Rapid and sensitive LC–MS/MS method for the analysis of antibiotic linezolid on dried blood spot

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ABSTRACT

Linezolid is a new drug from the oxazolidinone class of antibiotics used against mycobacteria and multi-drug resistant (MDR) Gram-positive bacterial infections, which may are also glycopeptide-resistant. The drug usage in pediatric age needs an accurate drug monitoring for effective patient management. The aim of this study was to evaluate the use of dried blood spot (DBS) specimens to determinate linezolid levels during treatment. Advantages of DBS include short collection time, low invasiveness, ease and low cost of sample collection, transport and storage. The analysis was performed in LC–MS/MS operating in positive ion mode and multiple reaction monitoring (MRM) mode. The calibration curve in matrix was linear in the concentration range of 1–100 mg/L with correlation coefficient value of 0.9987. Intraday and interday coefficients of variation were within 3.6% and 13.0%, respectively. We also tested the thermal and temporal drug stability in dried blood spots at four different temperatures to evaluate the risks of sample delivery in different conditions. The short term stability studies showed that linezolid concentration remained stable for at least one month under all the conditions tested.

This new assay has favorable characteristics being highly precise and accurate and allows a fast linezolid analysis with a total run time 22 min long, in gradient analysis. Concentration data for plasma and DBS samples from patients after treatment were compared showing a good correlation.

Correlation between DBS data and serum samples measured by HPLC–UV was satisfactory.

The benefit for patients is the ability to monitor the treatment with a simple and convenient sample collection at home.

1. Introduction

Linezolid is the first member of a new class of antibiotics, the oxazolidinones [1]. Linezolid exhibits a broad spectrum of activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci (CoNS), glycopeptide-resistant enterococci and penicillin-resistant *Streptococcus pneumoniae* [2,3]. It is also active against mycobacterial species, including *Mycobacterium tuberculosis* and *Nocardia spp* [2]. An advantage of linezolid on glycopeptides in the clinical practice is its availability as intravenous (iv) and oral formulations which allow the switch to an oral treatment after induction iv treatment.

In the United States, linezolid was licensed by the FDA in adults and children in 2002. On the contrary, in most European countries linezolid use in the pediatric setting remains off-label and the clinical experience with this antibiotic is still limited [2]. Recent data suggest that linezolid is a safe and effective agent for the treatment of serious Gram-positive bacterial infections in neonates and children, however, at present, linezolid is reserved for those neonates and children who are intolerant to or fail conventional agents [2]. Linezolid resistance even though is rare, with rates lower than 0.1%, it is already described in the pediatric population, so this treatment should be chosen for selected conditions [2].

A linezolid-containing regimen can be also a valuable option for treating MDR and extensively drug-resistant tuberculosis in children as well as disseminated non-tuberculous mycobacterial infections [2,4].

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Most information regarding the pharmacokinetic (PK) profile, efficacy, and tolerability of linezolid is derived from adult studies [3].

Recent results in infants and children indicate that PK of linezolid is strongly age-related since children <12 years have a faster clearance and shorter elimination half-life than children >12 years and adults. Moreover, there are data on PK in infants <1 year of age and in preterm infants but results have a limited significance due to the low number of subjects evaluated. As a result, there is an agreement that linezolid should be administered at a dosage of 10 mg/kg every 8 h in children ≥1 week-11 years of age and at a dosage of 10 mg/kg every 12 h in preterm infants <1 week of age [3]. Since linezolid is known to be 100% available following oral administration, the dosage does not need to be changed when switching iv to oral administration [5]. However, linezolid PK studies in children showed an inter-individual variability in children, particularly after oral administration [6,7].

Therefore, a therapeutical drug monitoring (TDM) may be very useful in children receiving oral administration of this drug.

The published HPLC–UV and LC–MS/MS methods proved to be precise and accurate, but required large volume of biological fluids and are therefore unsuitable for sample collection in neonates in whom obtaining blood samples is not convenient or possible.

An attracting and potential method to easily obtain samples for PK studies and TDM in children is collection of whole blood samples onto filter paper (dried blood spot or DBS) [7–12]. DBS are commonly used for neonatal screening of metabolic diseases, cystic fibrosis and hypothyroidism in micro-blood samples. Some studies recently evaluated the use of DBS for TDM and toxicology in adults [7–12]. Some authors, also from our group, applied the DBS technique to evaluate drug concentrations in newborns, infants and children [7–12]. These reports showed that the accuracy of TDM studies using DBS is comparable to that of traditional TDM studies on plasma which require larger blood volumes.

In the present study we developed and validated a LC–MS/MS based method on DBS for the quantification of linezolid in children's blood samples, and it was compared with a previous validated HPLC method used in a reference clinical–toxicological laboratory.

2. Experimental

2.1. Patients and samples collection

Patients were enrolled in this prospective, open-label study followed from the Pediatric Infectious Diseases Unit, Anna Meyer Children's University Hospital, Florence, Italy. Only children with infections unresponsive to treatment and who need to be treated with linezolid for clinical reasons were enrolled. Paired serum and DBS samples before and/or after administration of linezolid (10 mg/kg every 8 h administered intravenously or orally). Samples were collected after 3 days from starting linezolid hematocrit was obtained from each patient simultaneously to serum/DBS collection. In all individuals linezolid was used add-on medication. The study protocol was approved by the local Ethical Committee and written informed consent was obtained from children's parents/guardians.

2.2. Standards

Chemical standard of linezolid was kindly supplied by Pfizer (Groton, CT, USA). Stock solution of 2 g/L was made in water and stored in different aliquots at −20 °C. Dilutions were made using HPLC grade water. All chemicals and solvents were of the highest purity available from commercial sources and used without any further purification.

2.3. Sample preparation

Blood spot samples were stored at 4 °C in a sealed plastic bag containing desiccant until analysis. One 32 mm diameter disk (containing about 3.3–3.4 μL of blood) was punched from each DBS sample and extracted with 300 μL of a mixture 30:70:0.05 (v/v/v) of water, acetonitrile and formic acid, respectively. Samples were extracted in an orbital shaker and kept at 37 °C for 25 min.

For the setting-up of this study, a pooled mixture of blood samples was spiked with linezolid and 20 μL were spotted on filter paper (Whatman 903, GmbH, Dassel, Germany).

2.4. Validation procedures

In order to achieve accurate quantization of analytes in complex matrices a stable isotope-labeled internal standard may be more appropriated, but unfortunately labeled linezolid is not commercially available. In addition the use of structural analogous as internal standard could results in a different detector response because of ion suppression from matrix components or low extraction recoveries.

Therefore we used a calibration curve prepared in duplicate by spotting on filter paper spiked human control blood to obtain concentrations of 0, 15, 5, 10, 20, 50 and 100 mg/L.

As reported in the bioanalytical validation procedure [13], three replicates of three different concentrations of linezolid (1, 50, 100 mg/L), were analyzed in ten different runs for determining the accuracy and precision. These concentration levels represent the entire calibration curve range in particular: three times the LLOQ (low QC), near the center of the standard curve (middle QC) close to the highest concentration level (high QC). Average recovery of linezolid from DBS samples was determined by comparing responses with those obtained by injection of the same amount of drug, added before or after the extraction at two different concentrations (5 and 50 mg/L). To calculate the linear regression, the peak size was plotted against the drug concentration in mg/L.

The short-term stability study on DBS samples was evaluated up to one month after storage at −20 °C, +4 °C, room temperature and +37 °C.

2.5. Mass spectrometry

The samples were measured using an Agilent (Waldbronn, Germany) 6430 bench-top Triple-Quad Mass Spectrometer equipped with the eletrospray source operated in MRM in positive ion mode. The capillary voltage of the mass spectrometer was set to 4000 V and the Fragmentor voltage was set to 110 V, for each transition, the drying gas flow was 9 L/min of nitrogen heated at 325 °C. The following transitions were monitored: m/z 338.3 > 235.2 (quantifier), m/z 338.3 > 296.2 (qualifier) and m/z 338.3 > 195.1 (qualifier). Optimal collision energies were found at 20, 18, 18 V, and the resulting cell acceleration voltage was +7 V for all transitions.

The quantitation experiments were undertaken by using a Series 1290 Infinity LC System (Agilent Technologies, Waldbronn, Germany) UHPLC capillary pump coupled to an Agilent Micro ALS autosampler, both being fully controlled from the Mass Hunter data system. Liquid chromatography was performed using an Agilent Zorbax Eclipse Plus C18, rapid resolution 18 μm, 21 mm × 50 mm HPLC column (Agilent Technologies, Waldbronn, Germany). The column was maintained at 60 °C during the run and the column flow was set at 0.4 mL/min. The chromatographic separation were obtained using a fast gradient starting from a 90% aqueous solution containing 0.1% formic acid and 10% acetonitrile containing 0.1% formic acid. The 90% of organic solvent was reached in 15 min and the system reconditioned to the starting condition in 0.7 min. The total run time was 22 min long and the linezolid retention time

was fixed to 115 min The eluent from the column was directed to the electrospray source without split ratio. One μL of the extracted sample was injected for the LC–MS/MS experiments. All samples were kept stored at +4 °C in the autosampler tray to avoid degradation.

System control and data acquisition were performed with Mass Hunter (Version B.0400) software including the Qualitative package (for chromatographic and spectral interpretation) and the Quantitative Software (for quantitative information generation). Calibration curves were set up with the Mass Hunter Quantitative program using a linear least-square regression non-weighted.

2.6. HPLC–UV

Linezolid serum concentrations were determined by a validated HPLC–UV assay [14]. Briefly samples were prepared by mixing aliquots (50:50) of the specimen with acetonitrile and centrifuging at $5000 \times g$ for 5 min. The eluent was evaporated to dryness, the residue was reconstituted in mobile phase and 100 μL were injected into the HPLC.

The stationary phase was a Pinnacle 2 C18 5ODS, 100 mm × 46 mm (Restek Corporation, Bellefonte, PA, USA). The mobile phase was 1% ortho-phosphoric acid, 30% methanol, 2 g/L heptane sulphonic acid, adjusted to pH 5 by the addition of 10 M sodium hydroxide, with a flow rate of 10 mL/min and UV absorbance detection at 254 nm. The validated lower limit of quantification was 0.06 mg/L. A Series 200 auto-sampler, UV detector and pump were used (PerkinElmer Instruments, Shelton, CT, USA). Assay reproducibility was: intra-day <6%; inter-day <10%. The correlation between drug concentration and area value was good for both aqueous and serum samples across the concentration range ($r = 0.995$ for both).

3. Results and discussion

3.1. Patients

Fifteen paired serum and DBS samples were obtained from 9 patients, 6 (67%) males, median age (7 years). One child presented MRSA orbital cellulitis, one was treated for MRSA mastoiditis, two for MRSA pneumonia, and one had a CoNS superinfection during severe chickenpox Linezolid was also used in three patients with MDR tuberculosis and in another one for the treatment of an intracranial abscess not responding to conventional therapy.

3.2. Optimized extraction from DBS

The DBS use is becoming a valid alternative compared to a traditional blood sample, in particular for pediatric application, where is usually stressful and unethical to obtain a sufficient number of blood samples. In this scenario the analysis based on few drops of blood can be appealing also for pharmacokinetic studies involving children.

The extraction mixtures were selected after test of different solvent compositions and the 70–30 acetonitrile/water proportion yield the best extraction recovery. Each DBS was twice extracted sequentially and the second extraction represents less than 5% of the first one. We tested also different extraction times (25, 45 and 60 min) and no variations were recorded. Additionally, extraction temperature was test at 25 °C, 37 °C and 60 °C. The best extraction yield occurred at 37 °C.

To be able to evaluate punching location impact and the homogeneity of drug distribution, experiments on several spots from the same drop of blood have been performed; no significant differences

were detected. This fact is due to the low spotted blood volume on paper (20 μL) generating an homogenous spot [15,16].

3.3. Chromatographic conditions

The chromatographic conditions were selected through a number of preliminary studies of optimization of parameters in order to speed-up the running time (22 min) in gradient mode. To maintain the needed resolution a 18 μm stationary phase column was used, and the column oven was set to 60 °C. Sample dilution rate (90 times) and injection volume (1 μL) were chosen to prevent column overload. The sample injection volume was enough to achieve the needed sensitivity, reducing the matrix injection into the system.

Under the conditions of our assay, the dead-volume of the entire system (including the tubing) was less than 130 μL, and the retention time of the solvent front (unretained peak) was 0.3 min. Considering the Linezolid retention time (115 min), it was retained long enough from the column to avoid the salt suppression. Moreover the column showed robust performances regardless of the salt or any other interfering component and no deterioration in column efficiency was observed after the analysis of 500 DBS samples.

3.4. Method validation

The specificity is provided by the MS/MS measurement combined to the high retention time stability. Fig. 1 shows the MS/MS spectrum obtained by fragmenting the precursor ion (338.3 Th) of linezolid under the above described conditions. From these experiments, the resulting most selective ion-pair transition for the quantitative experiment (SRM) is 338.3 > 235.1. We have chosen two additional transitions as qualifiers: 338.3 > 296.1 and 338.3 > 194.9.

As suggested from Li and Tse [16] we evaluated the recovery as shown in Fig. 2. We compared two concentrations (5 and 50 mg/L) in three replicates, obtaining 15% recovery for both levels.

The non-weighted regression equation for our LC–MS/MS method was $y = 258x + 184$; $R^2 = 0.9980$. The mean correlation coefficient for regression lines, generated on 10 different days was $R^2 = 0.9987$ (SD ± 0.0009, range 0.9973–0.9996). A correlation coefficient of >0.995 is generally considered as the evidence of an acceptable fit of the data to the regression line.

In order to assess the method suitability on a wide range of concentrations, ten replicates of three different calibrators were used (1, 50 and 100 mg/L) resulting an intra-day repeatability below 11% for all values (Table 1). The inter-day repeatability, obtained by processing three replicates of each concentration level (1, 50 and 100 mg/L) in ten separate assays for two weeks, was better than 13% (Table 1).

Using the proposed method the estimated limit of detection (signal to noise ratio >5) in DBS was 0.2 mg/L, the limit of quantitation (signal to noise ratio >15) was 0.4 mg/L.

3.5. Short term stability studies

Stability studies showed no significant differences between the room and refrigerated temperatures, highlighting that molecule is stabilized on paper. Also, linezolid concentrations on dried blood spot were not significantly different under conditions of storage at 37 °C for a month (Table 2). This means that, for therapeutic linezolid monitoring, the DBS could be sent by mail even from tropical countries where ambient temperature is elevated. The overall results in determining stability at different temperatures are reported in Fig. 3.
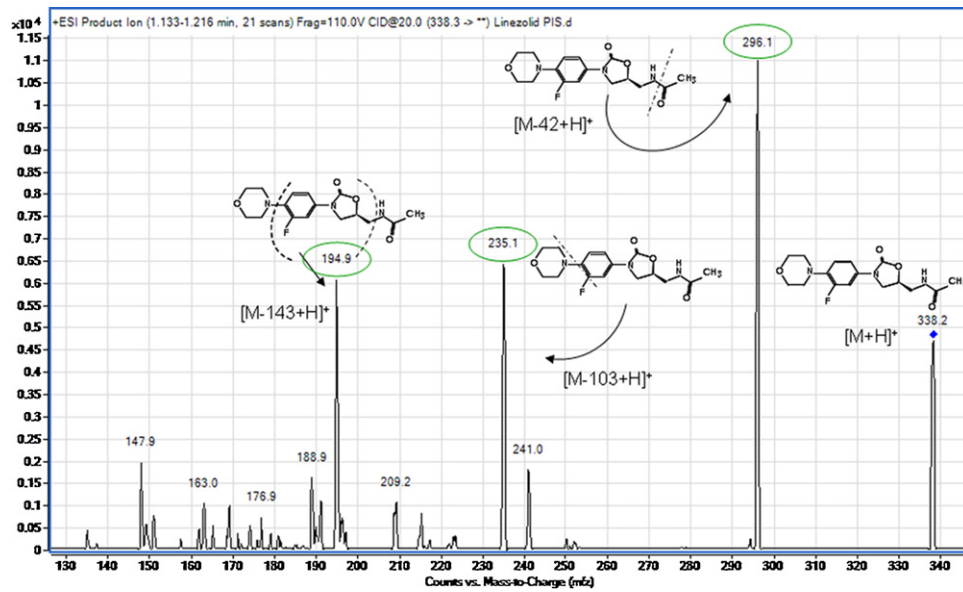
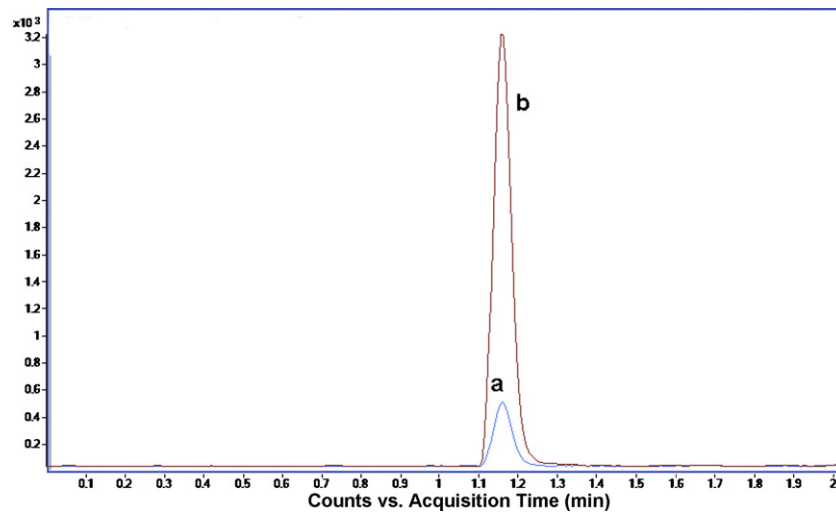Fig. 1. Product ion scan and molecular structure of linezolid.

Fig. 2. Comparison between the chromatogram from extracted DBS fortified with 5 mg/L of linezolid (a) versus a linezolid standard solution (5 mg/L) in acetonitrile:water (70:30, v/v) added to an extracted DBS blank sample (b), considering the same matrix effects on both samples.

3.6. Evaluation of hematocrit impact in linezolid value obtained on DBS

In this study, 15 DBS and matched plasma samples, collected from 9 patients, were compared. For each samples hematocrit (HCT) was measured and hematocrit-corrected DBS was calculated according to the equation $C_{plasma} = C_{DBS} \times (100 - HCT)/100$. This equation is used as a conversion factor to express levels determined on DBS in the equivalent plasma. In addition, DBS values were corrected using the theoretical hematocrit (mean per age) in order to determinate if the method could be used when real HCT is not available. We observed a

Table 1
Inter-day and intra-day imprecision of linezolid measurements on DBS. Inter-day and intra-day imprecision of the dried blood spot assays were determined as described in Section 2.

Expected concentration ng/μL	Mean	Standard deviation	CV%	% Accuracy
Inter-day (n = 10)				
1	1.2	0.2	13.0	119.0
50	49.7	0.8	1.5	99.4
100	100.7	0.9	0.9	100.7
Intra-day (n = 10)				
1	0.9	0.1	5.1	94.0
50	44.1	2.3	5.2	88.3
100	96.0	4.7	4.9	96.0
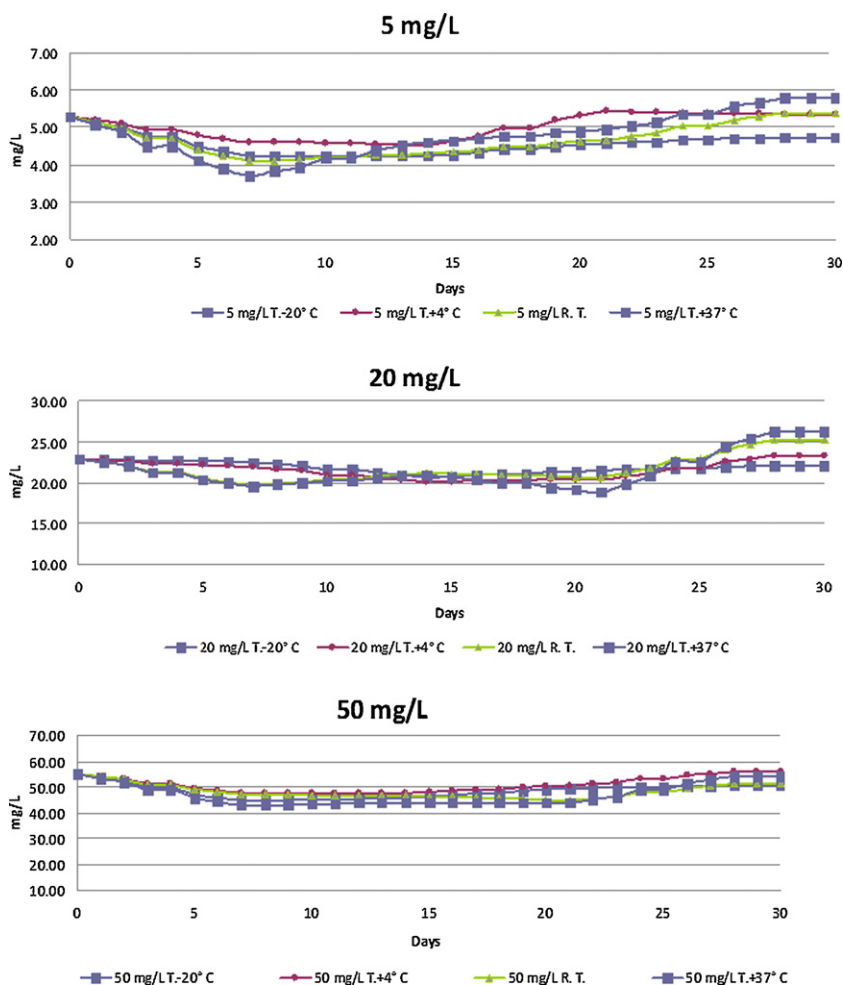
Fig. 3. Stability of linezolid investigated at 4 different temperatures: −20 °C, +4 °C, room temperature and +37 °C for a month by repeat injection of 3 DBS spiked samples (5, 20, and 50 mg/L).

good correlation between linezolid and the levels measured in the corresponding DBS both if the real HCT ($R^2 = 0.9268$) (Fig. 4a) or the theoretical one have been used ($R^2 = 0.9849$) (Fig. 4b). Fig. 5 shows a Bland–Altman plot of the percent difference in linezolid concentration (DBS vs plasma) between the two matrices versus the mean linezolid concentration. All the differences in concentration fell within ±196 standard deviations.

Table 2
Stability of linezolid in dried blood spot at room temperature, +4 °C, +37 °C and −20 °C. Values are the mean of triplicate measurements.

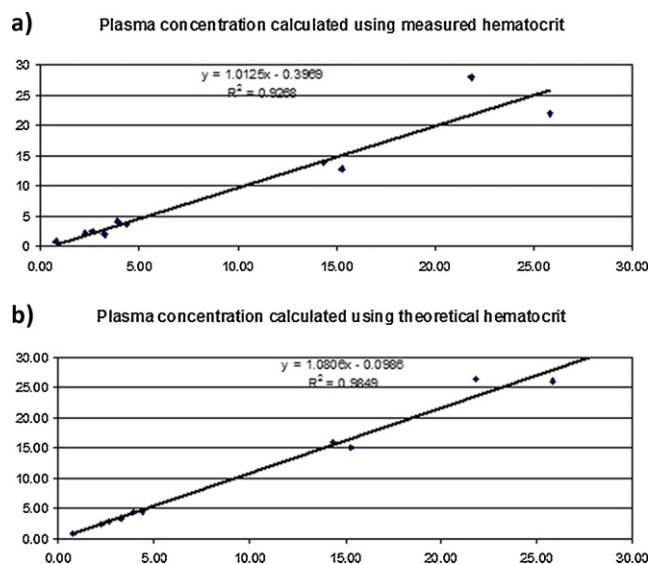| Expected concentration (mg/L)	Storage temperature	Average, analyses in triplicate for 30 days (mg/L)	DS	CV%	Accuracy
5	−20 °C	4.6	0.2	5.1	92.2
20	−20 °C	21.9	0.7	3.2	109.6
50	−20 °C	49.2	2.6	5.3	98.4
5	+4 °C	5.1	0.4	8.3	100.9
20	+4 °C	21.7	1.3	5.9	108.4
50	+4 °C	51.4	3.4	6.5	102.8
5	Room	4.8	0.5	10.4	95.0
20	Room	21.9	2.1	9.7	109.5
50	Room	49.0	2.5	5.1	97.9
5	+37 °C	4.9	0.7	15.2	97.2
20	+37 °C	21.7	2.9	13.4	108.5
50	+37 °C	48.0	4.7	9.7	96.0

Fig. 4. Comparison between the values concentration obtained from the plasma and calculated after DBS analysis, corrected using the hematocrit ratio. In the upper part the measured hematocrit was used and in the lower part the calculation were performed using theoretical hematocrit (mean per age).
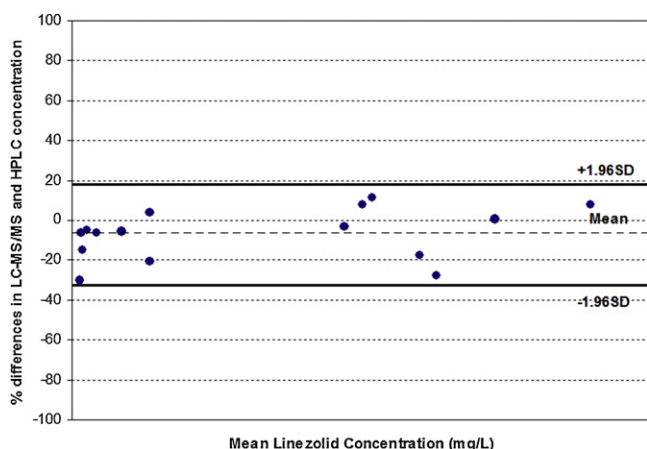
Fig. 5. Percent difference in linezolid concentration measured in plasma and from DBS versus mean concentration. The mean difference is −695%. All differences are contained within 196 standard deviations (S.D.).
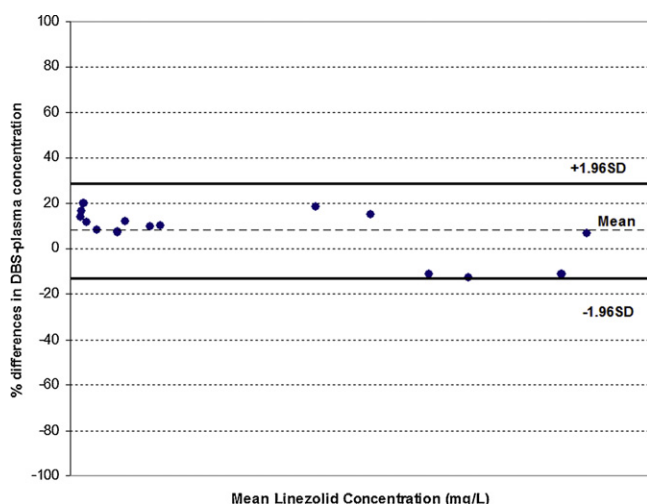

Fig. 6. Bland–Altman plot between HPLC and LC–MS/MS values obtained in plasma and DBS corrected by hematocrit value. The mean difference is +764%. All differences are contained within 196 standard deviations (S.D.).

3.7. Comparison between LC–MS/MS and HPLC–UV measurements

To compare the two methods we used a Bland–Altman plot, where the percent differences in linezolid concentration fell within 196 standard deviations (Fig. 6). On the basis of our study data the DBS technique was well correlated with a standard analytical method such as HPLC.

4. Conclusions

DBS sampling offers many advantages for collection, handling, shipment and storage of specimens. Blood samples are easily recovered, involving minimal sample manipulation and making it easy their use in numerous clinical applications especially for therapeutic drug in pediatric patients.

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