated with saturation of plasma or serum proteins that bind rifampicin. Clearly the redevelopment of rifampicin as a TB drug warrants the development of an analytical method for protein-unbound rifampicin concentrations achieved after administration of high doses of the drug.

Previously we measured free rifampicin concentrations after standard doses using ultrafiltration at room temperature. In ultrafiltration, centrifugal forces are employed as the driving force for the passage of serum water across a filter membrane [14]. The aim of the current project was to develop and validate a new ultrafiltration method for the measurement of the free, active concentration of rifampicin at 37 °C, enabling for PK and PK-PD studies with higher doses of rifampicin.

2. Materials and methods

2.1. Chemicals and materials

Rifampicin (cas.nr. 13292-46-1, purity 98%, see Fig. 1 for chemical structure), acetic acid (100%), ammonium acetate (> 98%), acetonitrile and methanol of LC-MS quality were purchased from Merck (Darmstadt, Germany). Ascorbic acid was purchased from Bufa (IJssestein, The Netherlands). HPLC quality water was obtained with a Veolia Purelab flex 4 system from Veolia (Ede, The Netherlands). Centrifree YM-30 ultrafiltration filter devices were from Millipore B.V. (Amsterdam, The Netherlands). Drug free human serum was obtained from Sanquin blood bank (Nijmegen, The Netherlands) and was stored at –40 °C.

2.2. Preparation of stocks

Two rifampicin stock solutions containing 1000 mg/L of rifampicin with 200 mg/L ascorbic acid were prepared in methanol: HPLC quality

water (4:1%v/v). Ascorbic acid is added to rifampicin to protect it from degradation [15]. Stocks were stored at –40 °C.

2.3. Preparation of calibration stocks for the calibration curve

For the preparation of the calibration curve, one of the rifampicin stock solutions was diluted with methanol to achieve six calibration stocks of 1.3–2.6–7.8–26–78–260 mg/L (calibration stocks 1–6) and were stored at –40 °C until analysis. All stocks were shown to be stable for at least 12 months.

2.4. Calibration curve for unbound rifampicin in serum ultrafiltrate

Calibration curve standards could not exist of serum samples spiked with known amounts of rifampicin that are subsequently being ultrafiltrated, as the analytical method aims to measure unbound concentrations of rifampicin and these concentrations cannot be set (or ‘spiked’) in serum. Therefore the calibration curve consisted of blank serum ultrafiltrate spiked with rifampicin. Blank ultrafiltrate was obtained by ultrafiltration of drug free human serum.

The calibration curve was made freshly on the day of analysis. First, solutions of rifampicin in blank ultrafiltrate were prepared. For standard 0, 200 µL blank ultrafiltrate was used. For standards 1–6, 10 µL of one of the calibration stocks 1–6 was added to 190 µL blank ultrafiltrate. For standard 7, 20 µL calibration stock 6 was mixed with 180 µL blank ultrafiltrate. The resulting calibration curve concentrations of rifampicin were 0.065–0.13–0.39–1.3–3.9–13–26 mg/L. Secondly, 200 µL volumes of each of these solutions of rifampicin in ultrafiltrate were transferred into maximum recovery[®] vials (Waters) with 400 µL of methanol: ascorbic acid 20 mg/mL in water (40:1%v/v). The vials were closed with a polypropylene screw cap with silicon/PTFE septum to prevent evaporation. The use of methanol in the samples was pivotal to keep rifampicin dissolved and prevent it from sticking to inserts and vials, whereas ascorbic was essential to prevent disintegration of the drug.

2.5. Internal quality control samples: stocks and sample preparation

Although serum could not be spiked with predetermined protein-unbound concentrations of rifampicin, it was deemed desirable to have internal quality control over the sample preparation procedure. To this end, internal quality control (QC) samples were prepared from the second stock solution (see above) resulting in total (protein-bound plus unbound) concentrations of 1.5, 10 and 61 mg/L in serum, designated as QC Low, Medium and High respectively. The QC samples were stored at –80 °C and found to be stable for at least 26 months.

Sample processing for these QC samples was identical to sample

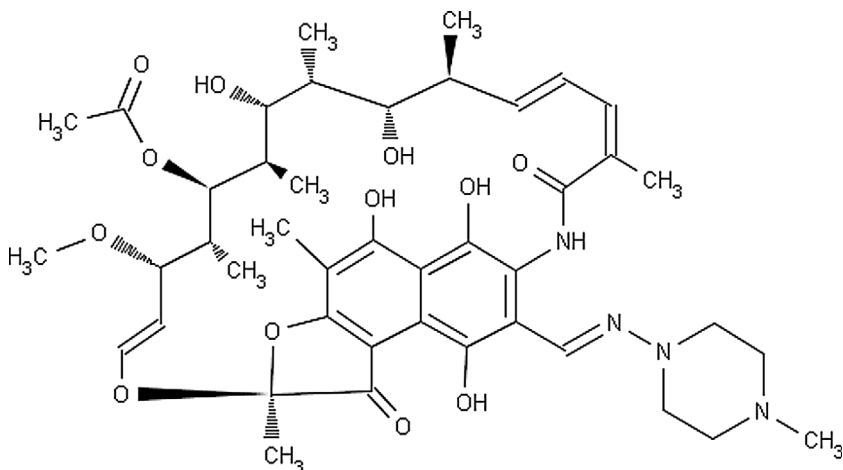


Fig. 1. Chemical structure of rifampicin.

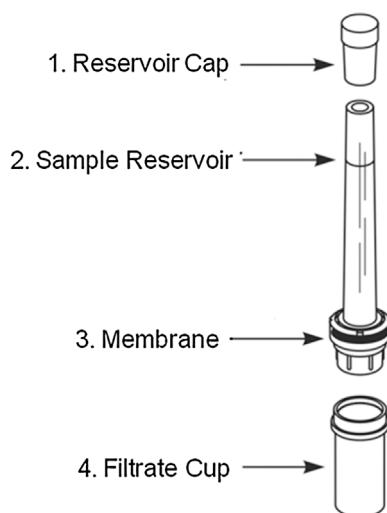


Fig. 2. Centrifree Ultrafiltration Device.

processing of patient samples. Since there was no known nominal concentration of unbound rifampicin in these samples, the mean protein-unbound concentrations of rifampicin of these QC samples as measured during validation ($n = 15$) were taken as internal QC concentration values. During analysis of samples for pharmacokinetic research, QC Low, Medium and High samples are to be analyzed within each run and a maximum deviation of 15% from the concentrations measured during the validation will be accepted.

2.6. Sample preparation

Whole blood with rifampicin was centrifuged within 4 h after blood withdrawal and the resulting serum was prepared further or stored at $-80\text{ }^{\circ}\text{C}$. A volume of 0.5 mL of serum (sample, QC or drug free human serum) was pipetted into the sample reservoir of a Centrifree YM-30 tube (Fig. 2, part 2). For 60 min the tube was equilibrated at $41\text{ }^{\circ}\text{C}$ at $1 \times g$ within a Rotanta 460 R centrifuge with fixed angle rotor 5645 (radius 132 mm, angle 25°) to reach a sample temperature of $37\text{ }^{\circ}\text{C}$. The centrifugal force was only $1 \times g$ during this equilibration period, as the centrifuge had to be put 'on' to develop any temperature, yet the ultrafiltration process should not start in this temperature equilibration period. Then for 20 min the tube was centrifuged at $41\text{ }^{\circ}\text{C}$ at $1650 \times g$ whereas the sample retained its temperature of $37\text{ }^{\circ}\text{C}$. The setting of the Rotanta 460 R centrifuge at $41\text{ }^{\circ}\text{C}$ resulted in a sample temperature of $37\text{ }^{\circ}\text{C}$, based on validation of sample temperatures with a calibrated thermometer. Finally, 200 μL of the clear ultrafiltrate in the ultrafiltrate cup (Fig. 2, part 4) was transferred into a maximum recovery[®] vial (Waters) with 400 μL of methanol: ascorbic acid 20 mg/mL water (40:1%v/v). The vials were closed with a polypropylene screw cap with silicon/PTFE septum to prevent evaporation. Each of the standards, samples and QC's were mixed before injection into the UPLC system.

2.7. UPLC-UV analysis

The Acquity UPLC system consisted of a model BSM solvent delivery pump, a model SM autosampler, a model CM column manager and a model TUV UV detector. Separation was carried out on a BEH C18 column ($100 \times 2.1\text{ mm}$ ID; particle size, $1.7\text{ }\mu\text{m}$) with the column temperature set at $45\text{ }^{\circ}\text{C}$. The mobile phase consisted of 35% acetonitrile and 65% 0.05 M acetate buffer pH 4.0. Flow rate was 0.5 mL/min and the total run time was 5 min. UV detection was set at 334 nm. Analytical runs were controlled and processed by Empower software (all by Waters, Etten-Leur, The Netherlands).

2.8. Validation procedures

The validation of the assay in ultrafiltrate was based on the most recent versions of the guidelines on bioanalytical method validation of the European Medicines Agency [16] and the FDA [17]. In addition, we studied validation parameters specifically related to ultrafiltration.

2.8.1. Selectivity

Various drugs potentially co-administered with rifampicin were tested for interference at their therapeutic serum concentrations. Furthermore, serum of six patients without TB medication was evaluated for interference by endogenous substances. The presence of interfering components was accepted if the response was less than 20% of the lower limit of quantitation (LLOQ) for rifampicin.

2.8.2. Carry-over

Carry-over was assessed by injecting blank samples after the calibrator at the higher limit of quantitation (HLOQ). Carry-over in the blank sample following the highest concentration calibrator had to be less than 20% of the LLOQ.

2.8.3. Accuracy, precision and linearity

Five replicates of samples of rifampicin in ultrafiltrate at the LLOQ and HLOQ were analyzed during three different days, in order to determine accuracy and within-day and between-day precision of the method *without* the ultrafiltration step. To analyse these samples, six calibration concentration levels were used, in addition to the blank sample which was not incorporated in the calibration line. In each validation run the six calibrators were analysed in duplicate. For the LLOQ, the percent deviation from the nominal concentration (accuracy) and the relative standard deviation (precision) had to be less than 20% and for the HLOQ both these measures had to be less than 15%.

For each replicate measurement, the concentrations measured in the LLOQ and HLOQ samples were divided by the nominal concentrations. To assess accuracy, the mean ratio of measured concentrations versus nominal concentrations ($n = 15$ at each concentration level, i.e. at the LLOQ or HLOQ) was calculated and multiplied by 100.

One-way Analysis of Variance (ANOVA) was used to assess the within-day and between-day precision at each of the two (LLOQ or HLOQ) concentrations, using the run day as the classification variable. The error mean square or mean square within groups (ErrMS), the day mean square or mean square among groups (DayMS), and the grand mean (GM) of all 15 measurements across the three run days were obtained from the ANOVA. The estimate of the within-day and between-day precision at every concentration was calculated as follows:

$$\text{Within-day precision} = ((\text{ErrMS})^{0.5}/\text{GM}) \times 100\%$$

$$\text{Between-day precision} = (((\text{DayMS}-\text{ErrMS})/n)^{0.5}/\text{GM}) \times 100\%$$

in which n is the number of replicate measurements within each day

We also wanted to validate the within-day and between-day precision of the analytical method *with* the ultrafiltration step. To this end, the three internal QC samples in serum were evaluated for within-day and between-day precision, by performing the analytical method including the ultrafiltration step on five replicates of each QC sample and during three different days. Relative standard deviations for within-day and between-day imprecision had to be less than 15%. Accuracy for resulting unbound concentrations could not be assessed in these internal QC samples, as the nominal value for the unbound concentrations cannot be preset or spiked.

Linear regression analysis was used to assess the linearity of the calibration curves in each validation run. Duplicate responses were used for each concentration, then transformed with a log-log fit and submitted to linear regression analysis. The blank was not taken into consideration.

A significant F-value would indicate linearity between analytical

response and concentration and the correlation coefficient r (or coefficient of determination, r^2) was used as a measure for the strength of the association.

2.8.4. Dilution integrity

After ultrafiltration, QC High was diluted twice and four times with blank ultrafiltrate, and each dilution was performed and measured five times. Since there was no known nominal concentration of unbound rifampicin, the mean concentration found during validation ($n = 15$) was taken as nominal (target) value. The percent deviation between the mean concentrations after dilution as compared to the nominal value before dilution and the relative standard deviation in measurement of each diluted sample had to be less than 15%.

2.8.5. Stability

The stability of processed samples in the autosampler was tested. All processed samples (standards, blank, LLOQ, QCH, QCM, QCL and HLOQ) obtained at the first day of assessment of accuracy and precision (see above, five replicates of rifampicin in ultrafiltrate at the LLOQ and HLOQ levels and five replicates of the three internal QC samples in serum) were re-analyzed after three days. For each sample, the percentage of concentrations obtained after three days in the autosampler compared to the initially measured concentration was calculated.

2.8.6. Additional validation parameters – recovery of the ultrafiltration process

In addition to validation parameters required by these guidelines, parameters associated with the use of the ultrafiltration device Centrifree YM-30 were validated.

The recovery of the ultrafiltration process of rifampicin was assessed to find out if all rifampicin could be accounted for and no rifampicin was lost during ultrafiltration, also considering the limited solubility of rifampicin. Recovery was assessed for QC Low, QC Medium and QC High by

- measuring the amount of protein-bound rifampicin (in μg) as present in the sample reservoir of the Centrifree YM-30 filter device (part 2 in Fig. 2) after ultrafiltration;
- measuring the amount of protein-unbound rifampicin (in μg) as present in the filtrate cup of the Centrifree YM-30 filter (part 4 in Fig. 2) after ultrafiltration;
- adding up protein-bound and unbound amounts of rifampicin after ultrafiltration;
- and comparing the total amount of rifampicin after ultrafiltration to the total amount before ultrafiltration.

Each of the amounts in μg was calculated by multiplication of measured concentrations and volumes (amount = concentration * volume).

In a formula:

Recovery=100%

$$* \frac{(\text{vol}_{\text{bound}} \times \text{RIF conc}_{\text{bound}}) + (\text{vol}_{\text{unbound}} \times \text{RIF Conc}_{\text{unbound}})}{\text{vol}_{\text{ultrafiltrated serum}} (=0.25\text{ml}) \times \text{RIF Conc}_{\text{total}}}$$

Each of the items in this formula was assessed as follows:

- vol. bound: the sample reservoir was weighed after ultrafiltration and empty. The difference in these weights equals to the weight of the bound fraction. The volume of the bound fraction was next calculated by the weight of the bound fraction divided by its density (1,05 g/ml, see below).
- vol. unbound: the filtrate cup was weighed after and before ultrafiltration. The difference in these weights equals to the weight of the unbound fraction. The volume of the unbound fraction was next calculated by the weight of the unbound fraction divided by its

density (1,01 g/ml, see below).

- RIF conc. unbound: The unbound rifampicin concentrations in the filtrate cup were measured with the method described in this article.
- RIF conc. total: The total rifampicin concentrations before ultrafiltration were analyzed with a validated method with the same UPLC-UV analysis but with another pre-treatment of the samples. To 100 μL plasma, 200 μL methanol: acetonitrile, 1: 1 and 10 μL ascorbic acid 20 mg/mL was added. The sample was thoroughly mixed and centrifuged for 5 min. Then 100 μL 0.05 M acetate buffer pH 4.0 was added before mixing and centrifuging again. Ten μL of the clear supernatant was injected. This assay was linear over the concentration range of 0.125–30 mg/L plasma. Accuracies in the LLOQ, QC Low (2.94 mg/L), QC Medium (11.0 mg/L), QC High (22.1 mg/L) and HLOQ were 101%, 100%, 101% 101% resp. 99%%. Within- and between-day coefficients of variation were < 3.0 resp. < 0.9% in plasma. Samples > HLOQ were pre-diluted.
- RIF conc. bound: The rifampicin concentrations in the bound fraction on the sample reservoir were analyzed with the same validated method for total rifampicin. The bound fraction on the sample reservoir was loosened from the sample reservoir by centrifugation of the reservoir upside down, followed by application of the method for total concentrations on the sample.

The density of the bound fraction was assessed by weighing five replicates of a set volume of bound fraction from the sample reservoir. The density of the unbound fraction was assessed by weighing five replicates of ultrafiltrate out of the filtrate cup.

2.8.7. Additional validation parameters – batch-to-batch difference in ultrafiltration devices

The batch-to-batch difference of the Centrifree YM-30 ultrafiltration device was validated. Unbound rifampicin in QC High and Low were analysed in fivefold with two different batches. The difference in mean concentrations measured with the two batches had to be less than 15%.

3. Results

3.1. Selectivity and carry-over

Most drugs tested did not interfere with the retention of rifampicin (Table 1), except for amphotericin B. Serum of six patients without TB medication was evaluated and found to be free from potential endogenous or other interferences.

Typical chromatograms of unbound rifampicin serum samples are shown in Fig. 3.

Carry-over in the blank sample following the high concentration calibrator proved not to be greater than 20% of the LLOQ.

3.2. Accuracy, imprecision and linearity

The results of the determination of accuracy and imprecision of the assay without (i.e. after) the ultrafiltration step are presented in Table 2a. These results show that the method is accurate at the LLOQ and HLOQ (accuracy of 102 and 97%) and precise. The maximum within-day and between-day coefficients of variation occurred at the LLOQ and were 8.8% and 5.6%, respectively.

Table 2b shows the results of analysis of five replicates of serum QC Low, Medium and High samples, using the whole method with the ultrafiltration step on three different days. Mean protein-unbound concentrations of 0.144 mg/L, 1.11 mg/L and 13.9 mg/L were measured in QC Low, Medium and High and these measures will be used as internal QC target values during routine analysis. For a new batch of QC's these target values have to be established again considering that every serum may give different target values. Of note, the free fraction of protein-unbound versus total rifampicin concentrations decreased from QC Low to QC High (Table 2b). Within-day and between-day precision of the

Table 1
Possible co-administered drugs tested.

acetaminophen	domperidone	lamivudine	rilpivirine
acyclovir	efavirenz	lidocaine	ritonavir
amphotericin B ^a	emtricitabine	lopinavir	saquinavir
amikacin	erythromycin	methadone	SQ109
amoxicillin	ethambutol	moxifloxacin	stavudine
atazanavir	etravirine	naproxen	sulfamethoxazole
carbamazepine	famotidine	nelfinavir	N4-acetylsulfamethoxazole
clarithromycin	phenobarbital	nevirapine	sulfametrole
clindamycin	phenytoin	ofloxacin	N4-acetylsulfametrole
clofazimine	fluconazole	oxazepam	tenofovir
caffeine	folic acid	oxcarbazepine	trimetoprim
dapsone	ganciclovir	pentamidine	valproic acid
mono-acetyl dapson	indinavir	pyrazinamide	zalcitabine
darunavir	isoniazid	pyrimethamine	zidovudine
didanosine	itraconazole	raltegravir	
dolutegravir	ketoconazole	rifabutin	

^a Co-elutes with rifampicin.

whole method with the ultrafiltration step complied with the preset requirements (CV < 15).

The calibration curves were linear over the concentration range of 0.65–26 mg/L unbound rifampicin by using log peak height vs log concentration ($F = 22133$, $P = 5.71E-21$). The regression coefficients (r^2) of all three calibration curves during validation of unbound rifampicin in serum were 0.9996 ± 0.0001 .

3.3. Dilution integrity

The percent deviation between the mean concentration of ultrafiltrated QC High after dilution compared to the nominal QC High concentration before dilution was 3%, both after twice and four times dilution of the QC High ultrafiltrate. The relative standard deviations in measurement of each diluted sample were 2.3% and 1.3% after two-fold and four-fold dilutions, respectively.

3.4. Stability

Processed samples of unbound rifampicin proved to be stable with mean concentrations of 101% of the initial concentrations after 3 days.

3.5. Additional validation parameters

Results presented in Table 3 show that no rifampicin is lost during sample pre-treatment. For QC Low, QC Medium and QC High the recoveries were 96%, 95% and 95% with a precision coefficient of variation of 1.4%, 2.5% and 6.8%.

The difference in mean concentrations of protein-unbound rifampicin for QC High and QC Low measured with two batches of the Centrifree YM-30 ultrafiltration devices was 3.4% and 1.6%.

4. Discussion

We described and validated a bio-analytical method using UPLC-UV for the measurement of protein-unbound rifampicin in human serum after ultrafiltration at a sample temperature of 37 °C. Previously, we measured total and protein-unbound rifampicin plasma concentrations in malnourished and well-nourished TB patients and briefly described our bio-analytical method [13]. However, that method was not optimized to measure at a sample temperature of 37 °C while retaining compound solubility and stability. Furthermore, the current method also covers much higher protein-unbound concentrations, which are relevant after administration of rifampicin at higher doses [6–9]. Finally, the previous validation did not evaluate the presence of batch-to-batch differences in ultrafiltration devices that have been reported for a bio-analytical method measuring free concentrations of rifampentine

[18].

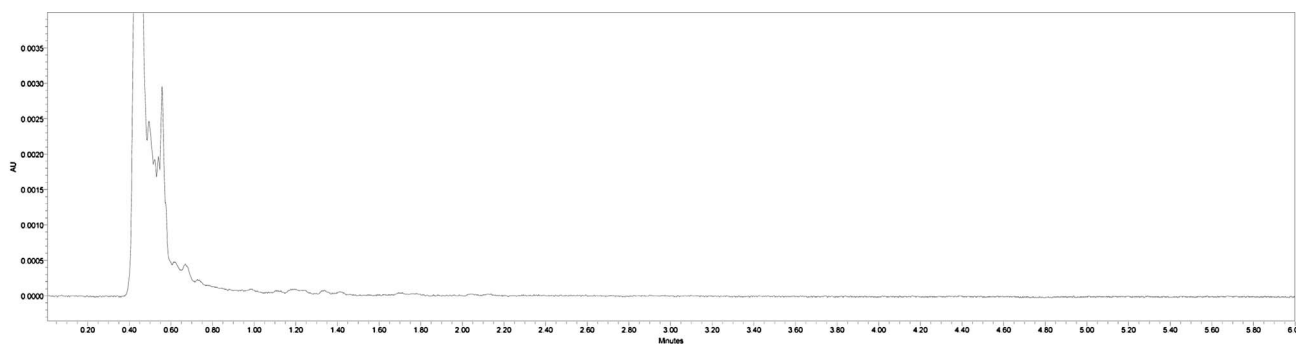
Our new analytical method measured free rifampicin concentrations using ultrafiltration. In ultrafiltration, centrifugal forces are employed as the driving force for the passage of plasma or serum water across a filter membrane [14]. Besides ultrafiltration other methodologies are available to determine plasma protein binding of drugs, such as ultracentrifugation and equilibrium dialysis. Equilibrium dialysis avoids the nonspecific binding and relatively large plasma volumes often associated with ultracentrifugation and ultrafiltration [19]. However, equilibrium dialysis can be time consuming and labour-intensive in terms of preparation time and time to equilibration, making it less attractive for pharmacokinetic assessments.

Clearly, measurement of protein-unbound concentrations with ultrafiltration involves more than straightforward ultrafiltration and subsequent measurement of drugs. Ultrafiltration brings along specific analytical challenges and certainly warrants a well-considered validation.

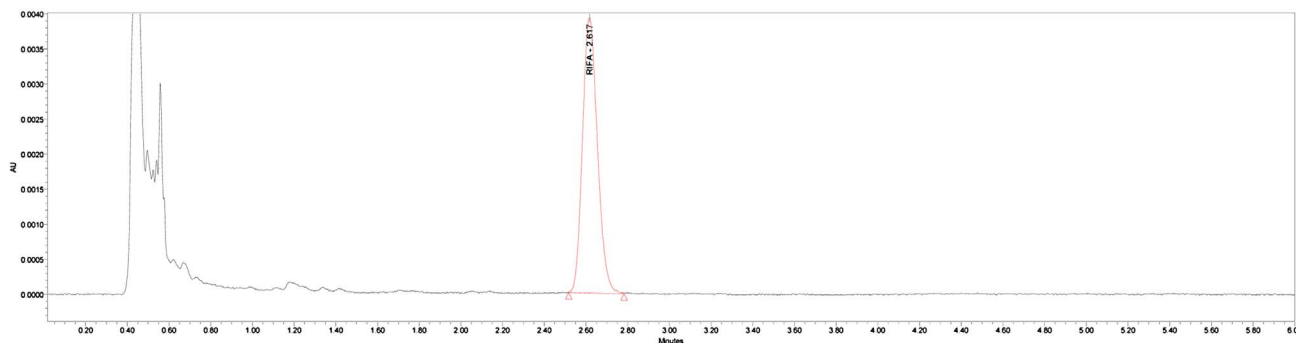
First, it is not possible to assess the accuracy of bio-analytical techniques that evaluate protein-unbound plasma or serum concentrations of drugs. This is because it is impossible to spike serum with a certain protein-unbound concentration of a drug and there is no way to retrieve information on the true equilibrium between protein-bound and protein-unbound drug in these samples. We still wanted to have internal control over the ultrafiltration step during routine analysis and assessed protein-unbound rifampicin concentrations in three internal QC serum samples, to be used as target values in routine analysis. Replicate analysis of these internal QC samples also allowed us to assess within-day and between-day precision of the analytical method with the ultrafiltration step included. Of course we also assessed accuracy and precision for the analytical steps after ultrafiltration.

Secondly, various experimental conditions and additional validation parameters have to be considered when developing ultrafiltration methods. We paid a lot of attention to performing ultrafiltration at body temperature, as it is described that temperature could influence the equilibrium of bound versus unbound drug in serum or plasma [20]. It is hypothesized that the unbound drug fraction at physiological temperature (37 °C) could be larger compared to room temperature, due to a decrease in the binding force at higher temperatures [21]. In our recent publication on the effect of nutritional status on protein binding of rifampicin administered in standard doses, we reported a small increase in free fraction (+1.1%) for plasma samples measured at 37 °C compared to results of the same method performed at room temperature. This increase in rifampicin free fraction in relation to a change in experimental temperature is in agreement with sparse data for other drugs as published in the literature [22].

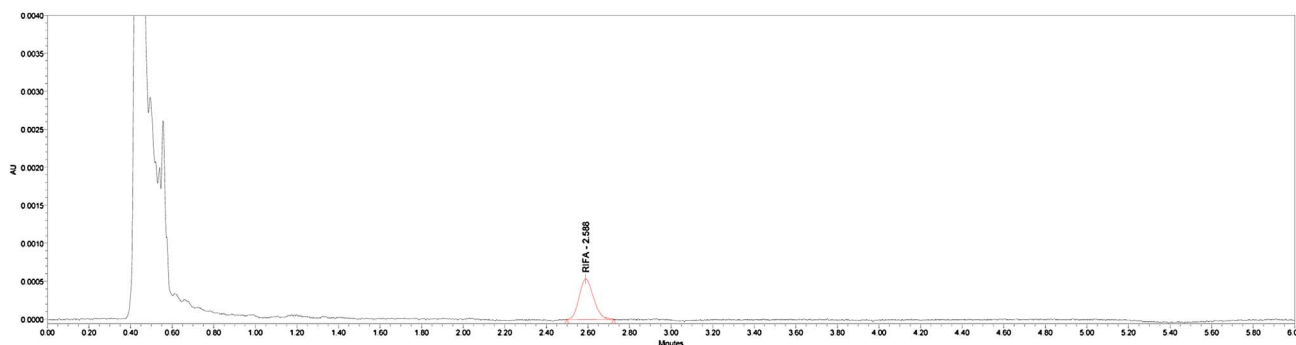
Of note, our work revealed that the temperature setting of a centrifuge does not represent the temperature within the centrifuged



panel A



panel B



panel C

Fig. 3. panel A: Chromatogram of a blank ultrafiltrate sample.

panel B: Chromatogram of a spiked calibration standard containing 1.3 mg/L rifampicin in ultrafiltrate.

panel C: Chromatogram of QC Low (1.5 mg/L of rifampicin) after ultrafiltration.

sample. In order to validate our sample temperature before and during centrifugation, we performed experiments in which a volume of 0.5 mL water was pipetted in six Centrifree YM-30 devices, which were set in different positions in the rotor to reach a sample temperature of 37 °C and retain this temperature during ultrafiltration. The experiments revealed that the time for equilibration within our specific centrifuge was 60 min at $1 \times g$ at a set temperature of 41 °C. Next, the samples were centrifuged for 20 min at $1650 \times g$, according to the manual of the Centrifree YM-30 device, at a set temperature of 41 °C. Under these conditions, the temperature of all samples remained 37 °C and the same settings were used for the validation of the method.

Sample pH is another parameter that may affect the free fraction [22]. The pH of blood in the body is kept constant at pH 7.4 with the help of carbonate as the main regulatory buffer system. Ex vivo, pH may increase due to loss of carbon dioxide during storage or sample processing and ultrafiltration can increase the pH of the sample up to pH 8 in our hands. We have not evaluated whether such a pH difference is

relevant to the free fraction of rifampicin and this is a limitation of our analytical method.

As additional validation parameters, we evaluated the recovery of the ultrafiltration process and demonstrated that no rifampicin was lost during ultrafiltration. We also assessed the batch-to-batch variation in analytical results when using different batches of ultrafiltration devices.

As a third item related to ultrafiltration, we would like to highlight that we encountered specific challenges during method development related to solubility and stability of rifampicin after ultrafiltration. More specifically, we observed decreased solubility of rifampicin in ultrafiltrate compared to plasma. Probably because of the absence of proteins after ultrafiltration, rifampicin preferred to stick to every insert or vial available to us rather than remain in solution in ultrafiltrate, especially in low volumes. This did not result in linear calibration curves. By adding 200 μL of ultrafiltrate to a maximum recovery vial with 400 μL of methanol, the calibration curve became linear. The second challenge we encountered was the instability of rifampicin in

Table 2

Accuracy and precision of rifampicin determination in serum and ultrafiltrate.^a (A) Accuracy and precision of rifampicin measurement in ultrafiltrate (analytical method *without*, i.e. after ultrafiltration). (B) Precision of rifampicin measurement in serum (whole analytical method *with* ultrafiltration)

(A)						
Sample	Concentration UF ^b spiked (mg/l)	Accuracy (n = 15) (%)	Within-day imprecision (CV%)	Between-day imprecision (CV%)		
LLOQ	0.0639	102	8.8	5.6		
HLOQ	25.6	97	5.3	2.1		
(B)						
Sample	Measured Serum concentration spiked (mg/l)	Measured unbound concentration ^c (mg/l)	Free fraction (%) ^d	Accuracy (n = 15) (%) ^e	Within-day imprecision (CV%)	Between-day imprecision (CV%)
QC Low	1.70	0.144	8.5	–	3.8	0.0 ^f
QC Medium	10.8	1.11	10.3	–	3.2	1.4
QC High	64.1	13.9	21.7	–	3.1	2.8

^a At each concentration level, five replicates of samples were analyzed during three different days (n = 15 measurements in total). Analysis of variance was used to assess the within-day and between-day precision at each concentration level (see text).

^b UF: ultrafiltrate.

^c The measured protein-unbound concentrations of rifampicin in QC samples are to be used as internal QC target concentration values during routine analysis. See text.

^d The free fraction increases (and the corresponding protein binding decreases) with increasing total (bound plus unbound) serum concentrations, probably due to saturation of protein binding sites. See text.

^e It is not possible to assess the accuracy of bio-analytical techniques that evaluate plasma or serum protein binding. This is because it is impossible to spike serum with a certain protein-unbound concentration of a drug. See text.

^f In case the between-day imprecision is 0.0%, no additional variation upon the within-day imprecision is observed as a result of performing the assay on different days.

the processed sample during the chromatographic quantification. Since ascorbic acid was added as a stabilizer in our method for total (bound plus unbound) rifampicin, we also supplemented the processed unbound rifampicin sample with ascorbic acid, enabling that no cooling was needed to prevent degradation of rifampicin during analysis.

The developed method is suitable for measurement of protein-unbound, active concentrations of rifampicin at various doses. The LLOQ for rifampicin in ultrafiltrate was set at a concentration of 0.065 mg/L, which corresponds to total (unbound plus bound) concentrations of 0.325–0.65 mg/L, based on 80–90% protein binding [13,23]. The HLOQ for unbound rifampicin was 26 mg/L, which would amount to a total concentration ranging from 130 to 260 mg/L. Evidence from the validation of our method suggests, however, that saturation of proteins may occur at higher concentrations, as reflected in an *increase* in free fraction from 9% to 22% (and a corresponding *decrease* in protein

binding from 91% to 78%) when total concentrations rise from 1.7 to 64 mg/L (Table 2b). In any case, the upper limit of the range of our method will certainly exceed protein-unbound peak concentrations in African TB patients that received up to 35 mg/kg of rifampicin for two weeks [7], ensuring that the current method can be applied to assess free rifampicin concentrations of individuals taking higher than standard dosing of rifampicin.

5. Conclusions

An assay was developed and validated for the determination of protein-unbound (active, free) rifampicin in human serum by ultrafiltration at a sample temperature of 37 °C. Rifampicin could be kept in solution in ultrafiltrate by addition of methanol and was stable after addition of ascorbic acid. Subsequent analysis took place with

Table 3

Recovery of the ultrafiltration of rifampicin (RIF).

QC	weight bound (g)	RIF Conc. bound (µg/mL)	weight unbound (g)	RIF Conc. unbound (µg/mL)	RIF Conc. total (µg/mL)	Recovery ^a	Mean	CV
Low	0.209	3.94	0.285	0.143	1.70	97.20%	95.8	1.4
	0.224	3.67	0.268	0.143				
	0.224	3.65	0.269	0.147				
	0.217	3.72	0.253	0.15				
	0.219	3.63	0.273	0.144				
Medium	0.230	22.1	0.259	1.06	10.8	94.60%	94.5	2.5
	0.233	21.4	0.257	1.10				
	0.212	23.4	0.276	1.09				
	0.206	24.1	0.284	1.15				
	0.21	25.0	0.289	1.11				
High	0.217	137	0.27	12.9	64.1	99.30%	95.4	6.8
	0.215	130	0.265	14.0				
	0.236	131	0.266	13.8				
	0.196	127	0.27	13.8				
	0.212	130	0.267	13.5				

^a See text for explanation.

$$\text{Recovery} = 100\% \times \frac{(\text{vol}_{\text{bound}} \times \text{RIF conc}_{\text{bound}}) + (\text{vol}_{\text{unbound}} \times \text{RIF conc}_{\text{unbound}})}{\text{vol}_{\text{ultrafiltrated serum}} (= 0.5 \text{ ml}) \times \text{RIF conc}_{\text{total}}}$$

The density of the bound and unbound fractions were 1.05 g/ml and 1.01 g/ml, respectively.

$$= 100\% \times \frac{(\text{weight}_{\text{bound}} / \text{density}_{\text{bound}} \times \text{RIF conc}_{\text{bound}}) + (\text{weight}_{\text{unbound}} / \text{density}_{\text{unbound}} \times \text{RIF conc}_{\text{unbound}})}{0.5 \times \text{RIF conc}_{\text{total}}}$$

ultraperformance liquid chromatography with UV detection. The assay can be used to measure unbound rifampicin concentrations when this drug is administered in high doses and such analyses are relevant in the redevelopment of this pivotal TB drug.

Conflicts of interest

All authors declare that they have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately have influenced, or be perceived to have influenced, this work.

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