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Linezolid in multidrug-resistant tuberculosis

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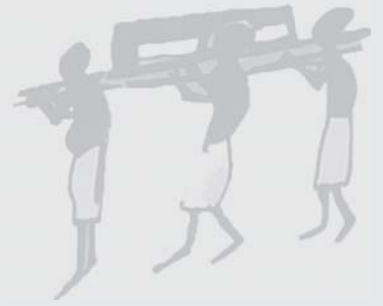
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Chapter 4A

Dried blood spot analysis for therapeutic drug monitoring of linezolid in MDR-TB patients

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ABSTRACT

Linezolid is a promising antimicrobial agent for the treatment of multidrug-resistant tuberculosis (MDR-TB), but its use is limited by toxicity. Therapeutic drug monitoring (TDM) may help to minimize toxicity whilst adequate drug exposure is maintained. Conventional plasma sampling and monitoring might be hindered by logistic problems in most parts of the world that may be solved by dried blood sampling (DBS). The aim of this study is to develop and validate a novel method for TDM of linezolid in MDR-TB patients using DBS.

Plasma, venous DBS and capillary DBS specimens were obtained simultaneously from eight patients receiving linezolid. A DBS method was developed and clinically validated by comparing DBS with plasma results using Passing-Bablok regression and Bland-Altman analysis.

This study showed that DBS analysis was reproducible and robust. Accuracy and between and within-day precision from three validation presented as bias and CV were less than 17.2% for lower limit of quantification level and 7.8% for other levels. The method showed a high recovery of approximately 95% and a low matrix-effect of less than 8.7%. DBS specimens were stable at 37°C for 2 months and at 50°C for one week. The concentration ratio of DBS/plasma was 1.2 (95% CI: 1.12 – 1.27). Linezolid exposure calculated from DBS and plasma showed good agreement.

In conclusion, DBS analysis of linezolid is a promising tool to optimize linezolid treatment in MDR-TB patients. Easy sampling procedure and high sample stability may facilitate TDM, even in underdeveloped countries with limited resources where conventional plasma sampling is not feasible.

INTRODUCTION

Linezolid is used as a second line drug in the treatment of multidrug-resistant tuberculosis (MDR-TB) due to its efficacy *in vitro* (21), *in vivo* (9) and in patients (1, 2, 14, 17, 34) against *Mycobacterium tuberculosis*. The World Health Organization (WHO) classifies linezolid as a reserve anti-tuberculosis drug for the treatment of multidrug-resistant/extensively drug-resistant tuberculosis (MDR/XDR-TB) (33). Linezolid is usually added to a treatment regimen consisting of anti-tuberculosis drugs for which the *Mycobacterium tuberculosis* is still susceptible. However, treatment with linezolid may be limited by toxicity, such as time- and dose-dependent neuropathy or myelosuppression (17, 29), urging dose reduction or cessation of treatment with linezolid. Therapeutic drug monitoring (TDM) can be used to implement dose reductions to limit toxicity, whilst preventing inadequate exposure. Efficacy predicting pharmacokinetic / pharmacodynamic (PK/PD) parameters, such as the area under the concentration-time curve to MIC ratio (AUC_{0-24h}/MIC), might be helpful in evaluating linezolid dosages (1, 7, 26, 32). The AUC_{0-24h}/MIC has been shown to be the best predictive model in a murine model (32), but evidence from human data are lacking. Further PK/PD data from TB-programs or large studies are needed for the development of evidence based PK/PD parameters, such as an AUC_{0-24h}/MIC ratio target.

Linezolid treatment has been evaluated for TB treatment, in several case series (17, 23). However, neither drug susceptibility testing (DST) nor drug exposure assessment was performed for linezolid, making it difficult to draw conclusions on efficacy (5). For instance, drug-interactions with other antimicrobial agents might have occurred and may have had an impact on linezolid exposure (6, 15). In addition, conventional drug exposure evaluation for TB drugs using plasma samples might have been hindered in these studies by logistical challenges (30). The use of dried blood spot (DBS) sampling may provide a helpful alternative to conventional plasma sampling through simplified sampling procedure and increased sample stability. DBS sampling has been applied in the treatment of other infectious diseases like malaria and HIV (30). Other advantages may include a lower required blood sample volume and lower biohazard risk of DBS samples compared to conventional plasma samples (12, 18, 30). Compared to conventional sampling, DBS sampling may be hindered by inter and intra-patient hematocrit (Hct) variation causing different blood viscosity yielding a proportional analytical bias with Hct value. Furthermore, Hct may affect the drug blood / plasma partition ratio complicating the comparison with conventional plasma samples. In the development of a bioanalytical method for linezolid using DBS analysis important

patient related factors like blood spot volume, Hct value (3, 24) and difference between capillary and venous blood, have to be assessed during validation (12, 18, 25, 30). To enable individualized linezolid treatment the aim of this study was to develop and validate a method for DBS analysis and evaluate it in MDR-TB and XDR-TB patients.

MATERIALS AND METHODS

Patients

From September 2010 to March 2012, MDR-TB patients (≥ 18 years) were recruited from the Tuberculosis Centre Beatrixoord, University Medical Center Groningen (Haren, The Netherlands). Eligible for inclusion were patients receiving treatment with anti-tuberculosis drugs for which routine therapeutic drug monitoring was scheduled. Patients with bleeding disorders were excluded from the study. The study procedures were reviewed and approved by the local Ethics Committee. Patients receiving linezolid were included after providing written informed consent.

Sampling was performed at least one week after the start of linezolid treatment to ensure the steady-state was achieved. Venous blood samples were obtained our before drug intake and at 1, 2, 3, 4 and 8 hours after dosing according to a previous study (2) and local procedures for TDM of TB drugs to be able to calculate drug exposure and other PK parameters. Venous dried blood spot (vDBS) specimens were prepared by pipetting 50 μL venous blood onto Whatman 31 ET CHR paper. The remaining venous blood was centrifuged at 3000 rpm for 5 minutes at room temperature to attain plasma which was stored at -20°C until analysis. DBS specimens were obtained through a finger prick by dropping the blood directly on dried blood spot paper. DBS samples were obtained before drug intake, 2 and 8 hours after dosing, representing low, high and medium linezolid blood levels respectively. Both the vDBS and DBS samples were left to dry at room temperature and stored in sealed plastic bags with desiccant sachets at -20°C until analysis.

DBS analysis

To quantify DBS samples an 8 mm-diameter disc was punched out of each blood spot. Extraction of these discs was performed by sonication with a frequency of 47 kHz during a period of 20 minutes using 500 μL of extracting solvent consisting of cyanoimipramine

0.3 mg/L (internal standard) and EDTA 1 g/L in water. From this solution, a volume of 200 μ L was added to 750 μ L of acetonitrile. The samples were vortexed for 1 minute and subsequently centrifuged at 11000 rpm for 5 minutes. An injection volume of 5 μ L was analyzed using a validated LC-MS/MS analysis method (16). The plasma samples were prepared and analyzed using the same method.

DBS method validation

The DBS analytical method was validated in accordance with the recommendation of US Food and Drug Administration's (FDA) *Guidance for Industry Bioanalytical Method validation* (27). For the validation, blood was prepared by mixing plasma, red blood cell and linezolid stock solution to achieve blood at desire concentration and Hct. Subsequently, the validation DBS samples were prepared by pipetting 50 μ L of blood onto the paper. Linearity was assessed with $1/x^2$ weighting over a concentration range of 0.05 – 40 mg/L. Clinical relevant concentrations were well within the range of the assay standards (2). The within-day and between-day accuracy and precision were evaluated on four validation levels of LLOQ (lower limit of quantification), LOW, MED and HIGH at concentrations of 0.05, 0.25, 15 and 30 mg/L, respectively. Each validation level was analyzed in fivefold on three consecutive days. The matrix effect and the recovery of linezolid from DBS were determined using a common method (18, 31). The stability of DBS specimens was assessed by storing validation DBS at ambient condition and 37°C after one week, two weeks and two months. As a worst case scenario the stability of DBS specimens was also assessed at 50°C after one day, two days and one week. The stability was evaluated at LOW and HIGH levels in fivefold by comparing the analytical result with the nominal concentrations. In addition to the criteria suggested in the FDA guideline (27), the impact on assay accuracy and precision due to the variations of Hct and blood spot volume were evaluated. For these purposes, Hct of 20, 25, 30, 35, 40, 45 and 50%, and blood spot volumes of 30, 50, 70 and 90 μ L were assessed. During the method validation, blood spot volume and Hct were standardized at 50 μ L and 35%, respectively. The set of Hct of 35% reflects the Hct in tuberculosis patients (3).

Pharmacokinetic and pharmacodynamic evaluation

Pharmacokinetic parameters were evaluated using a non-compartmental model of the KINFIT module of MW Pharm (version 3.9; Mediware, The Netherlands). The AUC_{0-12h} was calculated using the trapezoidal rule from 0 up to 12 hours and the AUC_{0-24h} by doubling the

AUC_{0-12h} . The maximum concentration (C_{max}) was defined as the highest observed linezolid concentration with t_{max} as corresponding time. The elimination half-life ($t_{1/2}$) was calculated by dividing the natural logarithm of 2 ($\ln 2$) by the elimination constant (k_e) as calculated by MW Pharm. The apparent clearance (Cl) of linezolid was calculated by dose/ AUC_{0-12h} . The volume of distribution (V_d) was calculated by dividing Cl with k_e .

The drug susceptibility testing of the *Mycobacterium tuberculosis* isolates was performed at the Dutch National Mycobacteria Reference Laboratory (National Institute for Public Health and the Environment; RIVM) using the Middlebrook 7H10 agar dilution method (28). The AUC_{0-24h}/MIC ratio, often used as a predictive pharmacodynamic parameter for efficacy, was calculated (32).

Statistics

In the method validation, the bias was defined as the difference (in percentage) between analytical result and the nominal concentration. The method was clinically validated by comparing the concentrations of DBS and vDBS with plasma concentrations using Passing-Bablok regressions and Bland-Altman analysis by applying the software tool Analyse-it 2.20® (Analyse-it Software, Ltd). Conversion factors, calculated from geometric mean (v)DBS/plasma concentration ratios, were used to calculate converted DBS and vDBS concentrations(4). Subsequently, the converted concentrations were used to calculate the AUC_{0-12h} of DBS and vDBS. The agreement between AUC_{0-12h} value of converted DBS and plasma was evaluated using Bland-Altman analysis. Spearman correlation and Wilcoxon signed-rank test was applied to other comparisons.

RESULTS

Patients

Eight patients with a median (IQR) age of 29 (24 – 33) years were included in this study. The baseline characteristics are presented in Table 1. The median (IQR) of Hct was 37.4 (33.0 – 41.4) %. At time of the study three of eight patients received linezolid 300 mg twice a day and five patients in a dose of 600 mg twice daily. Isolates of seven patients showed resistance to first-line drugs isoniazide, rifampicin, ethambutol, pyrazinamide, and streptomycin. The isolate of one patient showed resistance to all first-line drug except pyrazinamide. All DSTs

Table 1 Baseline demographics of study patients (n=8)

Parameter	Value
Age (y) ^(#)	29 [24 – 33]
Sex (n)	
Male	2
Female	6
Bodyweight (kg) ^(#)	60.5 [55.8 – 61.5]
Height (m) ^(#)	1.69 [1.67 – 1.76]
Body mass index (kg/m ²) ^(#)	20.1 [19.0 – 21.4]
Ethnicity (n)	
Caucasian	4
African	3
Asian	1
Co-morbidity (n/N)	
HIV	1/8
Hemoglobin (g/L) ^(#)	7.0 [6.4 – 8.8]
Hematocrit (v/v*100%) ^(#)	37.4 [33.0 – 41.4]
TB treatment other than linezolid (n)	
Moxifloxacin	7
Amikacin/Kanamycin	6
Ethambutol	5
Cotrimoxazole	3
Clofazimine	2
Ertapenem	2
Pyrazinamide	1

^(#): median [interquartile range].

revealed resistance for rifabutin, whereas one isolate showed fluoroquinolone-resistance and three protonamide-resistance. None of the patients experienced significant discomfort from the finger pricks during DBS sampling which was supported by the fact that all completed the three consecutive samples in this study.

DBS method validation

The DBS assay method showed linearity over the analytical concentration range. The pooled correlation coefficient was $r^2 = 0.9947$. The regression equation is: concentration = $(0.1635 \pm 0.0025) \times \text{response} + (0.0001 \pm 0.0003)$. Within-day and between-day accuracy and precision showed CVs within accepted range. Within-day CVs ranged from 1.6% to 13.8% and between-day CVs from 3.5% to 10.2%. The mean measured concentration was within 98.7%

to 106.3% of the nominal concentration. The bias caused by variable matrices, i.e. DBS and EDTA matrices, was less than 8.7%. The recovery of DBS extraction was between 94.1% and 97.2%. No significant linezolid degradation was observed after storing DBS at 50°C for at least one week and at 37°C or ambient temperature for two months as biases were less than 15%.

Variation of blood spot volume between 30 µL to 90 µL had a minor impact on the assay accuracy as the bias ranged from -11.6% to 7.1%. The variation of Hct from 20% to 50% yielded biases within -7.6% to 6.8% and -12.5% to 5.7% for MED and HIGH level. Larger biases of -17.8% to 11.9% were observed at the LOW concentration level (0.25 mg/L) (Table 2).

Comparisons of DBS, vDBS and plasma analysis

Significant proportional biases were observed in Passing-Bablok regressions in which the slope of regression line between DBS and plasma was 1.28 (95% CI: 1.13 – 1.44) and vDBS and plasma was 1.46 (95% CI: 1.40 – 1.54). The intercepts were -0.42 (95% CI: -1.72 – 0.17) and -0.67 (95% CI: -1.36 – -0.09), respectively (Figure 1). In Bland-Altman analysis, the geometric mean concentration ratios of DBS and vDBS versus plasma were 1.20 (95% CI:

Table 2 Summarized results of the validation of DBS analysis

Validation criteria	Validation levels (n=5)			
	LLOQ	LOW	MED	HIGH
Nominal concentrations (mg/L)	0.05	0.25	15	30
Reproducibility ^(#)				
Accuracy (% Bias)	4.5	6.3	3.2	-1.3
Within-day precision (% CV)	13.8	4.0	4.1	1.6
Between-day precision (% CV)	10.2	3.5	6.1	7.7
Overall precision (% CV)	17.2	5.3	7.4	7.8
Matrix effect (%)		2.9	8.7	1.9
Recovery (%)		95.5	94.1	97.2
Effect of blood volume (range of % Bias) ^(#)		-2.9 – 4.1	-11.4 – 7.1	-11.6 – 9
Effect of hematocrit (range of % Bias) ^(#)		-17.8 – 11.9	-7.6 – 6.8	-12.5 – 5.7
Stability ^(#)				
1 week at 50°C (% Bias)		6.7		-3.4
2 months at 37°C (% Bias)		-10		-5.9
2 months at ambient temperature (% Bias)		-2.5		-2.0

^(#): data from 3 separated validation days; ^(#): comparison with samples of standardized hematocrit (35%); ^(#): comparison with sample of standardized blood spot volume (35 µL); ^(#): present data from the last time of the period only.

1.12 – 1.27) and 1.36 (95% CI: 1.32 – 1.40), respectively. The ratio of vDBS/plasma was higher than that of DBS/Plasma (Wilcoxon signed-rank test, $n=24$, $p<0.01$). 95% limits of agreement were shown with less than 5% of the values falling out of the ranges (Figure 2).

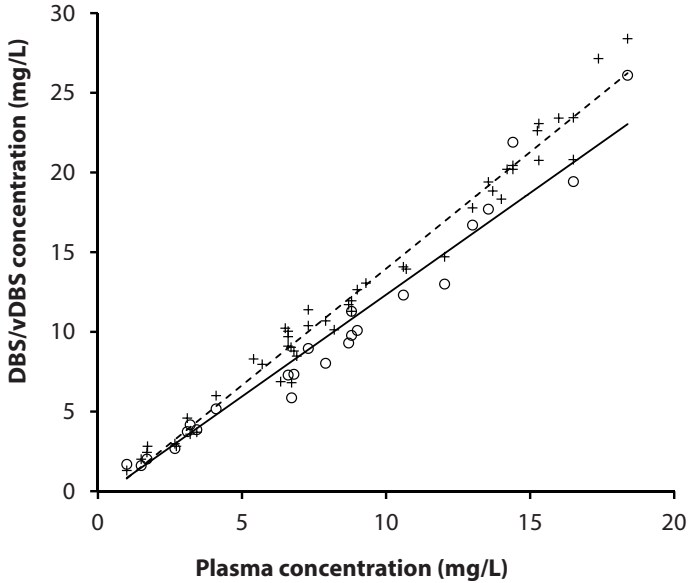


Figure 1 Passing-Bablok regression between measurements in DBS/vDBS and plasma.

-----: vDBS-plasma Passing-Bablok regression: slope = 1.46 (95% CI: 1.40 – 1.54), intercept = -0.67 (95% CI: -1.36 – -0.09); ———: DBS-plasma Passing-Bablok regression: slope = 1.28 (95% CI: 1.13 – 1.44), intercept = -0.42 (95% CI: -1.72 – 0.17).

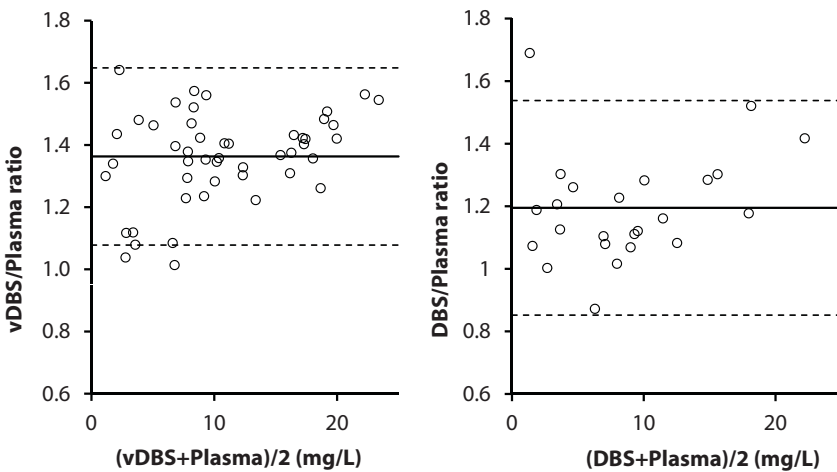


Figure 2 Bland-Altman plot of concentration ratios of DBS and vDBS vs. plasma.

———: Mean ratio; -----: Limit of agreement (mean ratio \pm 1.96 \times SD ratio).

Pharmacokinetic and pharmacodynamic evaluation

A median (IQR) plasma AUC_{0-12h} of 50.9 (50.5 – 54.9) mg^*h/L was observed following a dose of 300 mg and 126 (121.6 – 127.6) mg^*h/L following a dose of 600 mg. Linezolid pharmacokinetic parameters are shown in Table 3. The concentration-time curves of plasma, DBS and vDBS are presented in Figure 3.

The AUC_{0-12h} values of DBS and vDBS were calculated using the converting factors 1.20 and 1.36 for DBS and vDBS respectively. The subsequent result showed a good agreement with

Table 3 Steady state pharmacokinetic parameters of linezolid in plasma (n=8)

Parameter	300 mg linezolid twice a day (n=3)	600 mg linezolid twice a day (n=5)
AUC_{0-12h} (mg^*h/L)	50.9 [50.5 – 54.9]	126.9 [121.6 – 127.6]
C_{max} (mg/L)	8.8 [7.8 – 8.9]	16.5 [14.4 – 16.5]
T_{max} (h)	1.9 [1.9 – 4.8]	1.9 [1.7 – 3.0]
$T_{1/2}$ (h)	4.6 [4.0 – 6.9]	7.5 [7.3 – 7.9]
Cl (L/h)	4.9 [3.8 – 5.1]	3.1 [3.0 – 3.1]
Vd (L)	32.6 [29.4 – 34.4]	34.8 [32.9 – 41.6]

Data are presented as median [interquartile range].

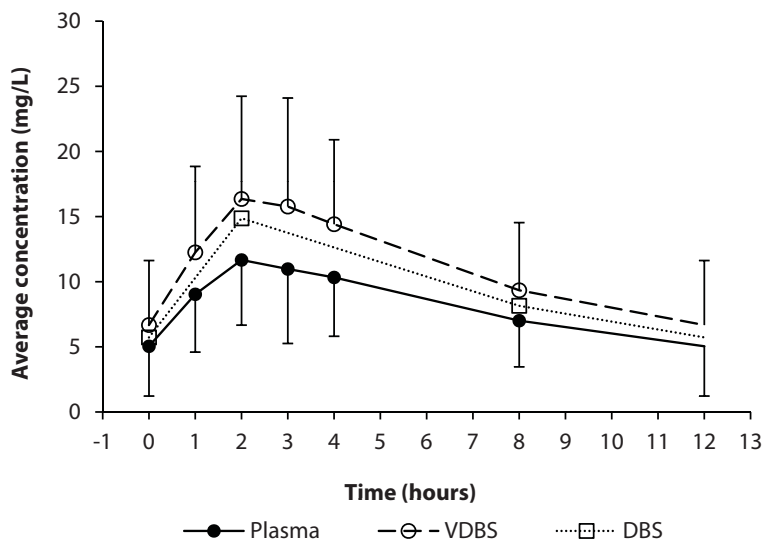


Figure 3 Concentration-time curves of linezolid in plasma, vDBS and DBS.

Plasma and vDBS data are presented as mean and SD. For visual purposes, the DBS data are presented as mean without error bar.

plasma. All the values were within the 95% limit of agreement (Figure 4). The individual data for each patient for AUC_{0-12h} attained from plasma and converted (v)DBS concentrations and the respective AUC_{0-24h}/MIC values are presented in Table 4. Patients that received a linezolid dose of 300 mg twice daily (n=3) had a median (IQR) plasma AUC_{0-24h}/MIC ratio of 236 (219 – 322) mg*h/L and patients that received 600 mg twice daily (n=5) had a median (IQR) plasma AUC_{0-24h}/MIC ratio of 508 (486 – 1398) mg*h/L.

Table 4 Pharmacokinetic and pharmacodynamic parameters of linezolid using plasma, vDBS and DBS concentrations

Patient	Dose (2d.d)	MIC (mg/L)	AUC_{0-12h} (mg*h/L)			AUC_{0-24h}/MIC		
			Plasma	vDBS ^(#)	DBS ^(#)	Plasma	vDBS ^(#)	DBS ^(#)
1	300	0.5	50.1	46.7	51.5	201	187	206
2	300	0.25	50.9	54.2	50.7	407	433	405
3	600	0.5	121.6	118.1	112.1	486	472	449
4	600	<0.125	127.6	130.9	114.2	>2042	>2094	>1827
5	600	0.5	126.9	132.0	140.2	508	528	561
6	600	0.5	66.6	69.4	64.2	266	278	257
7	300	0.5	58.9	46.1	42.4	236	184	170
8	600	0.25	174.7	183.1	189.6	1398	1465	1517

^(#): relative AUC_{0-12h} and AUC_{0-24h}/MIC calculated using conversion factors (i.e. 1.20 for DBS and 1.36 for vDBS).

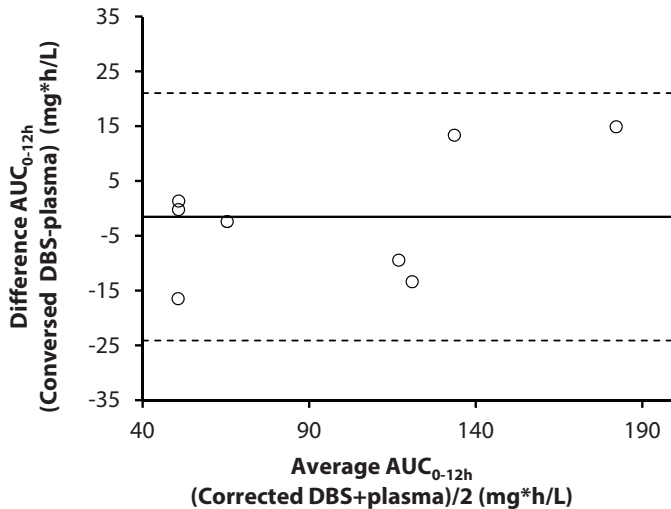


Figure 4 Bland-Altman plot of AUC_{0-12h} from corrected DBS vs AUC_{0-12h} plasma samples.
 — : Mean difference; - - - : Limit of agreement (mean difference \pm 1.96 \times SD difference).

DISCUSSION

This study showed that DBS analysis is an easy tool to individualize MDR-TB treatment with linezolid. In addition, this report presents a novel, validated method of analysis of linezolid in dried blood spots, with specimens that proved to be very stable over time.

In previous studies on DBS analysis of other drugs, several technical factors were pointed out that have to be considered when interpreting DBS analysis, such as the effect of Hct and blood spot volume (12, 13, 18, 30). For the analysis of linezolid in DBS, the effect of Hct seemed to be of minor concern. In this study, biases fell within accepted ranges for Hcts between 20 – 50%. These Hcts cover an even broader range than clinical Hcts found in TB patients in literature, i.e. $35.4 \pm 6.7\%$ (3), and in this study $37.4 \pm 4.4\%$. Based on these findings, the standardization of Hct at 35% during DBS validation is acceptable. Furthermore, variation of blood spot volume between 30 – 90 μL had little effect as biases were within 15%.

Despite the minor influence of technical factors, i.e. Hct value and blood spot volume, physiological factors are also mentioned in literature to possibly limit the applicability and interpretation of DBS analysis (13). Such a factor might be differences between plasma concentration and whole blood concentration. This study shows that concentration of linezolid is higher in blood than in plasma. This is caused by different binding capacity to plasma proteins and blood cells. Furthermore, concentrations of linezolid were higher in vDBS than in DBS. This might be caused by differences between the capillary and venous blood (13, 25, 30). Nevertheless, the concentration of DBS and vDBS specimens, both showed good correlation with plasma concentration. To compensate for these differences, we propose conversion factors of 0.83 (1 / 1.20) for DBS and 0.74 (1 / 1.36) for vDBS to calculate corresponding plasma values. After the conversion, good agreement between $\text{AUC}_{0-12\text{h}}$ of DBS and plasma was observed.

A meta-analysis showed that a ≤ 600 mg linezolid daily dose resulted in lower frequency of either adverse event or adverse events necessitating treatment discontinuation than the dose of > 600 mg daily (8). Among the published data, the lowest rate of adverse effects was observed with a dose of 300 mg once daily (17). Nevertheless, lowering the dose clearly results in lower exposure to the drug (2, 19). In combination inter-patient variability and possible drug-drug interactions, under or overexposure may occur. Therefore, treatment with a fixed dose may be questionable (6, 11, 22, 26). The application of TDM for linezolid can help avoid under- or overexposure which may occur in 30 to 40% of the cases (20).

In this study, all patients had *Mycobacterium tuberculosis* isolates with a MIC ≤ 0.5 mg/L for linezolid. With a dose of 600 mg (n=5) twice daily, very high AUC_{0-24h}/MIC ratios were reached (10), so dose reductions could be implemented to prevent time- and dose-dependent toxicity. Furthermore, a high correlation of AUC_{0-24h}/MIC values between converted DBS and plasma (Spearman's rho = 0.976, n=8) was observed. This suggests that TDM using DBS may result in identical interventions compared with conventional plasma sampling. Therefore, adaptive dosing of linezolid to prevent potential toxicity and to assure therapeutic exposure is feasible using DBS.

The high stability of DBS specimens can minimize the logistic burden of conventional sampling in limited-resource areas. With a simple instruction, the DBS samples can be performed easily and sent to equipped facilities for analysis by mail (12, 30). This could enable the TDM in TB-programs worldwide including resource limited settings where MDR/XDR-TB epidemic is a growing problem. TDM using DBS for MDR/XDR-TB should be especially considered in areas where HIV or malaria co-infections are highly prevalent as DBS has been successfully applied to monitor the treatment of such diseases (30).

Since treatment of MDR/XDR-TB is long and complicated by adverse drug reactions, TDM of linezolid with DBS could be used to optimize drug exposure during treatment. In conclusion, this study presents a novel, validated analysis of linezolid in DBS specimens that is suitable for optimization of linezolid treatment of MDR-TB. Advantages include a very simple, low biohazard risk sampling method using a finger prick, easy logistics and very good stability of DBS specimens.

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