Exploring strategies to individualize treatment with aminoglycosides and co-trimoxazole for MDR Tuberculosis
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Dried Blood Spot Analysis for Therapeutic Drug Monitoring of Co-trimoxazole in Patients with Tuberculosis

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In preparation
ABSTRACT

Co-trimoxazole, consisting of sulfamethoxazole and trimethoprim, is used for the prevention of infections with Pneumocystis jiroveci pneumonia (PCP) in HIV-infected patients. In addition, data from HIV-TB co-infected patients suggest that co-trimoxazole may be of added value in the treatment of TB.

Unfortunately, the blood concentrations of sulfamethoxazole are highly variable between individuals, and this variation may attribute to its toxicity. Dried blood spots (DBS) have shown to be a reliable alternative to venous blood sampling, notably due to simple collection strategy and superior sample stability.

We developed a liquid chromatography tandem mass spectrometry analysis of sulfamethoxazole and its toxic metabolite, sulfamethoxazole-N-acetyl to quantify both compounds in DBS cards. The method was validated according to FDA and EMA guidelines and clinically validated.

The stability of the compounds on the dried blood spots was acceptable with a bias of maximal -7.5% and -7.0% after 14 days at 50 °C and 1 month at 37 °C, respectively. The median difference in the area under the curve calculated on DBS compared to plasma samples was -5.8% (IQR -6.25 - -0.13%).

This newly developed method showed to be reliable and robust and was fully validated. The stability of both compounds was sufficient to transport DBS cards from developing countries to a sophisticated laboratory to perform TDM. This method can be used in daily patient care and in future prospective pharmacokinetic studies exploring the use of sulfamethoxazole for TB treatment.

INTRODUCTION

Tuberculosis (TB) is a life threatening disease killing approximately 1.5 million people every year. TB is an infectious disease caused by Mycobacterium tuberculosis. Multidrug resistant (MDR) M. tuberculosis is resistant to at least isoniazid and rifampicin, the two most powerful first line anti-tuberculosis drugs. The spread of MDR-TB is increasing, with an estimated 310,000 new infections in 2011. Therefore, much effort is needed to find new treatment options.

A potential candidate for MDR-TB is co-trimoxazole; a classic drug used against many types of infections. Co-trimoxazole consists of trimethoprim and sulfamethoxazole. Trimethoprim is not active against M. tuberculosis but sulfamethoxazole showed in vitro activity against both TB and MDR-TB. A recent prospective cohort study showed that co-trimoxazole prophylaxis against Pneumocystis jiroveci pneumonia (PCP) in the treatment of HIV reduced the incidence of TB. These considerations make this drug a potential candidate for the treatment of TB. Co-trimoxazole has been shown to be effective in the prophylaxis of PCP in HIV-infected patients with a CD4 count below 200 /mm3. It is therefore implemented in HIV treatment guidelines as standard care until there is evidence of immune recovery. Although a fixed dose has been recommended, serum concentrations vary considerably between individuals. In patients with chronic liver disease, increased serum concentrations of sulfamethoxazole were found, correlating with a larger likelihood of toxicity. Furthermore, sulfamethoxazole is metabolized by N-acetyltransferase to sulfamethoxazole-N-acetyl, which has the tendency to precipitate due to concentration in the kidneys. This has a detrimental effect on the renal function. A general advice is to drink 2 litres of water per day to avoid this.
To ensure that therapy with sulfamethoxazole is effective, the exposure to the drug needs to be high enough. This exposure can be quantified using the area under the pharmacokinetic (PK) curve for 24 hours (AUC$_{0-24h}$). The plasma AUC$_{0-24h}$ is a surrogate marker for the concentration at the site of the infection and the exposure to the infecting micro-organisms. To have a surrogate marker for the efficacy and toxicity, the AUC$_{0-24h}$ needs to be related to the minimal inhibitory concentration of the micro-organism (MIC, AUC$_{0-24h}$/MIC).

Dried blood spot (DBS) monitoring is a simple procedure to collect blood from a patient. A DBS can be made with a few blood drops on a sheet of absorbent paper. The concentration of the drug of interest is subsequently measured in the dried blood spot. This method enables measuring blood levels of patients in outpatient clinics and in remote areas. The DBS can be easily stored and sent to a laboratory because of high sample stability. From our own experience, it takes only a week to send a DBS card through local post services from the rural country Ghana to our laboratory in The Netherlands with a 100% delivery rate up to now (n = 6, unpublished data). With the combination of high sample stability and easy sampling, therapeutic drug monitoring is feasible in rural parts of the world with a high frequency of HIV and/or TB.

Another additional advantage is reduced discomfort for patients. Moreover, medical personnel can take DBS samples after limited additional training. DBS analysis of anti-TB drugs is increasingly proposed to replace venous sampling for therapeutic drug monitoring (TDM). Several analytical DBS procedures for TB drugs have already been published.

Although DBS seems a simple procedure in the field, method validation is not and requires additional validation for the effect of the haematocrit value and blood spot volume. A higher haematocrit value results in a higher blood viscosity and affects the flux and diffusion properties of the blood. This may result in a positive analytical bias.

The objective of this study was to develop and validate a simple method to detect and quantify sulfamethoxazole and sulfamethoxazole-N-acetyl simultaneously in dried blood spots suitable for TDM. The DBS procedure was also evaluated in a prospective study.

**PATIENTS AND METHODS**

**Patients**

TB patients between 18 and 64 years of age with culture confirmed drug-susceptible *M. tuberculosis* were eligible for this prospective clinical trial. Co-trimoxazole 960 mg daily was added for 4 to 6 consecutive days to the standard treatment of TB to obtain a steady state concentration situation. On the 5th (± 1) day, three dried blood spots were collected 1h, 5h and 8h post-dosage at the same time point as regular venous serum samples were taken. The study was approved by the local ethical committee (METC 2013/195) and registered at Clinicaltrials.gov (NCT01832987). Patients that participated in the study gave written informed consent.

**Materials**

Sulfamethoxazole was purchased from Sigma Aldrich (MO, USA). Sulfamethoxazole-N-acetyl was obtained from Santa Cruz (TX, USA). The deuterated internal standards sulfamethoxazole-D4 and sulfamethoxazole-N-acetyl-D4 were purchased from Alsachim (Illkirch Graffenstaden, France) and Santa Cruz (TX, USA), respectively.

Ultrapure water was produced with a Milli-Q system (Millipore Corporation, MA, USA). Ammonium acetate and acetic acid were both obtained from Merck (NJ, USA), while trifluoroacetic
acid and acetonitrile LC-MS were purchased from Biosolve (Dieuze, France). Methanol was retrieved from Merck (NJ, USA). Whatman DMPK type C cards (Whatman, Kent, UK) were used for spotting the blood.

**LC-MS/MS**

The LC-MS/MS apparatus and conditions were identical to the serum and plasma analysis, as published earlier. In short, liquid chromatography separation took place with a Scientific Hypurity Aquastar C18 (50*2.1 mm, 5μm particles) column in a system with a Finnigan Surveyor MS Pump Plus and Finnigan Surveyor Autosampler Plus. Gradient elution with three mobile phases was applied to reduce the run-time to three minutes with a flow rate of 500 μL/min.

A TSQ Quantum Access Max (TSQ Quantum, Thermo Scientific, San Jose, CA, USA) tandem quadrupole mass spectrometer was used for both the serum and DBS MS/MS analysis. The spray voltage was 3500V, sheath and auxiliary gas pressure was set to 35 and 10 bar and the capillary temperature was set to 350 °C.

To perform the analysis, the most sensitive ion transitions were used: 254.0 → 156.1 (sulfamethoxazole), 258.1 → 160.1 (sulfamethoxazole-D4), 296.1 → 198.0 (sulfamethoxazole-N-acetyl), 300.1 → 202.1 (sulfamethoxazole-N-acetyl-D4).

**Preparation of solutions and QCs**

The internal standard solution was composed of 0.2 mg/L sulfamethoxazole-D4 and 2.0 mg/L sulfamethoxazole-N-acetyl-D4 in methanol/water (80/20). Quality control blood samples were prepared in blood consisted of LLOQ (2 mg/L), LOW (10 mg/L), MED (40 mg/L) and HIGH (80 mg/L) samples of sulfamethoxazole and sulfamethoxazole-N-acetyl. DBS QC samples were produced by pipetting 50 μL of the quality control blood samples on a DBS card and drying it for three hours.

**Sample preparation**

A venous dried blood spot (VDBS) was made by pipetting 50 μL of the venous plain blood on a DBS paper. To analyse both VDBS and DBS, a paper disc of 8 mm was punched out each blood spot on the DBS. The IS solution was added (250 μL) and the sample was vortexed. After vortexing, the sample was placed in an ultrasonic bath for 10 minutes. Afterwards, the sample was vortexed again for one minute and subsequently centrifuged for 5 minutes at 10,000 rpm. Of the extract, 200 μL was transferred in another vial, 500 μL ultra-pure water was added and 10 μL was subsequently injected on the column.

**Method validation**

Each calibration line consisted of eight different concentrations, as shown in table 1. Each concentration was prepared and measured in triplicate. Both sulfamethoxazole and sulfamethoxazole-N-acetyl were measured in the range of 2.0 – 100.0 mg/L. Calibration curves were corrected for the internal standard based on peak area. The slope, intercept and correlation and regression coefficient were calculated by ANOVA.

Validation was performed according to the FDA and EMA guidelines. Selectivity was determined by analysing six blank blood spots retrieved from volunteers on our lab.
All samples were tested for peaks on the retention times of sulfamethoxazole and sulfamethoxazole-N-acetyl. Ion suppression was tested using a constant infusion test.

Accuracy (coefficient of variation (CV)) was investigated by the analysis of LLOQ (2.0 mg/L), LOW (10.0 mg/L), MED (40.0 mg/L) and HIGH (80.0 mg/L) concentrations of both analytes in fivefold on three consecutive days. Precision (bias (%)) was examined within-run and between-run with the data from the accuracy determination. Dilution integrity was also tested during these three consecutive days by spiking a DBS card with 200 mg/L in fivefold with sulfamethoxazole or sulfamethoxazole-N-acetyl. The extract was diluted 1:10 and analysed. The within-day and between-day precision were calculated using ANOVA.

The matrix effect was determined by spiking extracts of blank DBS with LOW, MED and HIGH QC concentrations of sulfamethoxazole or sulfamethoxazole-N-acetyl and comparing them with the peak area of spiked extraction fluid. The recovery was calculated by dividing the peak area of LOW, MED and HIGH concentration spiked DBS by the peak area of spiked extract of a blank DBS. Process efficacy was determined by dividing the peak area of a DBS spiked with LOW, MED and HIGH QC concentration divided by the peak area of spiked extraction fluid.

The stability assessment was performed by analysing spiked dried blood spots (LOW and HIGH QC concentrations) stored for 14 days at 20, 37 and 50 °C and one month at 20 and 37 °C. The peak area of analysed dried blood spots were compared with the peak area of freshly prepared samples.

The influence of the haematocrit (Hct) value on the accuracy and precision was additionally assessed. In tuberculosis patients, an Hct value of 35% was expected. We tested therefore Hct values of 20, 25, 30, 35, 40, 45 and 50%. The influence of the volume of the blood spot was also assessed by varying the blood spot volumes from 30, 50, 70 to 90 μL.

Clinical validation

Venous blood, VDBS and DBS concentrations were compared with a Bland-Altman plot and Passing and Bablok regression. According to EMA guidelines, the difference between DBS and serum concentrations should be within 20% in 67% of the cases. Plots were constructed with SigmaPlot version 12 (Systat Software Inc., San Jose, CA, USA). Passing and Bablok regression was calculated using Medcalc version 15.8 (MedCalc Software bvba, Ostend, Belgium). The area under the curve (AUC_{0-24h}) of sulfamethoxazole was calculated based on the measured DBS concentrations using a validated one-compartment population pharmacokinetic model. The AUC_{0-24h} based on the DBS samples was compared with the AUC_{0-24h} calculated with full curves.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope (± st. dev)</th>
<th>Intercept (± st. dev)</th>
<th>Corr. coefficient</th>
<th>Regr. coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethoxazole</td>
<td>0.392 (± 0.004)</td>
<td>-0.009 (± 0.023)</td>
<td>0.999</td>
<td>0.997</td>
</tr>
<tr>
<td>Sulfamethoxazole-N-acetyl</td>
<td>0.112 (± 0.002)</td>
<td>0.005 (± 0.008)</td>
<td>0.998</td>
<td>0.996</td>
</tr>
</tbody>
</table>
RESULTS

Method validation

No peaks were observed in six lots of blank samples at the retention times of the analytes. No ion suppression was observed. The details of all three calibration lines are displayed in table 1. The correlation and regression coefficients were all >0.99. The accuracy in measuring sulfamethoxazole and sulfamethoxazole-N-acetyl varied between -14.2 – 2.4% (as shown in table 2). The coefficient of variation between the measurements was 2.9 – 6.9% within-day and 0.0 – 9.5% between three days. The mean bias in determining the dilution integrity of sulfamethoxazole and sulfamethoxazole-N-acetyl was 1% and -4% with an overall CV of 9.5% and 6.6%, respectively. Both accuracy and precision fell within the FDA and EMA defined limits of 15% (LLOQ: 20%).

Table 2. Validation results

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Concentration level</th>
<th>Nominal concentration (mg/L)</th>
<th>LLOQ</th>
<th>LOW</th>
<th>MED</th>
<th>HIGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethoxazole</td>
<td></td>
<td></td>
<td>2.0</td>
<td>10.0</td>
<td>40.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Sulfamethoxazole-N-acetyl</td>
<td></td>
<td></td>
<td>2.0</td>
<td>10.0</td>
<td>40.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Accuracy (bias (%))</td>
<td>sulfamethoxazole</td>
<td>-6.9</td>
<td>-2.4</td>
<td>0.9</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sulfamethoxazole-N-acetyl</td>
<td>-14.2</td>
<td>-3.0</td>
<td>0.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Within-day precision (CV (%))</td>
<td>sulfamethoxazole</td>
<td>3.3</td>
<td>2.9</td>
<td>3.5</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sulfamethoxazole-N-acetyl</td>
<td>6.9</td>
<td>6.9</td>
<td>5.8</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Between-day precision (CV (%))</td>
<td>sulfamethoxazole</td>
<td>9.5</td>
<td>4.5</td>
<td>3.7</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sulfamethoxazole-N-acetyl</td>
<td>0.0</td>
<td>3.0</td>
<td>3.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Matrix effect (bias (%))</td>
<td>sulfamethoxazole</td>
<td>n.d.</td>
<td>0.8</td>
<td>2.1</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sulfamethoxazole-N-acetyl</td>
<td>n.d.</td>
<td>0.1</td>
<td>3.1</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Recovery (bias (%))</td>
<td>sulfamethoxazole</td>
<td>n.d.</td>
<td>86.4</td>
<td>96.1</td>
<td>87.5</td>
<td></td>
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<tr>
<td></td>
<td>sulfamethoxazole-N-acetyl</td>
<td>n.d.</td>
<td>93.2</td>
<td>103.7</td>
<td>89.6</td>
<td></td>
</tr>
<tr>
<td>Autosampler stability (7 days) (bias %)</td>
<td>sulfamethoxazole</td>
<td>n.d.</td>
<td>-1.6</td>
<td>n.d.</td>
<td>-2.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sulfamethoxazole-N-acetyl</td>
<td>n.d.</td>
<td>-3.0</td>
<td>n.d.</td>
<td>-5.4</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Influence of the haematocrit and blood spot volume on the measured concentration. SMX: sulfamethoxazole, SMXA: sulfamethoxazole-N-acetyl
The results of the matrix effects and recovery evaluation are shown in table 1. The limits of the FDA and EMA were met at all three concentration levels. The recovery and matrix effects varied from 86.4 – 103.7% and 0.1 – 6.7%, respectively, and complied with the FDA and EMA defined limits.

The autosampler stability of sulfamethoxazole and sulfamethoxazole-N-acetyl was determined after 7 days. The bias was -1.6 – -5.4% compared to the nominal concentration.

The stability of the two analytes on the DBS is evaluated at various storage conditions during 14 days and one month. After 1 month at 37°C, the bias calculated for sulfamethoxazole and sulfamethoxazole-N-acetyl -7.0 – 3.9%. Storage at 50°C for 14 days resulted in a deviation of -7.5 – -2.9% in comparison with the nominal concentration.

**Influence of haematocrit and blood spot volume**

The influence of the haematocrit (hct) value is displayed in figure 1. At a hct value of 20%, bias varied from -15.3 – -8.8% for all analytes. A bias of 4.0 – 5.1% was found at a relatively high haematocrit value of 50%. The bias caused by varying the blood spot volume was -3.2 – 6.1%, as shown in figure 1.

**Clinical validation**

In total 12 patients were prospectively enrolled in this study. The median age was 31 (IQR: 26 - 52) years with a median height of 175 cm (IQR: 168.2 – 180.0) and a median body weight of 61.5 kg (IQR: 56.4 – 67.2). All received 960 mg co-trimoxazole once daily.

Overall, 21 DBS samples of 9 patients were used to validate the method of analysis. The median haematocrit-corrected DBS concentrations were 20.8, 25.4 and 19.7 mg/L at 1, 5 and 8 hours post-dose. Median serum concentrations were 18.5, 35.0 and 30.8 mg/L, respectively. The correlation between serum and haematocrit-corrected DBS concentrations was best described by: serum = -5.36 + 1.57 x DBS (Passing and Bablok regression). The 95% confidence interval of the intercept and slope was -11.43 – 0.40 and 1.31 – 1.85, respectively. All haematocrit-corrected DBS sulfamethoxazole concentrations are displayed in figure 2a. After correction using the proposed regression formula, three of all 21 DBS concentrations deviated more than 20% with the serum concentration (77.7%, 79.0% and 129.4%). In addition, we calculated the regression formula with only the samples 5 and 8 hours post-dose. This relationship was best described by: serum -2.88 (95% CI: -11.02 – 2.97) + 1.57 (95% CI: 1.30 – 1.89) x DBS. None of the back calculated serum concentrations at 5 and 8 hours post-dose differed more than 20% with the actual serum concentration using this regression formula.

For sulfamethoxazole-N-acetyl, the median haematocrit-corrected DBS concentrations were 3.3, 4.7 and 5.9 mg/L at 1, 5 and 8 hours post-dose. Median serum concentrations were quantified at 6.0, 10.0 and 9.9 mg/L. The correlation between the haematocrit-corrected DBS concentration and serum concentration was best described by: serum concentration (mg/L) = 0.50 (95% CI -6.99 - 2.59) + 1.77 (95% CI 1.26 – 3.15) x DBS concentration (mg/L), (Passing and Bablok regression) as shown in figure 2b. The difference between the corrected DBS and the serum concentration was >20% in 6 of 17 cases (71.1% – 154.5%).

The median difference in AUC_{0-24h} calculated on the DBS samples was -5.8% (IQR -6.25 - -0.13%) in comparison with full serum curves. A Bland Altman plot is displayed in figure 2c. Two DBS-based AUC_{0-24h} differed more than 20% (-23.7% - -22.8%). The coefficient of variation in the root mean squared error (CV(RMSE)) was 9.7%.
Figure 2a. Passing and Bablok correlation between sulfamethoxazole serum concentration and the haematocrit corrected DBS concentration (95% CI: dotted lines). Intercept: -5.36 (95% CI: -11.43 – 0.40), slope: 1.57 (95% CI: 1.31 – 1.85)

Figure 2b. Correlation between sulfamethoxazole-N-acetyl serum concentration and the haematocrit corrected DBS concentration (95% CI: dotted lines). Intercept: 0.50 (95% CI: -6.99 - 2.99), slope: 1.77 (95% CI: 1.26 – 3.15)
DISCUSSION

We developed a method to analyse sulfamethoxazole and sulfamethoxazole-N-acetyl in dried blood spots which was successfully validated based on the FDA and EMA guidelines on bioanalytical validation.\textsuperscript{28,29} Clinical evaluation showed that estimating the $\text{AUC}_{0-24h}$ with DBS samples provides comparable results with traditional venous blood sampling, indicating that this method is suitable for daily patient care.

Both the haematocrit and blood spot volume influence the quantification of analytes on DBS cards.\textsuperscript{24} The haematocrit value was obtained from all patients and was used to correct the analytical result. Bias caused by varying blood spot volumes was within 6\% and was considered negligible.

Due to compatibility with the serum and plasma analysis, we used identical mass transitions for all analytes of interest.\textsuperscript{26} One problem which occurred during the development of the analytical method was the limited separation of sulfamethoxazole and sulfamethoxazole-N-acetyl. This problem was solved by adding 500 $\mu$L ultrapure water to the extract, increasing the hydrophilicity of the sample, which improved the separation. We used a gradient elution in order to further improve separation, and to minimize the total time of analysis to three minutes. In addition, we optimized the sample preparation turnover by comparing a 10 minute with a 60 minutes ultrasonic bath. No difference in response was observed.

One of the major advantages of this dried blood spot technique is the simplified logistics since no precautions concerning storage environment are required. The bias in concentration after 14 days at 50 °C or 1 month at 37 °C did not exceed 15\%, indicating that extreme temperatures do not affect the analytical result. This indicates that this method is also suitable in countries with an extreme climate without cold chain transport requirements. With this method, TDM is within reach in developing countries when the DBS samples are transported to a sophisticated laboratory for analysis and TDM.

The clinical validation of the analysis of sulfamethoxazole on DBS cards resulted in three
deviations >20%, which meets the EMA requirements on cross-validation.\cite{28} When using a regression formula based on the samples withdrawn 5 and 8 hours post-dose, no deviations <20% are observed in the calculated serum concentration based on the DBS concentration versus the true serum concentration. This indicates that the peripheral distribution of sulfamethoxazole is not complete one hour after ingestion of the drug. When all DBS results were entered in our pharmacokinetic model, two deviations of -23% and -24% in the $AUC_{0-24h}$ were observed compared to the $AUC_{0-24h}$ calculated on full serum curves. Furthermore, the RMSE was calculated at 9.7%, indicating that predicting the $AUC_{0-24h}$ of sulfamethoxazole with this DBS method is reliable and suitable for clinical use.

The sulfamethoxazole-N-acetyl concentration determination is only to detect toxicity leading to renal toxicity. This analysis seems less accurate than the analysis of sulfamethoxazole itself. The difference between DBS concentration and serum concentrations might be caused by a large inter- and intraindividual variation in peripheral sulfamethoxazole-N-acetyl concentration. However, the concentration of the DBS cards were higher than the serum concentrations in cases where the deviation was >20%. Therefore, the sulfamethoxazole-N-acetyl concentration may be over-estimated using this methodology that may result in an overestimation of possible toxic concentrations. When an increase of sulfamethoxazole-N-acetyl is observed, additional clinical evaluation and reanalysis of sulfamethoxazole-N-acetyl in venous blood is needed to evaluate renal toxicity. Although serum creatinine is also feasible using dried blood spots,\cite{30} it is unfortunately no measure of renal toxicity as co-trimoxazole influences tubular excretion of creatinine.\cite{31} Therefore measurement of an additional marker, such as Cystatin C, is required to confirm renal toxicity.\cite{32} Cystatin C could also be quantified using DBS.\cite{33}

The method that we developed and validated is suitable for analysis of sulfamethoxazole and sulfamethoxazole-N-acetyl and is validated according the FDA and EMA guidelines on sensitivity, selectivity, linearity, accuracy, precision and stability. One-point sampling combined with this DBS method provides a convenient and reliable method to assess the individual pharmacokinetics of sulfamethoxazole in the treatment of various infectious diseases, such as PCP. In addition, this method can be used in further prospective trials to assess sulfamethoxazole exposure.
REFERENCES


