

University of Groningen

The influence of the sample matrix on LC-MS/MS method development and analytical performance

Koster, Remco Arjan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2015

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Koster, R. A. (2015). *The influence of the sample matrix on LC-MS/MS method development and analytical performance*. University of Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.



Chapter 3.3

Simultaneous determination of rifampicin, clarithromycin and their metabolites in Dried Blood Spots using LC-MS/MS

D.H. Vu
R.A. Koster
M.S. Bolhuis
B. Greijdanus
R. van Altena
D.H. Nguyen
J.R.B.J. Brouwers
D.R.A. Uges
J.W.C. Alffenaar

ABSTRACT

Introduction: Rifampicin (RIF) and clarithromycin (CLR) are common drugs for the treatment of infections like *Mycobacterium tuberculosis* and *Mycobacterium ulcerans*. Treatment for these diseases are long-term and the individual pharmacokinetic variation, drug-drug interactions or non-adherence may introduce sub-therapeutic exposure or toxicity. The application of therapeutic drug monitoring (TDM) can be used to ensure efficacy and avoid toxicity. With the use of dried blood spot (DBS), TDM may be feasible in rural areas. During DBS method development, unexpected interactions or matrix effects may be encountered due to endogenous components in the blood. Another complication compared to plasma analysis is that RIF can form chelate complexes with ferric ions or can bind with hemes, which are potentially present in the extracts of dried blood spots.

Methods: The investigation focused on the interaction between RIF and the endogenous components of the DBS. The use of ethylenediaminetetraacetic acid (EDTA) and deferoxamine (DFX) as chelator agents to improve recoveries and matrix effects were investigated. A rapid analytical method was developed and validated to quantify RIF and CLR and their active metabolites desacetyl rifampicin (DAC-RIF) and 14-hydroxycylarthyromcin (14OH-CLR) in DBS samples. A clinical application study was performed in tuberculosis patients by comparing DBS concentrations with plasma concentrations.

Results: The interaction between RIF and the DBS matrix was avoided using the complexing agents EDTA and DFX, which improved recoveries and matrix effects. The developed sample procedure resulted in a simple and fast method for the simultaneous quantification of RIF, CLR and their metabolites in DBS samples. High stability was observed as all four substances were stable at ambient temperature for two months. Deming regression analysis of the clinical application study showed no significant differences for RIF, DAC-RIF, CLR and 14OH-CLR between patient plasma and DBS analysis. The slopes of the correlation lines between DBS and plasma concentrations of RIF, DAC-RIF, CLR and 14OH-CLR were 0.90, 0.99, 0.80 and 1.09 respectively. High correlations between plasma and DBS concentrations were observed for RIF ($R^2=0.9076$), CLR ($R^2=0.9752$) and 14OH-CLR ($R^2=0.9421$). Lower correlation was found for DAC-RIF ($R^2=0.6856$).

Conclusion: The validated method is applicable for TDM of RIF, CLR and their active metabolites. The stability of the DBS at high temperatures can facilitate the TDM and pharmacokinetic studies of RIF and CLR even in resource limited areas. The role of EDTA and DFX as complexing agents in the extraction was well investigated and may provide a solution for potential applications to other DBS analytical methods.

INTRODUCTION

Rifampicin (RIF) and clarithromycin (CLR) are used for the treatment *Mycobacterial* infections. According to the tuberculosis (TB) treatment guideline of the World Health Organization, RIF is the back bone of the first line anti-TB drugs in the treatment of *Mycobacterium tuberculosis*. CLR is indicated for treatment of multidrug resistant (MDR) TB. In combination, RIF and CLR showed high efficacy for the treatment of *Mycobacterial ulcerans* which is the organism causing Buruli Ulcer disease [1]. RIF displays large pharmacokinetic variability that may result in subtherapeutic drug exposure [2, 3]. It is well known that RIF is a liver enzymes inducer while CLR is an inhibitor. Several studies suggested that RIF reduces the CLR plasma concentration while CLR, on the other hand, increases the RIF plasma level [4, 5]. Furthermore, the metabolism of RIF and CLR by cytochrome P450 results in active metabolites including 25-desacetyl rifampicin (DAC-RIF) and 14-hydroxyclearithromycin (14OH-CLR) [6-8]. Therapeutic drug monitoring (TDM) might help to assure adequate exposure and therefore may improve the treatment outcome of these drugs.

The common endemic areas for TB or Buruli Ulcer diseases often have limited resources. Conventional plasma sampling is often not feasible due to lack of equipment or cooled transportation [9]. Dried blood spot (DBS) sampling has many potential advantages such as prolonged sample stability, lower risk of infections and transport at ambient temperature [9, 10]. These advantages may facilitate the application and implementation of TDM even in resource limited areas. Methods of analysis for the determination of RIF or CLR in the biological fluids using high performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC-MS/MS) have been reported earlier [11, 12], including the simultaneous determination of rifampicin and clarithromycin [13, 14]. Only one report described the development of an analytical method to determine RIF in DBS using HPLC [15]. However, the described extraction method is time consuming, while the LLOQ of 1.5 mg/L is still too high regarding RIF trough levels ranging from 0.2–1.0 mg/L. In addition, the DBS method did not include the determination of DAC-RIF. The application of LC-MS/MS with high selectivity and sensitivity can help to deal with these limitations.

To extract DBS samples, hydrophilic or hydrophobic organic solvents can be applied [16-18]. If hydrophilic extraction is implemented, further liquid-liquid extraction is not required but endogenous components from the DBS matrix will contaminate the extract [19]. The endogenous components in the blood may cause unexpected interactions or matrix effects during the analysis [20]. Another complication in DBS compared to plasma analysis is that RIF can

form chelate complexes with ferric ions or can bind with hemes which are potentially present in the extracts of dried blood spots [21]. This could negatively affect the analytical results. The application of ethylenediaminetetraacetic acid (EDTA) and deferoxamine could prevent RIF from forming chelate complexes or bind with hemes. In addition, EDTA has previously been successfully applied to precipitate endogenous components [22]. All considered, the use of a chelator like EDTA could improve the robustness of an analytical DBS method.

In addition to the standard validation criteria, the influence of the hematocrit value and blood spot volume should be assessed during the method validation, as these parameters could be of influence on the analytical results [9, 10, 23]. Furthermore, before DBS is implemented in daily routine, the correlation between RIF in DBS and plasma concentrations should be demonstrated [9, 10, 23].

The aim of this study is to develop a rapid LC-MS/MS method for the determination of RIF, CLR and their metabolites in DBS that is suitable for TDM or clinical pharmacokinetic studies in rural areas.

MATERIALS AND METHODS

Chemicals, reagents and disposables

Clarithromycin ($C_{38}H_{69}NO_{13}$) and 14-hydroxyclearithromycin ($C_{38}H_{69}NO_{14}$) were provided by Abbott (IL, USA). Rifampicin ($C_{43}H_{58}N_4O_{12}$) and 25-desacetyl rifampicin ($C_{41}H_{56}N_4O_{11}$) were provided by Sanofi-Aventis (Frankfurt, Germany). The 2H8-Rifampicin and cyanoimipramine were supplied by Brunschwig Chemie (Amsterdam, The Netherlands) and by Roche (Woerden, The Netherlands), respectively. Purified water was prepared by a Milli-Q Integral system (Billerica, Massachusetts, USA). Acetonitrile (ACN) of ultra LC/MS grade were supplied by Biosolve (Valkenswaard, The Netherlands). Disodium ethylenediaminetetraacetic acid (EDTA), ammonium acetate, acid acetic and trifluoroacetic anhydride were of analytical grade and purchased from VWR (Amsterdam, the Netherlands). Deferoxamine mesilate (DFX) was obtained from Novartis Pharma (Arnhem, the Netherlands). Pooled plasma and packed red blood cells were obtained from the Department of Hematology, University Medical Center Groningen according to local regulations. Whatman 31 ET CHR paper sheets (Whatman, Kent, UK) were cut in to 4×6 cm paper cards which were used for the preparation of calibration and quality control (QC) DBS and patient sampling.

Equipment and conditions

Vortexing was performed with a Labtek multi-tube vortexer (Christchurch, New Zealand). Sonification was performed at 47 kHz using a Branson 5210 ultrasonic bath (Danbury, CT, USA). The punching machine (punch diameter of 8mm) was supplied by the Technical Support Facilities of the University of Leiden (Leiden, the Netherlands) and designed by P.M. Edelbroek PhD, (Heemstede, the Netherlands) and was used in an earlier study [19].

The LC-MS/MS system consisted of a Surveyor® MS pump and a Surveyor plus® autosampler connected with a Thermo Fisher Scientific TSQ Quantum Discovery, triple quadrupole mass spectrometry (Thermo Fisher, Waltham, USA). The autosampler and column were set at a temperature of 20°C. The chromatographic analysis was performed on a 50 mm×2.1 mm×3µm HyPurity C18 column (Interscience, Breda, the Netherlands). The analytes were eluted with a flow rate of 300 µl/minute using a solvent gradient as followed: 0–1 minute, ACN from 0% to 95%, water from 95% to 0%; 1–2.5 minute, ACN 95% and water 0%; 2.5–2.6 minute, decreased ACN to 0% and kept eluting by 95% water until 3.5 minutes. The aqueous buffer (ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoroacetic anhydride 2 mg/L at pH 3.5) was kept at 5% during the gradient.

The Thermo TSQ Quantum Discovery mass selective detector worked in positive ion mode and performed selected reaction monitoring (SRM) at a scan width of 0.5 m/z. The mass parameters for each analytes and the internal standards are presented in table 1. The ion spray voltage, sheath gas pressure, auxiliary gas pressure and capillary temperature were set at 3500 V, 35 arb (arbitrary units), 5 arb and 350°C, respectively. The Xcalibur software version 1.4 SR1 was used for peak height integration and quantification (Thermo Fisher, Waltham, USA).

Preparation of the samples

Preparation of the stock solutions

Two separate stock solutions were prepared in water at a concentration of 600 mg/L for RIF and 200 mg/L for the other three analytes. These stock solutions were used to prepare the calibration curve and QC samples. For spiking low concentrations, the stock solutions were diluted with water to 25 mg/L. This ensures that the spiked volume of the stock solution could not exceed 5% of the total volume. The stock solutions were stored at 4°C.

Table 1 The mass spectrometer conditions and concentrations of calibration and quality control samples

Substance	Mass transition (m/z)			CE (eV)	Calibration concentrations (mg/L)	QC sample concentrations (mg/L)				
	Parent	Product				LLOQ	LOW	MED	HIGH	OC
Rifampicin	823.3	791.2		17	0.15, 0.45, 1.5, 3.0, 9.0, 15.0, 24.0, 30.0	0.15	0.45	15.00	24.00	60.00
Desacetyl rifampicin	781.4	749.2		14	0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.15	0.50	5.00	8.00	20.00
Clarithromycin	748.5	590.2		18	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.05	0.15	5.00	8.00	20.00
14-Hydroxy clarithromycin	764.4	606.2		20	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.05	0.15	5.00	8.00	20.00
² H ₈ -Rifampicin	831.5	799.5		17	-	-	-	-	-	-
Cyanoimipramine	306.2	218.0		39	-	-	-	-	-	-

CE: collision energy.

Preparation of the Calibration Curve and QC's in Blood

The packed red blood cells (RBC) obtained from the Hematology department contained a preservation solution. To completely remove the preserving solution, RBC were centrifuged at 3,506 xg for 10 minutes and the upper layer was discarded. To wash the RBC, an equal volume of phosphate buffered saline with pH 7.4 was added and mixed by a rotating mixer for 5 minutes. The RBC suspension was centrifuged and the upper layer was discarded. The RBC washing procedure was subsequently repeated twice with phosphate buffered saline and lastly with plasma. Calibration and QC blood at the desired Hct values were produced by mixing washed RBC with plasma and appropriate volumes of stock solution. All the calibration and QC blood samples were prepared at the standardized Hct value of 35%, since the tuberculosis patient population showed to have a lower mean Hct than healthy volunteers [19]. The concentrations of each component in the calibration and QC samples are presented in table 1.

Preparation of DBS samples

The calibration and QC DBS samples were prepared by pipetting 50 μ l of blood onto the paper card and left to dry for 3 hours at ambient temperature. DBS cards were stored separately in sealed plastic bags with desiccant sachets at -20°C.

Development of the DBS extraction

The DBS at MED level were used for roughly evaluating the process efficiency of different methods of extraction. The analytical method for the simultaneous determination of RIF, CLR and their metabolites in plasma was successfully validated previously. Therefore, the developed DBS analytical method mainly focused on the extraction procedure, matrix effects and recovery [14]. Different extracting solvents including pure water, mixtures of 0%, 30% and 80% ACN in methanol were tested at a volume of 300 μ L for DBS at MED level. A sonication of 60 minutes at ambient temperature was used to accelerate the extraction and 5 μ L of extract was injected into the LC-MS/MS. The sample preparation was performed in three-fold to evaluate matrix effects and recovery in the method development [23]. Three solutions were prepared for each extraction solvent: The neat solution (extraction solvent) was spiked at the theoretical calculated MED concentration (solution A). Solution A was used to generate extracts of blank DBS (solution B). The extracts of DBS at MED level used blank extracting solvent (solution C). The average peak height responses were used to calculate recovery and matrix effects. The calculations of the recovery and matrix effects were as followed: $\text{recovery} = C/B \times 100$, $\text{matrix effect} = (B/A \times 100) - 100$.

The DBS aqueous extraction suffered from endogenous matrix effects and therefore required a cleaning process [9, 19]. A volume of 600 μL ACN was added to precipitate 200 μL of DBS aqueous extracts containing EDTA. The EDTA concentrations were tested in the range of 0.0 to 1.54 g/L (12 concentrations). UV-VIS absorption spectra with a wavelength range of 200–600 nm were obtained from the varying supernatants using a Varian UV-VIS spectrometer to indicate the cleanliness of these solutions.

Extraction procedure used for the method validation

The extracting solution consisted of $^2\text{H}_8$ -Rifampicin 0.25 mg/L (internal standard of RIF), cyanoimipramine 0.05 mg/L (internal standard of DAC-RIF, CLR and 14OH-CLR), EDTA 1 g/L and DFX 1 g/L in water. A disc with a diameter of 8 mm was punched out from the central part of DBS and transferred to a 1.5 mL Eppendorf plastic tube. An extracting solution volume of 300 μL was added and the extraction was accelerated by sonication for 20 minutes. A volume of 200 μL of the extract was transferred to a glass vial and a 600 μL of ACN was added to precipitate the endogenous components. The sample was vortexed for 1 minute, centrifuged at 10,146 xg for 5 minutes and 5 μL of the supernatant was injected into the LC-MS/MS system.

Method validation

The method was validated in accordance with the US Food and Drug Administration's Guidance for Industrial Bioanalytical Method validation [24]. The validated criteria included the selectivity, linearity, accuracy and precision, dilution integrity, carry-over, process efficiency and stability. In addition to these validation guidelines, the validation was extended with the assessment of the influence of Hct and blood volume of DBS, which are recommended for DBS analysis [19, 25]. The validation was performed with maximum tolerated bias and coefficient of variation (CV) of 20% for the lower limit of quantification (LLOQ) and 15% for the other validated concentrations.

Selectivity, specificity and carry-over

The selectivity and specificity were evaluated by comparing the responses of the LLOQ and blank DBS samples prepared from 5 batches of human blood. The average response of blank DBS samples was required to be within 20% of the average response of the LLOQ samples. The carry-over was assessed using the response ratio of a blank sample injected

after a HIGH QC and compared to a LLOQ response. The response was required to be less than 20% of the LLOQ QC.

Linearity, reproducibility and dilution integrity

On each of three consecutive validation days, a single calibration curve was analyzed to assess linearity. The calibration curves were constructed using $1/x^2$ weighted linear regressions. The analytical responses, which were the peak height ratios between analyte and the respective internal standard, were used for the quantification. Additionally, in each validation day, five QC's of LLOQ, LOW, MED, HIGH and OC (over the calibration curve) were analyzed in fivefold to evaluate the intra-day and inter-day accuracy and precision and dilution integrity. The precipitated supernatants from OC samples were diluted 10 times with extracts of blank DBS before injection. Accuracy and the inter-day and intra-day precision was estimated using one-way analysis of variance (one-way ANOVA).

Matrix effect, complexing effect and recovery

The matrix effects resulting from DBS endogenous components or ferric chloride were evaluated at three QC levels of LOW, MED and HIGH. The role of the complexing agents EDTA and DFX in recovering the response due to DBS matrix effects or ferric chloride was also investigated. Different solutions A, B, C, D, E, F, G, H, I and K were prepared and analyzed in five-fold as presented in table 2.

Table 2 Experimental design to investigate the matrix effect, complexing effect, recovery and process efficiency

Added components	Solutions (*)									
	A (μL)	B (μL)	C (μL)	D (μL)	E (μL)	F (μL)	G (μL)	H (μL)	I (μL)	K (μL)
Neat solution	200	200	200	200	200	200	200	200	200	
Ferric chloride 1 g/L		10			10			10		
EDTA 23 g/L				10	10	10	10	10	10	10
Deferoxamine 23 g/L							10	10	10	10
Blank DBS (disc)			1			1			1	
QC DBS (disc)										1
Water	30	20	30	20	10	20	10		10	210

Neat solution: aqueous solution containing the analytes at the theoretical concentrations of LOW, MED and HIGH level; QC DBS (disc): DBS at LOW, MED and HIGH concentrations; (*): These solutions were subsequently processed according to the described extraction procedure, with the added volume of 690 μL ACN.

To calculate the theoretical concentration of the neat solution, DBS with a blood volume of 10 μL was prepared and therefore the whole DBS could be punched for extraction. All prepared solutions were sonicated for 20 minutes and 690 μL of ACN was added to each sample. The prepared solutions yielded volumes of 230 μL instead of the normally used volume of 200 μL . To retain the ratio of 1:3 (aqueous:ACN) for the protein precipitation, the volume of ACN was raised to 690 μL . After vortexing for 1 minute and centrifuging at 10,146 $\times g$ for five minutes, the clear solution was transferred to a glass vial and injected into the LC-MS/MS. The mean responses of five replicate analyses obtained from their respective solutions were denoted as A to I. The matrix effects resulting from the DBS matrix and ferric chloride were calculated as followed: without presence of complexing agents: $(B/A \times 100) - 100$ and $(C/A \times 100) - 100$; with EDTA: $(E/D \times 100) - 100$ and $(F/D \times 100) - 100$ and with the mixture of EDTA and DFX: $(H/G \times 100) - 100$ and $(I/G \times 100) - 100$, where 0% represents no decrease of signal or matrix effects. Recovery and process efficiency were calculated as $K/I \times 100$ and $K/G \times 100$, respectively, where 100% represents optimal recovery or process efficiency.

Effect of hematocrit and blood spot volume

Hct and blood spot volume may affect the analytical results and therefore these parameters were evaluated [19, 25]. The effect of Hct was evaluated using QC blood at three Hct values of 20, 35, 50%. The effect of blood spot volume was assessed by preparing DBS with blood volumes of 30, 50 and 100 μL . At each Hct level and blood spot volume, three QC levels of LOW, MED and HIGH were analyzed in five-fold. The bias is calculated as the percentage deviation from the standard Hct of 35% or blood volume of 50 μL . Further, it was assessed if the bias caused by the Hct value could be corrected [19].

Stability

The stability of the analytes in DBS was tested with QC levels LOW and HIGH after storing at temperatures of 50°C for 1, 3, 7 and 15 days, at 37°C for 10, 20 and 30 days and at ambient temperature for 7, 30 and 60 days. The samples were prepared and analyzed in fivefold and the analytical result was compared with their nominal concentration.

Clinical application study

Adult tuberculosis patients in the TB unit of the Beatrixoord hospital who received rifampicin or clarithromycin were eligible for the clinical application study. The proposal was approved by the medical ethical committee of University Medical Center Groningen. Informed consents were obtained from all participating patients. To create a finger prick, the patient's finger was disinfected with 70% ethanol and, after the ethanol completely evaporated, a prick was made with a disposable contact activated lancet (Becton Dickinson, New Jersey, United States). A single drop of blood was deposited on a coded paper card. Three DBS samples at 0, 2 and 8 hours after dosing for clarithromycin or at 1, 2 and 4 hours after dosing for rifampicin were collected from each patient. The DBS samples were then left to dry at ambient temperature for 3 hours and stored separately in a sealed plastic bag with a desiccant sachet at -20°C until analysis. A venous blood sample was taken at the same time as DBS sampling using an EDTA vacutainer. After centrifuging for 5 minutes at 3,506 xg, the plasma was obtained and stored at -20°C until analysis with a validated method [14, 26]. The correlation between DBS and plasma analytical results were assessed using simple linear regression and Deming regression to compare the analyte concentrations in plasma and DBS samples by applying the software tool Analyze-it, version 2.20 (Analyze-it Software, Ltd.) in Microsoft Excel.

RESULTS

Method development

The extraction of DBS was intensively investigated by testing various extraction solvents. With the use of methanol or its mixture with ACN, a dramatic decrease in peak height of rifampicin was observed. The addition of ACN appeared to negatively influence the recovery. With 80% of ACN in methanol, recoveries of less than 3% for all four analytes were observed (table 3).

During method development it was observed that the rifampicin peak height decreased in the presence of DBS extracts or whole blood matrix. The same effect was observed if ferric chloride was added to the neat solution at a final concentration of 50 mg/L in the extract. The assumption is that rifampicin may bind with components in the DBS matrix in which Fe^{3+} was suggested to be a potential factor [22]. Therefore, the un-fragmented mass of RIF-Fe(III) ($m/z=879$, collision energy=0 eV) was checked and a small peak at the retention time of rifampicin was observed. The efforts to adjust the chromatographic conditions did not

Table 3 Effect of different extracting solutions on matrix effects and recovery at MED level

	Extracting solution (ACN: MeOH)	Rifampicin	Desacetyl rifampicin	Clarithromycin	14-Hydroxy clarithromycin
Matrix effect (%)	0:100	-85	-37	6	9
	30:70	-81	-26	12	13
	80:20	-83	-41	3	3
Recovery (%)	0:100	40	54	75	68
	30:70	32	44	65	59
	80:20	2	1	3	2

Data calculated from 3 replications.

succeed to retrieve the chromatographic response of rifampicin. In addition, complexing agents such as EDTA and DFX could not prevent the formation of these complexes during the extraction when methanol or ACN was used.

The aqueous extraction of DBS yielded a dark red extract which was not suitable to directly inject into LC-MS/MS system. The addition of ACN and methanol to the aqueous extracts did not sufficiently precipitate the endogenous components. However, the use of an aqueous extracting solution containing EDTA can initiate the precipitation of endogenous components. With the EDTA concentration range from 0 to 1.54 g/L, the precipitation depended on the EDTA concentration. As the concentration of EDTA increased, the amount of precipitated endogenous components in the DBS increased and therefore the supernatant was cleaner. At an EDTA concentration higher than 0.58 g/L, the precipitated extracts appeared to be totally colorless and UV-VIS spectra presented no significant absorbance peak (figure 1). Although the EDTA concentration of 0.58 g/L was sufficient to precipitate endogenous components in the DBS, the EDTA concentration of 1 g/L was used to compensate for potential patient variability in blood characteristics. The precipitation of endogenous components from the aqueous extracts of DBS using EDTA and ACN was simple, rapid and can be applied to analysis methods for other drugs also.

Although the precipitated extraction was optically clean with the presence of EDTA, the peak height of rifampicin was approximately 50% of the peak height of respective neat solution. It was recovered to approximately 100% after adding DFX to the extracting solution at a final concentration of 1 g/L.

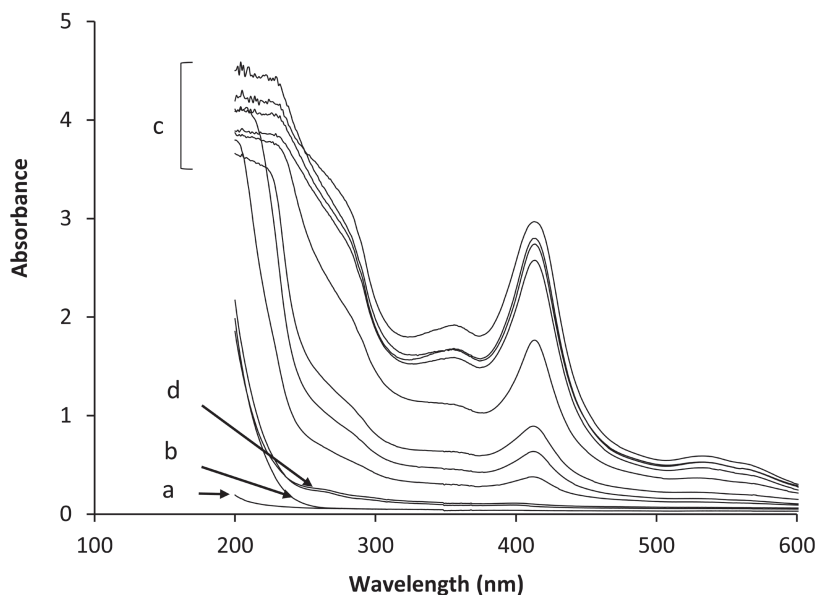


Figure 1 UV-VIS absorbance of the precipitated DBS extracts using different EDTA concentrations. A: ACN: water (v/v 3:1); B: Blank extract using EDTA 1.54 g/L in water; C: Extracts of DBS using EDTA concentrations of 0–0.38 g/L in water; D: extracts of DBS using EDTA concentrations of 0.58–1.54 g/L in water.

Method validation

Selectivity, specificity and carry over

The mean response of the blank samples accounted for less than 4.6% of the response of LLOQ samples. In addition, all six batches of human blood showed no signal higher than 20% response of the LLOQ sample prepared from the same matrix. A corresponding chromatogram is presented in figure 2. No carry-over was observed for all four analytes as the responses of the blank sample after injecting a HIGH QC sample were less than 20% of the response of LLOQ samples.

Linearity, reproducibility and dilution integrity

The calibration curves of RIF, DAC-RIF, CLR and 14OH-CLR showed to be linear with correlation coefficients (R^2) of 0.9953, 0.9971, 0.9987 and 0.9986, respectively. The linear model test based on ANOVA showed no significant lack of fit. The CV and bias ($n=3$) at each calibration level were all less than 15%.

The reproducibility of the method was assessed as the accuracy and the within day and between day precision. The accuracy and precision estimated by one-way ANOVA analysis

were tolerated with criteria of the FDA guidelines in which acceptable bias and CV are less than 20% for LLOQ and 15% for other validation concentrations (table 4).

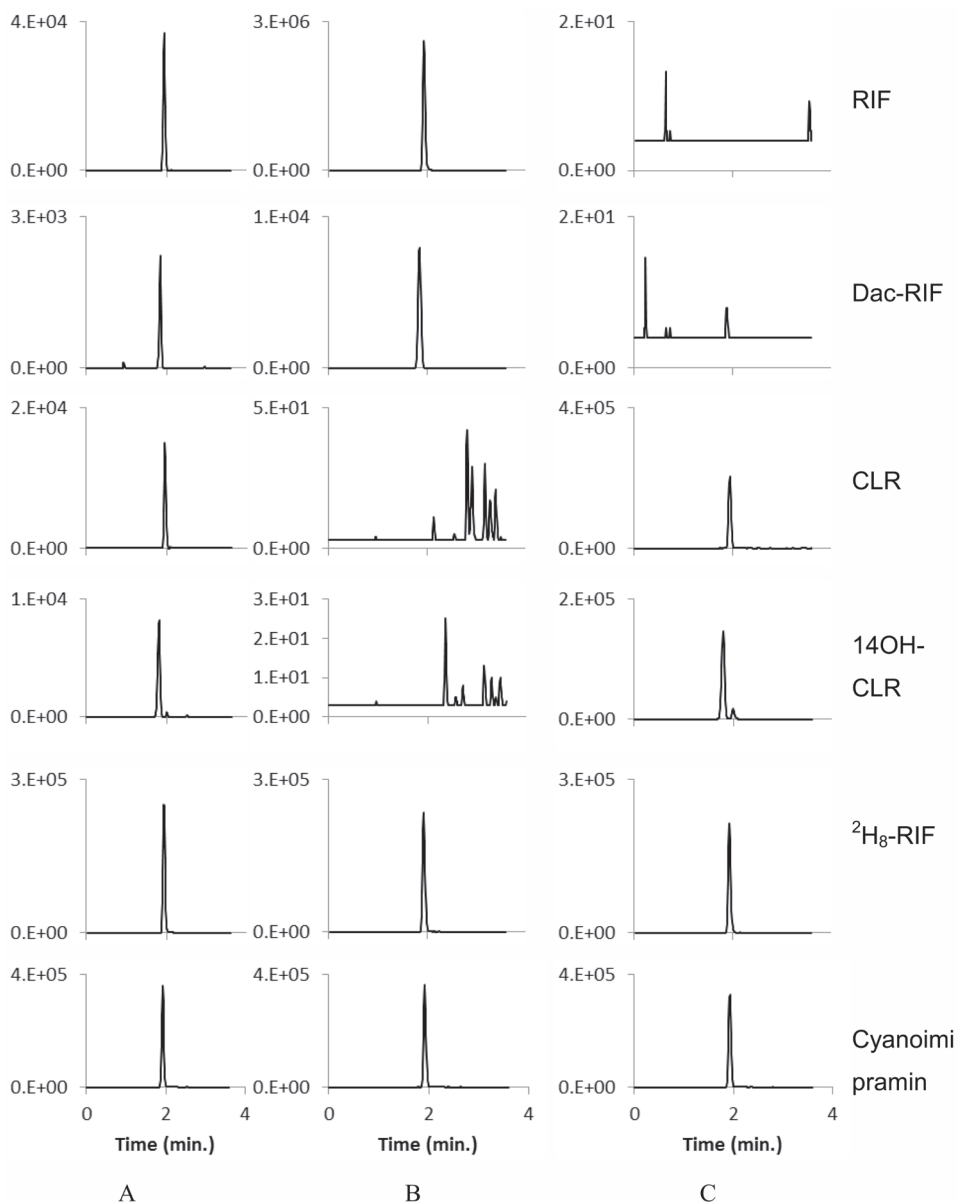


Figure 2 Representative chromatograms.

A: LLOQ DBS sample; B: DBS sample of a TB patient at $t=2h$ after administered rifampicin (DBS concentration RIF=11.00 mg/L, DAC-RIF=0.51 mg/L); C: B: DBS sample of a TB patient at $t=2h$ after administered clarithromycin (DBS concentration CLR=0.80 mg/L, 14OH-CLR=0.92 mg/L).

Table 4 Accuracy, precision and the dilution integrity (n=5)

QC level	Rifampicin					Desacetyl rifampicin				
	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC
Nominal concentration (mg/L)	0.15	0.45	15.0	24.0	60.0	0.15	0.5	5.0	8.0	20.0
Accuracy (% bias)	-1.1	1.9	1.6	-0.5	4.0	0.1	5.0	1.4	-0.1	12
Within-run precision (% CV)	5.4	2.1	3.2	2.7	3.8	12.5	6.0	4.8	4.2	4.1
Between run precision (% CV)	7.2	2.6	2.4	3.7	6.2	11.0	4.8	0.0	0.0	7.1
Overall precision (% CV)	9.0	3.3	4.0	4.6	7.3	16.6	7.7	4.8	4.2	8.2
QC level	Clarithromycin					14-Hydroxy clarithromycin				
	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC
Nominal concentration (mg/L)	0.05	0.15	5.0	8.0	20.0	0.05	0.15	5.0	8.0	20.0
Accuracy (% bias)	0.3	10.5	3.6	0.2	-4.0	-3.9	4.7	4.7	2.7	-7
Within-run precision (% CV)	9.1	3.7	2.6	2.3	3.2	10.7	5.6	3.4	3.3	3.2
Between run precision (% CV)	0.0	4.8	0.6	0.0	5.9	6.4	7.9	3.7	0.0	6.2
Overall precision (% CV)	9.1	6.0	2.7	2.3	6.7	12.5	9.7	5.0	3.3	7.0

OC: Over the calibration curve concentration (diluted 10 times).

The accuracy and precision of the 10 times diluted OC samples were within the acceptance of FDA guidance demonstrating the dilution integrity of the method (table 4).

Matrix effect, complexing effect and recovery

With the presence of ferric chloride or DBS, substantial matrix effects of at least -72% were observed, which resulted in a dramatic decrease in peak heights of RIF. This effect was neutralized by incorporating complexing agents during the extraction. With the presence of EDTA and DFX, the observed matrix effects due to DBS were +2%, +6% and -4% for the LOW, MED and HIGH levels of RIF respectively. Similar matrix effects of at least -48% were observed with DAc-RIF. The used complexing agents reduced the matrix effects. However, the matrix effects were not fully excluded as -11% to -33% matrix effects were still observed in DBS. The responses of CLR and 14OH-CLR were not influenced by the DBS matrix or the

presence of the complexing agents. High recoveries of 88% to 102% were obtained at three QC levels for CLR and 14OH-CLR. Lower recoveries of RIF and DAC-RIF between 70% and 91% were observed yielding lower process efficiencies. Nevertheless, compared to methanol or its mixtures with ACN the aqueous extraction with the use of EDTA and DFX produced 51% higher recoveries for RIF and 31% higher recoveries for DAC-RIF at the MED levels (tables 3 and 5).

Effect of hematocrit and blood spot volume

At all three QC levels of CLR and 14OH-CLR, Hct of 20% and 50% generated minor bias, which was within the acceptance limit of 15%. For RIF and DAC-RIF however, a bias of -18.4% was observed at a Hct of 20% and a bias of 28.0% was observed for a Hct of 50%. When the analytical results were corrected for their respective Hct value according to Vu et al. [19], the biases were reduced to -8.9% and 15.8% respectively.

Table 5 Matrix effects, complexing effects, recoveries and process efficiencies

Matrix	Calculation formula (*)	Rifampicin			Desacetyl rifampicin		
		LOW (%)	MED (%)	HIGH (%)	LOW (%)	MED (%)	HIGH (%)
(1) Ferric chloride	$(B/A \times 100) - 100$	-72	-83	-83	-48	-52	-49
(2) DBS	$(C/A \times 100) - 100$	-40	-66	-72	-32	-47	-50
(3) Ferric chloride with EDTA	$(E/D \times 100) - 100$	-32	-4	-9	-58	-33	-14
(4) DBS with EDTA	$(F/D \times 100) - 100$	-58	-58	-63	-59	-62	-49
(5) Ferric chloride with EDTA and DFX	$(H/G \times 100) - 100$	-3	8	-9	-31	-13	-4
(6) DBS with EDTA and DFX	$(I/G \times 100) - 100$	2	6	-4	-33	-22	-11
(7) Recovery	$K/I \times 100$	70	91	87	70	85	82
(8) Process efficiency	$K/G \times 100$	71	96	83	47	66	73
		Clarithromycin			14-Hydroxy clarithromycin		
		LOW (%)	MED (%)	HIGH (%)	LOW (%)	MED (%)	HIGH (%)
(1) Ferric chloride	$(B/A \times 100) - 100$	5	15	15	6	1	2
(2) DBS	$(C/A \times 100) - 100$	-2	6	1	-15	-7	-9
(3) Ferric chloride with EDTA	$(E/D \times 100) - 100$	17	10	8	-4	4	6
(4) DBS with EDTA	$(F/D \times 100) - 100$	6	14	11	-22	-4	-2
(5) Ferric chloride with EDTA and DFX	$(H/G \times 100) - 100$	9	10	3	5	7	4
(6) DBS with EDTA and DFX	$(I/G \times 100) - 100$	0	3	2	-15	-3	-1
(7) Recovery	$K/I \times 100$	99	102	95	88	92	90
(8) Process efficiency	$K/G \times 100$	99	105	98	75	89	90

The variation in blood spot volume resulted in from -7.8% to 8.9% for all four analytes at QC MED and HIGH. At LOW QC the blood spot volume of 30 μ L resulted in negative biases as low as -23.6, -19.4, -17.6 and -20.6% for RIF, DAc-RIF, CLR and 14OH-CLR, respectively.

Stability

A high stability was observed with CLR and 14OH-CLR in DBS in which no significant degradation occurred at ambient temperature, 37°C and 50°C for 60 days, 30 days and 15 days, respectively. RIF and DAc-RIF showed to be stable at ambient temperature for up to two months. At higher temperatures of 37°C and 50°C, RIF and DAc-RIF were stable for 10 days and for 3 days, respectively. Longer storage at temperatures over 37°C resulted in more than 15% degradation (table 6).

Clinical application study

Thirteen patients receiving rifampicin agreed to join the study. One patient experienced vomiting on the sampling day and was excluded. From the remaining twelve patients, eight pairs had RIF's concentrations under LLOQ (0.2 mg/L for plasma and 0.15 mg/L for DBS) both in plasma and in DBS. The DAc-RIF concentrations lower than LLOQ (0.2 mg/L for plasma and 0.15 mg/L for DBS) were observed in 16 pairs of samples. Two pairs of samples with DAc-RIF concentrations above LLOQ for DBS (0.19 mg/L and 0.23 mg/L) but below LLOQ for plasma were not included in the regression analysis. Four MDR-TB patients treated with CLR provided 12 pairs of DBS and plasma samples. Under LLOQ levels (0.1 mg/L for plasma and 0.05 mg/L for DBS) were observed in two pairs of samples of CLR and 14OH-CLR. The correlation results obtained with Deming regression are shown in figure 3. The slopes of the correlation lines between DBS and plasma concentration of RIF, DAc-RIF, CLR and 14OH-CLR were 0.90, 0.99, 0.80 and 1.09 respectively.

DISCUSSION

The result of this study revealed that concentrations of RIF, CLR and their active metabolites can be measured in DBS which present a good correlation with plasma. Therefore, this method is considered suitable for therapeutic drug monitoring and pharmacokinetic studies. The validated analytical method was reproducible and the DBS specimens showed high stability. In addition, a simple extraction was developed in which problems due to the interaction between the analytes and the DBS matrix was minimized.

Table 6 Long term stability under different storage temperatures (n=5)

Temperature	Time (days)	Rifampicin		Desacetyl rifampicin	
		LOW	HIGH	LOW	HIGH
Ambient	7	5.5 (4.3)	-0.6 (3.4)	1.2 (12.7)	1.1 (5.9)
	30	-2.6 (5.2)	-1.7 (2.6)	2.4 (3.2)	-0.6 (5.7)
	60	-4.3 (2.4)	-14.5 (2.2)	-3.3 (4.5)	-11.5 (5.0)
37°C	10	0.6 (4.1)	-14.1 (3.4)	-4.8 (5.8)	-11.1 (1.7)
	20	-8.9 (5.8)	-19.2 (1.7)	-8.0 (7.1)	-20.3 (2.5)
	30	-15.0 (6.8)	-28.2 (5.3)	-15.7 (8.9)	-26.4 (4.0)
50°C	1	-1.2 (6.0)	0.8 (2.6)	5.8 (6.4)	3.2 (3.7)
	3	-9.8 (4.7)	-9.1 (2.1)	-11.9 (7.3)	-5.5 (5.0)
	7	-15.7 (4.5)	-19.0 (5.5)	-18.4 (10.1)	-14.0 (5.3)
	15	-20.2 (5.0)	-24.2 (5.4)	-31.3 (12.2)	-25.8 (7.0)
		Clarithromycin		14-Hydroxy clarithromycin	
		LOW	HIGH	LOW	HIGH
Ambient	7	4.8 (7.7)	2.4 (3.8)	9.2 (9.7)	4.3 (3.3)
	30	9.2 (3.8)	0.1 (3.8)	4.5 (5.2)	2.1 (5.0)
	60	7.8 (4.8)	-5.5 (1.8)	3.0 (8.9)	-3.0 (3.9)
37°C	10	10.7 (6.3)	-7.0 (3.2)	1.4 (3.3)	-0.6 (1.5)
	20	8.0 (6.1)	-7.7 (4.8)	-1.7 (4.5)	-6.4 (4.5)
	30	13.9 (6.5)	-6.7 (3.4)	-0.2 (5.7)	-4.4 (3.4)
50°C	1	8.2 (4.1)	-0.8 (2.6)	1.5 (4.9)	3.6 (1.4)
	3	-0.7 (4.1)	3.9 (1.2)	5.7 (5.2)	8.8 (2.8)
	7	2.0 (2.8)	0.0 (5.0)	5.0 (2.6)	6.0 (7.0)
	15	9.4 (4.4)	-1.9 (3.8)	-6.9 (6.4)	-1.1 (3.0)

Results are presented as %Bias (%CV) of five replications. The bias was calculated from the nominal concentration.

The development of a simple extraction method was challenged by low recoveries and substantial matrix effects observed for RIF and DAC-RIF during extraction with ACN, methanol or their mixtures. Consequently, in the pre-validation experiment, non-linear calibration curves and irreproducible results were observed. Experiments during method development revealed that significant matrix effects occurred if DBS or blood was added to the extraction. The complex formation between RIF and endogenous components in blood extracts was assumed in a previous suggested theory [21, 22]. This complex was unstable and showed to have a slightly different retention time. During ionization, the analytes were released from their complexes. A shoulder was observed next to the RIF and DAC-RIF's peak and thus deteriorating the chromatographic quality of the analysis and lowering the peak height at the

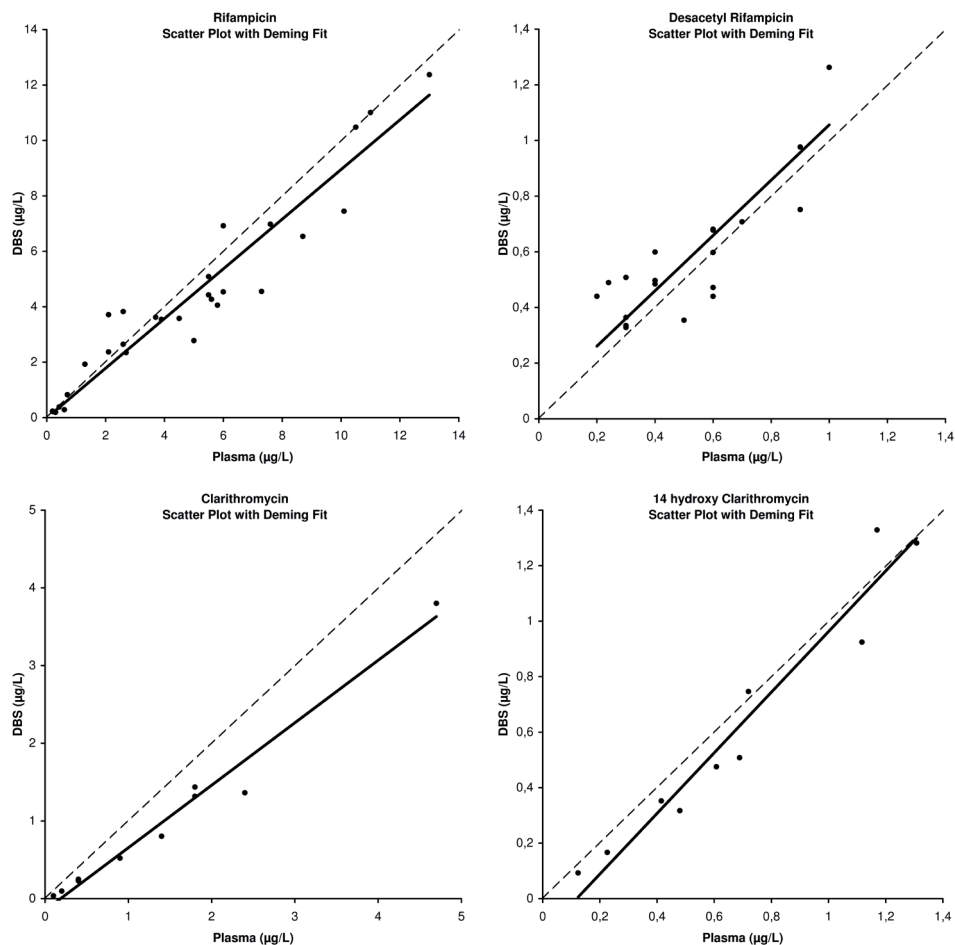


Figure 3 Clinical application study of venous blood and DBS for rifampicin, desacetyl rifampicin, clarithromycin and 14 hydroxy clarithromycin.

The dotted line is the identity line, the continuous line is the Deming regression line. Simple linear regression coefficients and Deming regression equations are the following. rifampicin: $R^2=0.9076$ ($n=28$), $y=0.90x-0.01$ (95% confidence interval (CI) slope: 0.78–1.01, intercept: -0.53–0.51); desacetyl rifampicin: $R^2=0.6856$ ($n=19$), $y=0.99x+0.06$ (95% CI slope: 0.46–1.52, intercept: -0.20–0.32); clarithromycin: $R^2=0.9752$ ($n=10$), $y=0.80x-0.15$ (95% CI slope: 0.53–1.08, intercept: -0.41–0.11) and 14 hydroxy clarithromycin: $R^2=0.9421$ ($n=10$), $y=1.09x-0.13$ (95% CI slope: 0.81–1.37, intercept: -0.29–0.03).

RIF's retention time. The incorporation of complexing agents such as EDTA and DFX aimed to prevent the formation of these complexes during the extraction was unsuccessful with the extraction using methanol or ACN. During method development it was observed that an aqueous extraction provided an appropriate environment for interaction between the DBS matrix and complexing agents. Furthermore, EDTA added to the aqueous extraction

solvent improved the precipitation of the DBS extract, resulting in a cleaner supernatant. Without EDTA, the response of RIF and DAC-RIF was negatively influenced by the dirty extract while no such effect was observed with CLR and 14OH-CLR. Interestingly, the efficacy of the precipitation appeared to be EDTA concentration-dependent. An EDTA concentration higher than 0.58 g/L showed to be sufficient for a good precipitation. The incorporation of EDTA recovered the response of the neat RIF's solution containing ferric chloride but not of the DBS extract, where the complexing effects were again strongly present at -58% to -63%. It was supposed that RIF may interact with other endogenous components in the DBS matrix rather than only the ferric ions. It was reported that DFX as a ferric chelating agent improves the analytical response of artemisinin derivatives [27]. The addition of both DFX and EDTA to the extraction solutions recovered the responses of RIF in the DBS extraction to approximately 100% (table 5).

The successful combination of EDTA and DFX for the extraction might be explained by their chelating properties. During the complex formation DFX completely covers the surface of Fe^{3+} , while EDTA is not able to completely shield the surface of the Fe^{3+} ion and forms an open (basket) complex. Although EDTA has a high stability constant for the formation of the EDTA and Fe^{3+} complex, other metal ions also form complexes with EDTA, making EDTA not a very specific chelating agent. DFX on the other hand, is known for its strong binding affinity to Fe^{3+} and less affinity to other metals, making it a specific chelating agent for Fe^{3+} . This makes DFX better suitable as a complexing agent. However, the contribution of EDTA in the developed method is 2-fold. First, it can form complexes with Fe^{3+} . Second, it aids in the precipitation of dissolved matrix after the DBS extraction which is performed by the addition of ACN [28].

The DBS sampling without a volumetric device is confronted with an analytical bias resulting from the variation of Hct or blood volume of the spot. It is generally known that as Hct values increase, and thus viscosity of the blood, DBS will become smaller and higher concentrations will be measured for the same fixed area punch. On the other hand, lower concentrations will be measured in fixed size spots at lower Hct values. As expected, this study also showed significant biases at the extreme Hct values. The correction for the Hct value can reduce this bias to an acceptable range [19]. Since the Hct values of patients may not be available for this correction, the bias up to 28% for RIF should be taken into account when interpreting analytical results to make a clinical decision. Similarly, biases of approximately 20% were observed with the blood spot of 30 μ L at LOW QC concentration. This may result from a chromatographic effect in which the analytes stay more in the centre part of the DBS. As an

acceptance criterion for the approval of a DBS during routine analysis, a minimal spot size, corresponding with a 50 μL DBS, should be applied. The use of a volumetric capillary may be considered to reduce this kind of bias [10, 23].

The clinical application study showed no significant differences between patient analysis of plasma and DBS for RIF, DAc-RIF, CLR and 14OH-CLR. The slope of CLR (0.80) showed the highest deviation from the identity line, indicating a systematic difference between plasma and DBS. The 95% confidence intervals of the Deming regression analysis showed no significant deviation from the ideal correlation. For CLR, the differences in concentrations between plasma and DBS can be clinically relevant, and based on the current results it is advised to compensate for these differences with the use of the Deming fit correlation equation. Although DAc-RIF showed no systematic difference between plasma and DBS, the correlation coefficient had a low R^2 of 0.6856. This may result in substantial errors once plasma exposure is predicted from DBS concentrations. Since DAc-RIF is equally active as RIF, the sum of both concentrations can be used to evaluate whether patient concentrations are within therapeutic range. However, DAc-RIF concentrations are about tenfold lower than their respective RIF concentrations, and will therefore be less likely to affect potential dose adjustments based on TDM. It was noticed that DBS and plasma samples under LLOQ levels were 100% matched for CLR, RIF and 14OH-CLR and 89% matched for DAc-RIF. These results showed that DBS sampling can also be useful to monitor the non-adherence of patients or patients with very low bioavailability. The results of DBS as a consequence can be used to predict the plasma exposure which is well investigated in pharmacokinetics studies. However, it would be advisable to assess a larger population for an extended clinical application study in the future.

One of the most attractive issues of DBS in bioanalysis is the stability. This study showed that RIF, CLR and metabolites were stable up to 2 months at ambient temperature of approximately 25°C. All four substances also showed to be stable at 37°C, which mimics a tropical climate and is common in the high burden areas like South Africa and Asia. In these conditions, CLR and 14OH-CLR were not affected after 2 months and RIF and DAc-RIF were stable after 10 days of storage only. CLR and its metabolite 14OH-CLR also showed a high stability at a high temperature of 50°C as no significant degradation was observed after 15 days. RIF and its metabolite DAc-RIF showed to be stable after 3 days of storage at 50°C. Although RIF and DAc-RIF were found to be less stable than CLR, 10 days at 37°C or 3 days at 50°C could be enough for transporting sample even by normal post.

In conclusion, a rapid LC-MS/MS method was developed and thoroughly validated to simultaneously quantify RIF, CLR and their metabolites in DBS. The role of EDTA and DFX as complexing agents in the extraction was well investigated and may provide a solution for potential applications to other DBS analytical methods. The clinical application study showed a good correlation between the results of DBS and conventional sampling. The long term stability of the DBS at high temperature could facilitate the TDM and pharmacokinetic studies of RIF and CLR even in resource limited areas.

REFERENCES

1. Nienhuis WA, Stienstra Y, Thompson WA et al. Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. *Lancet*. 375(9715), 664-672 (2010).
2. van Crevel R, Alisjahbana B, de Lange WCM et al. Low plasma concentrations of rifampicin in tuberculosis patients in Indonesia. *Int. J. Tuberc. Lung Dis*. 6(6), 497-502 (2002).
3. Wilkins JJ, Savic RM, Karlsson MO et al. Population pharmacokinetics of rifampin in pulmonary tuberculosis patients, including a semimechanistic model to describe variable absorption. *Antimicrob. Agents Chemother*. 52(6), 2138-2148 (2008).
4. Wallace R, Brown B, Griffith D, Girard W, Tanaka K. Reduced Serum Levels of Clarithromycin in Patients Treated with Multidrug Regimens Including Rifampin Or Rifabutin for *Mycobacterium-Avium* *Mycobacterium-Intracellulare* Infection. *J. Infect. Dis*. 171(3), 747-750 (1995).
5. Alffenaar JW, Nienhuis WA, de Velde F et al. Pharmacokinetics of rifampin and clarithromycin in patients treated for *Mycobacterium ulcerans* infection. *Antimicrob. Agents Chemother*. 54(9), 3878-3883 (2010).
6. Rodvold K. Clinical pharmacokinetics of clarithromycin. *Clin. Pharmacokinet*. 37(5), 385-398 (1999).
7. Acocella G. Clinical Pharmacokinetics of Rifampicin. *Clin. Pharmacokinet*. 3(2), 108-127 (1978).
8. van Ingen J, Egelund EF, Levin A et al. The Pharmacokinetics and Pharmacodynamics of Pulmonary *Mycobacterium Avium* Complex Disease Treatment. *Am. J. Respir. Crit. Care Med*. (2012).
9. Vu DH, Alffenaar JW, Edelbroek PM, Brouwers JR, Uges DR. Dried blood spots: a new tool for tuberculosis treatment optimization. *Curr. Pharm. Des*. 17(27), 2931-2939 (2011).
10. Edelbroek PM, Heijden JV, Stolk LM. Dried Blood Spot Methods in Therapeutic Drug Monitoring: Methods, Assays, and Pitfalls. (1536-3694) (2009).
11. Srivastava A, Waterhouse D, Ardrey A, Ward SA. Quantification of rifampicin in human plasma and cerebrospinal fluid by a highly sensitive and rapid liquid chromatographic-tandem mass spectrometric method. *J. Pharm. Biomed. Anal*. 70, 523-528 (2012).
12. Calleja I, Blanco-Prieto MJ, Ruz N, Renedo MJ, Dios-Vieitez MC. High-performance liquid-chromatographic determination of rifampicin in plasma and tissues. *J. Chromatogr. A*. 1031(1-2), 289-294 (2004).
13. Oswald S, Peters J, Venner M, Siegmund W. LC-MS/MS method for the simultaneous determination of clarithromycin, rifampicin and their main metabolites in horse plasma, epithelial lining fluid and broncho-alveolar cells. *J. Pharm. Biomed. Anal*. 55(1), 194-201 (2011).

14. de Velde F, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* 877(18-19), 1771-1777 (2009).
15. Allanson AL, Cotton MM, Tettey JN, Boyter AC. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. *J. Pharm. Biomed. Anal.* 44(0731-7085; 4), 963-969 (2007).
16. Gitau EN, Muchohi SN, Ogutu BR, Githiga IM, Kokwaro GO. Selective and sensitive liquid chromatographic assay of amodiaquine and desethylamodiaquine in whole blood spotted on filter paper. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 799(1570-0232; 1), 173-177 (2004).
17. Koal T, Burhenne H, Romling R, Svoboda M, Resch K, Kaefer V. Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 19(0951-4198; 21), 2995-3001 (2005).
18. Barfield M, Spooner N, Lad R, Parry S, Fowles S. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 870(1570-0232; 1), 32-37 (2008).
19. Vu DH, Koster RA, Alffenaar JW, Brouwers JR, Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* 879(15-16), 1063-1070 (2011).
20. Fujimoto T, Tsuda Y, Tawa R, Hirose S. Fluorescence polarization immunoassay of gentamicin or netilmicin in blood spotted on filter paper. *Clin. Chem.* 35(0009-9147; 5), 867-869 (1989).
21. Ascenzi P, Bolli A, di Masi A et al. Isoniazid and rifampicin inhibit allosterically heme binding to albumin and peroxynitrite isomerization by heme-albumin. *J. Biol. Inorg. Chem.* 16(1), 97-108 (2011).
22. Ke J, Yancey M, Zhang S, Lowes S, Henion J. Quantitative liquid chromatographic-tandem mass spectrometric determination of reserpine in FVB/N mouse plasma using a "chelating" agent (disodium EDTA) for releasing protein-bound analytes during 96-well liquid-liquid extraction. *J. Chromatogr. B Biomed. Sci. Appl.* 742(2), 369-380 (2000).
23. Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed. Chromatogr.* 24(1099-0801; 0269-3879; 1), 49-65 (2010).
24. US Department of Health and Human Services Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation (2001).
25. Timmerman P, White S, Globig S, Ludtke S, Brunet L, Smeraglia J. EBF recommendation on the validation of bioanalytical methods for dried blood spots. *Bioanalysis.* 3(14), 1567-1575 (2011).
26. Vu DH, Koster RA, Wessels AM, Greijdanus B, Alffenaar JW, Uges DR. Troubleshooting carry-over of LC-MS/MS method for rifampicin, clarithromycin and metabolites in human plasma. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* 917-918, 1-4 (2013).
27. Lindegardh N, Hanpithakpong W, Kamanikom B et al. Quantification of dihydroartemisinin, artesunate and artemisinin in human blood: overcoming the technical challenge of protecting the peroxide bridge. *Bioanalysis.* 3(14), 1613-1624 (2011).
28. Flora SJ, Pachauri V. Chelation in metal intoxication. *Int. J. Environ. Res. Public. Health.* 7(7), 2745-2788 (2010).

