



## Determination of moxifloxacin in dried blood spots using LC–MS/MS and the impact of the hematocrit and blood volume

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### ABSTRACT

Moxifloxacin (MFX) is a potential oral agent use in the treatment of multidrug-resistance tuberculosis (MDR-TB). Due to variability in pharmacokinetics and *in vitro* susceptibility of causative bacteria, therapeutic drug monitoring (TDM) of MFX is recommended. Conventional plasma sampling for TDM is facing logistical challenges, especially in limited resource areas, and dried blood spots (DBS) sampling may offer a chance to overcome this problem. The objective of this study was to develop a LC–MS/MS method for determination of MFX in dried blood spots (DBS) that is applicable for TDM. The influence of paper type, the hematocrit (Hct) and the blood volume per spot ( $V_b$ ) on the estimated blood volume in a disc ( $V_{est}$ ) was investigated. The extracts of 8 mm diameter discs punched out from DBS were analyzed using liquid chromatography tandem mass spectrometry (LC–MS/MS) with cyanoimipramin as internal standard. The method was validated with respect to selectivity, linearity, accuracy, precision, sensitivity, recovery and stability. The effect of Hct and  $V_b$  on LC–MS/MS analytical result was also investigated. The relationship between MFX concentrations in venous and finger prick DBS and those in plasma was clinically explored.  $V_{est}$  was highly influenced by Hct while the effect of  $V_b$  appeared to be different among paper types. Calibration curves were linear in the range of 0.05–6.00 mg/L with inter-day and intra-day precisions and biases of less than 11.1%. The recovery was 84.5, 85.1 and 92.6% in response to blood concentration of 0.15, 2.50 and 5.00 mg/L, respectively. A matrix effect of less than 11.9% was observed. MFX in DBS was stable for at least 4 weeks at room condition (temperature of 25 °C and humidity of 50%). A large range of Hct value produced a significant analytical bias and it can be corrected with resulting DBS size. A good correlation between DBS and plasma concentrations was observed and comparable results between venous DBS and finger prick DBS was attained. This fully validated method is suitable for determination of MFX in dried blood spot and applicable for TDM.

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### 1. Introduction

Fluoroquinolones play a crucial role in MDR-TB treatment regimen [1]. Moxifloxacin (MFX) is one of the most promising drug of this group with high *in vitro* and *in vivo* activity and is well tolerated [2–4]. Furthermore, MFX may be useful in extensively drug-resistant tuberculosis (XDR-TB) [5]. MFX appeared to be effective in shortening tuberculosis treatment if it is added to or substituted for an agent in the standard regimen [6]. In selected patient populations (e.g. HIV and tuberculosis meningitis), MFX is a potential candidate to become part of the routine treatment [7,8].

Although MFX is frequently used in the treatment of tuberculosis, the optimal dosage of MFX in tuberculosis treatment is not clearly defined [9]. The *in vitro* pharmacodynamic infection model showed optimized efficacy using a dosage of 800 mg per day, while 400 mg per day is given in daily practice [1,7,9]. Furthermore, a significant drop in area under the curve (AUC) of MFX of approximately 30% is observed if rifampicin as an enzyme inducer is administered concomitantly [10]. Taking these facts in consideration, a TDM may help to avoid too low blood levels and to improve the treatment outcomes [11]. Unfortunately, facilities for determination drug level in remote rural areas are not available. As blood samples are normally unstable at room temperature and cooled shipment is not feasible, the application of DBS sampling is a potential solution to overcome these logistical problems. Although MFX is stable in plasma at the room temperature for at least 5 days [12] long distance transport may take more time or room temperature may be exceeded during transport. DBS sampling has also other advantages including easily

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to perform; lower risk of infection and the required blood sample volume is smaller [13,14]. Although the influence of Hct and volume of the bloodspot ( $V_b$ ) were emphasized as potential confounding factors, these may vary for each drug. Therefore Hct and  $V_b$  need to be investigated in DBS method development [13–19].

Up to now, DBS method had been developed for the pharmacokinetics and TDM of a number of drugs [13,14,20]. As DBS proved its value in TDM of HIV drugs it may also help to optimize the treatment of tuberculosis (TB), especially with MDR-TB.

Clinical validation is highly recommended for DBS method development because Hct, viscosity, and components of blood may vary between patients. In the clinical validation the relation between the plasma concentration and the concentration of the drug in whole blood (plasma–blood cell partition coefficient) can be determined. The result obtained with DBS can be translated to the reference value which have been determined in serum or plasma value using the blood/plasma ratio [14].

The objectives of our study are to develop a LC–MS/MS method for determination of MFX in DBS, and to investigate the effect of influencing factors on DBS method development.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Moxifloxacin hydrochloride was provided by Bayer AG (Berlin, Germany). The internal standard, cyanoimipramine, was supplied by Roche (Woerden, The Netherlands). Acetonitrile (ACN) Lichrosolve and water for LC/MS were purchased from Bio-Solve (Valkenswaard, The Netherlands). The chemicals, including methanol (MeOH) Lichrosolve and trifluoroacetic anhydride, were of HPLC or analytical grade and were obtained from VWR (Amsterdam, The Netherlands). Three types of paper, including Whatman 31 ET CHR, Whatman 903 and Whatman N<sub>0</sub>3 were used. The punching machine (punch diameter = 8 mm) was supplied by Technical Support Facilities of the University of Leiden (NL) and designed by P.M. Edelbroek PhD, (Heemstede, the Netherlands) [14]. Packed red blood cells and pooled human serum were provided by the Department of Haematology, University Medical Center Groningen according to local regulations.

### 2.2. Sample preparation

Stock solutions of MFX were prepared as stock A and stock B by dissolving MFX HCl in water at concentration of 200 mg/L in order to make calibration standards and quality control (QC) samples. The stock solutions were diluted to working stock solutions of 10 mg/L (working stock A1 and B1).

Packed red blood cells (RBC) were centrifuged and the preserving solution was discarded. The subsequent cells were washed three times with physiological buffer and one time with serum before adding a precise volume of serum to produce blood with the Hct of 20, 35 and 50%.

Calibration blood was prepared at concentrations of 0.05, 0.15, 0.50, 1.00, 2.00, 3.50, 5.00 and 6.00 mg/L by adding stock solution A or A1. QC samples, including LLOQ (lower limit of quantitation), LOW, MED, HIGH, OC (over the calibration curve) at concentration of 0.05, 0.15, 2.50, 5.00 and 10.00 mg/L, respectively, were prepared from stock solution B and B1 by the same method. All tests for the validation were performed using the Hct of 35% except for the evaluation of the Hct effect itself.

To prepare a DBS, 50  $\mu$ L of blood was transferred onto paper by an Eppendorf pipette. It was left to dry for at least 3 h at room temperature and then preserved in a sealed plastic bags at  $-80^\circ\text{C}$ . Each QC level was prepared in six folds of which five were analyzed.

**Table 1**  
Solvent gradient programme.

Time (min)	Buffer (%)	Water (%)	Acetonitrile (%)
0	5	95	0
0.5	5	35	60
1.6	5	35	60
1.61	5	0	95
2.9	5	0	95
2.91	5	95	0
3.5	5	95	0

The extracting solution consisted of cyanoimipramine 0.03 mg/L in a mixture of methanol and water (9:1, v/v). An 8 mm diameter disc was punched out from the DBS and was then transferred to an 1.5 mL Eppendorf tube where 300  $\mu$ L of extracting solution was added. The extraction was accelerated by 60 min of sonication. After sonication 200  $\mu$ L of the extract was transferred to a polypropylene vial and 5  $\mu$ L was injected into the LC–MS/MS system.

### 2.3. Equipment and conditions

All samples were analyzed on a Thermo Fisher Scientific TSQ Quantum Discovery (Waltham, US) triple quadrupole LC–MS/MS with a Thermo Surveyor MS pump and a surveyor plus autosampler with a set temperature of  $+20^\circ\text{C}$ . Analyses were performed on a 50 mm  $\times$  2.1 mm HyPurity C<sub>18</sub> 5- $\mu$ m analytical column (Inter-science Breda, the Netherlands). The mobile phase at flow rate of 0.3 mL/min consisted of purified water, acetonitrile and an aqueous buffer (containing ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoroacetic anhydride 2 mL/L water). The buffer was maintained constant at 5% during the gradient (Table 1).

The Thermo TSQ Quantum Discovery mass selective detector was used in positive ion mode and performed selected reaction monitoring as scanning mode. The mass parameters of  $m/z$  402.0–358.2 (collision energy 19 eV) and  $m/z$  306.0–218.0 (collision energy 39 eV) were measured with scan width of 0.5  $m/z$  for MFX and cyanoimipramine detection. Ion spray voltage, sheath gas pressure, auxiliary gas pressure and capillary temperature were set at 3500 V, 35 arb (arbitrary units), 5 arb and  $350^\circ\text{C}$ , respectively. Peak height integration and quantification of the components were achieved with Xcalibur software version 1.4. SRI (Thermo Fisher Scientific, Waltham, US).

### 2.4. Method development and validation

#### 2.4.1. Method development

To determine MFX in a DBS, a fixed diameter disc is punched out from DBS. Normally, it is assumed that blood volumes in different discs are equal. However, paper type, blood viscosity, that mostly relates to Hct, and  $V_b$ , were possible factors introducing analytical bias [14,19,23]. As a consequence, we evaluated the variation in disc-weight and blood spreading with three types of paper including Whatman grade 3, Whatman 31ET CHR and Whatman 903.

From each of six paper cards taken randomly, six blank paper discs were punched out and scaled to determine disc-weight variation. In addition, a precise blood volume of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60  $\mu$ L with a Hct of 20, 35 and 50% was pipetted on the paper in 6 fold. From both sides of the blood spots, images were taken using a Nikon D60 camera. Areas of blood spots were measured by image analysis using ImageJ<sup>®</sup> software (version 1.42q). The  $V_{\text{est}}$  was calculated using the equation:

$$V_{\text{est}} = \left(\frac{1}{4}\right) \cdot \frac{\pi \cdot d^2 \cdot V_b}{S_{\text{DBS}}} \quad (1)$$

in Eq. (1),  $d$  is the diameter of the punch (8 mm);  $V_b$  and  $S_{DBS}$  are blood volume and respective DBS area. For each type of paper, linear regression analysis was performed to estimate the effect of the two parameters, Hct and  $V_b$ , on the variation of  $V_{est}$ . The changes of  $V_{est}$  over the investigated range of Hct and  $V_b$  were calculated as  $Error_{Hct} = \Delta_{Hct} b_{Hct} / \bar{V}_{est}$  and  $Error_{V_b} = \Delta_{V_b} b_{V_b} / \bar{V}_{est}$ , respectively. In which,  $\bar{V}_{est}$  is the average of  $V_{est}$ .  $\Delta_{Hct}$  and  $\Delta_{V_b}$  are the differences between the lowest and the highest values of Hct and  $V_b$  that were investigated, respectively;  $b_{Hct}$  and  $b_{V_b}$  are unstandardized regression coefficients of Hct and  $V_b$ .

To develop the extraction method, different extracting solutions including MeOH, ACN, water, the mixture of ACN:MeOH (16:84, v/v) and the mixtures of MeOH:water (10:90 and 20:80, v/v) were tested with a sonication time of 30 min. The extracting solution was selected based on the visual signs and the chromatographic response of the extraction. With this extracting solution, the sonication time of 0, 10, 30, 60 and 100 min was evaluated and the optimal value was selected. The DBS at concentration of 2.5 mg/L was used in the method development.

#### 2.4.2. Method validation

The method was validated in terms of linearity, selectivity and specificity, accuracy, precision, dilution integrity, carry-over, process efficiency and stability [21]. Validation was performed with a maximum tolerated bias and coefficient of variation (CV) of 20% for the LLOQ and 15% for the other validation concentrations. In addition, the influence of Hct and  $V_b$  were also evaluated.

On each of three consecutive days, a single calibration curve with eight concentration levels was analyzed. Calibration curves were then established using  $1/x$  weighted linear regression. Peak height ratios of MFX and the internal standard were used to calculate concentrations. Inter and intra-day reproducibility was evaluated at LLOQ, LOW, MED, HIGH and OC levels. The extract at OC level was diluted ten times with the extract of a blank DBS and the analytical result was then multiplied by ten to correct for the dilution. Selectivity and specificity were evaluated by analyzing blank and LLOQ DBS samples prepared from five different batches of real human blood. The carry-over was estimated by injecting a blank sample five times after analyzing a HIGH-level sample.

To calculate the process efficiency, 10  $\mu$ L of blank and QC (LOW, MED and HIGH) blood were used to make a spot. The whole spot was punched out and extracted. Process efficiency, which was defined as matrix effect and recovery, was calculated from peak height responses of three solutions (A, B and C). Solution A was the extract of 10  $\mu$ L-QC DBS in extracting solution. Solution B was the mixture of MFX stock solution and the extracting solution at concentrations equal to nominal values of solution A. Solution C was the extract of 10  $\mu$ L-blank DBS which was extracted by solution B. The matrix effect and recovery were determined as: matrix effect =  $(C - B)/B$ ; recovery =  $A/C$  [13].

In addition, the matrix effect was also investigated by injecting the extracts of five DBS samples derived from finger pricks of MFX free volunteers while MFX and cyanoimipramine neat solution were post-column infused [22].

The stability of processed samples after 24 and 48 h stored in the auto-sampler was evaluated by re-injecting the extracts of previous days and calibrated by a freshly prepared calibration curve. Long-term stability was investigated for  $-80^\circ\text{C}$ , room condition ( $25^\circ\text{C}$  and 50% of humidity), high temperature ( $50^\circ\text{C}$ ) and high humidity ( $\sim 100\%$  at room temperature) at 2 weeks and 4 weeks after DBS preparation. High humidity environment was created by storing DBS in a sealed plastic bag with wet tissues without contacting with DBS paper and monitored by a hygrometer.

#### 2.5. Influence of Hct and $V_b$

Tuberculosis patients have a relatively low Hct value of approximately  $35 \pm 6\%$  [23]. For this reason, Hct values of 20, 25, 30, 35, 40, 45 and 50% were evaluated using the same experiment described in Section 2.4.1. A linear regression equation between  $V_{est}$  and Hct was constructed. Furthermore, the QC level of LOW, MED and HIGH at each Hct value were prepared, analyzed and calibrated by calibration samples with a Hct of 35%. The result was then corrected by the following equation:

$$C_{corrected} = C_{observed} \frac{V_{std}}{V_{std} + b(Hct - 35)} \quad (2)$$

$C_{corrected}$  is the concentration after correcting for Hct;  $C_{observed}$  is the concentration before correcting for Hct;  $V_{std}$  is the  $V_{est}$  at standardized Hct (35%); Hct is the Hct of corrected sample;  $b$  is the regression coefficient between  $V_{est}$  ( $\mu\text{L}$ ) and Hct (%).

Blood volumes of 30, 50 and 100  $\mu\text{L}$  were used to make different DBS sizes. Discs were punched out from the central part of the DBS and analyzed in five fold. Calibration samples with a blood volume of 50  $\mu\text{L}$  were used to evaluate the influence of  $V_b$ .

#### 2.6. Clinical validation

The samples for clinical validation were taken from tuberculosis patients who received MFX 400 mg once daily orally as part of their treatment. The study protocol was approved by the local institutional ethics committee. Written informed consent was obtained from the patients. Venous blood sampling with a volume of about 3 mL was performed by nurses before the intake of the drug and at 1, 2, 3, 4 and 8 h after oral administration. At each sampling time, a venous DBS was prepared by pipetting 50  $\mu\text{L}$  of the venous blood onto the paper. The remaining venous blood was centrifuged at 3000 rpm and the plasma was withdrawn and stored at  $-20^\circ\text{C}$  until analysis. In addition, at the time of pre-dosing, 2 and 8 h post-dose, finger pricks were taken and blood was dropped directly on the paper to make the finger prick DBS. The DBS were left dried for at least 3 h then stored in a sealed plastic bag at  $-80^\circ\text{C}$  before analysis. The DBS samples were analyzed using the developed method and the plasma samples were analyzed by the routine analytical method in our laboratory [12]. The correlations between venous DBS, DBS from finger prick and plasma concentrations were evaluated by simple linear regression and Passing-Bablok regression with the help of Analyse-it<sup>®</sup> software.

### 3. Results

#### 3.1. Method development

The evaluation of the different types of filter paper showed that disc weight relates to the paper's thickness and its matrix. The variation in disc weight may contribute to the variation of  $V_{est}$  and subsequently to the analytical result. Among the three types of paper tested the Whatman 31ET CHR showed the smallest variation in disc weight with a CV of 2.3% (Table 2).

With the assumption that blood equally spreads from the center to peripheral of the DBS,  $V_{est}$  apparently represents blood volume in a punched disc. Linear regression analysis showed that Hct and  $V_b$  highly contributed to the variation of  $V_{est}$  but was not equal among different types of paper. Whatman 31ET CHR showed the highest  $R^2$  of 0.89 which means 89% of variation in  $V_{est}$  can be explained by the regression model for this type of paper. Furthermore, the effect of the  $V_b$  was of no significant influence ( $\beta_{V_b} = 0.03$ ,  $p = 0.246$ ) suggesting that blood equally spreads on this paper regardless the size of DBS. In contrast, Hct is an important predictor which explains 88% (equal to  $\beta_{Hct}^2$ ) of total variation of the  $V_{est}$ . If the results are

**Table 2**  
Variability in disc weight and the effect of Hct,  $V_b$  on  $V_{est}$  among 3 types of paper.

Paper	Disc weight (mg)		Effect of Hct and $V_b$ on $V_{est}$					
	Mean	CV (%)	Mean (SD)	$\beta_{Hct}$	$\beta_{V_b}$	$R^2$	Error <sub>Hct</sub>	Error <sub><math>V_b</math></sub>
N <sub>0</sub> 3	9.10	4.1	15.87 (1.22)	0.68*	0.41*	0.62	12.8%	9.8%
903	9.32	3.2	18.95 (2.48)	0.84*	0.13*	0.72	26.8%	5.2%
31ET CHR	9.59	2.3	19.95 (1.95)	0.94*	0.03	0.89	22.5%	0.9%

Hct was centralized at 50% and  $V_b$  was centralized at 35 (mL);  $\beta_{Hct}$ ,  $\beta_{V_b}$ : standardized regression coefficient of Hct and  $V_b$ ;  $R^2$ : total correlation coefficients of the model.

\*  $p < 0.001$ .

corrected for Hct, the accuracy of the DBS method can be improved. For the other two types of paper the lower  $R^2$  value suggested that the variation in  $V_{est}$  is less explained by the Hct and  $V_b$ . In addition, the contributions of these parameters are not unique because  $\beta_{Hct}$  and  $\beta_{V_b}$  vary among different paper types (Table 2).

The difference in Hct ranged from 20 to 50%. This produced an Error<sub>Hct</sub> of 12.8% with Whatman N<sub>0</sub>3, 22.5% with Whatman 31ET CHR and up to 26.8% with Whatman 903. With Whatman 31ET CHR the Error <sub>$V_b$</sub>  was only 0.9% and this suggests the bias was not explained by  $V_b$ . However, with Whatman N<sub>0</sub>3, the bias caused by  $V_b$  can reach 9.8%. The Whatman 31ET CHR showed a high correlation between the bias of  $V_{est}$  and Hct. Based upon these results the Whatman 31ET CHR was selected for the method development.

The extract in water was dark and not suitable for injecting into the LC–MS/MS system. We did not intend to develop another step to clean this extract because it required a more complicated procedure. In addition, MFX appeared to be poorly extracted by ACN or the mixture of ACN:MeOH (16:84, v/v) because a very low response was observed. The extract in MeOH was clear and produced a high chromatographic response. By adding 10 or 20% percent of water, the chromatographic response was improved. However, with 20% water, the extract became darker and therefore the mixture of 10% water in MeOH was selected. During the optimization of the extraction peak height responses increased with increasing sonication time. The maximum extraction performance was achieved at 60 min, where no significant difference was observed between 60 and 100 min ( $p = 0.43$ ) (Fig. 1). Therefore, 60 min of sonication was selected to be used in the method validation.

### 3.2. Method validation

#### 3.2.1. Selectivity and interference

No interfering peaks at the retention time of MFX and cyanoimipramine were observed in the chromatograms of 5 blank blood DBS samples. The responses of blank DBS samples were lower than 4% of those of the LLOQ DBS. These

results showed that the method is selective and specific (Fig. 2).

#### 3.2.2. Linearity, accuracy and precision

Calibration regression lines ( $n = 3$ ) were linear in the range of 0.05–6 mg/L with correlation coefficients ( $R^2$ ) of  $0.9986 \pm 0.0015$ . The attained regression equation is:  $y = 0.1376(\text{SD} = 0.0156)x + 0.00026$  ( $\text{SD} = 0.0014$ ).

The results of inter and intra-day reproducibility, with respect to bias and precision were within accepted range for all QC levels, with a maximum bias of  $-8.1\%$  and a maximum CV of 8.7%. The maximum bias and CV of the OC samples after correcting for the dilution were  $-11.1\%$  and 5.8%, respectively (Table 3).

During method development, carry-over was observed and re-injections of blank samples for at least 4 times were needed to totally eliminate the carry-over effect. Consequently, 5 injections of blank sample were used after a HIGH level sample to resolve carry-over during validation.

#### 3.2.3. Recovery

High recoveries of 84.5%, 85.1% and 92.6% for QC LOW, MED and HIGH were achieved and no significant matrix effect was observed (Table 4). Furthermore, no significant ion suppression or ion enhancement visually presented at the retention time of MFX (1.5 min.) or cyanoimipramine (2.0 min.) during ion suppression testing with post-column infusion (Fig. 3).

#### 3.2.4. Stability

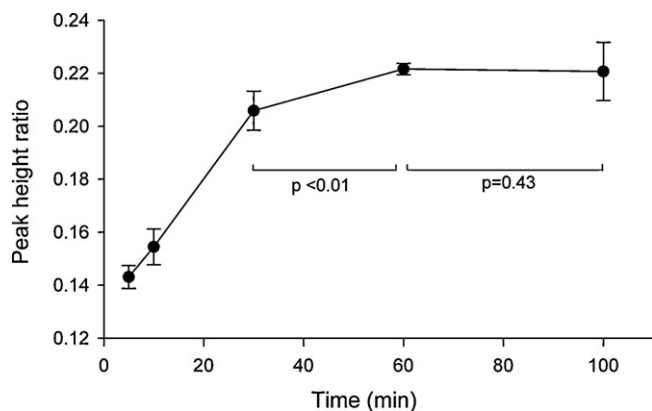
The stability of processed samples in the auto-sampler was evaluated at 24 and 48 h and complied with the validation criteria. For long-term stability, the DBS samples were stable at room conditions for 2 and 4 weeks. However, high humidity or high temperature significantly accelerated the degradation of MFX in DBS as the MFX amounts decreased up to  $-33.5\%$  bias (Table 5). This indicates that the samples should not be exposed to high temperatures and extremely high humidity.

### 3.3. Influence of Hct and $V_b$

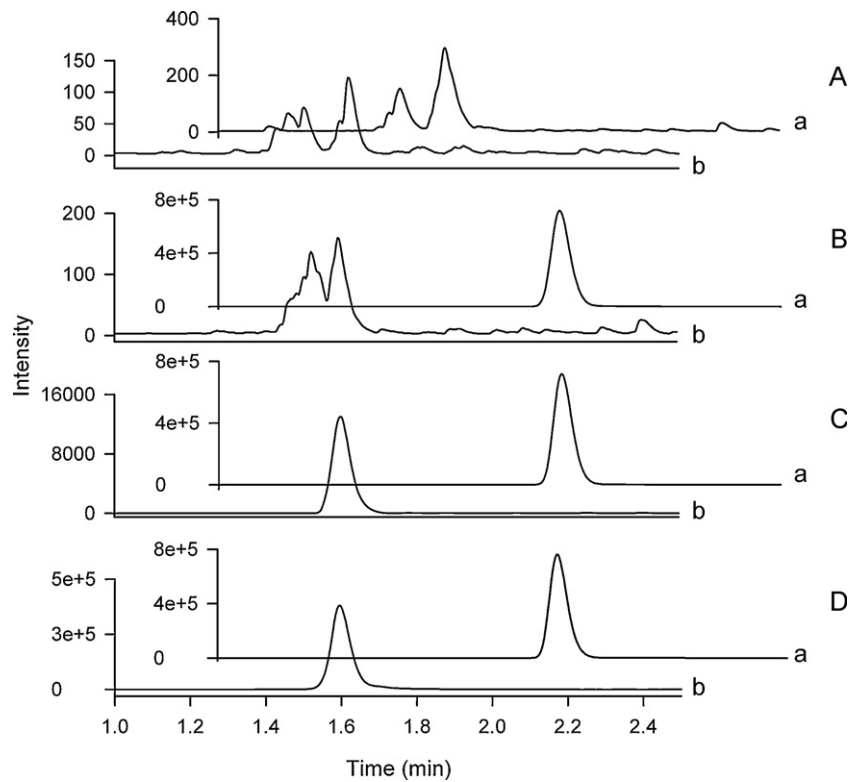
The regression equation between  $V_{est}$  and Hct was  $V_{est} = 19.98 + 0.1398 \times (\text{Hct} - 35)$  ( $R^2 = 0.81$ ).

From this result,  $V_{std}$  of 19.98 and  $b$  of 0.1398 were applied to Eq. (2). Extreme Hct percentages showed high analytical biases before correcting. The difference of uncorrected concentrations between lowest and highest Hct was approximately 40%. After correcting for Hct, the biases were lowered and fell within the accepted range of 15% (Fig. 4).

The  $V_b$  showed to have an effect on the concentration by less than 15% bias. The  $V_b$  showed to be directly proportional to the concentration of the DBS. Even though the volume of the DBS has a minor effect on the concentration of the punched area of the DBS, it does affect the accuracy of the analysis within validation requirements (Fig. 5).



**Fig. 1.** Influence of sonication time on the response of DBS extract ( $n = 5$ ). Result was presented as mean  $\pm$  SD.



**Fig. 2.** Represent chromatogram. Chromatogram of cyanoimipramin (a) and moxifloxacin (b) in a blank DBS extracted by blank extracting solvent (A); in a blank DBS (B), a LLOQ DBS (MFX = 0.05 mg/L) (C) and a patient finger prick DBS (MFX = 1.49 mg/L) (D) extracted by extracting solvent with internal standard (cyanoimipramin = 0.3 mg/L).

### 3.4. Clinical validation

For clinical validation, the plasma, venous DBS and DBS from finger prick samples were taken from 6 tuberculosis patients with hematocrit values of 26, 37, 33, 41, 35 and 38%. The simple linear regression showed excellent correlations between the plasma level and the DBS level: finger prick DBS,  $R^2 = 0.996$  ( $n = 18$ ); venous DBS,  $R^2 = 0.973$  ( $n = 36$ ). Using a Passing Bablok regression, the obtained slopes of regression lines between DBS and plasma concentrations was significantly higher than 1 (95% CI: finger prick

DBS, 1.32–1.77; venous DBS, 1.50–1.66), and thus shows systemic differences between DBS and plasma concentrations (Fig. 6). A comparable result between finger prick DBS and venous DBS concentrations was observed:  $y = 1.01x - 0.05$  (95% CI slope: 0.92–1.11 and intercept:  $-0.23$  to 0.01).

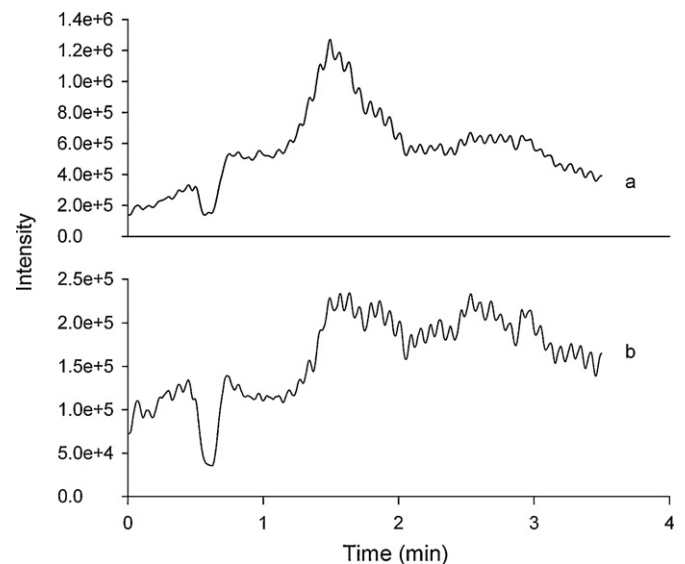
### 4. Discussion

We developed a method of analysis for routine monitoring of MFX using dried blood spot sampling. Our method

**Table 3**  
Inter-day and intra-day accuracy and precision ( $n = 5$ ).

	QC level (nominal conc.) (mg/L)	Mean conc. (mg/L)	Precision (CV %)	Accuracy (bias %)
Day 1	LLOQ (0.05)	0.053	6.8	5.8
	LOW (0.15)	0.146	8.7	-2.6
	MED (2.50)	2.572	2.4	2.9
	HIGH (5.00)	5.244	1.7	4.9
	OC (10.00)	10.343	5.5	3.4
Day 2	LLOQ (0.05)	0.051	4.9	2.8
	LOW (0.15)	0.138	3.5	-8.1
	MED (2.50)	2.360	3.3	-5.6
	HIGH (5.00)	4.705	2.4	-5.9
	OC (10.00)	8.892	2.3	-11.1
Day 3	LLOQ (0.05)	0.050	5.4	0.3
	LOW (0.15)	0.153	2.8	2.2
	MED (2.50)	2.388	5.5	-4.5
	HIGH (5.00)	4.968	3.2	-0.6
	OC (10.00)	8.955	5.8	-10.4
Inter-day	LLOQ (0.05)	0.051	5.8	3.0
	LOW (0.15)	0.146	6.9	-2.8
	MED (2.50)	2.440	5.4	-2.4
	HIGH (5.00)	4.972	5.1	-0.6
	OC (10.00)	9.397	8.7	-6.0

LLOQ: lower limit of quantification; MED: medium; OC: over the calibration curve.



**Fig. 3.** Representative ion suppression chromatograms obtained from post-column infusion experiments with blank DBS samples. (a) cyanoimipramin; (b) moxifloxacin.

**Table 4**  
Matrix effect and recovery of the DBS method ( $n=5$ ).

QC level (nominal conc.) (mg/L)	Solution	Response		Matrix effect (%) (C/B-1)	Recovery (%) (A/C)
		Mean	CV (%)		
LOW (0.15)	A	0.020	6.0	-3.4	84.5
	B	0.024	3.7		
	C	0.024	3.8		
MED (2.50)	A	0.335	2.5	-11.9	85.1
	B	0.446	5.1		
	C	0.393	1.8		
HIGH (5.00)	A	0.676	3.3	-5.1	92.6
	B	0.769	2.0		
	C	0.730	1.2		

A, B and C: response (peak high ratio) of solution A, B and C.

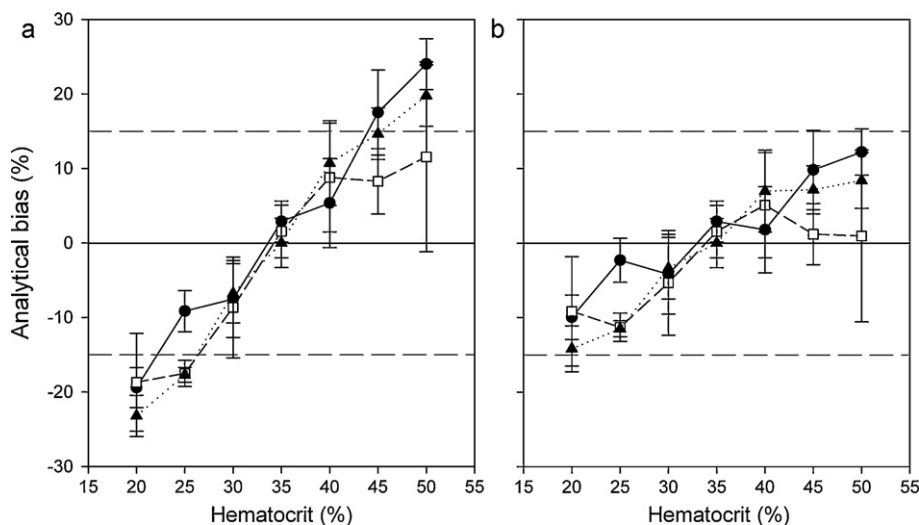
**Table 5**  
Stability after 2 and 4 weeks stored in different conditions ( $n=5$ ).

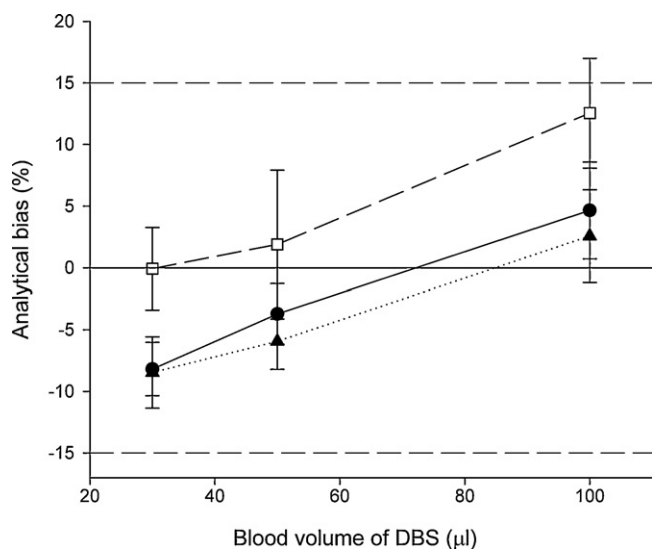
Condition	QC level (nominal conc.) (mg/L)	2-Week stability			4-Week stability		
		Mean conc. (mg/L)	CV (%)	Bias (%)	Mean conc. (mg/L)	CV (%)	Bias (%)
-80 °C	LOW (0.15)	0.156	3.5	4.1	0.161	4.3	7.4
	HIGH (5.00)	4.886	1.8	-2.3	5.324	4.9	6.5
Normal	LOW (0.15)	0.158	4.2	5.5	0.158	6.0	5.4
	HIGH (5.00)	4.660	2.9	-6.8	5.231	6.0	4.6
50 °C	LOW (0.15)	0.124	5.8	-17.1	0.112	4.5	-25.4
	HIGH (5.00)	3.869	1.4	-22.6	3.503	6.2	-29.9
Humidity	LOW (0.15)	0.117	31.9	-21.7	0.124	17.1	-17.4
	HIGH (5.00)	3.325	39.2	-33.5	4.518	14.4	-9.6

was based on punching and extracting a part of the collected blood spot using LC/MS/MS. Validation was performed according to the guidelines for bioanalytical method validation. Effect of Hct and  $V_b$  were evaluated as part of this validation. The method is suitable for clinical pharmacokinetic studies and routine monitoring of MFX in daily practice. To the best of our knowledge this is the first described validation using DBS for MFX.

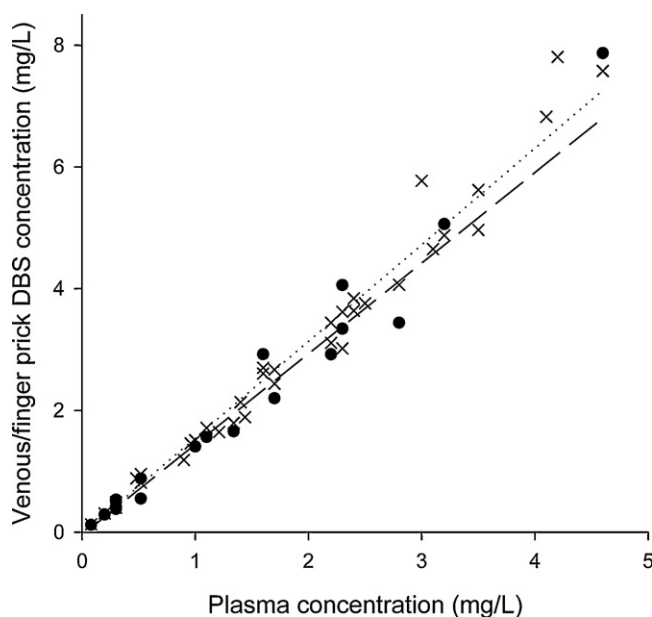
The Hct value and blood volume showed to have a relation with the size of blood spot. Blood with a high Hct shows an increased viscosity resulting in a smaller bloodspot. This factor needs to be addressed if the blood is not obtained with a volumetric capillary and only part of the spot is extracted and analyzed. However, sampling with a finger prick without volumetric capillary is easier and cheaper and can therefore be preferred in case of sampling

in remote rural areas. The impact of Hct and  $V_b$  on the accuracy of DBS analysis should be emphasised during method validation. Our results showed that with a Hct range from 20 to 50%, a significant difference of 22.5% in  $V_{est}$  was observed and therefore results should be corrected for Hct value. The correction can help to reduce the bias but seems not to totally eliminate it. The remaining small bias may be the result of other factors such as chromatographic effect in which the higher Hct, the higher concentration of MFX in the central part of DBS [13,14]. Wilhelm et al. concluded that Hct ranged from 20 to 70% produced no significant bias of cyclosporin A concentration in DBS [15]. Nevertheless, even though all biases were less than 15%, Hct of 20 and 70% seemed to produce a higher bias. In the clinical application of DBS, La Marca et al. suggested that ignoring the impact of Hct could lead to a serious error especially if Hct levels not within the normal range [20].

**Fig. 4.** Influence of Hct on analytical result and the correction ( $n=5$ ). Before correction for Hct: (a); after correction for Hct: (b); LOW: opened square, dash line; MED: closed rectangles dot line; HIGH: closed circles, solid line.



**Fig. 5.** Influence of blood volume on analytical result ( $n=5$ ). LOW: opened square, dash line; MED: closed rectangles dot line; HIGH: closed circles, solid line.



**Fig. 6.** Correlation between MFX concentration in venous/finger prick DBS and plasma. Correlation between venous DBS and plasma concentration (cross): Passing Bablok regression line (dot line,  $n=36$ ),  $y=1.59x-0.04$  (95% CI slope: 1.50–1.66, intercept:  $-0.15$  to  $0.03$ ); simple linear regression coefficient  $R^2=0.973$ . Correlation between finger prick DBS and plasma concentrations (closed circles): Passing Bablok regression line (dash line,  $n=18$ ),  $y=1.49x-0.05$  (95% CI slope: 1.32–1.77, intercept:  $-0.30$  to  $0.06$ ); Simple linear regression coefficient  $R^2=0.966$ .

In case of sampling in remote rural areas, patients' Hct values are not always available and in that case analytical bias caused by Hct seems unavoidable. However, as TB patients have a mean Hct value of 35% and our method is calibrated on this point, the resulting difference on the concentration of MFX is generally small.

In a clinical setting, it is difficult to control the size of the DBS without using a volumetric device. The effect of the size of the DBS on the bias of the result appeared to have less impact than the effect of the Hct. The concentration of the DBS showed to be directly proportional to the blood volume it was created with. Although the volume of the bloodspot showed to be of influence, the differences were well within 15% bias (Fig. 5). Because the discs were punched out from the central part of the DBS, the possible reason could be

the chromatographic effect of the paper in which MFX distributed more in the central and less at the peripheral part. This experiment proved that it is best to create bloodspots that show low variation in volume. On the other hand variation in volumes of 30–100  $\mu\text{L}$  between patient blood spots will still be within validation requirements.

An ion enhancement caused by ethylene diamine tetraacetic acid (EDTA) on MFX analysis was reported earlier [12]. We also experienced ion enhancement with DBS prepared from fresh frozen plasma that contained citrate as anticoagulant. For that reason, we selected serum to prepare DBS in which a matrix effect of less than 15% was observed.

In the clinical validation, it appeared that the MFX concentration in DBS was significantly higher than in plasma. This can be explained by the unequal distribution of MFX between plasma and blood cells caused by a difference in binding capacity to plasma proteins and blood cells [24]. The slopes of the regression lines of 1.49 (95% CI: 1.32–1.77) for finger blood/plasma ratio and 1.59 (95% CI: 1.50–1.66) for venous blood/plasma ratio showed a higher concentrations in blood than in plasma. Despite these differences, excellent regression correlations were observed. The ratio can be used to translate the DBS concentration into a plasma concentration. Comparable MFX concentrations between DBS from finger prick and venipuncture were observed and thus suggested a similar MFX concentration between the venous and the finger capillary blood. This result also confirmed that the DBS sampling from the finger prick blood without a volumetric device was as reliable as using pipette. Although the free drug concentration of MFX cannot be calculated based on a DBS result, it still enables the attending physician to make clinical decisions on dosing MFX in TB patients.

## 5. Conclusion

A rapid and fully validated LC–MS/MS method was developed for determining MFX in DBS.  $V_b$  is of minor influence compared to Hct value on the analytical result. MFX concentrations obtained with DBS are significantly higher than the plasma concentrations because of the blood/plasma ratio but show a good correlation. As MFX is stable in DBS at room conditions for at least 4 weeks, the method can facilitate pharmacokinetic studies and TDM of this drug in remote rural areas.

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