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Journal of Chromatography B, 857 (2007) 76–82
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A rapid and sensitive HPLC–MS method for the detection of plasma and cellular rifampicin

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Received 23 January 2007; accepted 1 July 2007
Available online 10 July 2007

Abstract

Rifampicin is active against both intracellular and extracellular *Mycobacterium tuberculosis*. The ability to measure rifampicin drug concentrations in both plasma and in cells may be useful in evaluating the suitability of dosage regimens for populations and individuals. Here a novel simple, precise and accurate method for the quantification of rifampicin in both cells and plasma is reported. Sample proteins were precipitated with acetonitrile containing the internal standard and then diluted with water. Aliquots of supernatant were then injected into the HPLC–MS system for chromatographic separation and detection. Rifampicin calibration curves encompassed concentrations from 100 to 12,800 ng/mL. Intra- and inter-assay precision and accuracy were determined using low, medium and high concentration quality control samples and was found to be within 10% in all cases. Rifampicin concentrations were found to be unaffected by freeze–thaw cycles, but were significantly affected by heat-inactivation (58 °C, 40 min). This assay was successfully utilised to determine the pharmacokinetic profile of rifampicin in plasma and peripheral blood mononuclear cells (PBMC) in 8 tuberculosis patients receiving rifampicin over an 8 h period.

Keywords: Rifampicin; HPLC–MS; Plasma; Cellular

1. Introduction

Rifampicin is an important anti-tuberculosis drug with both early bactericidal activity (EBA) and, more importantly, sterilising activity against *Mycobacterium tuberculosis* (MTB). Rifampicin exerts its anti-tuberculosis activity by targeting the bacterial DNA dependant RNA polymerase (encoded by *rpoB*) of both intracellular and extracellular MTB. The ability to kill MTB is related to the concentration of drug to which the bacterium is exposed [1]. As MTB is able to persist in plasma and within alveolar macrophages, it is important that both intracellular and plasma rifampicin pharmacokinetic information is available.

In order to determine drug concentrations within both cells and plasma, it is necessary to have an accurate, precise and specific analytical method, requiring only a small sample volume (cellular matrix tends to be volume limited). Ideally, the assay should have quick sample processing and be able to measure low rifampicin concentrations. A number of HPLC-based assays for rifampicin have been described [2–10], but these have lacked standardisation. For some assays an internal standard was not included [2–5], or the assays were not suitable for analysis of plasma (lacking an work-up procedure) [6,7] or else required large volumes of sample with relatively low sensitivity [8,9]. Only one previous HPLC–MS based assay has been reported [10], and lack of internal standard and validated work-up procedure does not make this suitable for analysing drug concentrations in plasma or cells.

Here a precise and accurate HPLC–MS method validated to internationally accepted criteria is described. The abilityof the

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doi:10.1016/j.jchromb.2007.07.005

assay to determine both plasma and cell associated rifampicin concentrations in tuberculosis patients receiving rifampicin is demonstrated.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Finnigan Spectrasystem P2000 Binary Gradient Pump and a Thermo Separation Products TCP Spectrasystem AS3000 autosampler (Thermo Fisher Scientific Hemel Hempstead, UK). The HPLC system was interfaced with a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (Thermo Fisher Scientific Hemel Hempstead, UK) operated under Xcalibur software (version 1.2, Thermo Electron Corporation, Hemel Hempstead, UK). A 2000-40M air compressor (JUN-AIR International A/S, Denmark) and a nitrogen generator system 75-72 (Parker Hannifin Corp, Kent, UK) were employed to provide nitrogen to the mass spectrometer. Analyte separation was achieved on a Betasil Phenyl-Hexyl analytical column (5 μm, 50 mm × 2.1 mm) (Thermo Electron Corporation, Runcorn, Cheshire, UK), protected with a betasil hexyl pre-column (5 μm).

2.2. Chemicals

HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR Laboratory Supplies (Poole, UK). Drug free plasma was obtained from the National Blood Transfusion Service (Liverpool, UK). [^{3}H]-rifampicin (specific activity: 15 Ci/mmol), was purchased from Moravek Biochemicals (Brea, California, USA). All other chemicals were obtained through Sigma–Aldrich (Poole, UK).

2.3. Preparation of quality control samples and internal standards

A rifampicin stock solution (1 mg/mL) was made up in MeOH (stored at −20 °C under dark conditions). On the day of study, the rifampicin stock solution was diluted in drug free blank plasma (to give a concentration of 100 μg/mL) and further diluted down to the top calibration standard (12800 ng/mL). A standard curve was generated (100–12800 ng/mL) by serial 1 in 2 dilutions of the top calibrator in drug free blank plasma.

To generate stocks of low (LQC: 150 ng/mL) medium (MQC: 1200 ng/mL) and high (HQC: 10,000 ng/mL) quality control samples, 50 mL of drug free blank plasma was spiked with the appropriate amount of rifampicin stock solution and stored in 500 μL aliquots (stored at −80 °C, under dark conditions).

An internal standard (IS: rifamycin SV) stock solution was accurately made up in ACN (stored at −20 °C, under dark conditions). On each experimental day, the working IS stock solution was diluted in ACN to 1 μg/mL. Stock rifampicin and internal standard solutions were stored at −20 °C under dark conditions for up to 6 months.

2.4. Sample treatment

50 μL (all in duplicate) of each of the standard curve concentrations, quality control sample (LQC, MQC and HQC) and test samples were transferred into clean 1.5 mL microfuge tubes. 150 μL of internal standard (1 μg/mL in ACN) was added and the tubes were vortexed vigorously to allow for maximal protein precipitation. Samples were centrifuged (10 min, 12000 × g) and supernatants (100 μL) transferred to an autosampler vial (with insert) and mixed with deionised water (150 μL). 10 μL of sample was injected into the HPLC–MS system for analysis.

2.5. Chromatographic and mass spectrometric conditions

Rifampicin and IS were separated and eluted using an isocratic mobile phase composed of 10 mM ammonium acetate (pH 4.0) and acetonitrile (60:40, v:v), at a flow rate of 0.4 mL/min. Mass spectral analyses for rifampicin and the IS were carried out using electrospray ionisation (ESI) in the positive ion mode (4.5 kV) with capillary temperature of 250 °C. For an optimal signal, sheath and auxiliary gas flows were set to 65 and 30 psi, respectively. For single ion monitoring (SIM) analysis, the first quadruple was set to scan for the 3 different mass-to-charge ratios (m/z) for rifampicin (m/z: 791.9 ± 0.3, 824.0 ± 0.3 and 846.0 ± 0.3) and one for the IS (m/z: 720.6 ± 0.3). The sum of the rifampicin ions detected representing $[MH]^+$ (m/z: 824.00), $[MNa]^+$ (m/z: 846.00) and a truncated rifampicin (without a methoxy group, $[MH–CH_3OH]^+$: m/z: 791.9) were used for rifampicin quantification.

2.6. Validation of rifampicin standard curve and quality control samples

Six assays containing a standard curve (in duplicate) and QC samples (in quadruplicate) were prepared on separate days and analysed by HPLC–MS. Using a 1/concentration^2 weighted quadratic regression standard curve, the nominal concentration of each of the QC samples (LQC, MQC and HQC) was determined.

2.7. Rifampicin recovery and stability

Drug free blank plasma was spiked with [^{3}H]-rifampicin (150, 1200, 10,000 ng/mL, 0.5 μCi/mL). For each drug concentration, 50 μL aliquots of spiked plasma were either diluted in PBS (100 μL) or precipitated with ACN (100 μL, as used during normal plasma work-up procedure) and centrifuged (3200 × g, 10 min). 50 μL of both the diluted and precipitated (supernatant) samples were removed for liquid scintillation counting and the recovery determined as the fraction of drug in the supernatant (of the precipitated sample) compared to the diluted plasma.

To determine the impact of plasma heat-inactivation (routinely 40 min, 58 °C) and freeze–thaw cycles on rifampicin stability, fresh drug free plasma samples were spiked with 150, 1200 and 10,000 ng/mL of rifampicin. Samples were then heat inactivated (40 min, 58 °C), or underwent 3 freeze (−80 °C) thaw (room temperature) cycles. Following sam-

ple treatment/storage conditions, the rifampicin concentrations were analysed in quadruplicate on three separate occasions and compared to the control sample that had been stored at −80 °C.

2.8. Plasma matrix effects on rifampicin HPLC–MS signal

To determine the potential of ion suppression by the matrix, mobile phase spiked with rifampicin and IS (both at 1000 ng/mL) was run through the HPLC–MS system. Drug free plasma samples were then precipitate by ACN (without IS) as described in the work-up procedure, and injected onto the column. The impact of the matrix was then determined on the SIM signal over time.

2.9. Determination rifampicin loss from PBMCs during cell isolation from blood

To determine the loss of rifampicin from cells during the PBMC isolation procedure, PBMCs were isolated from blood (50 mL) of six healthy volunteers by density gradient centrifugation on Ficoll-Hypaque. PBMCs were then washed 3 times (800 × g, 6 min) and resuspended in 5 mL of RPMI 1640 (supplemented with 10% FBS). The cell suspension was spiked with [^{3}H]-rifampicin (final concentration: 1000 ng/mL, 0.5 μCi/mL) and incubated (37 °C, 30 min). To determine the rifampicin content of the cells ('control'), the cell suspension (1 mL) was pelleted (3200 × g, 1 min), washed in ice cold PBS (3200 × g, 1 min), the cell count determined, and radioactivity determined by liquid scintillation counting. In parallel, the remaining 4 mL of the cell suspension was layered on Ficoll-Hypaque (2 mL, 4 °C) and centrifuged (800 × g, 25 min, 4 °C). PBMCs were then isolated and washed 3 times (800 × g, 5 min, 4 °C), before cell counting and liquid scintillation spectrometry. The recovery of rifampicin from PBMCs following isolation was calculated as the percentage of rifampicin present in the isolated PBMCs (cell count corrected) compared to that found in the 'control' (cell count corrected).

To determine the recovery of rifampicin from the cell pellet following ACN precipitation work-up procedure, PBMCs (10×10^6 cells) were isolated from six healthy volunteers and incubated with [^{3}H]-rifampicin (1000 ng/mL, 0.5 μCi/mL, 30 min). Cells were split into two aliquots (5×10^6 cells each), washed and pelleted (3200 × g, 1 min). Double distilled water (48 μL) was added to each cell pellet (cell volume of 5×10^6 PBMCs is 2 μL). The total [^{3}H]-rifampicin of one aliquot was then determined by liquid scintillation ('control'), while to the other aliquot ACN (100 μL) was added. This was followed by centrifugation (3200 × g, 10 min) and 50 μL (1/3 of total) of supernatant being taken for liquid scintillation counting. Recovery was determined as the percentage of drug found in the supernatant following precipitation (correcting for dilution) compared to the total 'control'.

2.10. Clinical samples

Eight patients (male (7/8), Caucasian (5/8), black African (2/8) and Indian (1/8)) were enrolled in a clinical study to determine plasma and cellular concentrations of rifampicin during therapy. Each subject provided written informed consent, and the study was approved by the local ethics committee. All subjects were over the age of 18 years, were able to give informed consent and received routine rifampicin (600 mg o.d.) therapy (for a minimum of 5 days) for the treatment of tuberculosis (pulmonary TB (4/8), extra-pulmonary TB (4/8)). None of the subjects suffered from hepatic impairment (as graded by elevated ALT) or anaemia (as graded by haemoglobin).

Venous blood samples (19 mL each) were obtained at 0.5, 1, 2, 4, 6 and 8 h post dose from each subject. 4 mL of heparinised blood was centrifuged (10 min, 2700 × g) to separate the plasma (approximately 2 mL) which was stored at −80 °C. Peripheral blood mononuclear cells (PBMCs) were isolated from the remaining 15 mL of heparinised blood by density gradient centrifugation on Ficoll-Hypaque Plaque (25 min, 800 × g). Cells were then washed 3 times in ice cold PBS, counted (by nuclear counter), pelleted and frozen down (−80 °C) for later HPLC–MS analysis. PBMCs were subsequently thawed and the volume calculated using a cell volume of 0.4 pL [11]. The sample was then made up to 50 μL with double distilled water and sonicated (1 min) in a water bath. The subsequent work-up procedure by protein precipitation was conducted in the same way as that for plasma samples. Cellular rifampicin concentrations were determined by HPLC–MS and corrected by taking into consideration the dilution of the PBMC into 50 μL.

2.11. Data analysis

HPLC–MS data acquisition and processing was performed by Xcalibur software. Standard curves were constructed using a 1/concentration^2 weighted quadratic regression of the peak area ratio (rifampicin: IS ratio) versus rifampicin concentration. Unknown and QC sample rifampicin concentrations were extrapolated from this standard curve. Nominal QC concentrations were determined in order to account for slight error or deviations in concentrations when the stocks were made up.

Assay precision was expressed as a coefficient of variance (%CV: [standard deviation/mean concentration] × 100) and assay accuracy as %bias ([absolute difference in measured concentration and nominal concentration] × 100/measured concentration). Rifampicin stability following heat-inactivation and freeze–thaw cycles was analysed by Mann–Whitney *U*-test (using StatsDirect statistical software, version. 2.5.6, Cheshire, UK). Plasma and cellular rifampicin concentrations from each individual patient were fitted to a one compartmental model, with first-order elimination and absorption, and a lag time. Pharmacokinetic parameters were determined using WinNonLin 5.1. (Pharsight Co. Mountainview, CA, USA).

3. Results

3.1. Detection and chromatography

Rifampicin and IS were detected by HPLC–MS over a run time of 6 min, with specific retention times of 1.1 and 1.7 min, respectively. Rifampicin was detected by scanning for the proto-

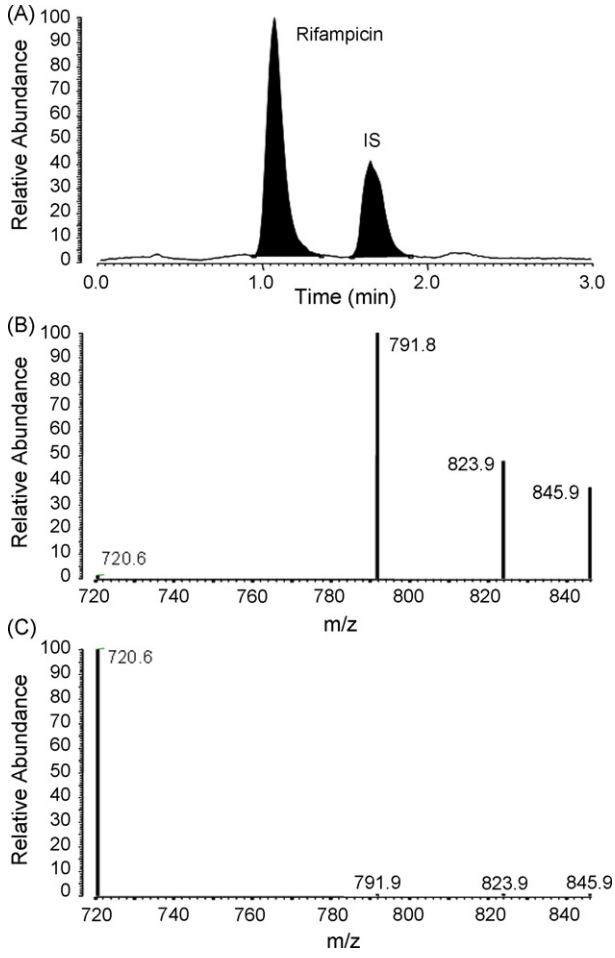

Fig. 1. (A) Representative HPLC–MS chromatogram depicting rifampicin and IS (rifamycin SV) peaks of a HQC sample following standard work-up procedure and detection by SIM. (B and C) represent the relative abundance of the ions measured by SIM at 1.07 min (rifampicin) and 1.66 min (IS), respectively.

nated molecule ($[MH]^+$, ±25% relative abundance), the sodium adduct (MNa^+, ±25% relative abundance) and a product ion (through ESI) that represents the loss of methoxy group (±50% relative abundance) (Fig. 1). The IS, rifamycin SV, was detected by SIM of the protonated molecule ($[MH]^+$).

3.2. Linearity and validation of standard curves

All standard curves generated between 100 and 12,800 ng/mL were adequately described using a 1/concentration^2 weighted quadratic regression. The correlation coefficient (r^2) for all validation standard curves was above 0.99. Furthermore, at least six points on the standard curve (other than zero) had both duplicates within 15% of the nominal concentration (20% for the lowest point on the standard curve). The lower and upper limits of quantification (LQC and HQC) were arbitrarily set as the bottom (100 ng/mL) and top (12,800 ng/mL) points of the standard curve. The limit of detection (LOD), defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1), was 40 ng/mL for this assay.

3.3. Accuracy and precision

Nominal LQC, MQC and HQC concentrations were calculated as 119, 1215 and 10,082 ng/mL, respectively. Accuracy was determined by calculating the percentage bias of the quality control samples (%bias = [measured concentration − mean nominal concentration] × 100/measured concentration). Precision was expressed as a coefficient of variance (%CV: [standard deviation/mean concentration] × 100) of the quality control samples. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations of six separate assays. Intra-assay variability was determined as the accuracy and precision of the eight individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was within 10% for the LQC, MQC and HQC concentrations (summarised in Table 1), which was within the general assay acceptability criteria for quality control samples [12].

3.4. Recovery, stability of plasma rifampicin

Rifampicin recovery for plasma spiked with 150, 1200, and 10,000 ng/mL of rifampicin was 89 ± 3%, 88 ± 3% and 91 ± 4%, respectively ($n = 6$). Determination of rifampicin stability following heat-inactivation showed that for all QC samples there was a small decrease in the measured drug concentration, a difference that was only significant for the low (±150 ng/mL) rifampicin concentrations QC ($P = 0.03$) (Table 2). Three freeze–thaw cycles had no significant impact on the rifampicin concentrations measured (Table 2).

Table 1
Inter- and intra-assay precision and accuracy for the measurement of rifampicin in human plasma as determined by HPLC–MS

QC level	Nominal concentration (ng/mL)	Accuracy (%bias[a]; mean ± STD)		Precision (%CV[b]; mean measured concentation ± STD)	
		Inter-assay	Intra-assay	Inter-assay	Intra-assay
Low	119.3	4.0 ± 2.3	3.1 ± 2.3	118.6 ± 5.8 (4.9)	117.9 ± 4.7 (4.0)
Medium	1215.6	2.1 ± 1.9	1.7 ± 1.1	1209.8 ± 36.5 (3.0)	1211.8 ± 25.9 (2.1)
High	10082.1	3.3 ± 2.9	3.5 ± 2.8	10189.8 ± 468.9 (4.6)	10119.6 ± 474.4 (4.7)

Intra-assay variability was determined on 8 separate QC samples analysed using the same assay, and inter-assay variability was determined using quadruplicate QC samples analysed on 6 separate assays.

[a] %Bias = |(measured concentration − nominal concentration)| × 100/measured concentration).

[b] %CV = (standard deviation/mean measured concentration) × 100.

Table 2
Rifampicin concentrations (ng/mL) were determined in freshly spiked human plasma (at a low, medium and high rifampicin concentration), and following heat-inactivation (58 °C, 40 min) and 3 freeze–thaw cycles (−80 °C cycles/room temperature)

Treatment	Low concentration	*P*-value	Medium concentration	*P*-value	High concentration	*P*-value
Untreated	148.1 ± 2.4		1155 ± 62		9678 ± 395	
Heat-inactivation	127.5 ± 3.6	0.03	1061 ± 60	0.11	9401 ± 325	0.34
3 × freeze/thaw	146.8 ± 6.4	0.97	1196 ± 54	0.34	9841 ± 253	0.69

Statistical analysis of treated vs. fresh rifampicin plasma samples was performed using a Mann–Whitney *U*-test.

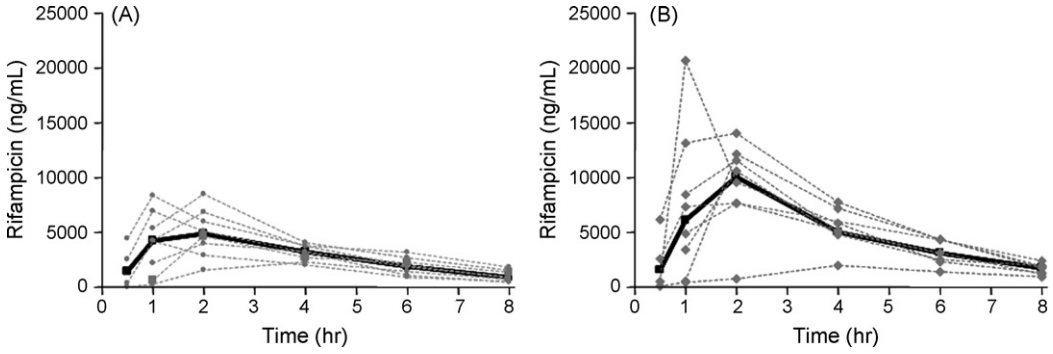

Fig. 2. (A) Plasma and (B) cellular rifampicin PK profiles in 8 patients receiving rifampicin (600 mg o.d.). Dashed lines indicate individual PK profiles whilst dark line indicates median PK profile.

3.5. Plasma matrix effects on rifampicin HPLC–MS signal

The injection of precipitated drug free plasma (matrix) did not cause ion suppression of rifampicin or rifamycin SV post solvent front (after 0.6 min) (data not shown).

3.6. Rifampicin loss from PBMCs during cell isolation from blood

The loss of rifampicin from PBMC during the isolation procedure (by density gradient centrifugation and wash steps) was 49% (recovery 51 ± 5%, $n = 6$). The work-up procedure for determining PBMC associated rifampicin following ACN precipitation did not lead to any drug loss (recovery 102 ± 5%, $n = 6$)

3.7. Quantification of patient samples

Using the HPLC–MS method, plasma and cellular rifampicin concentrations were determined over an 8-h period in 8 tuberculosis patients receiving rifampicin (Fig. 2). Using a first-order, compartmental (with lag) pharmacokinetic model, the maximum rifampicin plasma concentration (C_{max}: mean ± SD, 6.0 ± 2.5 μg/mL), time to maximum concentration (T_{max}: mean ± SD, 1.6 ± 0.9 h), apparent volume of distribution (V_d: mean ± SD, 83 ± 26 L), elimination half life ($T_{1/2}$: mean ± SD, 2.3 ± 0.7 h) and area under the curve (AUC_{0-8}: mean ± SD, 26 ± 9.3 h mg/mL) were calculated (Table 3). The cell associated C_{max} (mean ± SD, 11.2 ± 5.9 μg/mL), T_{max} (mean ± SD, 1.9 ± 1.0 h), $T_{1/2}$ (mean ± SD, 2.2 ± 0.8 h), AUC_{0-8} (mean ± SD, 45 ± 15 h mg/mL) and the cellular accumulation ratio (CAR: mean ± SD, 1.8 ± 0.7) were also determined (Table 3).

4. Discussion

Subpopulations of MTB residing in mononuclear cells could be a factor in the long duration of treatment currently required to achieve cure of patients. To get an idea of the effective concentration of rifampicin acting on internalised bacilli, it is important

Table 3
Plasma and cell associated pharmacokinetic (PK) parameters determined in 8 tuberculosis patients receiving rifampicin (600 mg o.d.)

PK parameter	Plasma		Cell associated	
	Mean (SD)	Median (range)	Mean (SD)	Median (range)
C_{max} (μg/mL)	6.0 ± 2.5	6.0 (2.4–10.0)	11.2 ± 5.9	11.8 (1.6–21.8)
T_{max} (h)	1.6 ± 0.9	1.3 (0.7–3.3)	1.9 ± 1.0	1.6 (0.7–4.1)
$T_{1/2}$ (h)	2.3 ± 0.7	2.2 (1.4–3.4)	2.2 ± 0.8	2.0 (1.4–4.0)
V_d (L)	83 ± 26	72 (53–128)		
AUC (h mg/L)	26 ± 9	25 (12–40)	45 ± 15	46 (15–65)
CAR			1.8 ± 0.7	1.6 (1.2–3.0)

PK parameters were determined from the measured plasma and cellular drug concentration profiles for each individual patient by first-order, compartmental (with lag) analysis using WinNonLin 5.1. (Pharsight Co. Mountainview, CA, USA). The cellular accumulation ratio (CAR) was calculated by dividing the cell associated AUC by the plasma AUC for each individual patient profile. (C_{max}: maximum concentration; T_{max}: time of maximum concentration; $T_{1/2}$: elimination half life; V_d: apparent volume of distribution; AUC: area under the drug concentration/time curve).

that cell associated drug concentrations can be measured as well as drug concentrations in plasma. To date, there are a number of published analytical methods [2–10], some that have been used to determine plasma rifampicin concentrations, but none used to determine cell associated rifampicin. Further, previous publications often have not been used on plasma samples [3,8,10], have not used and internal control [2–5], or have limited sensitivity [8,9]. Here a validated, accurate, precise and sensitive HPLC–MS method has been developed for the detection of rifampicin in both plasma and cells.

HPLC–MS allows for added specificity for the detection of rifampicin compared to that obtained with HPLC. In a previous publication [10], rifampicin was detected by HPLC–MS following direct injection of the drug and identical ions (*m/z*: $[MH]^+$, $[MNa]^+$ and $[MH–CH_3OH]^+$) were seen in the present study where rifampicin was extracted from plasma. None of the rifampicin ions were detected at any time in extracted drug free plasma samples.

The range of the standard curve used was designed to encompass rifampicin concentrations that cover a typical rifampicin 12 h pharmacokinetic profile as described previously [13], whilst going as low as possible to aid the detection of cellular rifampicin. The accuracy of the standard curve was ensured by the incorporation of quality control samples. As described in the FDA guidelines for industry [12], quality control samples may not deviate more than 15% from the nominal concentration (and 20% for the LLQC), a guideline followed for every assay. The HPLC–MS method was fully validated with inter- and intra-assay accuracy and precision falling within the FDA guidelines [12].

This assay requires only a small volume of plasma (50 μL). This may be of particular advantage for paediatric studies where children can only provide small volumes of blood, and when studying rifampicin concentrations in the cerebral spinal fluid (CSF) where sample volumes are limited. The sensitivity of the assay (LLQ of 100 ng/mL) also allows for the detection of rifampicin in pharmacological sanctuary sites (such as the CSF) where drug penetration is limited. Furthermore, the rapid and easy sample treatment by precipitation, as well as the short runtime of the assay (6 min) allows for rapid sample processing and analysis.

Mycobacterium tuberculosis is generally not present in large amounts in human plasma; however, the potential of HIV and/or hepatitis co-infection means that plasma samples need to be inactivated. Heat treatment (58 °C, 40 min) is routinely used to inactivate plasma samples from both HIV and Hepatitis C, however, data shown here indicate that the process of heat-inactivation significantly affects the stability of rifampicin. Therefore, it is advised that samples are treated with care and inactivated by the addition of acetonitrile during rifampicin work-up procedure. Further, it is of essence that all studies are performed away from direct light as rifampicin is known to be light sensitive. The process of freeze–thawing of rifampicin containing plasma samples had no effect on the measured drug concentration.

Previous studies have also utilised HPLC tandem mass spectrometry for the determination of cell associated HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTI) [14–17], in a similar way to that performed here. For this study, the maximum total blood volume provided by each patient was limited to 120 mL equating to 6-time points. As therapeutic ranges for rifampicin plasma concentrations in healthy volunteers have been described previously [13], published data was used to simulate an optimal limited sampling strategy using WinNonLin, allowing us to determine the optimal 6-time points to give the most accurate AUC_{0-8} for both plasma and cell associated rifampicin ($t = 0.5$, 1, 2, 4, 6 and 8 h).

Using the validated HPLC–MS assay in combination with an optimised sampling strategy the pharmacokinetic profile of rifampicin was determined in both plasma and within PBMCs. The plasma rifampicin pharmacokinetic profiles seen in this study are similar to those described previously [13,18,19], with a similar apparent volume of distribution and elimination half life. However, this is the first study, to our knowledge, that attempts to determine the accumulation of rifampicin in PBMCs *in vivo* in tuberculosis patients. The biggest obstacle to determining *in vivo* cellular rifampicin concentrations is definitely the loss of rifampicin during the isolation procedure for PBMC. Despite our best efforts, performing all experiments as quickly as possible and at 4 °C at all times, we consistently achieved a recovery of 51% (±5%). This loss is largely due to the movement of drug from PBMCs into the Ficoll-Hypaque over the 25 min centrifugation step. Unfortunately, there is currently no method available where cell isolation is not necessary and therefore this will be a common problem affecting all studies looking at cellular rifampicin *in vivo*. It must be noted therefore that the loss of drug from the cell during isolation will lead to an underestimate of the actual drug content of PBMC. Our finding that rifampicin accumulates around 1.7-fold within PBMCs *in vivo*, and 2.5–5-fold within PBMCs and cell line *in vitro* is in keeping with this observation [1,20]. It must be remembered though that the drug distribution of rifampicin in a cell may vary from compartment to compartment, and that the concentration measured in the whole cell is not necessarily the same as that at the site of the intracellular bacilli. Further, it must be noted that the susceptibility of intracellular bacilli to rifampicin may be different to that of extracellular bacilli [1], therefore intracellular bacilli may still be harder to eradicate than extracellular ones.

In summary, a novel validated HPLC–MS method is described that is sensitive, accurate, precise, uses a small sample volume and has a simple work-up procedure. This assay has been utilised successfully to determine the pharmacokinetic profile of plasma and cell associated rifampicin in tuberculosis patients.

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